G-PROTEIN SIGNALLING IS ESSENTIAL FOR *DROSOPHILA* OOGENESIS

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Abstract

Starting from a gal^4/UAS enhancer trap screen Dr Zhao isolated two cDNAs encoded by a gene expressed in anterior dorsal follicle cells. I sequenced these and identified them as members of a new family of RGS proteins responsible for the negative regulation of G-Protein signalling. This gene was also identified by Granderath et al. (Development 126,1781-1791 (1999)) who showed that it is involved in glial cell formation, and named *locomotive (loco)*. At least two transcripts of *loco* are expressed in oogenesis, *loco-c2* is observed in the anterior dorsal follicle cells and is downstream of the EGFR signalling pathway initiated in the oocyte. *loco-c3* is a new transcript of *loco,* which is expressed in the nurse cells from stage 6 onwards.

Disruption of *loco* by mutagenesis resulted in a range of egg phenotypes, including eggs that are ventralized to varying degrees, and eggs that are shorter than wild type. Antisense technology enabled us to establish that disrupting *loco* in follicle cells results in ventralized eggs, while disrupting *loco* in nurse cells results in short eggs due to defective dumping of the nurse cell cytoplasm into the oocyte. I propose that *loco* plays a role in the regulation of the EGFR signal in the dorsal follicle cells and is required for normal dorsal/ventral polarity in the egg and embryo.

Abbreviations

ATP	Adenosine-5'-triphosphate
bp	base pair
°C	degrees Centigrade
Ci	Curies
cDNA	Complementary deoxyribonucleic acid
cfu	colony forming units
cm	centimetre
DAB	3', 3'-Diaminobenzidine tetrahydrochloride
dATP	Deoxyadnenosine-5'-triphosphate
dCTP	Deoxycytosine-5'-triphosphate
dGTP	Deoxyguanosine-5'-triphosphate
dTTP	Deoxythymidine-5'-triphosphate
ddATP	2' (3'-di) Deoxyadnenosine-5'-triphosphate
ddCTP	2' (3'-di) Deoxycytosine-5'-triphosphate
ddGTP	2' (3'-di) Deoxyguanosine-5'-triphosphate
ddTTP	2' (3'-di) Deoxythymidine-5'-triphosphate
dNTP	deoxynucleotide-5'-triphosphate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylenediamine-tetra-acetic acid
g	gram
g	gravitational force
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HRP	Horse radish peroxidase
IPTG	Isopropyl-?-D-thio-galactopyranoside
kDa	kiloDaltons
kb	kilobase pairs
Klenow	Large fragment of DNA Polymerase I
l	litre

M	moles per litre
MOPS	Morpholinopropanesulphonic acid
mRNA	messenger RNA
nt	nucleotide
OD	Optical density
OLB	Oligo labelling buffer
pfu	plaque forming units
PCR	Polymerase chain reaction
pH	-Log10 [hydrogen ion concentration]
RT-PCR	Reverse transcription polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
rpm	revolutions per minute
SDS	Sodium dodecyl sulphate
Tris	Tris(hydroxymethyl)-amino-methane
Tris-HCl	Tris solution, pH adjusted with HCl acid
X-Gal	5-bromo-4-chloro-3-indolyl-?-galactopyranoside
TCA	Trichloroacetic acid
uv	ultra-violet
V	Volts
v/v	volume per volume
w/v	weight per volume
W	Watts
pmol	picomoles
ng	nanogram
nmol	nanomoles
µCi	microCuries
µg	microgram
µl	microlitres
µM	microMolar
µmol	micromole
mA	milliAmpere
mg	milligram

ml	millilitre
mm	millimetre
mM	milliMolar
msec	milliseconds
~	approximately

Amino Acid Abbreviations

A	Alanine
R	Arginine
N	Asparagine
D	Aspartate
C	Cysteine
Q	Glutamine
E	Glutamate
G	Glycine
H	Histidine
I	Isoleucine
L	Leucine
K	Lysine
M	Methionine
F	Phenylalanine
P	Proline
S	Serine
T	Threonine
W	Tryptophan
Y	Tyrosine
V	Valine

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Chapter 1 Introduction

1.1 Introduction

Oogenesis is the process by which eggs are formed. This process is central to life in all multicellular organisms. A fertilised egg has the ability to give rise to a complete organism, with all its different cell types organised into tissues, organs and systems. To achieve this, the oocyte has to be highly ordered, and in many organisms it contains localised maternal products, that are responsible for the early events and cell divisions, in the embryo. Without these early cues, placed correctly, an embryo would not be able to develop properly. This highly organised patterning is established within the oocyte, during oogenesis and is usually dependent on co-operation between oocyte, other germ line cells and associated somatic cells. This thesis reports studies on a gene involved during oogenesis in the dorsal-ventral patterning of the egg and embryo of *Drosophila melanogaster*.

Drosophila oogenesis has been studied at great length, and this has led to insight into several aspects of development. Morphogenesis, cell migration and cell specialisation, are all characteristics exhibited in *Drosophila* oogenesis. For example subsets of the follicle cells become morphologically changed (e.g. stretched follicle cells) while specific subsets take on specific fates, (e.g. the dorsal anterior follicle cells and border cells). Cell determination can be observed when one of the 16 germ line cells becomes the oocyte and the other 15 cells become the nurse cells. Chromosome structure is involved in nurse cell formation, as they adapt to their role of manufacturing all materials for the oocyte. The cytoskeletal network plays a vital role in the transport of nutrients and RNA into the oocyte, and in localisation within the oocyte of maternal products, essential for anterior-posterior axis formation and germ cell formation. Programmed cell death is essential for nurse cells and follicle cells, when the oocyte reaches maturity. As well as answering a number of developmental questions, *Drosophila* oogenesis has also increased our understanding of oogenesis in other organisms. It is becoming increasingly apparent that many aspects of development, such as signalling pathways, are conserved across species, with a few modifications. It is this similarity that is so fascinating.

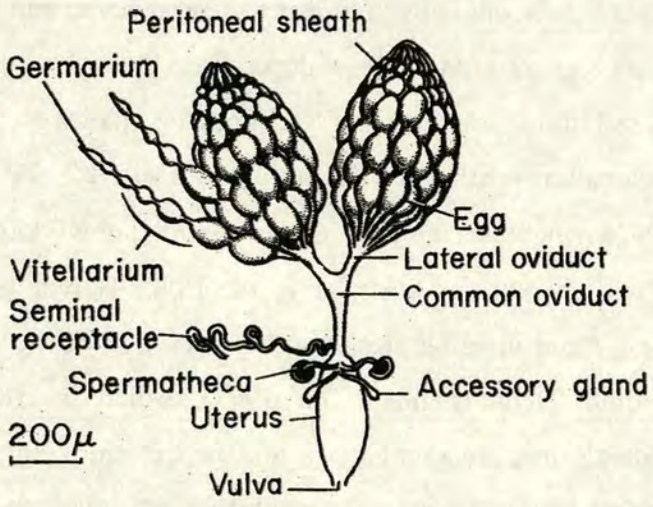
Drosophila oogenesis has proved a productive system to work on. All stages of oogenesis can be observed within a single ovary and the different cell types are easily observed due to their size and morphological differences. This, coupled with the capabilities of *Drosophila* genetics, has enabled this field of research to progress at a rapid rate. Axis formation offers opportunities to study both intercellular and intracellular signalling and over the last few years there has been an explosion in the understanding of dorsal-ventral patterning within the egg chamber. The study of dorsal-ventral axis formation has revealed a lot of information about signalling pathways, such as the Transforming Growth Factor α (TGFα)/ Epidermal Growth Factor Receptor (EGFR) pathway (Gonzalez-Reyes *et al.* 1995; Goode *et al.* 1996; Morimoto *et al.* 1996; Neuman-Silberberg and Schupbach, 1994; Sapir *et al.* 1998; Wasserman and Freeman M, 1998; Zhao and Bownes M, 1999). Epidermal growth factor signalling has being demonstrated in several different organisms and is used in many cell types, allowing adjacent cells to signal to one another, and influence cell behaviour. The resultant response to the signal depends on the cell type and the stage of development. In addition to the EGFR signalling pathway there is the Spatzle/Toll/Dorsal signalling pathway (DeLotto and DeLotto, 1998; Stein and Nusslein-Volhard, 1992), which is analogous to the mammalian NF kappaβ/I kappaβ or Dorsal/Cactus signalling pathway (Geisler *et al.* 1992; Belvin and Anderson, 1996) in both vertebrates and invertebrates. Another very well conserved signalling pathway is Fringe/Notch /Delta (Panin *et al.* 1997), which is crucial for limb development in mammals and imaginal discs and hence limb morphogenesis in insects (Irvine and Vogt, 1997). All these key pathways are involved in the correct dorsal ventral axis specification in the egg and the embryo of *Drosophila melanogaster*.

1.2 Overview of oogenesis

In a *Drosophila* adult female, each ovary is composed of parallel bundles of developmentally ordered egg chambers, each of which supports the development of a single oocyte (Fig 1.1). These bundles, called ovarioles, are divided into anterior and

posterior compartments. Oogenesis is initiated in the anterior compartment of the ovariole, the germarium (Fig1.2). Within the germarium, the germ line stem cell divides to form a daughter stem cell and a cystoblast. The cystoblast proceeds through four mitotic divisions to form a cyst of 16 cells, one of which becomes determined to form the oocyte, while the remaining 15 become the nurse cells found in each egg chamber. During oogenesis, the nurse cells synthesise maternal components for transport to the oocyte. Cytokinesis is incomplete at each of the cystoblast divisions, which leaves the 16 germ line cells interconnected by large cytoplasm bridges called ring canals, which are maintained until the completion of oogenesis and are the basis for most of the transport from nurse cells into the oocyte.

Figure 1.1 Adult Female Ovaries



From King, 1970

Legend

This figure illustrates the Female reproductive system, containing two ovaries made up of bundles of ovarioles. Each ovariole consists of a germarium at the anterior tip, which contains the germline stem cells. The germarium is followed by a string of progressively more developed oocytes, leading up to mature eggs which are ready to be deposited into the oviduct.

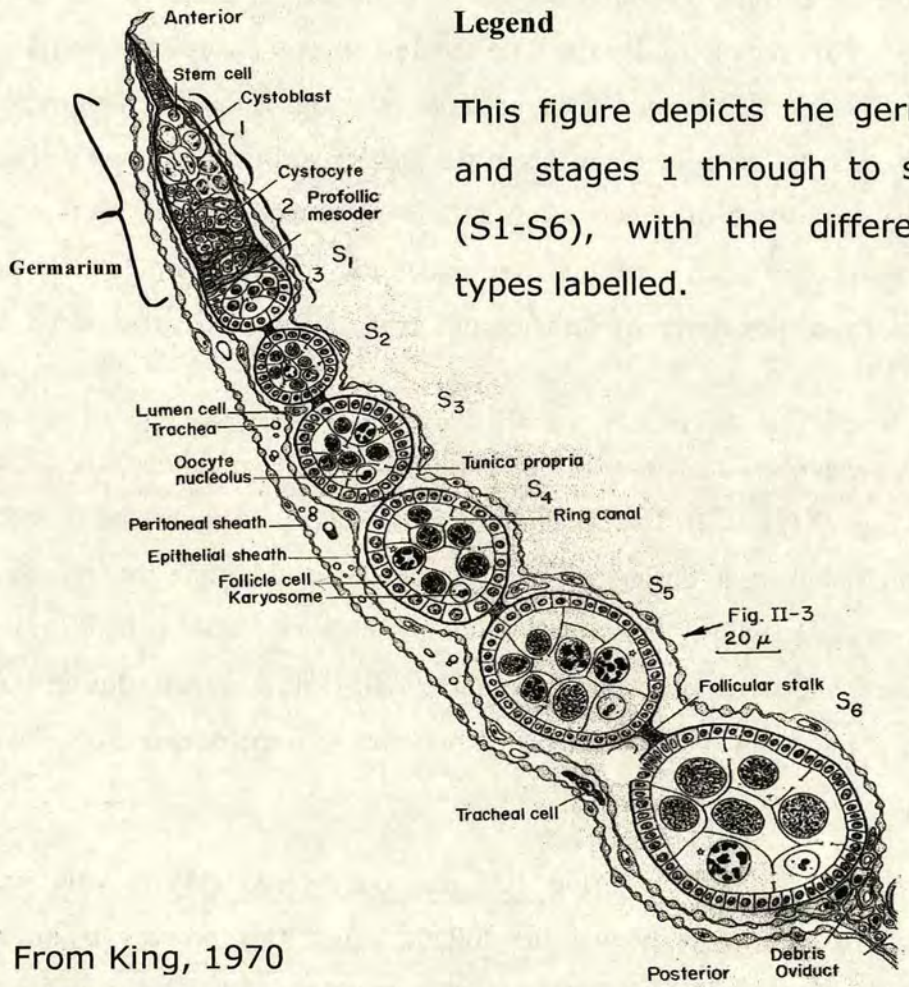
Germaria are divided into four cytologically distinct regions, which contain developmentally arrayed germ line cysts (Fig 1.2). The stem cells and the cystoblast lie within germarial region 1, whereas newly formed 16-cell cysts are located in region 2a. When cysts progress to region 2b, they become lens shaped and span the width of the germarium. The future oocyte is positioned at the centre of the lens-shaped cysts from region 2b. By the time the 16-cell cyst occupies region 3 of the germarium, the oocyte is located at the posterior pole; this is the first sign of polarity within the embryo. The oocyte remains at the posterior of the germ line cell cluster through the completion of oogenesis. In region 2a, somatic follicle cells begin to migrate around the 16-cell germ line cyst. When they reach region 3, the cysts are surrounded by a monolayer of follicle cells and are referred to as stage 1 egg chambers.

Stage 2 egg chambers bud from the germarium and enter previtellogenesis, which lasts until stage 7 (Fig 1.2). The developing egg chambers are connected by stacks of interfollicular stalk cells during stages 2 through 6; in this time the egg chamber increases in size whilst remaining roughly spherical. The oocyte grows at approximately the same rate as a single nurse cell. Oocyte growth during stages 2 through 6 is the result of the transport of nutrients into the oocyte from the nurse cells.

During stages 7 through 10a (Fig 1.3), the oocyte endocytoses yolk proteins synthesised by the fat body and the follicle cells. This process is known as vitellogenesis. Consequently, oocyte growth is more rapid than nurse cell growth, and by stage 10 the oocyte occupies the entire posterior half of the egg chamber (Fig 1.3). The morphogenetic molecules that specify the embryonic axes are asymmetrically positioned within the oocyte during these stages. mRNA of *bicoid*(Berleth T., *et al.* 1988), the primary anterior morphogen, is localised to the anterior cortex. Vasa (Hay *et al.* 1990), Staufen proteins (St Johnston *et al.* 1991), and *oskar* mRNA (Lehmann *et al.* 1986), which are required for pole cell formation and posterior patterning, are positioned at the posterior pole. *gurken* mRNA (Neuman-Silberberg *et al.* 1993), which plays a key role in dorsal-ventral axis

specification, accumulates between the dorsally located oocyte nucleus and the cortex and this location is dependent on germ cell-follicle cell interactions.

Figure 1.2 Oogenesis Stages 1-6



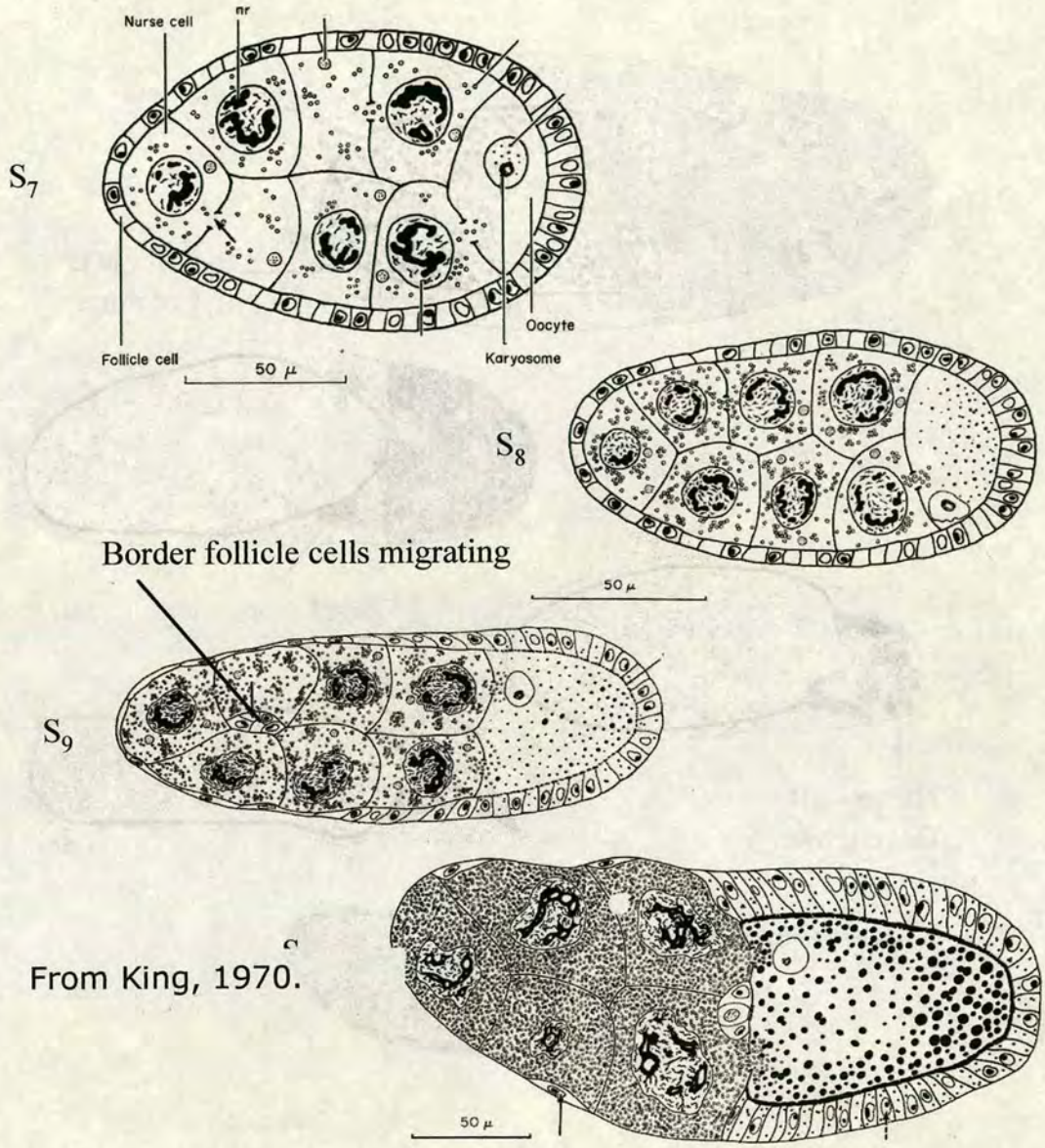
Legend

This figure depicts the germarium and stages 1 through to stage 6 (S1-S6), with the different cell types labelled.

From King, 1970

The follicle cells are very dynamic. They surround the whole egg chamber during stages 1-8 (Fig 1.2, 1.3) and at around stage 9, they rapidly migrate posteriorly to leave a very thin layer of cells over the nurse cells (stretched follicle cells) and a columnar layer around the oocyte (Fig 1.3). At about the same time, follicle cells from the anterior of the egg chamber migrate through the nurse cells to the anterior border of the oocyte. These become the border cells. During stage 10B, the anterior columnar follicle cells migrate centripetally along the dorsal ventral axis

Figure 1.3 Oogenesis Stages 7-10a

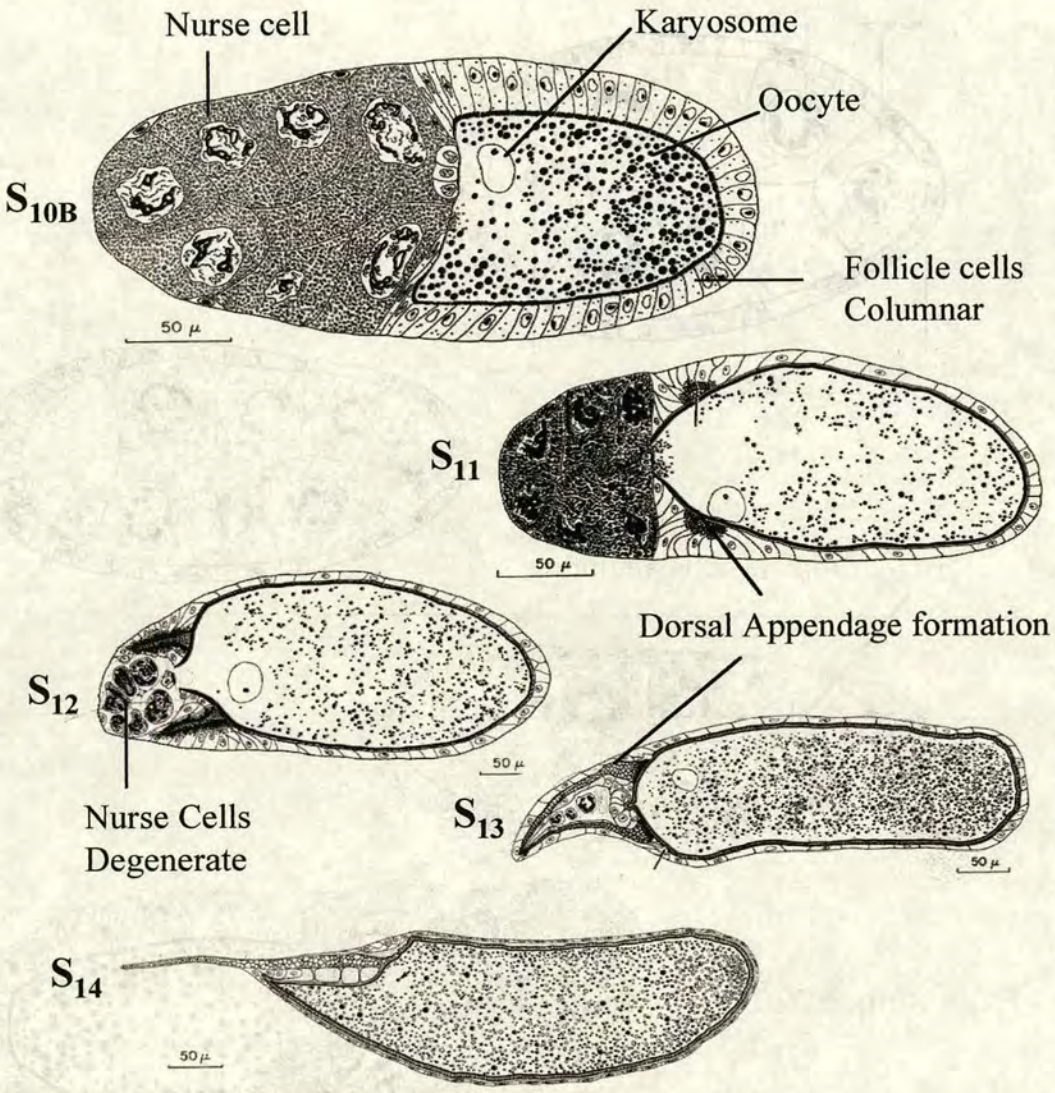


From King, 1970.

Legend

This figure represents oogenesis stages 7-10a. A 50μ scale is marked next to each stage. The cell types are marked on Stage 7.

Figure 1.4 Oogenesis Stages 10b-14



Legend

This figure represents oogenesis stages 10a-14. A 50μ scale is marked next to each stage. The cell types are marked on Stage 10b.

to cover the anterior end of the oocyte. Up to this stage the oocyte is surrounded by a layer of follicle cells, that secrete the vitelline membrane and chorion to protect the mature egg.

During stages 10B to 12, nurse cell cytoplasm is rapidly transferred through the ring canals into the oocyte (Fig 1.4). The transfer of most nurse cell cytoplasm into the oocyte occurs within 30 minutes. This process is known as "dumping". During stages 13 and 14, these ooplasmic movements stop and the meiosis 1 spindle assembles. The oocyte remains in metaphase of the first meiotic division until it enters the oviduct, when both egg activation and fertilisation initiate embryonic development.

At stages 13 and 14, the nurse cells and follicle cells shrink and undergo apoptosis. This leaves the mature egg, wrapped with a complete eggshell (chorion) and its specialised structures: a pair of dorsal appendages (filaments) at the anterior end of the egg, facilitating embryonic respiration; the operculum, used for the larvae to hatch and the micropylar apparatus, for sperm entry.

Table 1.1 Stages of oogenesis

Stage	Length	Average size	Identification
1	10 hr		16 cell cyst within region 3 of germarium; oocyte and nurse cells similar in size up to stage 8
2	8 hr	25 x 25 μm	oocyte and nurse cell nuclei similar in size; nurse cell nuclei begin polyploidisation 8C
3	8 hr	35 x 35 μm	karyosome and endobody are visible in germinal vesicle
4	6 hr	40 x 50 μm	nurse cell nuclei contain similar amount of DNA and appear polytene
5	5 hr	55 x 75 μm	Nurse cell nuclei no longer polytene; posterior nurse cells have more DNA than anterior ones
6	3 hr	60 x 85 μm	egg chamber still oval; follicle cell divisions cease; nurse cell ploidy equal
7	6 hr	70x115 μm	egg chamber elongated; nurse cells have higher ploidy at posterior; no yolk visible in oocyte
8	6 hr	190 μm	yolk granules visible in oocyte; follicle cell layer still uniform
9	6 hr	275 μm	border cell migration in progress; most follicle cells migrate over oocyte; secretion of vitelline membrane begins; oocyte is about 1/3 of the size of egg chamber
10A	6 hr	430 μm	follicle cells form columnar epithelium over oocyte; centripetal migration not visible; oocyte is 1/2 size of egg chamber
10B	4 hr	450 μm	centripetal migration in progress; dorsal follicle cells thicker than ventral; vitelline membrane extends into opercular region
11	0.5 hr	490 μm	nurse cell dumping onset; oocyte larger than nurse cells
12	2 hr	540 μm	nurse cell dumping complete; 15 nurse cell nuclei remain at anterior; dorsal appendages not visible
13	1 hr	650 μm	gradual disappearance of nurse cell nuclei; dorsal appendages visible; oocyte elongates
14	>2 hr	700 μm	no nurse cell nuclei remain; dorsal appendages complete their elongation

Modified from Spradling (1993)

1.3 The development of the ovary

1.3.1 Embryonic origin of the ovaries

There are special cytoplasmic determinants located at the posterior of the egg and early embryo. This posterior cytoplasm or pole plasm contains polar granules that act to induce the differentiation of the cells formed at the posterior to become the pole cells. At the 8th syncytial blastoderm nuclear division, 10 or more nuclei in contact with the posterior become cellularised. These are the first cells to form within the embryo and go on to divide and form a cluster of 40 to 60 cells. It is these pole cells that go on to form the germ line of the embryo and eventually adult.

The formation and localisation of the pole plasm depends on genes such as *oskar*, *vasa, valois, staufen, tudor* and *mago nashi* (Lehmann and Nusslein-Volhard, 1986; Hay *et al.* 1990; St Johnston *et al.* 1991; Boswell and Mahowald, 1985; Newmark and Boswell, 1994). The formation of the pole plasm is linked with segmentation, as *nanos* (Lehmann and Nüsslein-Volhard, 1991; Wang and Lehmann, 1991), the posterior determinant, is also localised in the pole plasm.

During the course of embryo development, the pole cells are carried into the embryo as a result of gastrulation, and midgut invagination. At 7 hours (25°C), the pole cells travel between the cells of the future midgut and arrive between the endodermal cells and the syncytial yolk sac membrane. The pole cells subsequently become associated with mesodermal cells on both sides of the embryonic gut to produce the gonads. These mesodermal cells are thought to be derived from the fifth abdominal segment (Mahowald and Kambysellis, 1980). Interestingly pole cell migration requires *nanos*, which is also critical for abdomen formation (Lehmann and Nusslein-Volhard, 1991). Although *nanos* is localised to the pole plasm, it is not involved in pole cell specification. However, in the absence of *nanos,* pole cells fail to migrate and therefore do not become functioning germ cells.

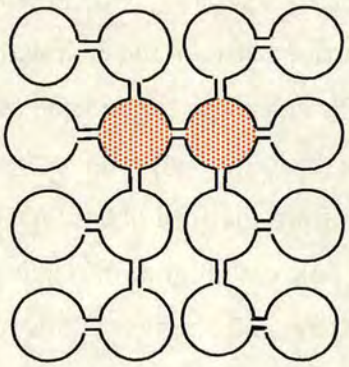
The ovaries first become identifiable in 1st instar larvae. From this point until mid 3rd instar the ovaries remain morphologically unchanged but increase in size. The

larval ovary is made up of somatic mesodermal cells and germ line derived oogonia. There appears to be a simple polarity during this stage as the larger germ line cells are located in the middle region while smaller somatic cells occupy the poles.

1.3.2 Oocyte specification

How is one of the sixteen cystocytes selected to become the oocyte? The first apparent selection of the oocyte appears around germarial region 2, as some mRNAs and proteins are transported into one of the 16 cystocytes. On an observational level the two oldest cystocytes form synaptonemal complexes, but eventually the complexes are only retained in one cell, which becomes the oocyte (Fig 1.5). This determination may suggest that there is an oocyte determining factor that is asymmetrically distributed. Since the fusome is asymmetrically distributed during cystoblast mitosis, it may serve as the oocyte determining factor. The fusome is a germ line specific organelle. Electron microscopy has shown that the fusome is a distinct region of cytoplasm rich in fibrils and vesicles but excluding mitochondria and ribosomes.

Figure 1.5 Oocyte selection



Legend

The two germ line cells with 4 ring canals are highlighted in red. One of these cells go on to become the oocyte.

1.3.3 Oocyte and the nurse cell differentiation

Chromosome structure plays an important role in nurse cell specialisation. During early stages of cyst development the oocyte chromosomes undergo synapses and

recombination. At around stage 3 the nucleus condenses and becomes transcriptionally inactive until just before the oocyte matures. The nurse cell chromosomes become polyploid and these banded polytene chromosomes progressively disperse to form large nuclei. These changes are thought to facilitate high levels of ribosome synthesis and other components required for oocyte growth.

1.3.4 Somatic cell differentiation in early oogenesis

The follicle cells are descendants of somatic stem cells. By region 2 of the germarium, they completely surround the 16 germ cell cyst. It has been identified that two somatic stem cells are located near the border of germarium at around 2a/b. A single division of both somatic stem cells results in the regeneration of 2 stem cells and 2 daughter cells. The daughter cells go through 4 rounds of division, resulting in 16 follicle cells surrounding the cystocyte at stage 2b. It should be noted that those somatic divisions are not co-ordinated with those of the germ line and in agametic flies the somatic stem cells continue to divide in the absence of the germ line cells (Spradling, 1993). A mechanism may operate to maintain a balance between germ line and somatic cell populations.

1.3.5 Cyst envelopment and follicle cell differentiation

The envelopment of the germ line cyst starts in region 2a when somatic follicle cells migrate from the wall of the germarium to surround the 16 cell cyst. This is the first migration of the somatic follicle cells. The individual cysts are not separated until region 2b when the follicle cells migrate inwardly (Fig 1.2). The follicle cells are only ever seen migrating after the 16 cell cyst has been formed, never when the 8 cell one is present. This is thought to be due to a signal from the cystocyte indicating that it has reached the 16 cell stage (Spradling, 1993).

1.3.6 Follicle cell migration and eggshell morphogenesis

Initially it was thought that the role of follicle cells was merely to secrete the eggshell, protecting the embryo as it developed. As more has been understood about

the follicle cells, several further roles have emerged. The follicle cells produce some of the yolk proteins for the oocyte. Probably more interesting is the signalling that is occurring between the follicle cells and oocyte and the follicle cells and nurse cells. These signals are responsible for various morphological changes within the follicle cell layer and the establishment of positional information within the follicle cells, that is manifested in the overall shape of the egg and its structures.

For example, the micropyle is produced by the border cells that migrate from the anterior pole of the egg chamber to reach the anterior oocyte at stage 10 (Fig 1.3). The dorsal appendages are progressively produced, from base to tip, by groups of anteriorly migrating follicle cells. The follicle cells therefore produce the eggshell by precisely migrating, changing cell shapes and integrating their structural gene expression. These changes require interactions between individual follicle cells and between the follicle cell layer and the underlying germ line cells. The follicle cells also play a role in establishing the axis, through signalling that is occurring between the follicle cell layer and oocyte.

The follicle cells are very dynamic and undergo a number of migrations. At certain stages follicle cells move as a sheet of cells and at other stages only specific subsets of cells move, in order to form specific structures of the eggshell. As the follicle cells form an epithelial sheet, this makes them ideal to study cell migration and morphogenesis.

The first migration of follicle cells is from the wall of the germarium to surround the 16 cell germinal cyst in region 2. Later in oogenesis, during stage 9, a group of 6-10 follicle cells at the anterior tip of the egg chamber leave the epithelial layer and migrate between the nurse cells to arrive at the oocyte border. At the same time the majority of the follicle cells migrate posteriorly to end up forming a thick columnar epithelium over the oocyte whilst the remaining follicle cells stretch over the nurse cells to form the stretched follicle cells. As the nurse cells dump the remainder of their content, the oocyte becomes larger, while the columnar follicle cells over the oocyte stretch and move anteriorly. This movement is passive as the follicle cells remain fixed in relation to the oocyte. At stage 12 the anterior dorsal follicle cells,

either side of the dorsal midline, migrate anteriorly to secrete dorsal appendages. At stage 12-13 other subsets of anterior follicle cells secrete the operculum and micropyle.

1.3.7 Dorsal-anterior follicle cell migration and dorsal appendage formation

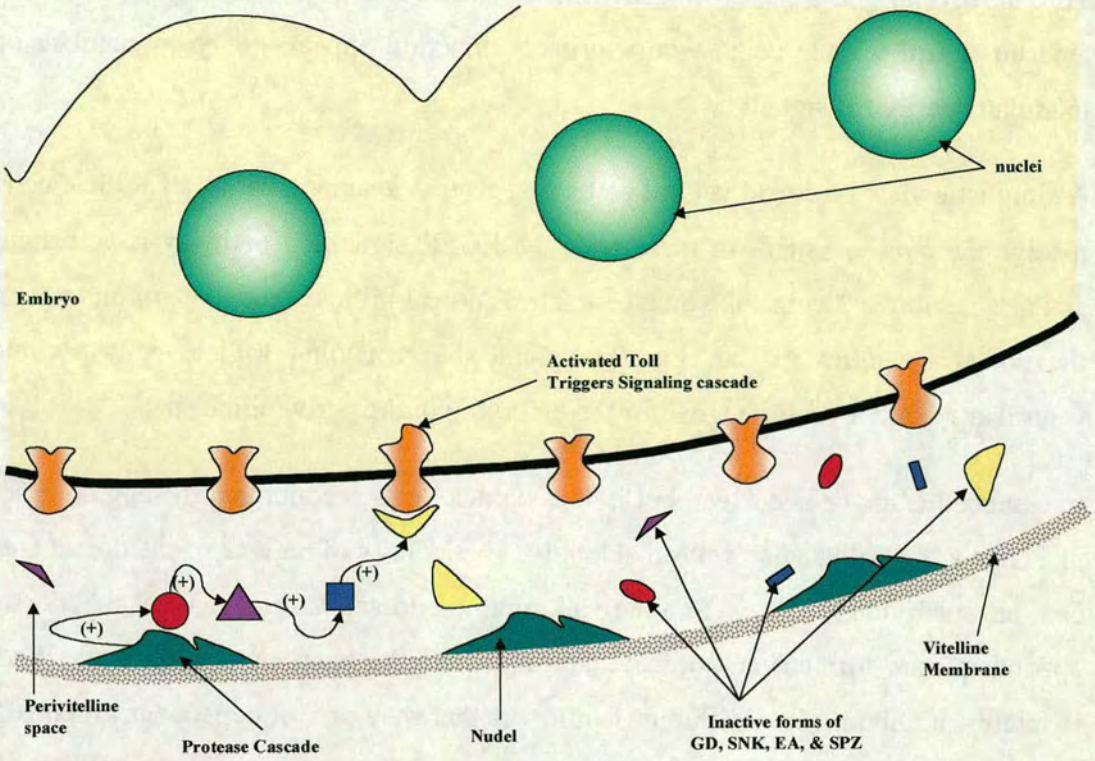
At the dorsal-anterior region of the mature egg of *Drosophila*, lies a pair of chorionic appendages, also called the dorsal appendages or dorsal filaments. Each dorsal appendage is formed by a group of follicle cells that start migrating over the anterior part of the oocyte at stage 11. Staining of a dorsal appendage marker, the Broad-Complex (BR-C), show that there are about 55-65 follicle cells in each group (Deng and Bownes, 1997). Filament-producing cells begin to secrete the filament bases to attach to the main body of the eggshell before they begin migrating towards the anterior. As cells migrate past the growing end, they join a cylinder of cells secreting chorion proteins and commence secretion themselves. The dorsal appendages complete their elongation at stage 14. All references describing this migration cite King (1970).

The production of the dorsal appendages is dependent upon the EGFR signalling pathway, which induces dorsal-ventral polarity of the eggshell and the embryo. Thus, the regulation of appendage formation provides a good model for the study of signal instructed morphogenesis. In strong *grk* mutants, the dorsal appendages are absent. In *fs(1)K10* mutant egg chambers, *grk* mRNA is not localised resulting in a ring being formed around the anterior, which induces the dorsal appendages to fuse at the ventral side. The downstream effector gene that directly regulates dorsal appendage formation is likely to be *Broad-Complex*, a gene complex encoding a family of zinc-finger transcription factors (Deng and Bownes, 1997).

1.3.8 Nurse cell-oocyte transport

The oocyte is dependent on the nurse cells for its growth and development. The nurse cells produce proteins, mRNA, ribosomes and mitochondria, all of which are

Figure 1.10 **The Embryo Signal**



Legend

This figure illustrates what is happening in the perivitelline space after the egg has been fertilised. Nudel is thought to be bound to the vitelline membrane, and to start a protease cascade that results in the sequential activation of Gastrulation defective (GD), Snake (SNK), Easter (EA) and Spatzle (SPZ). The active form of Spatzle is then able to bind Toll activating the ventral signalling pathway within the embryo.

1.4.2.3 Regulation of the EGFR signal

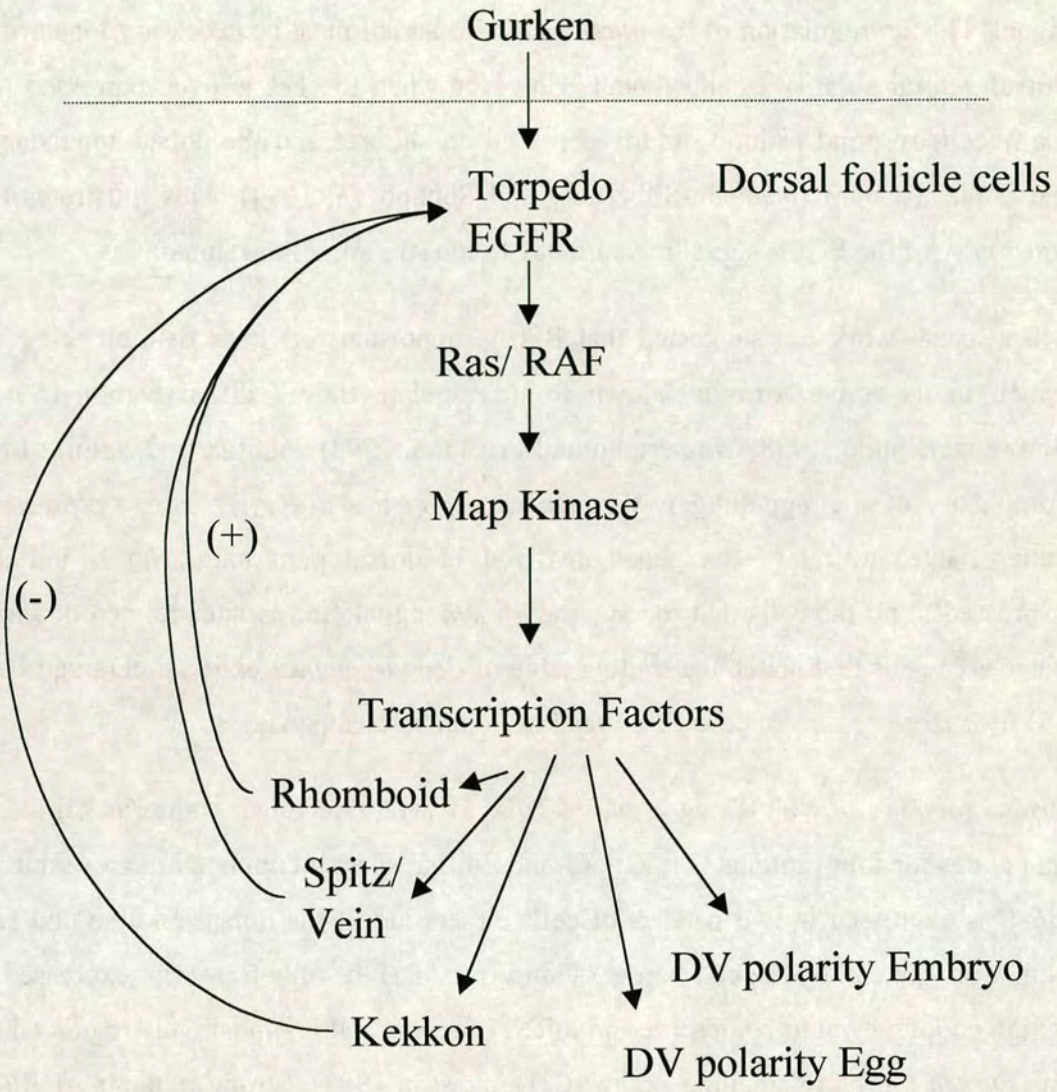
To date several genes have been identified downstream of the EGFR signal in the anterior dorsal follicle cells, whose primary function appears to be to regulate or modulate the EGFR signal.

A simplistic view of dorsal ventral patterning, is that a subpopulation of follicle cells receive the *gurken* signal. In these cells the EGFR signalling pathway is activated, and as a result these cells take on a new fate as dorsal follicle cells, and go on to form the dorsal structures of the egg. By default the remaining follicle cells become ventral and build a ventral signal for the embryo into the perivitelline space.

In essence the above is correct, but it does not take into account that the egg does not just have a "dorsal" and a "ventral" identity. The identity of regions of the dorsal area can be subdivided further into dorsal midline, dorsal lateral (where the dorsal appendages are formed) and dorsal anterior (where the operculum is formed). What is interesting about these different features is that they are all dependent on correct levels of activated EGFR, at exact time points, as the dorsal anterior region of the egg is being determined. We know that EGFR is initially activated in a single group of anterior dorsal follicle cells at stage 10, by the Gurken ligand, and that this signal is then refined by different genes activated downstream of the EGFR signal within the follicle cells.

One of the components is *rhomboid* (*rho*) a transmembrane protein (Ruohola-Baker *et al.* 1993). *rho* is expressed in the dorsal follicle cells, in response to the *grk* signal. The loss of *rho* expression results in ventralisation, whereas over expression of *rho* in all follicle cells causes dorsalisation (Ruohola-Baker *et al.* 1993). Surprisingly, this dorsalisation is dependent on *grk* and *top*. As *rho* is expressed in the dorsal follicle cells, it must act downstream of Grk. However the dorsalisation produced by over expression of *rho* requires Grk, suggesting that Grk lies genetically downstream of *rho* (Ruohola-Baker, *et al* 1993).

Figure 1.11 Signalling in dorsal follicle cells



Legend

This flow diagram illustrates the main components involved in the regulation of the EGRF signalling pathway in the anterior dorsal follicle cells. Gurken protein binds EGFR receptor Torpedo. Activated EGFR induces a Ras/Raf and Map Kinase pathway. This results in several transcription factors being activated and several genes being expressed. Some of the genes include *Spitz, Vein, Kekkon* and *Rhomboid,* all play a role in the modulating the EGFR signal. The modulation of the EGFR signal is critical for correct dorsal –ventral axis formation of the egg and embryo.

These results rule out a model in which *rho* is part of a simple linear signalling pathway, and suggests that *rho* may act in a feed back loop that amplifies the dorsal signal. This up regulation of the *gurken-torpedo* signal must be necessary for normal dorsal ventral polarity establishment. However when the *grk* is over-expressed too many cells respond resulting in an increased dorsal area and the dorsal appendages move further apart (Neuman-Silberberg and Schupbach, 1994). This illustrates the sensitivity of the EGFR signalling pathway to the strength of the signal.

More recent work has suggested that Rho is important for the activation of Spitz, which in its active form is known to up regulate the EGFR activation (Sapir, Schweitzer, Shilo, 1998; Wasserman and Freeman, 1998). Another indication of the complexity of the signalling is that the gene *rho* has a very dynamic expression pattern, like several of the genes involved in dorsal patterning. *rho* is initially expressed in all the cells that receive the *gurken* signal, and is later refined down to patches of cells that match the trailing edge of *Broad-complex* expression (stage 10b-11). It is these groups of cells that later form the dorsal appendages.

Broad-complex (*BR-C*) (Deng *et al.*1997) is a C2H2 zinc finger transcription factor that codes for four proteins, Z1, Z2, Z3 and Z4 that share a common amino terminus. *BR-C* is expressed in two patches of cells either side of the dorsal midline and two cells from the centripetal cell border. Transcript Z1 is the only transcript expressed at a high enough level to be observed in mRNA *in-situs* and it is positively regulated by the *gurken-torpedo* signalling pathway (Deng *et al.*1997). Strong mutants of *BR-C* have eggs that lack dorsal appendages, and weak mutants reduced dorsal appendages, so *BR-C* is directly required for dorsal appendage morphogenesis. *BR-C* expression analysis does suggest that *BR-C* responds to the Torpedo signal in a dose dependent manner, with BR-C being repressed at high levels of activated EGFR. The transcription factor *pointed (pnt)* is responsible for the repression of *BR-C* in anterior dorsal most follicle cells (Deng *et al.*1997). Deng and Bownes (1997) also established that *dpp* was responsible for the repression of BR-C in anterior most follicle cells. This is an interesting observation as a recent paper by Peri and Roth (2000), shows that *dpp* is needed in conjunction with *gurken* to form dorsal

appendages. This is illustrated by ectopically expressing *dpp* in the posterior follicle cells during stages 1-6, when *gurken* is active. This results in a ring of dorsal appendage material forming at the posterior (Peri and Roth, 2000). This implies that an interaction is needed between anterior (*dpp*) and dorsal (*gurken*) to form anterior-dorsal identity.

Pointed (*pnt*), (Morimoto *et al.* 1996) is a gene encoding a transcription factor with ETS DNA binding domains. It encodes two different transcripts, *pnt1* and *pnt2* (Klämbt, 1993). Transcripts, *pnt1* and *pnt2* have mutually exclusive exons, which has enabled their specific expression patterns to be elucidated. At around stage 9-10 during oogenesis, *pnt1* is expressed in the anterior dorsal most follicle cells around the dorsal midline. *pnt2* begins with a similar expression pattern to *pnt1,* which later changes to form a pattern similar to that of *BR-C.* Only the role of *pnt1* has been described as it is only when this transcript is disrupted that a dorsal ventral phenotype is observed. In *pnt1* absence a single fused broad appendage is formed, indicating a loss of midline follicle cells (Morimoto *et al.* 1996), whereas over expression of *pointed* results in loss of dorsal anterior follicle cells. The broad appendage phenotype observed in the absence of *pointed,* has led to the idea that *pointed* is responsible for dorsal midline fate through down-regulation of the EGFR signalling pathway in the dorsal-most cells (Deng and Bownes, 1997). It must be remembered that *pointed* encodes a transcription factor and is probably achieving this outcome through affecting a series of genes.

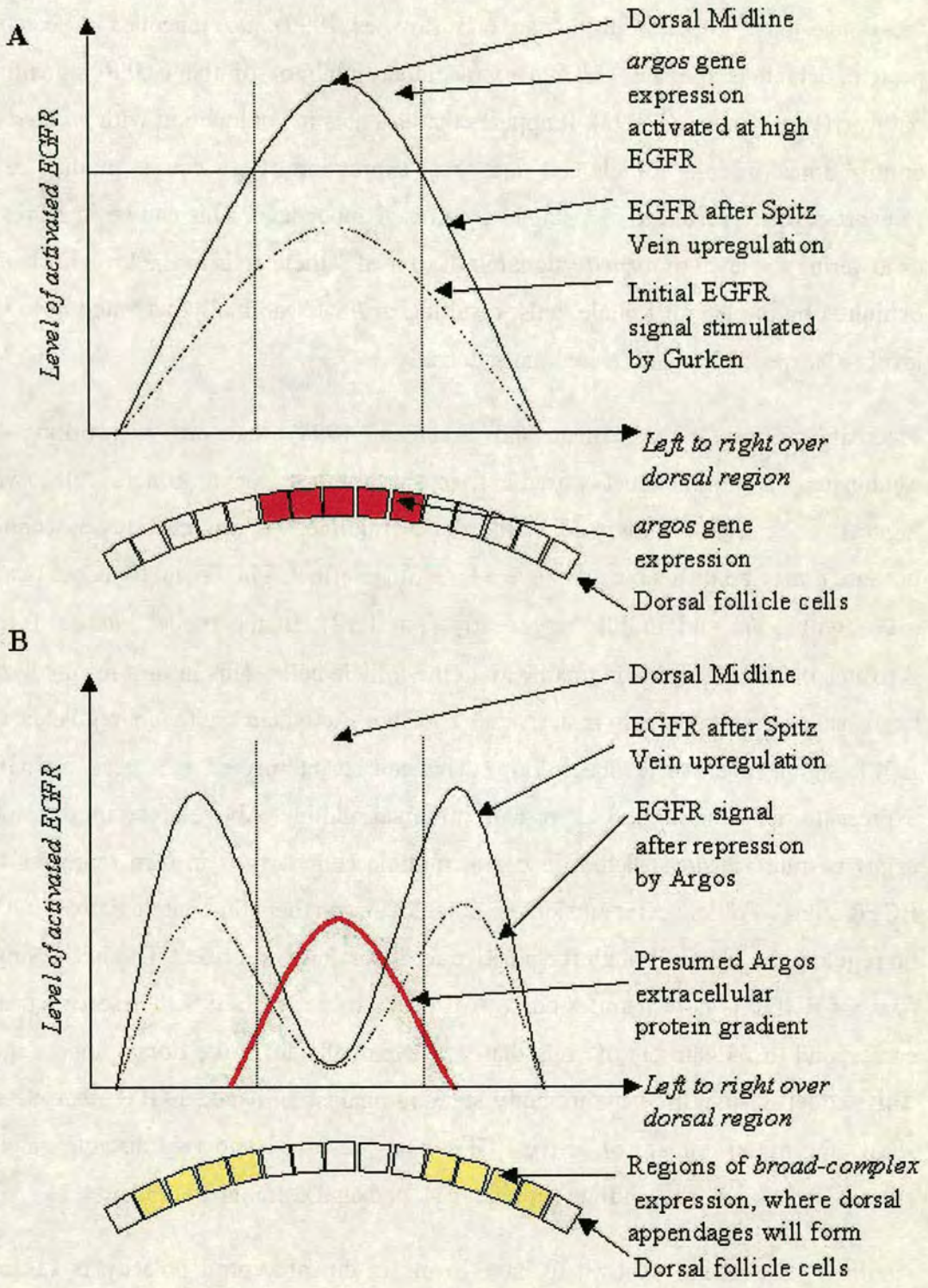
The role of *pnt2* has not been established its only difference being that its transactivation activity can be stimulated by MAPK while *pnt1* appears to be constantly active. It is thought that *pnt1* may be regulated by *pnt2* or may act in parallel (Morimoto *et al.* 1996). However *pnt2* must play a role in dorsal appendage formation, by virtue of its expression in cells that form the dorsal appendages.

Deng *et al.* (1997) first suggested that the activated EGFR in the anterior dorsal follicle cells could be subdivided into specific domains, from high activation in the

Legend 1.12

This figure illustrates how the initial EGFR signal (A) is split into two peaks that match where the dorsal appendages form (B). In A the EGFR signal is activated in the dorsal follicle cells, this leads to the downstream activation of *spitz* and *vein,* which in turn lead to the up-regulation of the EGFR signal. Above a certain threshold of EGFR activation *argos* gene expression is activated (red follicle cells). Argos is a secreted protein and is thought to form a protein gradient (B, red line). Argos represses the EGFR signal in the dorsal most follicle cells. This leaves two patches of activated EGFR in the follicle cells, either side of the dorsal mid line (B). EGFR is up-regulated, by Vein and Spitz as before and leads to downstream genes such as *broad-complex* being activated in two patches either side of the dorsal midline. It is these cells that go on to form the dorsal appendages.

Figure 1.12 **Freeman Model**



dorsal midline cells to lower activation laterally. *argos* (*aos*) is genetically downstream of *torpedo/EGFR* and is responsible for down regulation of its signal in the dorsal-most follicle cells (Zhao and Bownes 1999). *aos* encodes a secreted protein, which is the only known extracellular inhibitor of the EGFR signalling pathway(Freeman *et al.* 1992). It appears that *aos* acts in conjunction with *pointed* to control dorsal midline follicle cell fate. Over expression of *aos* causes midline cells to express *BR-C*, resulting in a single large fused appendage. This can be interpreted as lowering the level of *torpedo* signal in the dorsal follicle cells to the level normally exhibited by the lateral follicle cells, resulting in *BR-C* (normally activated at lower levels of torpedo) being activated more dorsally.

Freeman proposes (Wasserman and Freeman, 1998) that *aos* is working by establishing a morphogenetic gradient, in the anterior dorsal follicle cells, with highest concentration expressed in the dorsal midline. As *aos* encodes a secreted protein, it may be diffusible and have a long range effect. The Freeman model ties in *argos* with *spitz* and EGFR very neatly (Fig 1.12). In the model *gurken* TGFα activates the EGFR signalling pathway in the follicle cells. This in turn results in the expression of *spitz*, which is activated by Rho. Activated Spitz up regulates the EGFR signal (positive feedback loop). This can be interpreted as a peak of EGFR expression, with the highest expression at dorsal midline. Above a certain threshold *argos* is transcribed/translated, in dorsal midline cells. Argos in turn represses the EGFR signal. Follicle cells outside the range of Argos that still contain active EGFR, up regulate the signal through the positive feedback loop described. The initial single peak of EGFR is now manifested as two peaks of active EGFR. These two peaks correspond to the subsets of cells that will eventually form the dorsal appendages. This model is supported by antibody staining against activated EGFR, that clearly shows the initial domain of active EGFR being resolved into two discrete patches either side of the dorsal midline (Freeman M. personal communication).

Another gene recently shown to have a role in dorsal ventral polarity is *kekkon1* (Ghiglione *et al.*1999), which negatively regulates the EGFR signalling pathway. *Kekkon1* produces a transmembrane protein. The extracellular domain of Kekkon 1 is

thought to interact directly with the extracellular domain of Torpedo, suppressing the EGFR signal. As *kekkon* is downstream of activated EGFR, it is effectively acting as part of a negative feed back loop. Interestingly, in the complete absence of *kekkon 1* gene activity, flies are viable, fertile and do not exhibit any overt morphological defects, yet when under constitutive expression (under the regulation of *GAL4*) the eggs are ventralized (Ghiglione *et al.*1999). This presumably means its function is redundant.

Yet another gene involved in dorsal ventral patterning is *brainiac* (*brn*) (Goode *et al.* 1996). *brainiac* encodes a novel putative secreted protein. Its mRNA is expressed though the whole of oogenesis in the nurse cells and is most abundant at around stage 10. Although little is known about how *brn* works, evidence so far suggests that *brn* functions via a parallel, but overlapping pathway to the *grk-torpedo* signalling pathway. The conclusion has been drawn from *brainiac* and *gurken* double mutant analysis (Goode S. *et al.* 1996).

Some other genes that are also involved in patterning of the follicle cells are *mirror* *fringe* and *CF2* (Bownes unpublished data, Jordan *et al.* 2000, Hsu *et al.*1996). These genes are not involved in the regulation of the EGFR signal, but are important nevertheless. *fringe* codes for a secreted protein and in the wing imaginal disc is known to affect the cells adjacent to where it is expressed (Irvine KD and Wieschaus, 1994). In oogenesis *fringe* is expressed in all follicle cells except where the EGFR signalling pathway is activated (Bownes unpublished data). However anti-sense *fringe* heatshock analysis results in over proliferation or delamination of anterior dorsal follicle cells (Bownes unpublished data). This is interesting as these cells do not express *fringe,* yet they are responding to disruption of *fringe* expression. This evidence suggests that *fringe* may be responsible for how the anterior dorsal follicle cells would normally migrate, or adhere. This may include the direction in which the follicle cells migrate.

mirror encodes a homeodomain transcription factor, which is expressed in anterior dorsal and centripetal follicle cells (Jordan *et al.* 2000) and it is downstream of the EGFR signalling pathway. The *mirror* expression pattern is complementary to that of

fringe and led to the idea that *mirror* might be responsible for the repression of *fringe* in the anterior dorsal follicle cells, as *mirror* represses *fringe* in wing disc development. When *mirror* is ectopically expressed in the anterior follicle cells, *fringe* expression is lost from the anterior follicle cells (Bownes unpublished data). This evidence indicates that in oogenesis *fringe* is repressed by *mirror*. It was also shown that, when *mirror* is ectopically expressed in the posterior follicle cells, *fringe* expression is unaffected (Bownes unpublished data). This data may initially look as if it conflicts with *mirror* being responsible for repression of *fringe*. However it highlights one of the problems with ectopic misexpression of genes. Cells have a history and this may affect how they respond to an ectopic signal they would not normally receive.

It is also known that the zinc finger transcription factor *CF2* (Hsu *et al.* 1996) participates in dorsal ventral patterning. *CF2* is expressed in all columnar follicle cells. *CF2* is down regulated in anterior dorsal follicle cells by downstream components of the EGFR signal pathway. It is thought that CF2 is phosphorylated in the cytoplasm by MAPK, thus blocking nuclear import and resulting in its degradation (Mantrova *et al.* 1998). Disruption of *CF2* results in dorsal ventral patterning defects. When CF2 is ectopically expressed, moderate ventralisation of the egg and embryo is observed and, when using antisense mRNA to knock out protein expression, the converse is observed and eggs and embryos are moderately dorsalised (Hsu T *et al.* 1996). It is thought that CF2 acts as a repressor of dorsal follicle cell fate, specifically as a repressor of *rho* gene expression as ectopic expression of CF2 results in suppression of rho function (Mantrova *et al.* 1998). It is apparent that CF2 is necessary for correct dorsal ventral patterning in the egg and embryo, as disruption of CF2 results in loss of embryonic dorsal ventral axis and disruption of anterior-dorsal eggshell patterning.

1.5 G-Protein Signalling pathways

As the gene studied in this Thesis was found to be an Regulator of G-Protein Signalling (RGS) protein, the following section summarises G-Protein signalling.

A large number of hormones, neurotransmitters, chemokines, and sensory stimuli exert their effects on cells and organisms by binding to G-Protein coupled receptors. Heterotrimeric G-Proteins transduce ligands binding to these receptors into intracellular responses, which underlie physiological responses. As a result G-Proteins play important roles in determining the specificity and temporal characteristics of cellular responses to signals. The signalling of G-Proteins is often rapid and transient, and the key to this ability is for it to switch quickly between its GDP- and GTP bound forms.

1.5.1 Structure of Gα and mechanism of action

G- Proteins are heterotrimers in their inactive form, made up of α, β and γ subunits. Although there are many gene products encoding each subunit, four main classes of G-Protein can be distinguished by the Gα subunit, which can be divided into several classes (Hamm, 1998; Bohm *et al.* 1997). The G s α, G i/o α, Gqα and G12α can be classed on the basis of their amino acid sequence; Gs which activates adenylate cyclase; Gi which inhibits adenylate cyclase; Gq which activates phospholipase C; and G12 and G13 of unknown function (Hamm, 1998, Bohm *et al.* 1997). The β and γ subunits do not vary as much.

Gα subunits are peripheral membrane proteins, typically myristoylated and often also palmitoylated at, and near, the amino terminus (Bohm *et al.* 1997). These modifications anchor Gα to the membrane surface. Gα subunits contain two domains. One domain, involved in binding and hydrolysing GTP (the G domain), is structurally identical to the superfamily of the GTPases including small G-Proteins

and elongation factors. Gα also contains a unique helical domain that buries the GTP deep in the core of the protein (Bohm *et al.* 1997).

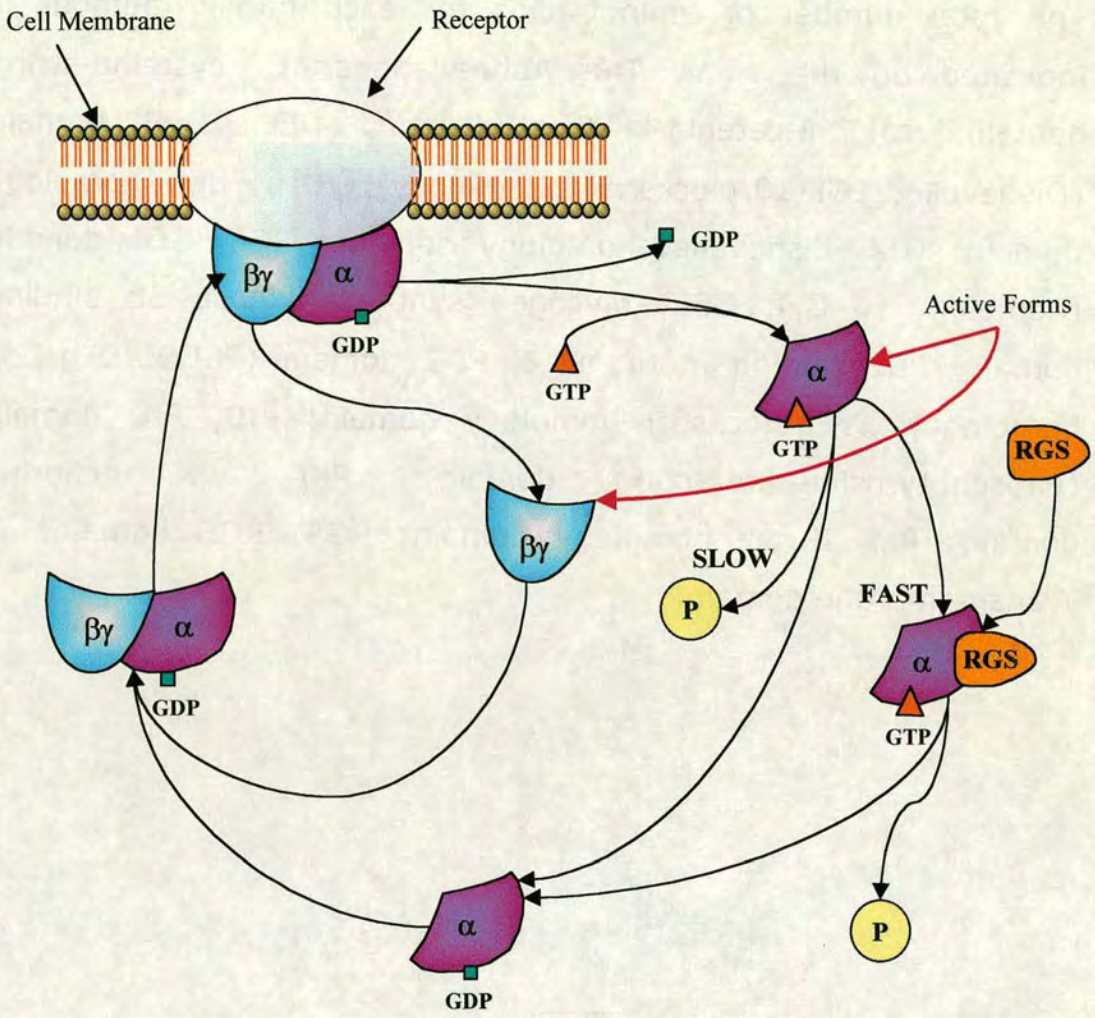
G-Proteins are usually associated with transmembrane receptors. On receiving an external signal, a conformational change in the receptor allows it to act as a guanine nucleotide exchange factor to stimulate rapid dissociation of GDP from the inactive Gα. The Nucleotide free Gα is then available to bind GTP, leading to the dissociation of Gα from Gαβγ heterotrimer, leaving both Gα and Gβγ free to interact with and modulate downstream components of signalling cascades (Fig 1.13). On the dissociation of the heterotrimer, the alpha subunit interacts with adenylyl cyclase, which generates cyclic AMP. The cAMP is then free to activate cAMP dependant kinase-pathways. Depending on whether the Gα is iα or sα, it negatively or positively regulates the signalling pathway.

Gα has a very slow dissociation rate, and termination of the signal depends on hydrolysis of the bound GTP (Bohm *et al.* 1997). However intrinsic Gα GTPase is too slow to account for rapid physiological responses. Rapid termination of Gα activity depends on a significant enhancement of the GTPase activity. A classic example of the fast response is the eyes response to light stimulus. When a light stimulus is removed, the eye responds within milliseconds due to the accelerated GTPase activity of transducin, as a result of the GAP activity of RGS Protein, (Druey *et al.* 1996). Transducin is the G-Protein responsible for phototransduction in retinal cones. RGS Proteins are a recently identified group of proteins capable of stimulating the GTPase activity of Gα and thereby causing desensitisation to a signal (Fig 1.12) (Berman *et al.* 1996, Druey *et al.* 1996).

1.5.2 Structure of RGS Genes

The RGS family signature is the homologous (45-80%), 120 residue RGS domain. This family was recognised a few years ago, when it was demonstrated through biochemical studies that RGS proteins negatively regulate several G-Protein

Figure 1.13 **RGS Proteins and G-Protein Signaling**



Legend

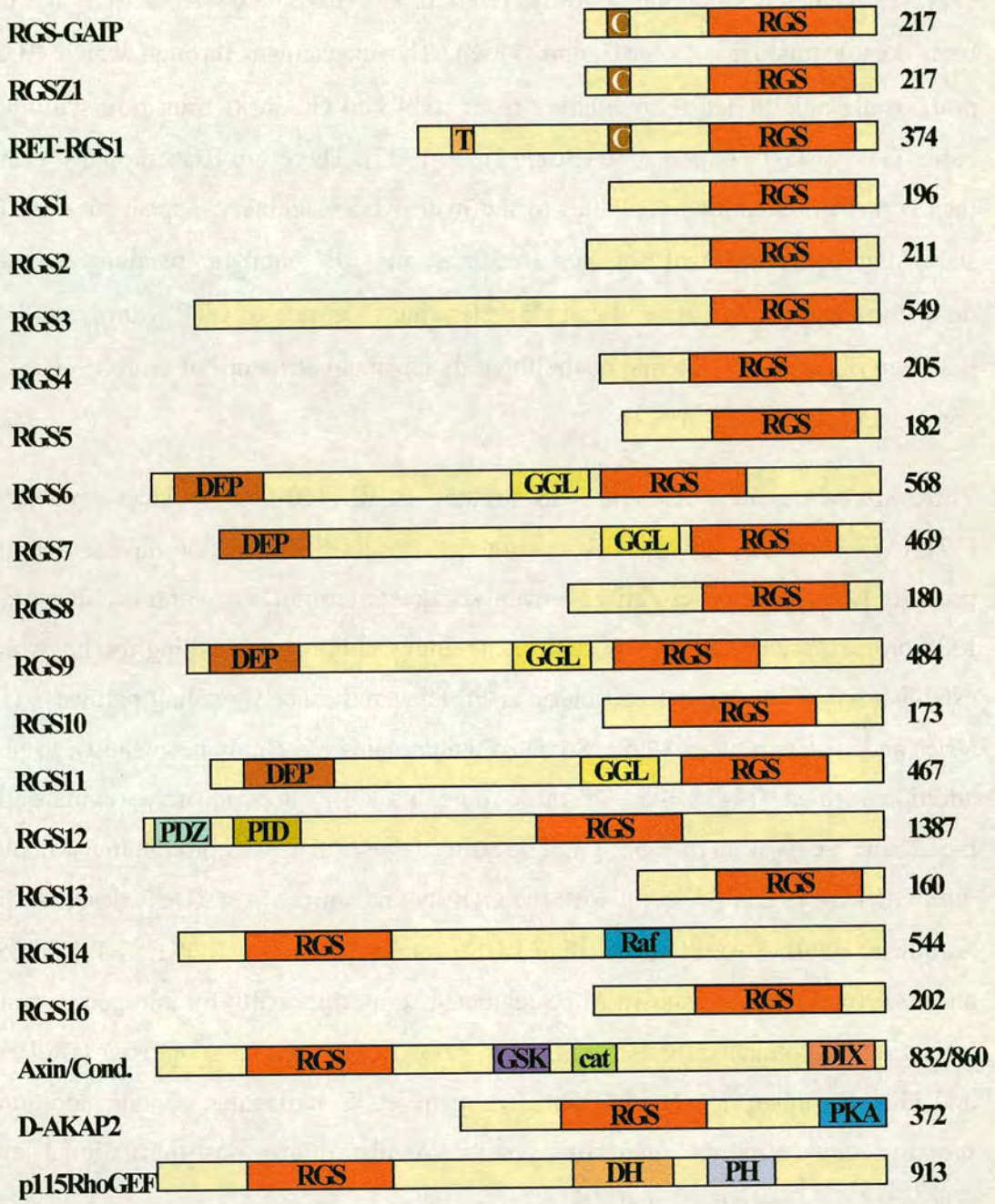
The Gαβγ heterotrimer is found bound to the Receptor in the inactive state. When the receptor is bound by its ligand the Gα subunit is activated and Gα and Gβγ dissociate into their active forms where they signal to downstream components of the signalling pathway. In the presence of RGS-Protein, Gα is quickly converted back to its inactive form, and re-associates with βγ and binds the receptor, ready for a new stimulus. In the absence of RGS-Protein, the response is much longer, due to the slow hydrolysis of GTP.

Legend 1.14

The total number of amino acids for each family member is indicated on the right. The Abbreviations: C, cysteine-string domain; cat, β-catenin-binding domain; DEP, DEP domain (Dishevelled/EGL-10/pleckstrin homology); DH, dbl homology domain; DIX, dishevelled-homology domain; GGL, GGL domain (homology to Gγ); GSK, glycogen synthase kinase 3b binding domain, PDZ-binding motif; PDZ, PDZ domain (PSD95/DLg/Zo1 homology); PH, pleckstrin-homology domain; PID, PID domain (Phosphotyrosine-interacting domain); PKA, PKA-anchoring domain; Raf, B-raf- homology domain; RGS, RGS domain; T, Transmembrane domain.

Figure 1.14

Schematic representation of mammalian RGS Proteins and their domains



From De Vries L and Gist Farquhar MG (1999).

mediated signalling pathways, at the level of interacting directly with the Gα subunit (Berman *et al.* 1996). Many RGS proteins subsequently tested *in vitro* for GAP activity interacted specifically with a subset of G-Proteins, that is the Gαi, Gαo and Gα12/13 mediated signalling pathways (Hamm, 1998). To date GAP activity has not been demonstrated for Gαs (Hamm, 1998). The mechanism through which RGS-proteins are able to act, is by binding more stabily to Gα in its transition state than either GTP or GDP bound state (Bohm *et al.* 1997). Therefore RGS proteins, rather than contributing catalytic residues to the hydrolytic machinery, appear to work by stabilising conformational changes that exist in Gαs' catalytic residues that are unique to the transition state, thereby accelerating Gαs' rate of GTP hydrolysis. This has been observed by looking at the three dimensional structure of an RGS and Gα complex, (Tesmer *et al.* 1997)

There are two main classes of RGS Protein: small (160-217) residues) and large (372-1387 residues). In small RGS proteins, the RGS domain dominates and the proteins have short N-termini and even shorter C-termini. By contrast, the larger RGS proteins are composite molecules containing additional signalling motifs, which establish links between heterotrimeric G-Proteins and other signalling pathways (De Vries and Gist Farquhar, 1999). Some of the domains have now been shown to play additional roles (Fig 1.14). Of these domains DEP domain may regulate the membrane localisation of RGS, GGL domain suggests that RGS protein in a complex with GB may interact directly with the GDP bound form of Gα (De Vries and Gist Farquhar, 1999). Several mammalian RGS proteins, including RGS1, RGS2, RGS3 and RGS16, have been shown to be regulated transcriptionally by mitogens, serum, inhibition of protein synthesis, and the activation of p53 tumour suppresser (De Vries and Gist Farquhar, 1999). Additionally, many RGS molecules contain additional protein sequence motifs, suggesting possible regulation at a post-translational level (De Vries and Gist Farquhar, 1999).

1.5.3 G-Proteins and their regulation in Drosophila

Despite the wealth of knowledge about G-proteins, at both a biochemical and structural level across various organisms from yeast to mammals, surprisingly little is known about G-Protein Signalling and its regulation in *Drosophila*, especially in a developmental context.

Wolfgang *et al* (1991) observed G-protein expression, using antibodies raised against *Drosophila* specific G-protein α subunits. They studied the spatial and temporal expression of all Gα families throughout the whole of *Drosophila* development, from embryo to adult. Of relevance to this project was the observation of specific Gα expression in the ovaries (Table 1.2). Both mRNA *in-situs* and antibody staining were carried out with 3 different Gα subunits O, s and i. GOα mRNA was detected in the oocytes and nurse cells, Gsα Protein was detected in the follicle cells, while Giα mRNA was detected in the nurse cells and oocytes, while protein was detected in the stage 10 nurse cells and anterior dorsal follicle cells (Stage 10)(Wolfgang *et al.* 1991). This data illustrates that several Gα subunits are present in oogenesis and G-Proteins are likely to play a dynamic roll in oogenesis.

Table 1.2 Gα expression in Oogenesis

GOα		***Gsα***		***Giα***	
mRNA	***Protein***	***mRNA***	***Protein***	***mRNA***	***Protein***
Oocyte and nurse cells	-	-	Follicle cells	Nurse cells and oocyte	Stage 10 oocyte Anterior /dorsal follicle cells

From Wolfgang *et al.* (1991)

1.6 Background on project.

The Bownes group have identified a number of genes expressed in specific subsets of follicle cells using the GAL4/UAS enhancer trap system (Deng *et al.* 1997). The GAL4 fly lines, when crossed to a *Lac-Z* reporter line, showed *β-galactosidase* expression in a very dynamic pattern at specific stages of oogenesis.

One enhancer trap line (C139), when crossed to *Lac-Z* had *β-galactosidase* expression in the anterior-dorsal follicle cells. This expression pattern suggests that the enhancer-trapped gene could be involved in dorsal patterning of the follicle cells. This line was selected for detailed analysis.

Further *Lac-Z* staining was also carried out on imaginal discs and testis. Staining was observed in the testis and part of the eye imaginal disc. This suggests that the enhancer trapped gene could also have a role in these tissues during development.

Plasmid rescue was carried out to obtain the genomic DNA flanking the P-element. The plasmid rescued genomic DNA was used to screen a *Drosophila* CantonS λFix genomic library (Stratagene) and two phage clones were isolated (λ652 & λ653) which contained DNA 5′ and 3′ of the insert. The two phage clones were used as probes for *in-situ* hybridisation to ovaries and an RNA transcript was detected in the anterior dorsal follicle cells, in a pattern similar to that detected by the LacZ staining. Thus it can be concluded that the gene of interest is near to the P-element and is expressed in a spatially restricted pattern.

An ovarian λZap cDNA library (a gift from Y.N. Jan, UCSF, San Francisco, California) was screened with the lambda clones and two cDNA clones were isolated. RNA *in-situ* hybridisation showed that both were expressed in dorsal/anterior follicle cells. The enhancer trapped gene is expressed in anterior-ventral follicle cells in *fs(1)K10* mutant ovaries in which *grk* transcript is not localised. This suggested that the enhancer-trapped gene was downstream of the *gurken-torpedo* signalling pathway and was then selected for further study in this

thesis. My aim was to analyse the regulation of expression and establish the function of this gene in oogenesis.

Chapter Two: Materials and Methods

2.1 Materials

2.1.1 Chemicals and radioactive isotopes

Chemicals were supplied by SIGMA, BDH, Fison, and Aldrich. Radioactive isotopes [32P]dCTP and [32P]dNTP were obtained from Amersham.

2.1.2 Restriction and modification enzymes

All restriction enzymes and buffers were obtained from Boehringer Mannheim, Gibco BRL, NBL, NEB, Pharmacia, Promega or USB.

Taq DNA polymerase, 10x Taq DNA polymerase buffer, and Q solution were supplied by Qiagen. T4 DNA ligase and 10x T4 DNA ligase buffer were obtained from Boehringer Mannheim and Promega.

Digoxygenin labelling and detection kit was purchased from Boehringer Mannheim.

2.1.3 Preparation of solutions and sterilisation

Typically, buffers and solutions were prepared with double distilled water (ddH_2O). Sterilisation was achieved either by autoclaving (15psi for 15min) or by passing through a 0.22μm pore sized filter. All necessary plasticware was sterilised by autoclaving and dried at 37°C.

Solutions required to be RNAase free were prepared by supplementing with 0.05% (v/v) DEPC (Diethyl pyrocarbonate [BDH]), incubated overnight at 37°C and then autoclaved to remove the DEPC. Chemicals reactive to DEPC were dissolved in RNAase free ddH_2O and then autoclaved as per normal. Glassware to be used in the manipulation of RNA was baked at 180°C for 8 hours. Plasticware to be used in RNA work was incubated in 0.1N HCl at room temperature overnight, followed by rinsing with RNAase free ddH_2O.

2.1.3.1 General solutions

ddH_2O (RNAase free)	Add 0.05% (v/v) DEPC (Diethyl Pyrocarbonate) to sterile ddH2O and incubate overnight at 37°C, then autoclave to remove the DEPC
70% (v/v) Ethanol	70 ml Ethanol made up to 100 ml with sterile ddH_2O
Ethidium Bromide	10 mg/ml in sterile ddH_2O
MOPS (for RNA agarose gels)	20mM Na-MOPS pH 7.0, 50mM sodium acetate, 10mM EDTA
Phenol/Chloroform	Re-distilled and pre-equilibrated (in 100mM Tris-HCl pH8.0) phenol was purchased from Sigma and mixed with Chloroform and Isoamyl Alcohol in the ratio 25:24:1 respectively. To prevent oxidation, 8-Hydroxyquinoline was added to 0.1% (w/v) and the solution was stored at 4°C
10% SDS	10% Sodium Dodecyl Sulphate in sterile ddH_2O
20 x SSPE	3.6M NaCl, 0.2M sodium phosphate, 0.02M EDTA pH7.7
Ribonuclease A (RNAase A)	20 mg/ml in 50% Glycerol, boiled for 5 min to inactivate contaminating DNAases, and stored at -20°C
Deoxyribonuclease I (DNAase I)	20 mg/ml in 50% (v/v) Glycerol, stored at -20°C
Proteinase K	20 mg/ml in 50% (v/v) Glycerol, stored at -20°C

Denhardt's (100x)	2% (w/v) bovine serum albumin; 2% (w/v) Ficoll; 2% (w/v) polyvinylpyrolidone. Stored at -20°C.
DNA loading buffer (6x)	0.25% (w/v) bromophenol blue; 0.25% (w/v) xylene cyanol FF; 30% (v/v) glycerol in ddH_2O. Stored at 4°C.
0.5M EDTA	0.5M Diaminoethanetetra-acetic acid, pH 8.0
Grinding buffer	100mM Tris-HCl, pH 8.5; 80mM NaCl; 50mM EDTA, pH 8.0; 5% (w/v) sucrose; 0.5% (w/v) SDS
PBS	137mM NaCl; 2.7mM KCl; 4.3mM Na_2HPO_4; 1.4mM KH_2PO_4
PBT	PBS with 0.1% (v/v) Tween-20
SSC (20x)	3M NaCl, 0.3M tri-sodium citrate
TAE	40mM Tris-acetate; 1mM EDTA; pH 7.7
TBE	89mM Tris-HCl; 89mM Boric acid; 2.5mM EDTA; pH 8.3
TE	10mM Tris-HCl; 1mM EDTA; pH 8.0
TENS	10mM Tris; 1mM EDTA; 0.1M NaOH; 0.5% (v/v) SDS
0.1M IPTG	1.2g isopropyl β-D-thiogalactopyranoside dissolved in ddH_2O. Filter sterilise. Store at -20°C, in darkness.
Ringer's Solution	6.5g NaCl, 0.14g KCl, 0.2g $NaHCO_3$, 0.12g $CaCl_2$, 0.01g NaH_2PO_4 per litre, sterilised by autoclaving.

2.1.3.2 Protein Gel Solutions

2x Polypeptide Sample Buffer

1.25ml 1M Tris-HCl pH 6.8, 2ml glycerol, 5ml 10% SDS, 0.5ml 0.1% Bromophenol Blue. Make up to 9ml with H_2O, then add 1ml 2-mercaptoethanol.

Protein Gel Acrylamide Stock

Protogel (National Diagnostics) 30% acrylamide solution, 2.7% crosslinked with methylene bisacrylamide (37.5:1 ratio), in distilled H_2O.

10% Separating Gel Solution

6.66ml Protogel, 2.5ml 3M Tris pH8.8, 200µl 10% SDS, 10.42ml H_2O. Polymerised with 200µl 10% APS and 20µl TEMED.

4% Stacking Gel Solution 650µl Protogel, 650µl 0.5M Tris pH6.8, 50µl 10% SDS, 3.59ml H_2O. Polymerised with 50µl 10% APS and 10µl TEMED.

10x Running Buffer 30g Tris, 144g glycine, 10g SDS. Made up to 1 litre with H_2O.

2.1.3.3 Protein Purification Solutions

Z-100 100mM KCl, 25mM HEPES pH7.9, 12.5mM $MgCl_2$, 1mM DTT, 0.1% NP-40, 10% glycerol, 1mM PMSF

Z-50 50mM KCl, 25mM HEPES pH7.9, 12.5mM $MgCl_2$, 1mM DTT, 0.1% NP-40, 10% glycerol, 1mM PMSF

2.1.3.4 Bradford Assay Solutions

0.5 mg/ml bovine serum albumin (BSA)

The concentration of BSA is determined using the A280 = 6.6 for a 10mg/ml solution of BSA (1cm pathlength).

2.1.3.5 Coomassie Brilliant Blue Solution

100mg Coomassie Brilliant Blue G250 was dissolved in 50ml of 95% ethanol. 100ml 85% phosphoric acid was added and the solution was made up to 1 litre with H_2O. The solution was then filtered through Whatman No. 1 filter paper and stored at 4°C.

2.1.3.6 β-galactosidase Staining Solutions

X-gal Stock — 8% (w/v) 5-bromo-4-chloro-3-indolyl-β-galactosidase, made up in dimethyl formamide and stored in a dark container at -20°C.

X-gal Staining Buffer

Solution A — 0.5M Na_2HPO_4/NaH_2PO_4

Solution B — 10mM Solution A, 150mM NaCl, 1mM $MgCl_2$, 7mM Potassium Ferrocyanide, 7mM Potassium Ferricyanide. Stored in a dark container at 4°C. Immediately prior to use, X-gal was added to a concentration of 0.2% (w/v).

2.1.3.7 Western Blot Solutions

Transfer Buffer — 25mM Tris pH 8.3, 192mM glycine, 20% methanol

2.1.3.8 Ponceau S Staining Solution

20ml Ponceau S Concentrate (Sigma) mixed with 180ml deionised water, stored at room temperature.

2.1.3.9 HRP Western Detection Solutions

Milk Buffer	5% Skimmed milk powder in PBS.
Developer	60mg 4-chloro-1-napthol dissolved in 20ml methanol. 100ml 1X milk buffer and 100µl hydrogen peroxide was added prior to use.

2.1.3.10 ECL Solutions

1.250mM Luminol	0.44g Luminol dissolved in 10ml DMSO, stored at 4°C in the dark
2.90mM p-Coumaric acid	0.15g p-Coumaric acid dissolved in 10ml DMSO, stored at 4°C in the dark
Solution A	1ml Luminol stock, 0.44ml p-Coumaric acid stock, 10ml Tris-HCl (pH 8.5) and made up to 100ml with water. Stored at 4°C in the dark
Solution B	10ml Tris-HCl (pH 8.5), 61µl 30% H_2O_2, and made up to 100ml with water. Stored at 4°C in the dark, and made weekly.
ECL detection solution	Solutions A and B mixed 1:1, just before use

2.1.3.11 In situ Hybridisation Solutions

pp (fix)	2g paraformaldehyde was dissolved carefully in 40ml H_2O (in 65°C water bath) by adding 30µl 10M NaOH and shaking occasionally. At high pH the paraformaldehyde dissolves easily. 5ml 0.5M PIPES pH7.0, 100µl 0.5M EGTA pH8 and 100µl 1M $MgSO_4$ were then added and the pH checked as ~6.8. The volume was adjusted to 50ml with water and stored at 4°C for up to 24 hours.

Hybrix DNA	50% formamide, 5x SSC, 50µg/ml sonicated, heat denatured salmon sperm DNA (ssDNA) 50µg/ml tRNA (Rnase free), 50µg/ml heparin, 0.1% Tween-20 pH 7.5 HCl.
Hybrix RNA	50% formamide, 5x SSC, 100µg/ml tRNA (Rnase free) 50µg/ml heparin, 0.1% Tween-20 pH 6.5HCl.
NMTT	100mM NaCl, 50mM $MgCl_2$, 100mM Tris (pH 9.5), 0.1% Tween-20, 1mM Levamisol (fresh).
NBT	75mg/ml NBT in 70% dimethylformamide
X-phosphate	50mg/ml X-phosphate in 100% dimethylformamide

2.1.5 Media

All media was prepared by departmental media staff, and sterilised by autoclaving (15psi/20mins).

Cornmeal fly food	per litre: 25g cornflour; 50g sugar; 17.5% (w/v) yeast; 10g agar; 4.5µg Nipagen fungicide.
Luria Broth (LB)	1% (w/v) Bacto-Tryptone; 0.5% (w/v) yeast extract; 1% (w/v) NaCl, pH 7.2.
Luria Agar	LB supplemented with 1.5% (w/v) agar.
SM (phage buffer)	100mM NaCl; 8.1mM $MgSO_4$; 50mM Tris, pH 7.5; 0.5% (w/v) gelatin.
Top agarose	LB supplemented with 0.7% (w/v) agarose

2.1.6 Antibiotics

Stock solutions of antibiotics were prepared according to the table below, and stored at -20°C in 1ml aliquots.

Table 1

Antibiotic	*Stock solution concentration*	*Working concentration*
Ampicillin	50mg/ml in ddH_2O	35-50μg/ml
Kanamycin	50mg/ml in ddH_2O	50μg/ml
Chloramphenicol	10mg/ml in ethanol	10μg/ml

2.1.7 Oligonucleotides

Primers were designed by using GCG software. Oligonucleotides were obtained from Oswel DNA services and Bioline. All oligonucleotides used for PCR or automated sequencing are tabulated below. All stocks were at 50pmol/μl.

Table 3

Name	*Sequence 5' to 3'*	*Description*	*Tm℃*
LOCOFPB1	GTGGATCCAGCTCCAATAGCCGC AATCTC	RT-PCR primer	55.9
LOCORPE2	CGGAATTCATATCGTTGTCGGAG GCGTTC	RT-PCR primer	56.0
LOCOFPB3	ATGGATCCATCGCAAGGAACCA GGAAC	RT-PCR primer	52.0

LOCORPE4	CGGAATTCACACTTTCTCCTCCT GCAC	RT-PCR primer	51.2
LOCOFPB5	ATGGATCCGCAGTTCATACGATC ACAACGC	RT-PCR primer	51.8
LOCOFPB6	ATGGATCCTGCCATTGTATTGCC CAC	RT-PCR primer	51.3
LOCOFPB7	ATGGATCCTTCGAAGCCATCGAC AGTG	RT-PCR primer	52.2
LOCOFPB8	GTGGATCCTTGTCTCAGCAGCTC GTCC	RT-PCR primer	54.6
LOCOGFP1	AGTAAGCAGCGCACATGCAC	Genomic primer	56.2
LOCOGRP2	GGCAGAAAGCGAAAACGTGAC	Genomic primer	55.3
LOCOGPF3	CCAGAATACCCATCGCAAG	Genomic primer	52.6
LOCOGRP4	TCAGACGGGCACGATAAAC	Genomic primer	51.9
96SCP1	GCAGCACCAAGTCGATCGAT	Loco sequence primer	52.6
96SCP2	CTACTGAGTGATCGACTGAT	Loco sequence primer	53.0
96SCP3	TTGCGCAGCTTGATACAGC	Loco sequence primer	52.1
96SCP4	AAGCTCAAGAAGAAGTCCAC	Loco sequence primer	50.3
T3	AATTAACCCTCACTAAAGGG	General sequence	52.0

		primer	
T7	GTAATACGACTCACTATAGGGC	General sequence primer	52.0
SP6	GTAAAACGACGGCCAGT	General sequence primer	50.0
REVERSE	GGAAACAGCTATGACCATG	General sequence primer	50.0

2.2 Drosophila methods

2.2.1 Maintenance of Drosophila stocks

Drosophila melanogaster stocks were maintained on cornmeal food at 18°C or 25°C. Benzyl benzoate strips (filter paper strips, soaked in 3% (v/v) benzyl benzoate) were added in the case of mite infestations.

2.2.2 Drosophila melanogaster stocks and strains

Strain/Class	*Designation*	*Comments*	*Reference*
Oregon R	OrR	A Drosophila melanogaster wild type strain	Hoigness and Prestidge, 1983
wr		white- (white eyed)	
Balancer	*3rd Chromosome*	***w-;+/+:TM3,sb/TM6,Tb***	
Deficiencies	Df(3R)EB6	94C2-5; 94E3	Mohler *et al.*, 1995

	Df(3R)5C1	93E-93F; 94C-94D ry[506]/TM3, Sb[1]	Granderath *et al.*, 1999
Δ2-3			
gurken (grk)	*P[grk+, w+]*	X7; 28-20/TM3	Neuman-Silberberg & Schüpbach, (1994)
	P[grk+, w+]	2 insertions on the X chromosome	Neuman-Silberberg & Schüpbach, (1994)
	grk^{WG}		Schüpbach & Wieschaus, (1991)
	grk^{HK}		Schüpbach & Wieschaus, (1991)
Torpedo	top^{QY1}		Schüpbach & Wieschaus, (1991)
fs(1)K10			Wieschaus *et al* (1978)
UAS lines	*UAS-pntP1*		Morimoto *et al.* (1996)
	UAS-pntP2		Morimoto *et al.* (1996)

2.2.3 Collection of staged eggs

About 300 flies (3-4 days after eclosion) were transferred into population cages which was covered with petri dish containing apple juice food with the yeast paste smeared in the middle. Eggs were washed off from the petri dish and collected in a sieve and rinsed thoroughly using distilled water.

In certain reactions (e.g. when the template was cloned genomic DNA), better results were obtained with the addition of Qiagen Q solution to a final 1x concentration.

An initial denaturation step of 2 minutes (15 minutes for Hot Start Taq) was carried out at 95°C. Subsequent steps were: 95°C denaturation, 15 seconds; 50°C-60°C annealing, 15 seconds; 72°C elongation, 30 seconds to 3 minutes. 30 cycles were used in all reactions. This was completed by a final 72°C elongation step of 7 minutes.

The annealing temperature was adjusted according to the primers used, and the extension time was modified according to the length of the fragment to be amplified (allowing 1 minute per 1 kb).

1/10th of the total reaction volume was analysed by electrophoresis.

2.5 RNA preparation and electrophoresis

2.5.1 Preparation of total RNA

Trizol system (Gibco BRL), a solution of phenol and guanidium isothiocyanate, which is based on the method of Chomczynski P and Sacchi N (1987), was used to extract total RNA. 0.5ml Trizol solution was added to up to 100mg tissue, homogenised, and a further 0.5ml of Trizol was added and mixed. The homogenate was incubated at room temperature for 5 minutes. 200µl of chloroform was added to the homogenate and mixed, followed by incubation at room temperature for 3 minutes. The aqueous and organic phases were separated by centrifugation at 17,000 rpm, for 15 minutes at 4°C. The aqueous phase was transferred to a fresh microfuge tube and 0.7 volumes of isopropanol was added and incubated at room temperature for 10 minutes. The RNA was precipitated by centrifugation at 17,000 g for 15 minutes at 4°C. The supernatant was removed and the RNA pellet washed with 1ml of 70% (v/v) ethanol (in DEPC water). The pellet was air dried then dissolved in DEPC sterile water.

2.4.9 DNA sequencing and sequence analysis

2.4.9.1 Automated DNA sequencing

DNA sequencing was performed using an ABI PRISM dye terminator cycle sequencing reaction ready kit (Perkin-Elmer Corporation), on an Omnigene thermal cycler (Hybaid Ltd.) and extension products separated on an ABI PRISM 377 DNA sequencer (Perkin-Elmer Corporation).

Approximately 0.4μg of double stranded DNA template was mixed with 3.2pmoles of an appropriate oligonucleotide primer and 8μl of terminator ready reaction mix in a final volume of 20μl. The mixture was overlaid with a drop of mineral oil, and subjected to thermal cycling as follows: 96°C for 30 seconds, 50°C for 15 seconds, 60°C for 4 minutes, repeated for 25 cycles.

Extension products were purified away from unincorporated terminators by addition of 2.0μl 3M sodium acetate pH 4.8, 50μl absolute ethanol, and incubation on ice for 10 minutes followed by 15 minutes centrifugation. The resulting pellet was washed with 70% (v/v) ethanol, air dried, and stored dry at 4°C prior to electrophoresis.

2.4.9.2 Sequence analysis

Sequence analysis was carried out on a UNIX mainframe computer using the Genetics Computer Group (GCG) package of programs, version 9 (Genetics Computer Group 1996). Sequence editing was performed using the Seqed program, manual sequences were input by hand while sequences generated by the automated sequencer were edited with GeneJockeyII (Biosoft) before transfer. Nucleotide and protein database searches were carried out using Fasta and Blast functions.

2.4.10 Polymerase chain reaction (PCR)

A typical 50μl PCR reaction consisted of the following: 25pmol each of primer 1 and primer 2; 0.2mM each dNTP (dATP, dCTP, dGTP, dTTP); 1x Taq polymerase buffer (Qiagen); 0.25U Taq polymerase (Qiagen); template DNA; and ddH_2O.

2.4.8 Recovery of DNA fragments from agarose gel

Two methods were used to recover the DNA fragments from the agarose gel.

2.4.8.1 QIAquick Gel Extraction

The DNA fragment was excised from the agarose gel with a clean razor blade and put into a microfuge tube containing 3 volumes (w/v) of buffer QX1 (Qiagen). The gel was dissolved at 50°C for about 10 minutes and the solution was transferred to a QIAquick spin column, which was put on a 2 ml collection tube. This was followed by spinning down (60 seconds, 13,000 rpm), washing with 0.75 ml of buffer PE (Qiagen), spinning down again and finally placing the spin column on a fresh microfuge tube. DNA was eluted by adding 50 µl TE or ddH_2O to the spin column and incubated at 65°C for 5 minutes. The spin column was then centrifuged at 13,000 rpm for 60 seconds and the eluate recovered.

2.4.8.2 Electroelution

A Model UEA Unidirectional Electroelutor (Sigma) was used to recover DNA from gels. The DNA band of interest was cut out of the gel with a clean razor blade and briefly soaked in electroelution buffer (20 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 5 mM NaCl) to remove the electrophoresis buffer. The gel slices were placed in the horseshoe shaped slots on the UEA platform. The electroelution buffer was added at a height to just barely cover the gel slice and 75 µl of the high salt buffer (7.5 M NH4Oac [for fragments <500bp use 10-13 M NH4Oac], 0.01% [w/v] bromophenol blue) was added into the V-channel. Typically, DNA fragments (0.5-3 kb) were eluted from the agarose gel for 30 minutes at 100 volts. After elution, the high salt buffer was carefully removed from the V channel into a fresh microfuge tube. The DNA fragment was precipitated by adding 0.6-1 volume of iso-propanol, followed by incubation at -70°C for at least 30 minutes, centrifugation, washing with 70% ethanol and resuspended in TE or other desired buffer.

centrifugation at 4,500xg for 5 minutes at 4°C. The cells were gently resuspended in 10ml of ice-cold TBF2 and incubated on ice for 15-60 minutes. Then the cells were divided in to 200µl aliquots, quick-frozen in a dry ice/isopropanol bath and stored at -70°C until they were needed.

2.4.7 Transformation of plasmid DNA into Escherichia coli

As Described in Promegas, Protocols and Applications Guide (Third Edition).

Where appropriate, 30µl of 2% (w/v in dimethylformamide) X-Gal and 20µl of 100mM IPTG were also spread over the surface of the plate to enable blue-white colour selection of colonies containing recombinant plasmids.

2.4.7 DNA gel electrophoresis

2.4.7.1 Preparation and running of agarose gels

The appropriate amount of agarose was melted in TAE or TBE in a microwave. EtBr was added to a final concentration of 0.5 µg/ml. DNA loading buffer was added to the samples to a final concentration of 1x, the samples loaded on the gel, and electrophoresis conducted at 50-100V in TAE or TBE until the appropriate degree of separation was obtained.

As a general rule, TAE was used when the DNA was to be recovered from, or used in, the gel fragment. TBE was used when electrophoresis was to be carried out over long time periods.

2.4.7.2 Molecular weight markers

GibcoBrl 1kb molecular weight markers or Bioline 100bp ladder were used to determine the size of electrophoresed DNA fragments, as directed by the manufacturer.

2.4.5.2 Ligation

Ligations were carried out in a total reaction volume of 20µl. 1 Unit of T4 DNA ligase was used per reaction, and 10x T4 DNA ligase buffer added to a final concentration of 1x. Reactions were incubated at RT overnight. The DNA was then EtOH precipitated and resuspended in ddH_2O, to a final concentration of 1ng/µl.

2.4.5.3 Exo-Mung Deletion

Restriction digest 5µg DNA (each time point) with two cutting sites adjacent to each other, one that generates a 3′ overhang and one that generates a 5′ overhang. Phenol/Chloroform, precipitate and resuspend in 10µl ddH_2O. Add 12.5µl 2X ExoIII buffer, 2.5µl Fresh β-mercaptoethanol, 30-40 units ExoIII enzyme (total volume 25µl). Carry out digest at 30°C, with time points every 30 seconds, stop reaction by placing on ice.

Add 20µl Mung Bean Buffer, 155µl ddH_2O and 25µl ExoIII reaction, heat the Tube at 68°C for 15 minutes then place on ice, add 1µl diluted Mung Bean Nuclease (20 units; dilute 1µl 105units/µl with 5µl 1x MB reaction Buffer) and incubate at 30°C for 30 minutes. This will blunt end the DNA.

Check the size of fragments at each time point by running 20µl of each reaction on an agarose gel, precipitate the desired size fragments, resuspend in 20µl and gel purify. Ligate and Transform.

2.4.6 Making competent cells for Transformation

5ml LB medium was inoculated with a single colony from an LB plate and incubated overnight at 37°C with shaking (approx. 225rpm). On the following day, 250ml LB medium, containing 20mM $MgSO_4$, was inoculated with 2.5ml of the overnight culture and grown in a 1 litre flask until the A600 reached 0.4-0.6. The cells were centrifuged at 4,500xg for 5 minutes at 4°C. The cell pellets were gently resuspended in 100ml of ice-cold TFB1, and all subsequent steps were performed on ice. The resuspended cells were incubated on ice for 5 minutes, then pelleted by

Oligonucleotides	1.0	40

Approximate nucleic acid concentration was also deduced by comparing the relative fluorescence of samples to those of a known concentration on an agarose gel.

2.4.3 Phenol-chloroform extraction

Protein impurities were removed from DNA preparations by phenol:chloroform extraction. An equal volume of pH equilibrated phenol was added to the DNA solution, which was then mixed thoroughly and centrifuged to separate the phases. The upper aqueous layer was transferred to a clean eppendorf tube. This step was repeated with an equal volume of 1:1 phenol:chloroform, followed by an equal volume of chloroform only.

2.4.4 Precipitation with ethanol or isopropanol

DNA was precipitated from solution using 0.6 volume of isopropanol or 2.5 volumes of 100% EtOH. In the latter case, 0.1 volume of 3M NaOAc, pH 7.0, was also added, where no salt had been used in previous steps of the protocol. The solution was centrifuged at maximum speed in a refrigerated benchtop centrifuge for 15 minutes at 4°C. The resulting DNA pellet was washed in 1ml 70% EtOH, and centrifuged for 5 minutes at maximum speed. The pellet was then air dried before being resuspended in an appropriate volume of ddH_2O.

2.4.5 Enzymatic reactions

2.4.5.1 Endonuclease restriction

Approximately 5 Units of restriction enzyme were used per microgram of DNA, and always constituted less than, or equal to, 1/10th of the total reaction volume. The appropriate 10x restriction buffer was added to a final concentration of 1x. Incubations were carried out at the recommended temperature (usually 37°C), for a minimum of 2 hours.

sample vortexed. Debris was then pelleted by centrifugation, and the plasmid DNA recovered from solution by EtOH precipitation.

2.4.1.3 Midi-preparation of plasmid DNA

Hybaid midi-prep kits were used to prepare up to 200µg plasmid DNA, following the manufacturer's instructions.

2.4.1.4 Preparation of DNA from recombinant bacteriophage

1ml aliquots of a 10ml overnight lysate were transferred to eppendorf tubes and centrifuged to pellet cell debris. The lysate was transferred to clean tubes, 1µl DNAseI added, and incubated at RT for 30 minutes. 200µl TES was then added, and the mixture incubated at 70°C for 15 minutes. 135µl KOAc was added, the solution mixed thoroughly, and incubated on ice for 15 minutes. The mixture was then centrifuged, and the supernatant phenol:chloroform extracted before being precipitated with isopropanol.

2.4.2 Estimation of the concentration of nucleic acids

Spectrophotometry was used to assess the concentration and purity of nucleic acids. Table 2.9 lists the typical concentrations of nucleic acids when reading the absorbance of the sample at λ260nm. DNA was judged to be free of contaminating protein if the A260:A280 ratio was greater than or equal to 1.8. RNA was judged if this ratio was larger or equal to 2.0.

Table Absorbency of nucleic acid solutions and inferred concentrations

Nucleic acid	Absorbency (A260)	Concentration (µg/ml)
Double stranded DNA	1.0	50
Single stranded DNA	1.0	40
Single stranded RNA	1.0	40

2.3.4 Liquid lysates

To obtain a homogeneous bacteriophage stock, 100µl plating cells were added to 3mls molten top agarose and poured onto an LB plate. When set, bacteriophage were streaked across the surface using a toothpick. The plate was incubated O/N at 37°C. Next day an individual plaque, and the bacterial cells surrounding it, were used to inoculate 10ml LB supplemented with 10mM $MgSO_4$. This culture was grown O/N at 37°C, at 180rpm. The following morning 0.1ml $CHCl_3$ was added to the culture, which was then shaken for a further 10 minutes. Recombinant bacteriophage DNA was then prepared as described in section 2.4.1.4

2.4 DNA methods

2.4.1 Preparation of DNA

2.4.1.1 Preparation of Drosophila genomic DNA

Approximately 15 flies were ground in a 1.5ml eppendorf tube containing 400µl Grinding buffer, using a plastic pestle. The homogenate was incubated at -70°C for 30 minutes, then at 65°C for 30 minutes. potassium acetate was added to a final concentration of 1M, followed by a 30 minute incubation on ice. Cell debris was pelleted by centrifugation (12krpm, 15 minutes, 4°C). The supernatant was transferred to a clean eppendorf tube and precipitated with 0.6vol of isopropanol. The DNA was pelleted by centrifugation (17krpm, 10 minutes, RT), and washed with 70% EtOH before centrifuging again (12krpm, 5 minutes, 4°C). The pellet was air-dried and resuspended in an appropriate volume of ddH_2O.

2.4.1.2 Mini-preparation of plasmid DNA

1.5ml of a 5ml overnight culture of transformed bacteria was centrifuged to pellet the bacterial cells. The pellet was then resuspended in 300µl TENS solution and vortexed until the mixture became sticky. 150µl 3M NaOAc, pH5.2-5.4 was added, and the

2.3.1 Long-term storage of bacterial cultures: stabs and glycerol stocks

1ml aliquots of LB agar in 1.5ml screw-top tubes were supplied by the media staff. Bacterial stabs were prepared by using toothpicks to puncture the agar with a single bacterial colony. The stabs were incubated for a few hours at 37°C, sealed with Parafilm, and stored at RT in the dark.

For glycerol stocks, 0.85ml of an O/N bacterial culture was added to 0.15ml sterile glycerol in a sterile 1.5ml screw-top tube. The mixture was vortexed before sealing with Parafilm, and stored at -70°C.

To recover the culture, 5ml LB (with antibiotic as required) was inoculated with the bacteria (from stab or glycerol stock) and grown O/N. Fresh agar plates were then streaked from this culture to obtain individual colonies.

2.3.2 Liquid cultures

5ml O/N cultures were prepared by inoculating 5ml LB with a single bacterial colony. If required, the appropriate antibiotic was added to the required concentration, and the culture then incubated O/N at 37°C, at approximately 180rpm.

O/N cultures were subcultured the next morning by diluting them 1:100 in fresh LB. The cells were then grown as before until the appropriate O.D. was attained.

2.3.3 Plating bacteria

Bacterial cells were grown and subcultured as described in section 2.3.3. When an O.D. (A600nm) of 1.0-1.2 was reached, the cells were centrifuged at approximately 5krpm. The bacterial pellet was then resuspended in 10mM $MgSO_4$. These plating cells were stored for up to two weeks at 4°C.

	($r_K^- m_K^-$ McrBC$^-$)	

Table: Plasmids for subcloning

The following plasmids were used as detailed in table.

Plasmid name	Features	Uses
pBluescript	Amp_r, Primer sites T3, T7, Reverse and -20, Blue –white colour selection	Subcloning cDNA fragments
pUAST	Places the cloned DNA fragment downstream of the Upstream Activation Sequence (UAS). Expression of the cloned fragment can be induced by the GAL4 transcription factor.	Creating microinjection constructs for sense and anti-sense misexpression experiments
pCasPer	Places the subcloned DNA under the control of the Drosophila Heat Shock promoter. Expression of the cloned fragment can be induced by heat shock.	Creating microinjection constructs for sense and anti-sense misexpression experiments
pΔ2-3	Helper plasmid for P-element transformation, encodes transposase	
pGEX-3X	IPTG, induction site, Amp_r,	Expression vector

2.2.5 Dissecting of ovaries

Flies 2-3 days old were transferred to fresh cornmeal food, which was topped with dry yeast, and incubated at 25°C for 32 hours before dissection. This ensured a complete range of oocyte stages.

2.2.6 Collection of virgin flies and crosses

Virgin flies were collected by either of the following two ways: (1) Dark pupae were picked out with a brush, sexed and placed into vials with fresh food individually. (2) Vials or bottles were emptied of flies, and the newly hatched flies (every 6 hours at room temperature) were anaesthetised and sexed. Same sex flies were placed together in vials containing fresh fly food.

Crosses of flies were generally carried out by placing 2-3 virgin female flies and 2-3 male flies into the same vial containing fresh fly food. They were incubated at 25°C or 18°C depending upon the genotypes of the flies.

2.3 Bacteria and bacteriophage methods

Cell Type	Genotype	Reference
XL1-Blue	*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac, [F', proAB, lacI^{q}ZΔM15, Tn 10 (tet^r)]*	Bullock, Fernandez and Short, 1987
BL21(DE3)	*F-, ompT, hsdS$_B$, (r$_B^-$,m$_B^-$), dcm, gal, λ(DE3)*	Studier and Moffat, 1986 Davanloo *et al.*, 1984
NM422	*F', lacI^{q}Δ(lacZ)M15 proA$^+$B$^+$ /supE thi Δ(lac$^-$proAB)/ Δ(hsdMS-mcrB)5*	

Apple Juice Media	9g Difco Bacto Agar and 10g sucrose was dissolved in 300ml H_2O by microwaving, 100ml apple juice was added when the solution had cooled to 60°C and the media was poured into petri dishes.
Tomato Juice Media	4g Difco Bacto Agar was dissolved in 165ml H_2O by microwaving, 40ml tomato juice and 0.5ml 10% Nipagin was added when the solution had cooled to 60°C and the media was poured into petri dishes.

2.2.4 Collection of early and late third instar larvae, pupae and sexed adults

Egg laying flies were placed in fresh food bottles for 4 hours and then removed. The bottle was then incubated at 25°C for 72 hours, 96 hours and 120-140 hours. Early third instar larvae were collected after 72 hours by floating them out of the food using a saturated sucrose solution, rinsed with distilled water and frozen in liquid nitrogen. Late third instar larvae were picked out with a paintbrush as they crawled up the walls of the bottles after incubation for 96 hours, placed in a microfuge tube, frozen in liquid nitrogen or dry ice/ethanol bath and stored at -70°C.

Pupae were collected by picking them off the inside of the food bottles after 120-140 hours incubation, placed in a microfuge tube and frozen in liquid nitrogen or dry ice/ethanol bath, and stored at -70°C.

Adult flies were collected ranging in age from newly eclosed to 10 days old flies. The flies were anaesthetised with di-ethyl ether, sexed, placed in a microfuge tube, frozen in liquid nitrogen or dry ice/ethanol bath and stored at -70°C.

2.5.2 RNA electrophoresis

All solutions were prepared with DEPC water. RNA was separated on 0.7% (w/v) to 1.0% (w/v) denaturing agarose gels in a MOPS buffering system. Agarose was dissolved in 10 ml 10 x MOPS and 73ml of water and cooled to about 55°C, then 17 ml of 37% (v/v) formaldehyde was added, the solution mixed and poured immediately into a gel tray. Gels were run in a 1 x MOPS buffer.

RNA samples were incubated at 65°C for 5 minutes in the following buffering system: RNA 5µl, formamide 12.5µl, 10 x MOPS 2.5µl, formaldehyde 4µl, then chilled immediately on ice. Prior to loading, 2.5µl RNA loading solution was added.

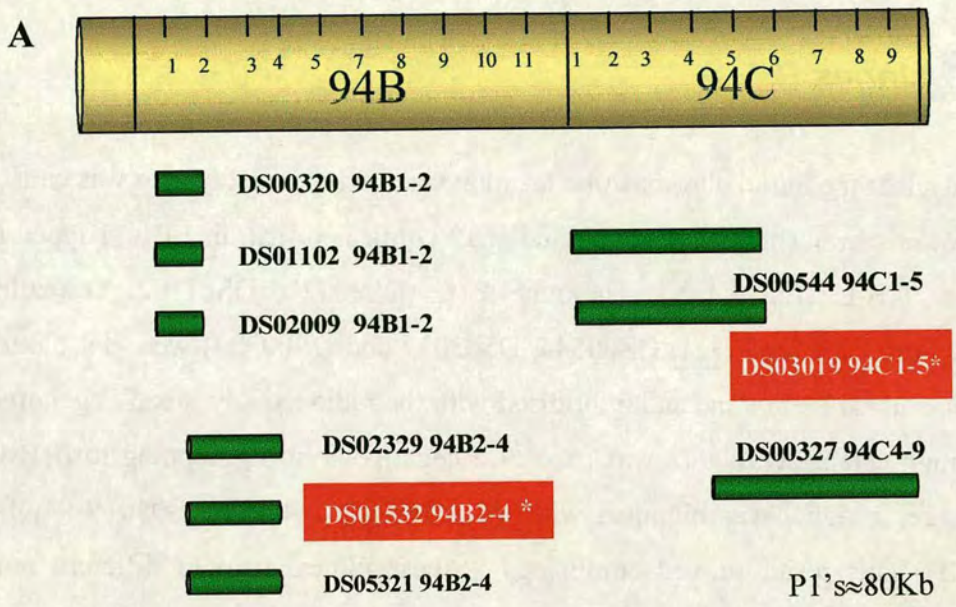
2.5.3 Reverse Transcription (RT) and RT-PCR

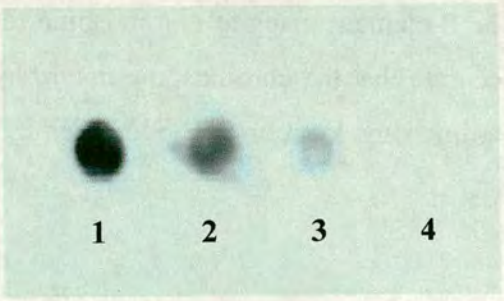
A typical 20µl RT reaction contained the following: 1-5µg template RNA; 1µM oligo(dT) primer (5' ggaattctttttttttttttttt 3'); 200U(Superscript)RNase H- reverse transcriptase (Gibco BRL); 1x first strand buffer (Gibco BRL);); 0.4mM dNTPs (dATP, dCTP, dGTP, dTTP); and ddH_2O.

The RNA template and the oligo(dT) primer were denatured together for 5 minutes at 70°C and snap cooled on ice before adding the other components of the RT reaction. This step removed secondary structures from the template:primer complex. All components bar the Superscript were mixed and incubated at 42°C prior to adding Superscript. The RT reaction was carried out at 42°C for 60 minutes, followed by a 10 minute heatshock at 70°C to deactivate any remaining Superscript.

1/20th of the total volume of the RT reaction was used as a template for subsequent PCR amplification. Standard PCR conditions were used, as described in section 2.3.9. 1/5 of the PCR reaction was analysed by electrophoresis.

Fig 3.1 **Mapping of fly line C139 rescue fragment**





1. Positive control
2. DS01532
3. DS03019
4. Negative control DS02943 61B-C

Legend

Part A represents the genomic region 94B-94C, below which are the nine P1s which map to the region. The two highlighted in red (DS01532 & DS03019) showed up positive when screened with the rescued genomic fragment. Part B is the dot blot showing that DS01532 and DS03019 hybridize to the genomic rescued fragment (dot 2 and 3 respectively). Dot 1 is a positive control of rescued fragment and dot 4 is a negative control, DS02943, that maps to 61B-C.

chromosome *in-situ* hybridization on the salivary glands. A band of staining was observed that was identified as region 94B on the right arm of chromosome three.

3.2.2 P1 clones

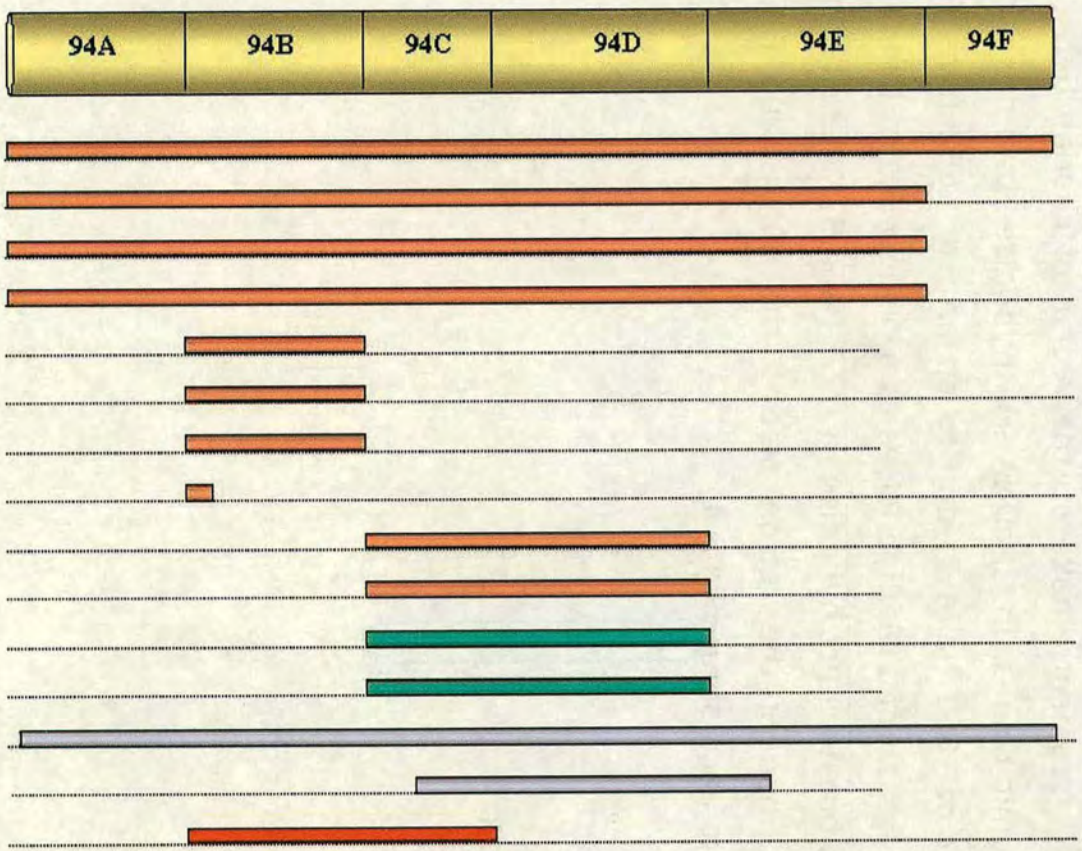
To establish that the initial chromosome location was correct, a P1 screen was carried out. A *flybase* search (http://flybase.bio.indiana.) confirmed that nine P1s mapped to the region 94B-C (Fig 3.1,A). The nine P1s, (DS00320, DS01102, DS02009, DS01532, DS02329, DS05321, DS00544, DS03019 and DS00327) were dot blotted on a nitrocellulose membrane and hybridised with the radioactively labelled genomic rescued fragment. P1 DS02943 was used as a negative control, mapping to 61B-C. On exposure, a signal was obtained with DS01532 94B2-4 and DS03019 94C1-5 (Fig 3.1,B). This result proved confusing, as these clones map to different non-overlapping positions (Fig 3.1,A). The experiment was repeated, confirming the initial result. This implied that the P1s were not accurately mapped. However, from P1 data it can be concluded than the P-element maps to chromosome region 94B2-94C-5. Although this is no more accurate that the chromosome *in-situ* hybridization data, it does agree with the initial chromosome location 94B, identified by Dr Zhao.

3.2.3 Fly Base Search

Having established the chromosome location as 94B2-94C, a *flybase* search (http://flybase.bio.indiana.) was carried out on chromosome region 94. This was done to identify all known genes in this region of the chromosome and any existing deficiency lines, insertions and inversions, YACS genes and P-elements lines that might prove useful, either in the generation of mutants or their characterisation. At this point little was known about the cDNA, and it was possible that it represented a

Fig 3.2

Genomic Map of chromosome region 3L, 94



fzo	*93-94*
cd	*94A-E*
obt	*94A-E*
wo	*94A-E*
94 Che	*94B*
Nc94B	*94B*
btn	*94B*
T-cp1	*94B1-2*
l(3)W33b	*94C-D*
l(3)ry107	*94C-D*
P{w82}1(3)w33B[1]	*94C-D*
P{ry11}1(30ry107[1]	*94C-D*
Df(3R)e-N19	*93B;94*
Df(3R)EB6	*94C2-5: 94E6*
Position of P-element	**94B2-C5**

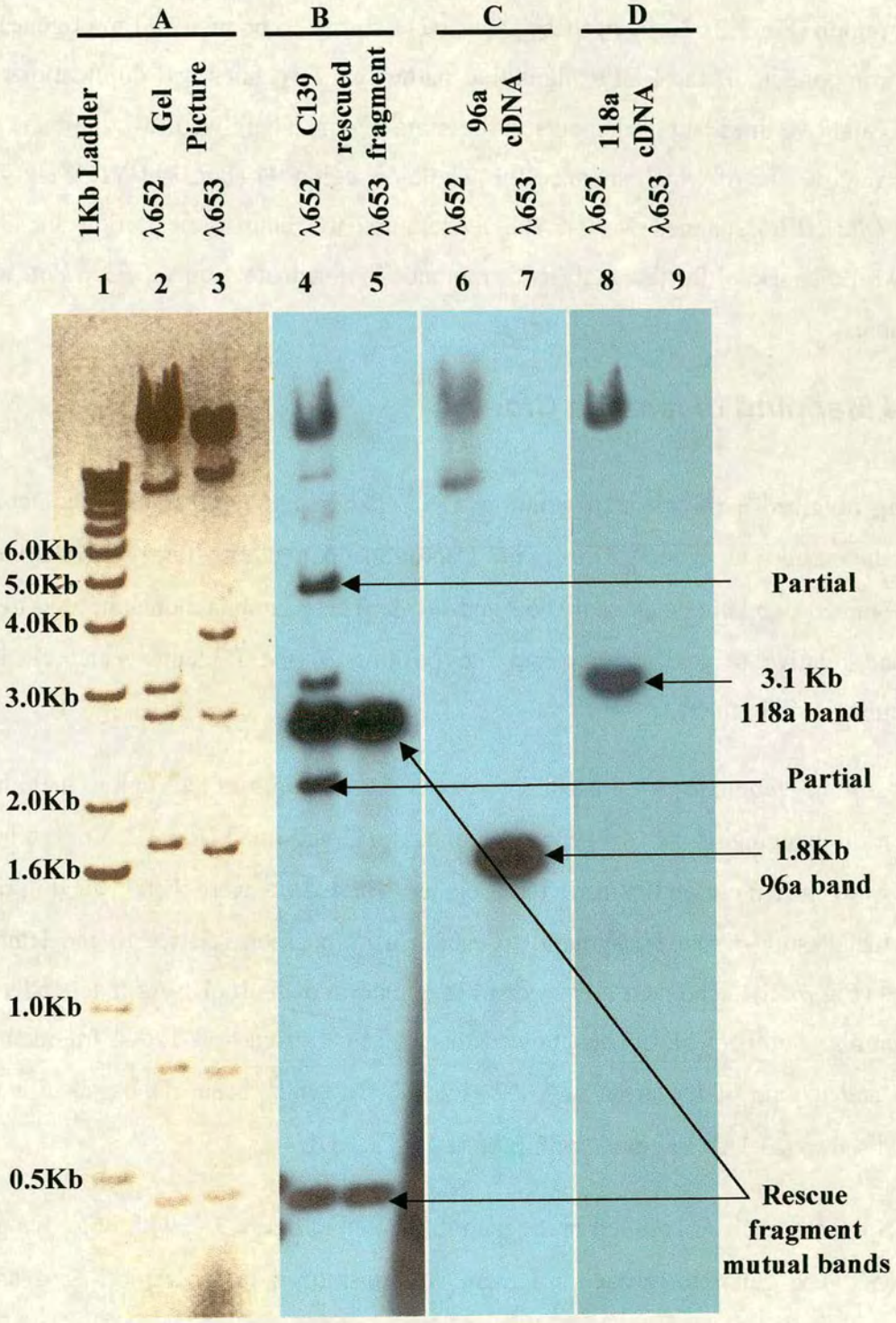
Legend

Map of chromosome region 3L,94 that was obtained at the start of the project from a *flybase* search (http://flybase.bio.indiana.). All genes are marked in brown, P-elements in green and deficences in blue. The mapped site of P-element C139 is marked in red.

Legend 3.3

A, illustrates an electrophoresis gel of EcoR1 digested DNA, with λ652 DNA in lane 2 and λ653 DNA in lane3, lane 1 being the marker (1Kb ladder, Gibco BRL approximate sizes of bands marked on gel). Adjacent are three autoradiographs (B, C & D) taken from the Southern blot of the gel hybridised in turn with the rescued genomic fragment (B), cDNA 96a (C) and cDNA 118a(D). In B, there are 2 bands common to both λ652 and λ653 (lanes 4,5 respectively), a 3Kb band and a 0.4Kb band. This suggests that these two fragments make up the region of overlap between the two genomic clones. In C, cDNA 96a hybridises to a distinct 1.8Kb fragment of λ653 (lane 7) and to an arm of λ652 (lane 6). The autoradiograph of 118a hybridises to a distinct 3.1Kb band in λ652 (lane 8), but does not hybridise to λ653 (lane 9). These results give a rough map of the region placing the cDNAs and P-element relative to each other (Fig 3.4).

Fig 3.3 **Mapping of genomic lambda clones**



gene that was already characterised, and for which mutants may have been available.

Eight genes were mapped to the region 94. The genes *94Che*, *Nc94B*, *btn* and *T-cp1* were mapped accurately to 94B, while the other genes were more generally mapped to the region (Fig 3.2). None of these genes were known to be involved in oogenesis, or as components of the EGFR signalling pathway. Two lines had duplications in 94C-D and two lines had P-elements in the same region. There were two deficiencies in the region, one of which spanned the whole of region 94 (Df*(3R)e-N19*), and the other *Df(3R)EB6* spanned 94C2-5-E6. I attempted to obtain these deficiencies, as they would be useful for the analysis of mutants. Unfortunately only *Df(3R)EB6* was available.

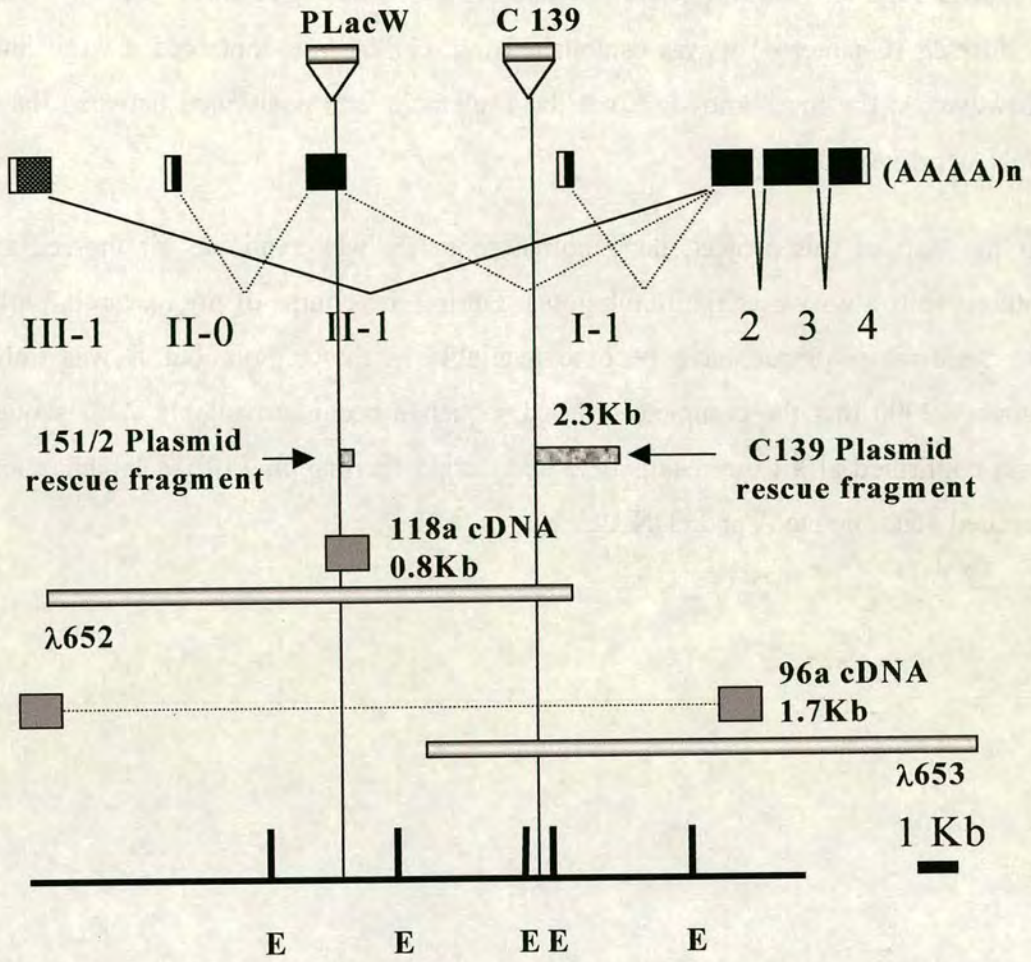
3.2.4 Mapping of lambda Clones.

Having obtained a fragment of genomic DNA flanking the P-element, Dr Debiao Zhao then used it to screen a *Drosophila* CantonS λFix genomic library (Stratagene), and obtained two lambda clones, λ652 and λ653. These lambda clones needed to be mapped relative to one another and the position of the P-element and cDNAs determined within them.

A mixture of techniques were used to carry out fine mapping of the region, including restriction mapping of the two genomic clones, λ652 and λ653 (Fig 3.3,A). Southern blots were carried out on the restricted λ clones. These blots were then probed in turn with the plasmid-rescued fragment, to establish its position relative to the lambda clones (Fig 3.3,B). The next step was a hybridization of both cDNAs independently to a similar Southern blot. This showed that 96a hybridised to a 1.8Kb fragment in λ653 and to one of the arms of λ652 (Fig 3.3,C), while 118a cDNA specifically hybridised to a 3.1Kb fragment within λ652 (Fig 3.3,D).

This Southern analysis resulted in the map illustrated in figure 3.4, with λ652 lying 5′ of λ653. The genomic rescued fragment was positioned in the region of overlap between λ652 and λ653, marking the position of the P-element. cDNA 118a lay

Fig 3.4 Genomic organisation of region surrounding P-insert C139



Legend

The organisation of λ652 and λ653 relative to each other, λ652 is 5′ of λ653, with a region of overlap in the middle containing the rescue fragment from fly line C139(site of P-element insertion).cDNA 118a is 5′ of the rescue fragment, within 5′ λ652, while cDNA 96a is 3′ of the rescue fragment, within λ653. The black bars at the bottom show the EcoR1 cutting sites (E). The positions of both the P-elements are shown, C139 from the original rescue and 151/2 a Dundee P-LacW flyline. The transcripts found to date are also marked on the map illustrating the organisation of the introns and exons(see Chapter 4).

within λ652, 5′ of the rescued genomic fragment, and cDNA 96a lay 3′ of the rescued fragment, within λ653. When RT-PCR and genomic PCR were later performed (Chapter 4) it was established that cDNA 96a contained a large intron. However, at the time, knowing that the P-element was positioned between the two cDNAs proved adequate.

At the start of this project, no genomic sequence was available for the region of interest (http://www3.ncbi.nlm.nih.gov/). During the course of my research, parts of the genomic sequence have become available in this region, but it was only in January 2000 that the complete ordered sequence became available. This sequence data confirmed all of the results obtained during the mapping of the lambda clones, rescued genomic DNA and cDNAs.

3.3 Mutants

3.3.1 Enhancer trap screen of Dundee based stocks

A saturation mutagenesis of chromosome three was carried out at Dundee University, using a *Lac-Z* P-element (Deak *et al*, 1997). This generated 2460 lethal or semi-lethal mutations. Gonzalez-Reyes A, Elliott H, Deng W and I (this was an Edinburgh, Cambridge and Dundee collaboration) screened fly lines for *Lac-Z* staining in the ovaries. Of the 2460 lines, approximately 400 had interesting *Lac-Z* staining patterns in the ovaries. Thirty nine of the fly lines had expression patterns in the anterior-dorsal follicle cells (Fig 3.5A). All these lines were screened initially by Southern analysis, using the λ clones (λ652 and λ653) as probes, and subsequently the cDNAs. A band shift on a Southern blot was obtained from line 151/2 when probed with cDNA 118a (Fig 3.5B). The lac Z staining pattern of line 151/2, shows staining in the anterior dorsal follicle cells and the nurse cells at stage 10 (Fig 3.5C). This is similar to the *in-situ* hybridisation pattern of *loco* (Chapter 5).

The Dundee lines contained the *P-lacW* P-element. This P-element contains a plasmid that can be rescued using either EcoR1 or SacII (Fig 3.6). Plasmid rescue was carried out on line 151/2, and a successful rescue was achieved with EcoRI. The genomic rescue fragment in the plasmid was approximately 200bp long. For confirmation, the fragment was used to probe a Southern carrying restricted lambda clones, λ652 and λ653, as well as cDNAs 96a and 118a. It was found to hybridise to cDNA 118a specifically, and the corresponding EcoR1 digested 3.1Kb λ652 fragment. This confirmed that the rescued genomic DNA was situated in the same region as cDNA 118a. The plasmid did not contain suitable primer sites for sequencing, so the rescue fragment was sub-cloned into pBluescript and sequenced, using the T3 and T7 primer sites. A sequence line up was carried out, using *gene jockey* software. This line up proved that the P-element Dundee line 151/2 inserted 3′ of exon II-0 in sequence present in the 3′ of cDNA 118a (Fig 3.4). Having obtained a potential mutant with a P-element lying in the coding region of the gene, we had to

Legend 3.5

Part A, is a list of all Dundee lines which were analysed to determine lac-Z expression pattern, confirming that all these lines exhibited staining in anterior dorsal follicle cells. These lines were then analysed by Southern blot to see if any contained a P-element insertion in the region surrounding cDNA 96a or 118a.

Part B is an autoradiograph of a Southern blot of genomic fly DNA that has been restricted with EcoR1 and probed with cDNA 118a. Fly line 151/2 has an additional band (band shift), indicating that there is possibly a P-element inserted in this region.

Part C shows the 151/2 lac-Z staining pattern in the anterior dorsal follicle cells.

Fig 3.5

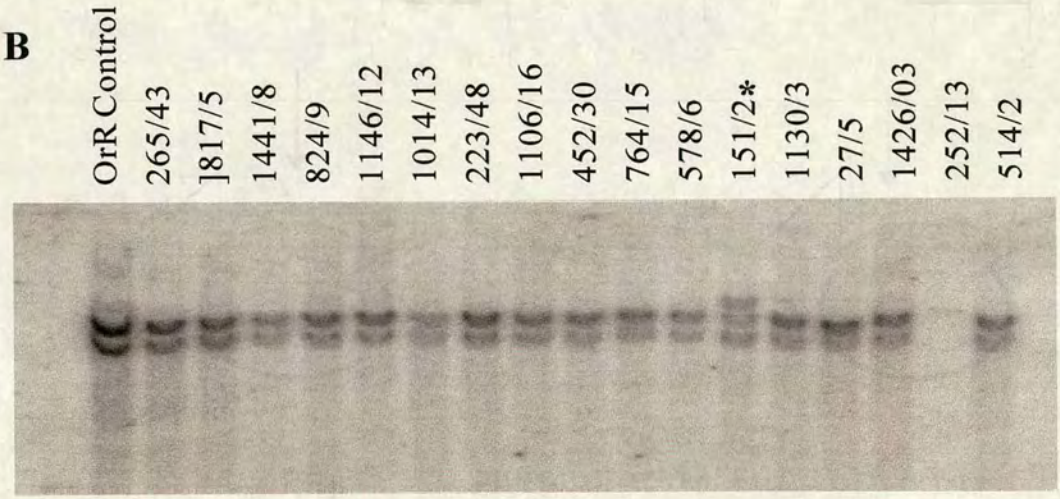
Isolation of mutant from Dundee lines

A

Dundee Lines Screened

151/2*	171/7	223/48	252/13	265/43	277/13
447/29	452/30	465/2	470/3	472/8	524/13
525/1	578/6	514/2	576/6	698/5	707/17
725/17	735/2	764/15	817/5	824/9	962/7
986/14	991/11	999/8	1003/5	1014/13	1049/13
1059/5	1106/16	1111/5	1127/11	1129/12	1130/3
1131/10	1146/12	1441/8			

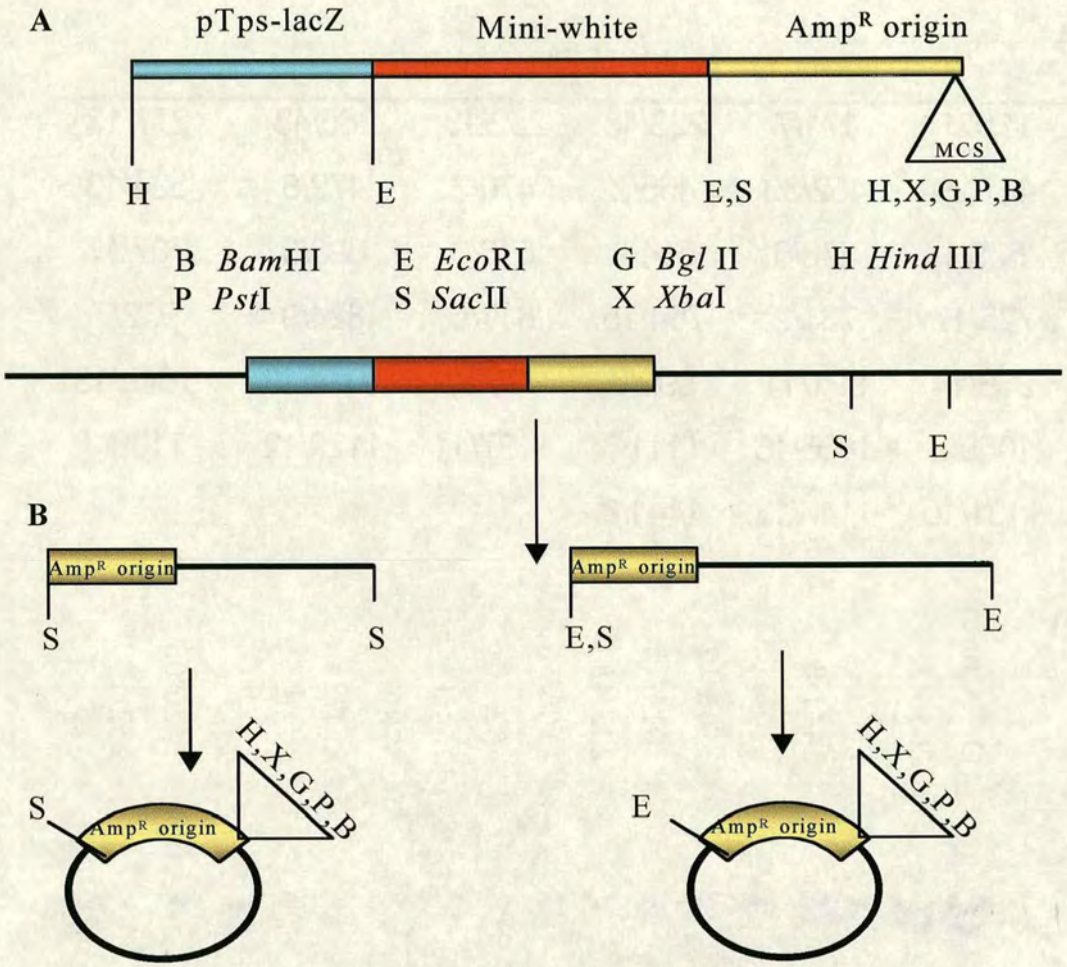
B



C

Lac Z staining pattern

Fig 3.6 Dundee line 151/2 plasmid rescue scheme



Legend

Part A shows the arrangement of P-element P-LacW in the Dundee fly lines, consisting of the Lac-Z reporter gene, mini white (which gives the flies their red eye colour) , and a plasmid that can be rescued. The relevant restriction sites are marked.

Part B illustrates how PLacW can be used to rescue genomic DNA. The genomic DNA is restricted either with EcoRI or SacII. The DNA is then ligated, resulting in circular fragments that can then be transformed into bacteria and selected with ampicillin. Therefore only bacteria containing P-element rescued fragments will grow.

Fig 3.7

Cross to establish P-element associated lethality of 151/2

Cross 1

♀ ♀ *w-* ; P{*w+*}(151/2) / TM6C, *Sb,Tb* X *w-* ; Δ2-3 *Dr*♂ ♂ / TM6B

F1: Collect male and virgin female red-drop eyed flies: *w-* ; P{*w+*}(151/2) / Δ2-3 *Dr*

Note : Only flies carrying both the P-element (red eyes) and source of transposase Δ2-3 *Dr* were selected.

Cross 2

♀ **or** ♂ *w-* ; P{*w+*}(151/2) / Δ2-3 *Dr* X *w-* ; TM3, *Sb* / TM6, *Tb* ♀ **or** ♂ respectively

F2: Collect male and virgin female white-eyed progeny, that are *Tubby* or *Stubble*, and keep each fly in separate vial:

♀ **or** ♂ *w-* ; P(excision) / TM3, *Sb* & *w-* ; P(excision) / TM6, *Tb*

Discard all flies with drop eyes

Notes : The selected F1 flies were crossed to a white eyed background to allow the movement of the P-element(which carries the red eyed marker,*w+*) to be followed. All flies containing the *Drop* eye marker were discarded as these flies contained the source of transposase (Δ2-3 *Dr*) .All white eyed flies were retained, and kept separate for single pair matings. As these are P-excisions lines

Cross 3

♀ or ♂ *w*- ; P(excision) / TM3, *Sb* or *w*- ; P(excision) / TM6, *Tb*

X

w- ; TM3, *Sb* / TM6, *Tb*

F3: Collect non-*Tubby* males and virgin females

w- ; P(excision) / TM3, *Sb*

Note: Flies selected from cross 2 were crossed to a 3rd chromosome balancer, in order to establish lines and ensure that none are lost. Non- *Tubby* progeny were collected, representing either homozygous flies or heterozygous flies for the balance TM3.

Cross 4

Cross, F3 siblings to obtain balanced fly lines over TM3, *Sb*

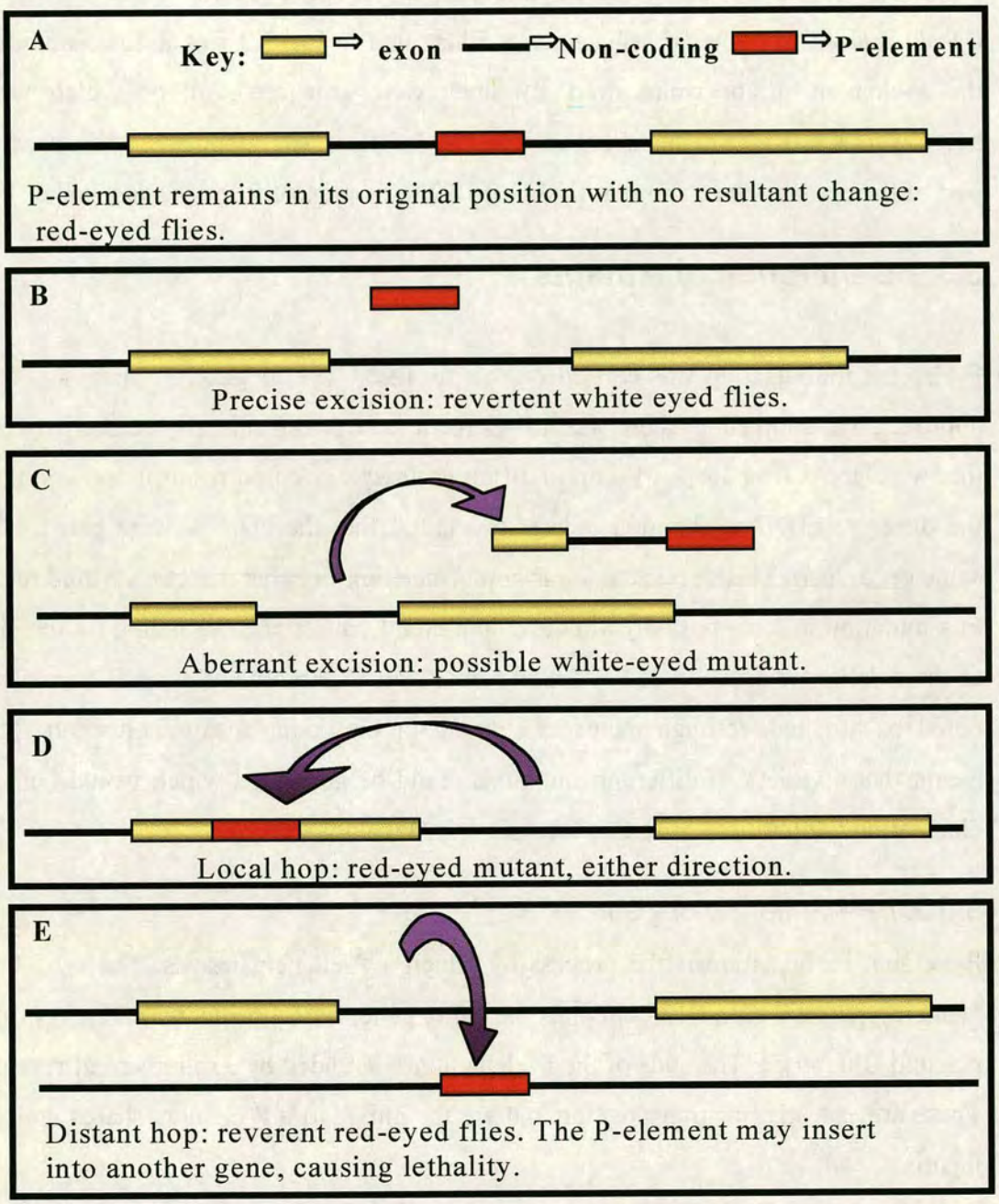
Note: This would ensure all lines balanced over TM3, which has the *Stubble* marker, identifiable on the adult. If any of the white eyed lines made are homozygous viable, eg do not carry *Stubble* marker, then the lethality not associated with the P-element.

Results

8 white eyed lines made, all of which were homozygous lethal, suggesting that the there is another site associated with the lethality of line 151/2.

Fig 3.8

Diagram showing the possible outcomes from the mobilisation of a P-element, close to a gene of interest



Legend

A-E represent the different outcomes of P-element C139 mobilisation, from its original site between the two cDNAs.

determine whether the homozygous lethality of the line was associated with the P-element. This was of special importance, as it had been found that lethality of a third of the Dundee lines was due to mutations at a location different to the P-element (Deak et al, 1997). The crossing scheme illustrated in Fig 3.7 was used to mobilise the P-element. Eight white eyed fly lines were generated, all of which were homozygous lethal. This established that the lethality was associated with another part of the chromosome, and that fly line 151/2 in its present form was unusable.

3.3.2 Generation of Mutants

P-element mobilisation was carried out on fly line C139 to generate mutants. This approach was adopted, as there was a P-element to hop conveniently located between the two cDNAs (Fig 3.4). A P-hop in different directions could result in mutations to the different cDNAs, although it later transpired that the cDNAs were part of the same gene, *loco*. This implies that a P-hop or deletion in either direction would result in a mutation in *loco*, possibly affecting individual transcripts, depending on the size of the deletion or position of P-element reinsertion. Alternatively, several transcripts could be affected, through incorrect splicing or disrupting a common exon. This meant that a variety of different mutations could be generated which would help in elucidating the multiple roles that *loco* plays in development.

3.3.2.1 P-element mobilisation

P-element mobilisation is the process by which a P-element moves. The GAL4 P-element present within C139 contains the *white* gene, GAL4 promoter and part of the plasmid Bluescript. The ends of the P-element are bounded by 31bp inverted repeats. These are essential for transposition and are the only part a P-element shares with P-factors.

In P-element mobilisation, the line containing the P-element (C139), is crossed to a strain which contains immobile P-factors (Δ2-3). The presence of transposase in the

germ line of the progeny of this cross results in the mobilisation of the P-element(C139) to new positions.

3.3.2.2 Outcomes of P-element mobilisation

During P-hop mutagenesis there are 5 possible outcomes. The first is that the P-element does not move and therefore no resultant change is observed (Fig 3.8A). The second outcome is that it excises precisely, the fly line reverting to its original white-eyed phenotype (Fig 3.8B). The third outcome is that the P-element excises imprecisely, either removing part of the P-element or the flanking genomic DNA (Fig 3.8C). In this case, if enough of the flanking genomic DNA is removed, a mutation in the adjacent gene will result. The fourth outcome is a local hop, where the P-element reinserts in the adjacent region, resulting in a red-eyed phenotype (Fig 3.8D). In this situation it is likely to affect the target genes function through inserting in a coding region, splice site or control region of the gene. The final outcome is a distant hop, where the P-element inserts at a distant site on the same chromosome, or on a different chromosome (Fig 3.8E). This will also result in red-eyed flies. A local hop is more likely to occur than a distant hop.

3.3.2.3 Crossing scheme

The following crossing scheme was used to generate mutants as it ensured that all lines generated were balanced (Fig3.9), reducing the likelihood of losing potentially interesting lines, which may be selected against. Virgin female w-;PgawB{w+}(c139) were crossed against several w-;Δ2-3Dr/TM6B males. The progeny with red dropped eyes w-;PgawB{w+}(c139)/Δ2-3Dr), were collected and the males and females were crossed against female and male w-;TM3/TM6 (chromosome 3 balancers), respectively. Every single progeny of this cross that did not contain the drop marker was used to set up individual lines, most of which were red-eyed (potential P-hops) and a few of which were white-eyed (precise and aberrant excisions). All of these individual flies were crossed against the opposite sex w-;TM3/TM6. The fly lines were balanced over TM3, as TM3 carries the Stubble marker, which could easily be used to screen for homozygous lethality. This

Fig 3.9

Cross scheme to generate new mutants

Cross 1

♀♀ ♂♂

w- ; PgawB{*w*+}(c139) X *w*- ; $\frac{\Delta 2\text{-}3\ Dr}{TM6B}$

F1: Collect male and virgin female red-drop eyed flies: *w*- ; $\frac{\text{PgawB}\{w+\}(c139)}{\Delta 2\text{-}3\ Dr}$

Note : Only flies carrying both the P-element (red eyes) and source of transposase Δ2-3 *Dr* were selected.

Cross 2

♀ **or** ♂ *w*- ; $\frac{\text{PgawB}\{w+\}(c139)}{\Delta 2\text{-}3\ Dr}$ X *w*- ; $\frac{\text{TM3, } Sb}{\text{TM6, } Tb}$ ♀ **or** ♂ respectively

F2: Collect male and virgin female coloured-eyed or white-eyed progeny, that are either *Tubby* or *Stubble*, and keep each fly in separate vial:

♀ **or** ♂

w- ; $\frac{\text{PgawB}\{w+\}(c139) \text{ or P(excision)}}{\text{TM3, } Sb}$ **or** *w*- ; $\frac{\text{PgawB}\{w+\}(c139) \text{ or P(excision)}}{\text{TM6, } Tb}$

Discard all flies with drop eyes.

Notes : The selected F1 flies were crossed to a white eyed background to allow the movement of the P-element(which carries the red eyed marker,*w*+) to be followed. All flies containing the *Drop* eye marker were discarded as these flies contained the source of transposase (Δ2-3 *Dr*) .All other flies were retained, and kept separate for single pair matings.

Cross 3

♀ or ♂ **or**

w- ; PgawB{*w*+}(c139) or P(excision) / TM3, *Sb* *w*- ; PgawB{*w*+}(c139) or P(excision) / TM6, *Tb*

X

w- ; TM3, *Sb* / TM6, *Tb*

F3: Collect non-*Tubby* males and virgin females

w- ; PgawB{*w*+}(c139) or P(excision) / TM3, *Sb*

Note: Flies selected from cross 3 were crossed to 3rd chromosome balancer, in order to establish lines and ensure that none are lost. Non- *Tubby* progeny were collected, representing either homozygous flies or heterozygous flies for the balancer TM3.

Cross 4

Cross, non- *Tubby* F3 siblings to obtain balanced fly lines over TM3*Sb*

Note: This would ensure all lines were balanced over TM3, and would have the stubble marker, identifiable on the adult. This will enable screen for homozygous lethality.

was achieved by picking out eight non-Tubby, normal pupae and putting them in a vial. Eight pupae were used, as this should ensure that flies of both sexes were present, thereby establishing the line.

3.3.3 Analysis of fly lines generated

3.3.3.1 Summary of P-hop data

Table 3.1 Summary of p-hop data

In table: HL represents homozygous lethal; HV represents homozygous viable; P/E represent presence of P-element or excision of P-element respectively and C/N represents complementation or non-complementation respectively.

	LINES GENERATED	HOMOZYGOUS LETHAL	LETHAL 67-85	REDUCED VIABILITY	REDUCED FERTILITY
RED	292	64	54	10	7
WHITE	107	9	5	7	7
TOTAL	399	73	59	17	14

466 fly crosses were set up. 67 lines were lost through crosses failing. This left 399 established fly lines for analysis, of which 292 were red eyed, containing the P-element and potential P-hops. The remaining 107 were-white eyed, where all or part of the P-element had excised.

Table 3.2P-Hop Data

LINE NAME	P / E	PHENOTYPE	COMMENTS ON VIABILITY/FERTILITY	COMPLEMENTATION 67/85	COMMENTS
S94FP16	P	HL	-	N	-
S94MP17	P	HV	Reduced Fertility	C	
S94MP34	P	HV	Few homozygotes	C	
S94MP45	P	HV	Few homozygotes	C	
S94FP46	P	HL	-	N	-
S94FP47	P	HL	-	N	-

S94FE48	E	HV	Reduced Fertility	C	
S94FP49	P	HL	-	N	-
S94MP57	P	HV	Few homozygotes	C	
S94FP65	P	HL	-	N	-
S94FP66	P	HL	-	N	-
S94FE67	E	HL	-	N	-
S94FE80	E	HV	Few homozygotes	C	
S94FP83	P	HL	-	N	-
S94FE85	E	HL	-	N	-
S94MP90	P	HL	-	C	-
S94FP106	P	HL	-	N	-
S94FP108	P	HL	-	N	-
S94FP112	P	HL	-	N	-
S94FP119	P	HL	-	N	-
S94FP120	P	HL	-	N	-
S94FP132	P	HL	-	N	-
S94FE133	E	HV	Few homozygotes	C	
S94FE135	E	HL	-	N	-
S94FE138	E	HV	Reduced Fertility	C	
S94FP139	P	HL	-	N	-
S94FP142	P	HL	-	N	-
S94FP148	P	HL	-	N	-
S94FP150	P	HL	-	N	-
S94FP154	P	HL	-	N	-
S94FP156	P	HL	-	N	-
S94FP157	P	HL	-	N	-
S94MP158	P	HV	Few homozygotes	C	
S94FP160	P	HL	-	N	-
S94FP163	P	HL	-	N	-
S94FP172	P	HL	-	N	-
S94FE184	E	HL	-	N	-
S94FP187	P	HL	-	N	-

S94FP191	P	HL	-	N	-
S94FP193	P	HL	-	N	-
S94MP194	P	HV	Reduced Fertility	C	
S94FP203	P	HL	-	N	-
S94FP205	P	HL	-	N	-
S94FP219	P	HL	-	N	-
S94FP221	P	HL	-	N	-
S94FP224	P	HL	-	N	-
S94FP225	P	HL	-	N	-
S94FP226	P	HL	-	N	-
S94FE230	E	HV	Few homozygotes	C	
S94FP231	P	HL	-	N	-
S94FP234	P	HL	-	N	-
S94FP246	P	HV	Abnormal wings	C	
S94FP258	P	HL	-	N	-
S94FP262	P	HL	-	N	-
S94MP277	P	HV	Few homozygotes	C	
S94FP284	P	HL	-	N	-
S94FP291	P	HL	-	-	-
S94MP295	P	HV	Few homozygotes	C	
S94FP305	P	HL	-	N	-
S94FP314	P	HL	-	N	-
S94FP318	P	HV	Reduced Fertility	C	
S94FE320	E	HV	Reduced Fertility	C	
S94FP321	P	HV	Abnormal wings	C	
S94FP322	P	HL	-	N	-
S94FP343	P	HL	-	N	-
S94MP345	P	HL	-	N	-
S94MP349	P	HV	Reduced Fertility	C	
S94MP358	P	HV	Reduced Fertility	C	
S94MP360	P	HV	Few homozygotes	C	

S94FE366	E	HL	-	C	
S94FE368	E	HV	Reduced Fertility	C	
S94ME370	E	HV	Few homozygotes	C	
S94ME371	E	HV	Reduced Fertility	C	
S94MP373	P	HL	-	C	
S94MP375	P	HL	-	C	
S94MP376	P	HL	-	C	
S94MP377	P	HL	-	C	
S94MP378	P	HL	-	C	
S94MP379	P	HL	-	C	
S94ME381	E	HV	Few homozygotes	C	
S94MP382	P	HL	-	C	
S94ME383	E	HV	Few homozygotes	C	
S94MP386	P	HV	Few homozygotes	C	
S94ME387	E	HV	Reduced Fertility	C	
S94ME388	E	HL	-	C	-
S94ME390	E	HL	-	C	Wing defect
S94MP391	P	HL	-	C	-
S94MP394	P	HV	Reduced Fertility	C	
S94ME395	E	HV	Few homozygotes	C	
S94ME399	E	HL	-	C	-
S94MP403	P	HL	-	N	-
S94FP409	P	HL	-	N	-
S94FP415	P	HL	-	N	-
S94FP418	P	HV	Few homozygotes	C	
S94MP419	P	HL	-	N	-
S94FP422	P	Hl	-	N	-
S94FP425	P	HL	-	N	-
S94FP431	P	HL	-	N	-
S94FE436	E	HV	Reduced Fertility	C	
S94MP449	P	HL	-	N	-

S94FP451	P	HV	Reduced Fertility	C	
S94FP453	P	HL	-	N	-
S94FP455	P	HV	Few homozygotes	C	
S94FP456	P	HL	-	N	-
S94FP462	P	HL	-	N	-

3.3.3.2 Screen for fly lines that are homozygous lethal or have reduced homozygous viability

When the mutagenesis was carried out there was no way of knowing that the mutants generated would be homozygous lethal. However I knew that *loco* was an RGS gene, and was likely to be involved in the regulation of several signalling pathways, at several stages of development. Ovary *in-situ* hybridisation had also shown that *loco* was expressed at multiple stages in oogenesis (Chapter 5). Hence, there was a high possibility that a mutant in *loco* would be lethal. With all fly lines balanced over TM3*Sb* they could be screened with ease for homozygous lethality. This was carried out by scoring a population of each fly line for the TM3*Sb* marker Stubble, which gives short bristles compared with wild type. If all the flies within the population carried this marker, it meant that they were all heterozygous for the balancer and C139, and that homozygous flies were not viable (hence homozygous lethal). This would also highlight fly lines which contained less than the expected number of homozygous flies. In a typical cross, a ratio 1:2:1 would be expected but, as TM3/TM3 are lethal, a ratio of 1:2 would be observed if the fly line was homozygous viable. Therefore if less than 1/3 of the flies observed were homozygous, the fly line exhibited a reduced homozygous viability. In practice, only fly lines exhibiting well below the expected 1/3 homozygotes were identified. This is because a relatively small number of flies were counted for each line (~50) and there was a great deal of variation. In many respects, the flies with a reduced homozygous viability are more interesting, because the ovarian phenotypes could be observed in these fly lines. Seventy two lines exhibited homozygous lethality. Sixty four of these fly lines were red-eyed and contained the P-element, or part of it. The remaining 8 lines were white eyed and represent deletions in the vicinity of the P-element, or part of the P-element

may still be present. As the P-element was in the middle of the gene, 5′ of exon I-1 (Fig 3.4), it is highly likely that some of these white-eyed lines are mutations in *loco*. There were 17 lines that exhibited well below the expected 1/3 ratio of homozygotes. These were classed as "reduced viability lines and 10 of these were red-eyed and 7 were white-eyed.

3.3.3.3 Screen for Reduced Fertility

I also screened homozygous females for impaired fertility by crossing a fixed number (3) of young homozygous females to heterozygous males (4-5) of the same line. Homozygous males were not used as they may also have had impaired fertility, which would have distorted the data. The crosses were compared, looking for lines which generated few offspring and 14 such lines were obtained, 7 red-eyed and 7 white-eyed. All fly lines that appeared normal were discarded.

3.3.3.4 Complementation analysis

Although several homozygous lethal lines were established, they still had to be identified as mutants in the RGS gene *loco*. Three deficiencies map to the region: *Df(3R)EB6*-94C2-5, *Df(3R)e-N19*-93B;94 and *Df(3R)5C1*-93E;94C-D (http://flybase.bio.indiana.edu:82/). *Df(3R)e-N19* was unavailable. The other two deficiencies were crossed to the 9 white-eyed lethal lines, all of which were viable over the deficiencies, which therefore did not cover the *loco* locus. As a suitable deficiency line was not obtained, one of the white-eyed homozygous lethal lines, 67, was crossed to all the remaining fly lines. It transpired that 59 of the 72 homozygous lethal lines fell into the same complementation group. The presence of 59 fly lines within the same complementation group is strong evidence that they represent mutants in the *loco* gene.

However, of the 8 white eyed lethal lines, only 2 were lethal over the lethal lines 67 or 85. This prompted complementation analysis to be conducted on all 8 white-eyed lethal lines (Table 3.3). This analysis illustrated that the flies fell into 2 complementation groups, which may represent deletions in either direction of the P-element.

Table 3.3 White eyed lethal line complementation analysis.

x	67	85	135	184	366	388	390	399
67	-	N	N	N	C	C	C	C
85		-	N	N	C	C	C	C
135			-	N	C	C	C	C
184				-	C	C	C	C
366					-	N	N*	N*
388						-	N	N*
390							-	N
399								-

N Non-complementing

C Complementation

* Represents occasional heterozygous flies observed.

3.3.3.5 Mutant phenotypes observed in reduced viability flies.

3.3.3.5.1 Fly line 80

Fly line 80 is a white-eyed excision line that exhibits reduced homozygous viability. I screened for an egg phenotype, which would indicate defective oogenesis. The vast majority of the eggs laid were normal, but a few ventralised eggs were observed (Fig 3.10,F). These eggs were the same length as wild type eggs but were thinner and

exhibited a radial symmetry, unlike the wild type (Fig 3.10A). These ventralised eggs always failed to hatch (Table 3.4).

3.3.3.5.2 Fly line 138

Fly line 138 is a white-eyed excision line that exhibits reduced homozygous viability. 5% of eggs laid were abnormal, exhibiting a range of phenotypes including, short eggs (Fig 3.10,C), ventralised eggs (Fig 3.10,F) and eggs with fused appendages (Fig 3.10 D). The low number of eggs counted reflects the reduced egg laying ability of this line.

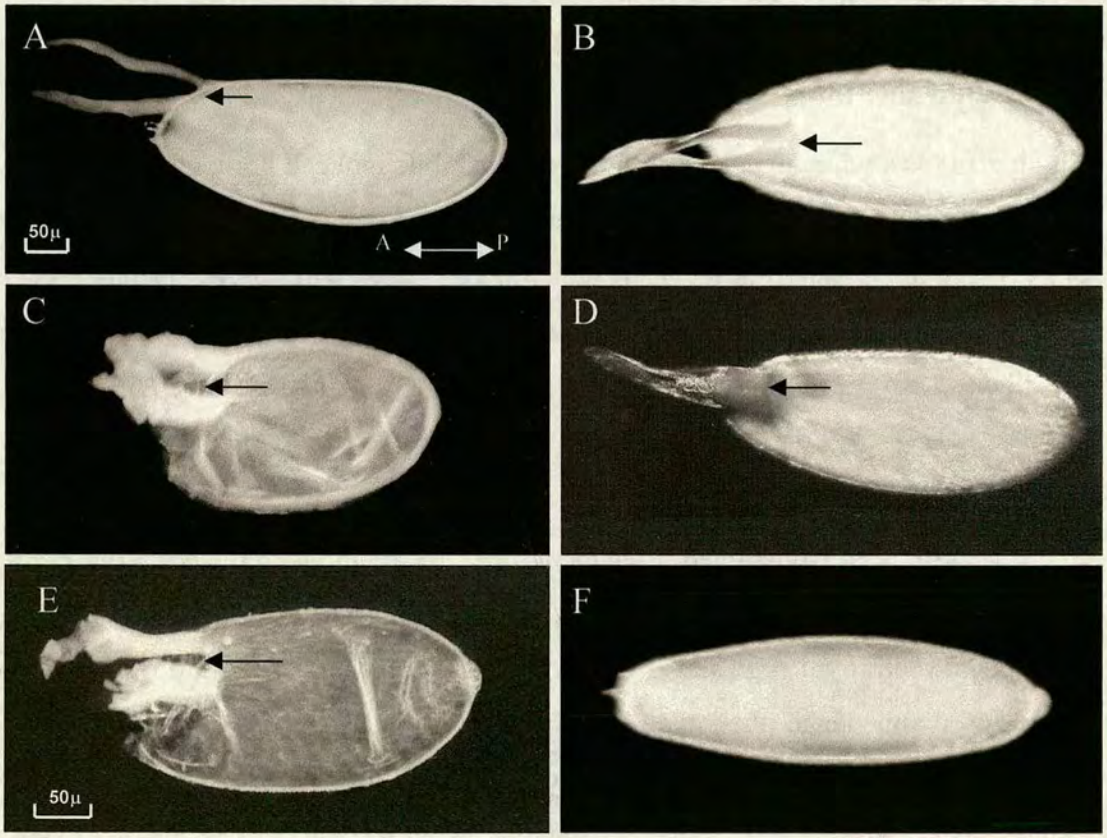
3.3.3.5.3 Fly line 230

Fly line 230 is a white eyed P-element line that exhibits reduced homozygous viability. 5% of eggs laid were abnormal, exhibiting a range of phenotypes including short eggs, ventralised eggs and eggs with fused appendages. Variation was observed in the fused appendage phenotype, which varied from fused at base (Fig 3.10,B) to fused along entire length (Fig3.10,D). Both the ventralised and short eggs failed to hatch (Table 3.4).

3.3.3.5.4 Fly line 295

Fly line 295 is a red eyed P-element line that exhibits reduced homozygous viability. 2.5% of eggs observed were abnormal, exhibiting a fused appendage phenotype (Fig

Fig 3.10 **Mutant Phenotypes**



Legend

All eggs have the anterior on the left and have their dorsal midline (if present) marked by a black arrow. A is a wild type egg, B is an egg with appendages fused at the base, D had its appendage fused along the whole length, while F is completely ventralised (note the difference in overall shape of egg). C and E are both short egg phenotypes and illustrate the range, from slightly shorter than wild type to approximately a quarter the length of wild type.

3.10B,D). This was the only phenotype observed. However, in this line a high proportion (25%) of the apparently normal eggs failed to hatch (Table 3.4).

3.3.3.5.6 Fly line 358

Fly line 358 is a red-eyed P-element line that exhibits reduced homozygous viability. In many respects the phenotypes observed in this line are similar to that of fly line 295, as the only egg abnormality was the fused appendage phenotype (Fig 3.10 B,D), although at a high frequency of 20%. The other similarity with line 295 was the high frequency 50.8% of death in normal looking eggs (Table 3.4).

3.3.3.5.7 Fly line 368

Fly line 368 is a white-eyed excision line that exhibits reduced homozygous viability. In this line, over 25% of the eggs observed exhibited a short egg phenotype, ranging from slightly shorter than wild type to a quarter of the normal length (Fig 3.10 C, E). All short eggs failed to hatch.

3.3.3.5.8 Fly line 371

Fly line 371 is a white-eyed excision line that exhibits reduced homozygous viability. In this line the flies had a greatly reduced fertility and 50% of the eggs observed were abnormal, showing either a short egg phenotype or a fused appendage phenotype (Fig 3.10 B, D, C, E).

3.3.3.5.9 Fly line 451

Fly line 451 is a red-eyed P-element line that exhibits reduced homozygous viability. The homozygous flies in this line are sterile. No normal looking eggs are laid and all eggs show a short egg phenotype (Table 3.4).

3.3.3.5.10 Fly line 455

Fly line 455 is a red-eyed P-element line that exhibits reduced homozygous viability. This line exhibited a range of phenotypes, fused appendage, ventralised and short eggs all being observed (Fig 3.10, Table 3.4).

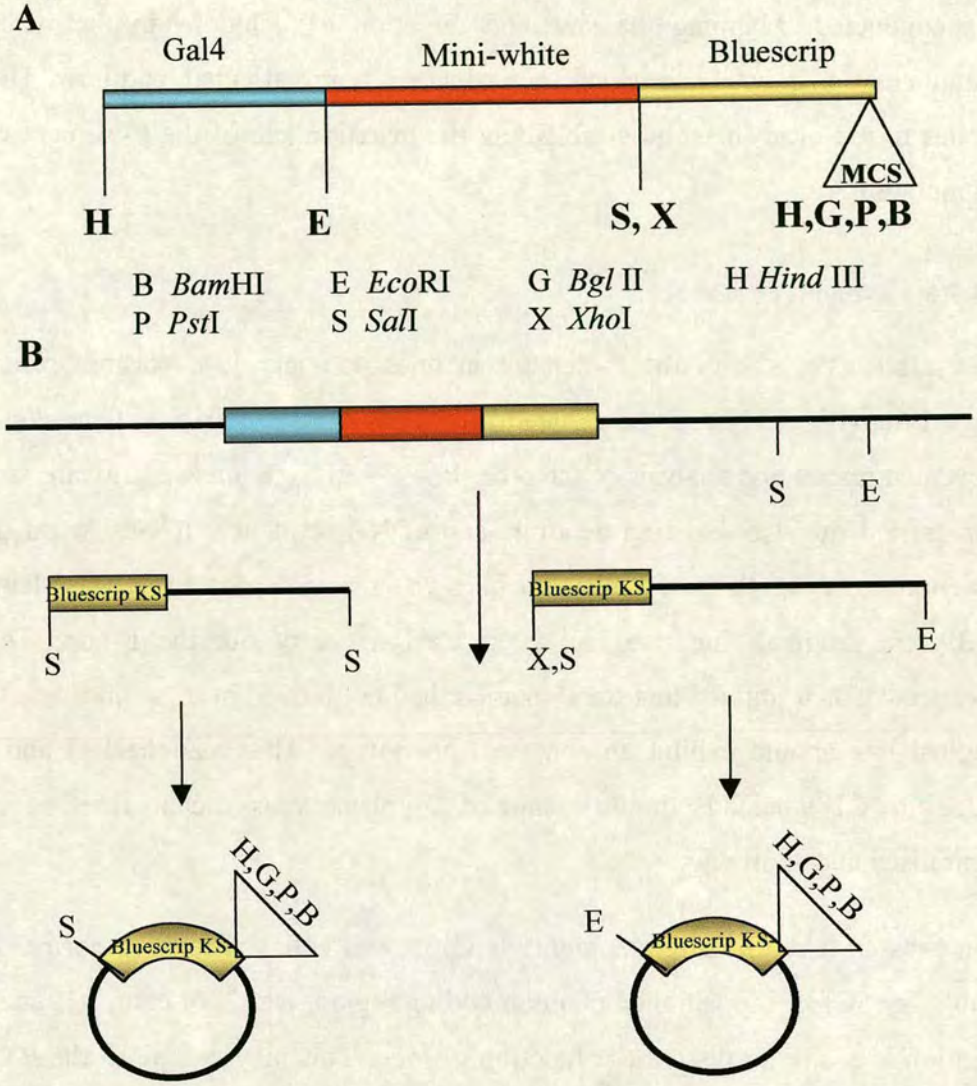
Table 3.4 Egg phenotypes observed in reduced viability lines

FLY LINE	AVERAGE NO OF EGGS LAID BY 5 FEMALES /5DAYS	NORMAL EGGS			FUSED APPENDAGES			VENTRALISED			SHORT EGG		
		total	dead	%	total	dead	%	total	dead	%	total	dead	%
80	195	192	13	6.3	-	-	-	3	3	100	-	-	-
138	97	92	18	19.6	2	2	100	2	2	100	1	1	100
230	515	489	53	10.8	9	4	44	14	14	100	3	3	100
295	212	207	55	26.6	5	3	60	-	-	-	-	-	-
358	326	262	133	50.8	64	36	56	-	-	-	-	-	-
368	267	212	67	31.6	-	-	-	-	-	-	55	55	100
371	20	10	2	20.0	5	2	40	-	-	-	5	5	100
451	50	-	-	-	-	-	-	-	-	-	50	50	100
455	206	167	12	7.1	4	0	0	3	3	100	32	32	100

3.3.3.6 Further analysis of reduced viability lines

It is difficult to interpret the information above, as various phenotypes were observed, with some lines only producing one phenotype and other lines containing the full range of phenotypes. An explanation for this is given in Chapter 4, where the analysis of *loco* and the transcripts generated shows that *loco* is a large gene (~20Kb), with several transcripts. Disruption of different transcripts to varying degrees could explain the phenotypes observed. Another observation was that none of the fly lines that exhibited abnormal egg phenotypes, showed a reduced viability, when heterozygous for white eyed excision line 67. However, complementation analysis has shown that the white-eyed fly lines fall into two groups. To try and

Fig 3.11 **P-GawB Plasmid Rescue**



Legend

Part A shows the arrangement of P-element P-gawB in the C139 flyline. This consists of the gal4 driver, mini white (which gives the flies their red eye colour), and Bluescript plasmid that can be rescued. The relevant restriction sites are marked. Part B illustrates how P-gawB can be used to rescue genomic DNA. The genomic DNA is restricted either with XhoI or SalI. The DNA is then ligated resulting in circular fragments that can then be transformed into bacteria and selected with ampicillin. Therefore only bacteria containing P-element rescued fragments will grow.

determine how these different mutants were affecting *loco,* some molecular analysis was conducted. Mapping the size and direction of deletions in the white-eyed mutants may help establish which transcripts are being affected, and how. The same applies to red-eyed lines, by establishing the insertion site of the P-element causing the mutation.

3.3.3.6.1 P-element rescue

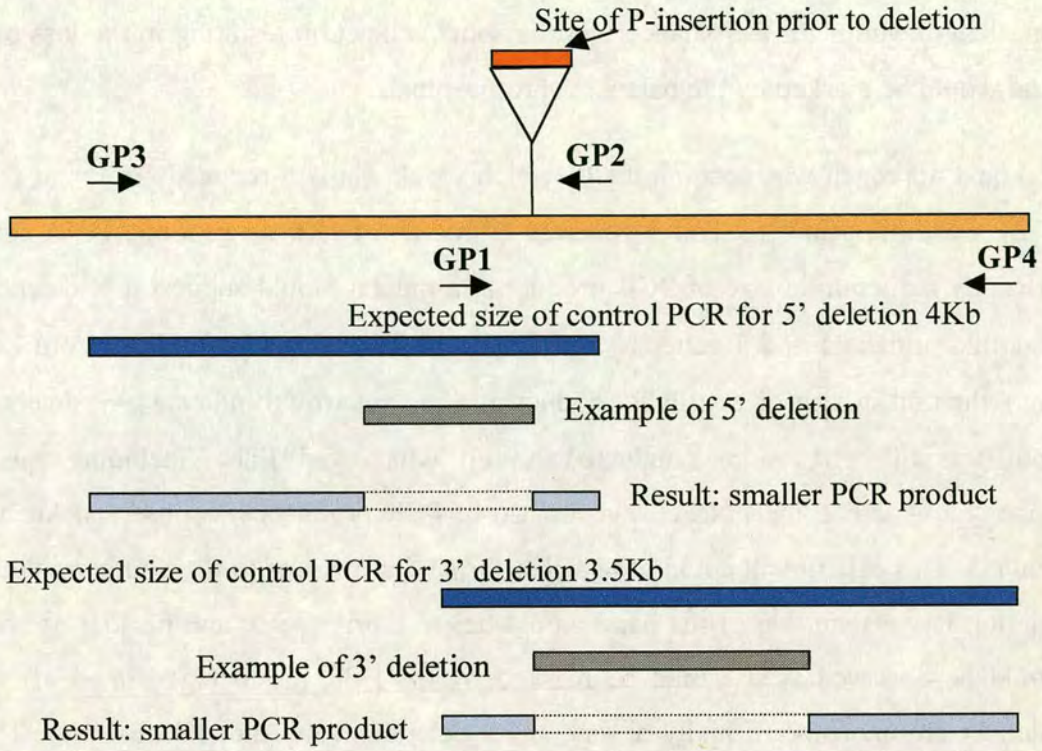
To establish the site of the P-element in lines 455 and 138, plasmid rescue was carried out (Fig 3.11) and the rescued fragments were sequenced. Time constraints prevented rescue and analysis of the other lines. With *gene jockey* software, a line up was carried out with existing genomic and cDNA sequence. It was found that the insertion site for both these P-element lines was the same and that it was identical to C139, the original line that had been used to carry out the P-hop. This was unexpected, as it implied that the P-element had not moved in these lines and that the original line should exhibit an abnormal phenotype. This was checked and it was found that C139 indeed exhibits a range of egg phenotypes, such as fused appendage, ventralised and short egg.

This was an embarrassing oversight, as C139 was believed to be a normal healthy viable line. C139 is positioned in a non-coding region, just 5' of exon I-1, and in this position it is able to disrupt the function of *loco.* This may be due to the P-element being inserted in a control region for one of the transcripts, possibly *loco-c1*, or it may affect proper splicing of one or more of the transcripts.

3.3.3.6.2 White eyed excision line analysis

Two approaches were used to ascertain the direction and size of the deletions in the white-eyed lines of interest. The first was by genomic Southern analysis, where I was hoping to observe a band shift that could be equated to deletion. Several different restriction enzymes were tried, but no band shifts were observed. This may have

Fig 3.12 **PCR based test for deletion in white-eyed lines**



Legend

Yellow line indicates the genomic region surrounding the P-insertion site. In the white-eyed line where the P-element has excised imprecisely, we would expect a deletion. This PCR method was used to determine the size of the genomic deletion and direction of deletion for particular white-eyed lines. No differences in PCR product size were observed, indicating that deletions were two small to be noticed, or larger than the PCR product.

failed for two reasons. A small deletion may be enough to disrupt the gene function but not large enough to be distinguished from normal bands. As all the flies used were heterozygous for the balancer chromosome, a deletion resulting in the loss of a band would be masked by the balancer chromosome.

The next approach was genomic PCR, which would show a reduced size band (Fig 3.12). Genomic primers 2 and 3 generate a product of 4Kb on genomic DNA from OrR, any reduction in size of PCR product in a mutant would suggest a 5′ deletion. Genomic primers 1 and 4 generate a product of 3.5Kb on genomic DNA from OrR any reduction in size of this PCR product in a mutant would indicate a 3′ deletion. Both sets of PCRs were conducted on all white-eyed lines, including the 9 homozygous lethal lines. No change in size of PCR products was observed for any mutants. This experiment could have failed for similar reasons to the Southern blot: a deletion larger than that of the band would delete a primer site and no loss of band would be observed as it would be masked by the PCR product generated off the balancer chromosome. Equally a very small deletion would not be observed. This could be resolved by further experiments.

3.4 Discussion

The aim of the work presented in this chapter was to generate mutants in the gene *loco*. This has proved successful as several mutants have been generated (more than can feasibly be analysed during a three year period)! The P-element rescue proved that the P-element line C139 initially used for the P-hop exhibited an egg phenotype, laying eggs that were either shorter than wild type or had reduced dorsal identity. This same observation was made in the other reduced viability lines, although not all lines exhibited the same range of phenotypes, or the same level of fertility. Unfortunately, further analysis was not possible to determine the nature of these different mutations and to separate the different functions of *loco*. However, the phenotypes observed can be divided into two specific groups. Some mutants that affect overall dorsal-ventral polarity of the egg, producing fused appendages and loss

of appendage phenotypes. The other group of mutants are the short egg phenotypes, that include all eggs that are of reduced length.

3.4.1 Ventralised phenotypes

Several of the lines exhibited phenotypes, ranging from partially fused to completely missing dorsal appendages. The interpretation of this phenotype has to be approached cautiously, as affecting downstream components of the *gurken-torpedo* pathway will result in a phenotype that is ventralised to some degree and there could be several reasons why this is observed. It could be due to loss or reduction of the dorsal midline cell type, as is observed in *argos* mutants {Zhao and Bownes 1999; Wasserman and Freeman, 1998). However this is not probable as *loco* mutants also exhibit a phenotype that completely lacks a dorsal appendage. This could result from complete loss of the dorsal cell type, resulting in a completely ventralised eggshell, or the failure of dorsal follicle cells to migrate. For loco mutants it is the first of these two reasons. The explanation is in the overall shape of the egg. In eggs where dorsal appendages have failed to form, the overall shape of the egg is recognisable as normal (i.e. dorsal surface is flatter than ventral surface, see Chapter 1). You would also see a thickening of chorion where you would expect the dorsal appendages to form. When there is complete loss of dorsal cell type, the complete egg is ventralised, being symmetrical around the anterior-posterior axis (rotational symmetry), and long thin eggs are observed (Fig 3.10F). The fused appendage phenotype can be interpreted as eggs that have a reduced dorsal area. This range of dorsal phenotypes is hardly surprising when you consider the gene in question. An RGS gene is a regulatory gene, therefore varying reductions in its level of expression, will result in different levels of modulation of the signal.

3.4.2 Short egg phenotype.

Short eggs are the result of dumping not occurring properly. Dumping happens at stage 13/14 in oogenesis, in a period of about half an hour, when the nurse cells

dump the rest of their content into the oocyte. There are two processes, which when affected can result in a short egg phenotype.

Prior to nurse cell dumping, the nurse cell cytoskeleton undergoes some rearrangement, and anchors the nuclei in the centre of the nurse cells. When the nurse cell cytoskeleton is disrupted, the nurse cell nuclei are not anchored properly and during dumping, the nuclei are drawn towards the ring canals and block them, preventing flow of cytoplasm into the oocyte (Cooley & Theurkauf 1994).

Also, as the nurse cells and oocyte become larger, the connecting ring canals grow and several genes have been identified that affect this growth of ring canals (Cooley 1998). In these mutants, the cytoplasm is unable to flow from the nurse cells into the oocyte, resulting in a dumpless phenotype and short eggs.

Chapter Four: Sequence and Transcripts

4.1 Introduction

The cDNAs 96a and 118a, obtained from the initial library screen, mapped approximately 4Kb apart, with the P-element C139 positioned between them (Chapter 3). At the time the research was started, there was no indication that the two cDNAs were part of the same gene. The cDNAs were subcloned and sequenced and the sequence compared to all existing genes in the database for homology at the protein level. cDNA 96a exhibited sequence similarity RGS genes (Regulator of G-Protein Signalling). RGS genes are involved in the negative regulation of G-Protein signalling pathways, by accelerating Gα subunits rate of GTP hydrolysis. At this time, no RGS genes had been identified in *Drosophila*. However RGS genes had been identified in several other organisms, so its presence in *Drosophila* was to be expected. cDNA 118a exhibited no sequence similarity to any existing sequence in the database.

Subsequent to characterising cDNA 96a as an RGS gene, it was also identified in glial cells and named *locomotive* (*loco*)(Granderath *et al* 1999), on account of mutant larvae that have problems crawling. Granderath *et al* (1999) identified 2 transcripts *loco-c1* and *loco-c2,* which share 3 exons positioned at the 3′ end of the transcripts. cDNA 118a is identical to an exon specific to *loco-c2*, while cDNA 96a shared the 3 core exons and had a unique 5′ exon. This group had also sequenced the cloned genomic region and, although they had not released this sequence data, did show the basic structure of the cDNAs. I set out with this information to characterise the cDNAs, using RT-PCR and genomic PCR to establish the transcripts present in the ovary and the structure of those transcripts.

4.2 Sequencing cDNAs

To determine whether cDNAs 96a and 118a represented existing genes already mapped to the region (Fig 3.2 Chapter 3) some preliminary sequencing was done. This generated about 400bp of readable sequence, at either end of each cDNA. This

sequence was used to search the NCBI database (National Centre for Biotechnology and Information, http://www3.ncbi.nlm.nih.gov/), and no significant similarity was obtained to any existing sequence. Based on this initial sequencing, the cDNAs represented a new gene or genes. Therefore complete sequencing of both cDNAs was undertaken. It was essential that the finished sequence contained no errors, as this would limit our analysis of the transcripts and identification of the correct Open Reading Frame (ORF). To achieve accurate sequencing of the cDNAs, they were subcloned into small fragments, which were then sequenced in from the ends. This generated the extensive overlap necessary for a low error rate.

4.2.1 Subcloning and Sequencing of cDNA 96a

96a is a 1.7kb cDNA in bluescript (Stratagene cloning vector) and, for ease of sequencing it was subcloned into 3 smaller fragments, 96RevSac, 96Mid and 96RevHinc in the same vector (Fig 4.1A). The smaller cDNA fragments generated were also used for *in-situ* hybridisations, as it was thought that smaller probes would have better tissue penetration and therefore show clearer staining (Chapter 5).

The cDNAs were sequenced with bluescript specific primers, T3, T7, Reverse and –20 (Fig 4.1B). The sequence obtained was compiled with the sequence analysis software *Gene-Jockey*. With the sequence complete all potential ORFs were analysed. In cDNA 96a there was one potential ORF with no stop codon, starting at nucleotide 655 in frame 1 (Fig4.2). Protein homology searches were carried out in all reading frames (http://www.ncbi.nlm.nih.gov/blastx) and sequence similarity to RGS Proteins was identified in frame 1, confirming that frame 1 was the correct reading frame.

The highest region of sequence similarity observed between cDNA 96a and RGS genes covers nucleotides 1116 to 1479, with RAT RGS12 (Ac No AAC53176.1) exhibiting the highest sequence similarity at 51%. A Protein pileup was carried out with several mammalian RGS genes and 96a (Fig 4.3). Over this region, it can be

Legend 4.1

Part A summarises the subcloning of cDNA 96a. In 1, 96a restricted with SacII and HincII. The restricted DNA was run on a gel and the 2.9Kb bluescript fragment and the 616bp cDNA 96a centre fragment were excised and gel purified. These two fragments had compatible ends and were then ligated and transformed into XL1 bluescript, resulting in plasmid 96a Mid.

In 2, the cDNA 96a was restricted with EcoRI, dropping out the 1.7kb cDNA when run on a gel. The gel purified insert and bluescript where then re-ligated and transformed into XL1 bluescript. The clones obtained were screened by restriction digest for cDNA in opposite orientation. This was named 96a Rev.

In 3, 96aRev was digested with SacII, when run on a gel this drops out a 1222bp fragment. The remaining part of cDNA and bluescript was ligated and transformed into XL1 bluescript. This was named 96a Rev Sac.

In 4, 96aRev is digested with HincII, when run on a gel this drops out a 1199bp fragment. The remaining part of cDNA and bluescript was ligated and transformed into XL1 bluescript. This was named 96a Rev Hinc.

Part B shows how all the subcloned fragments fit together. They were all sequenced using Bluescript primer T3, T7, Reverse and -20. The sequence was then compiled in *Gene-Jockey* and checked for errors.

Figure 4.1 **Subcloning and Sequencing of cDNA 96a**

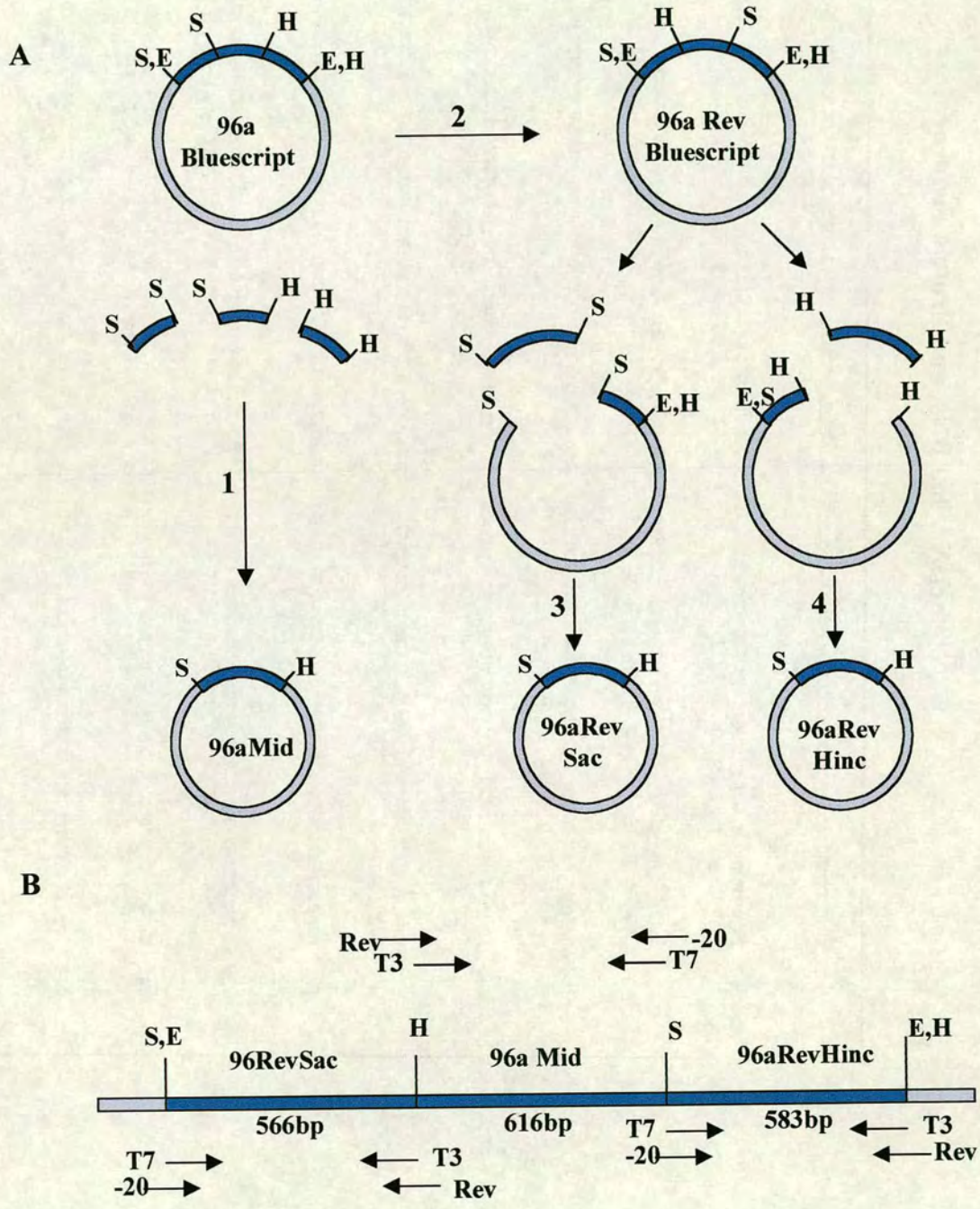
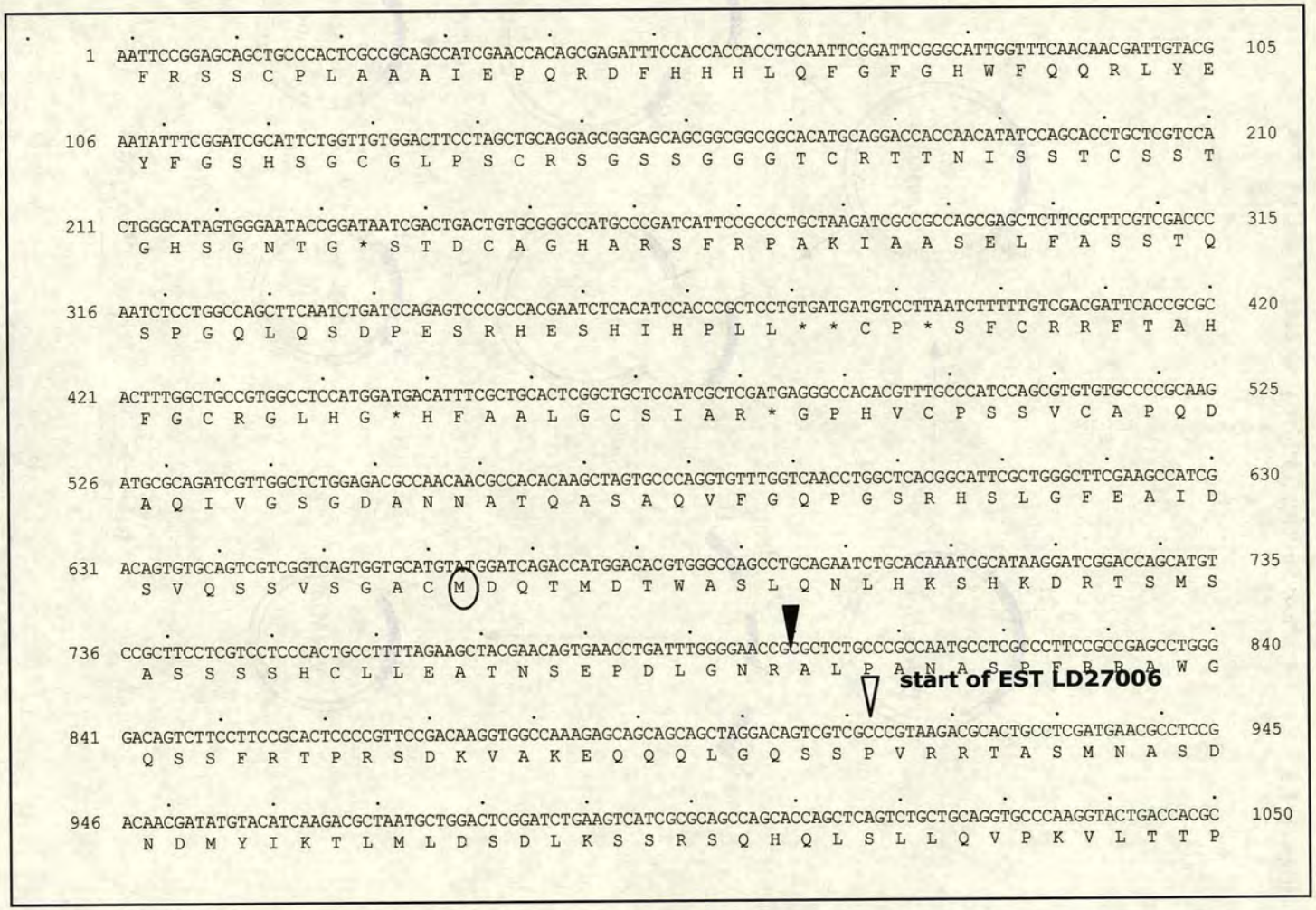
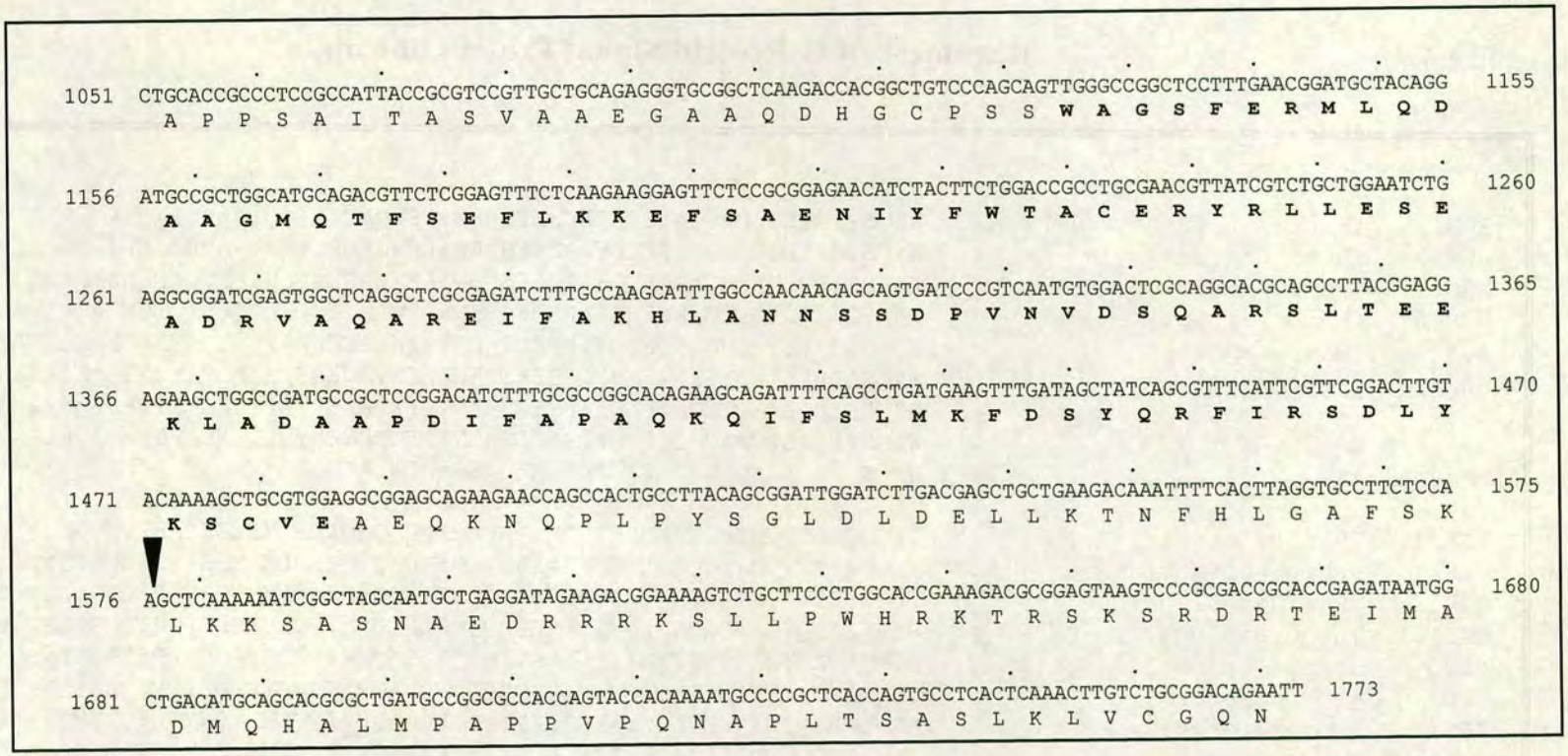


Fig 4.2

cDNA 96a DNA and Protein Sequence

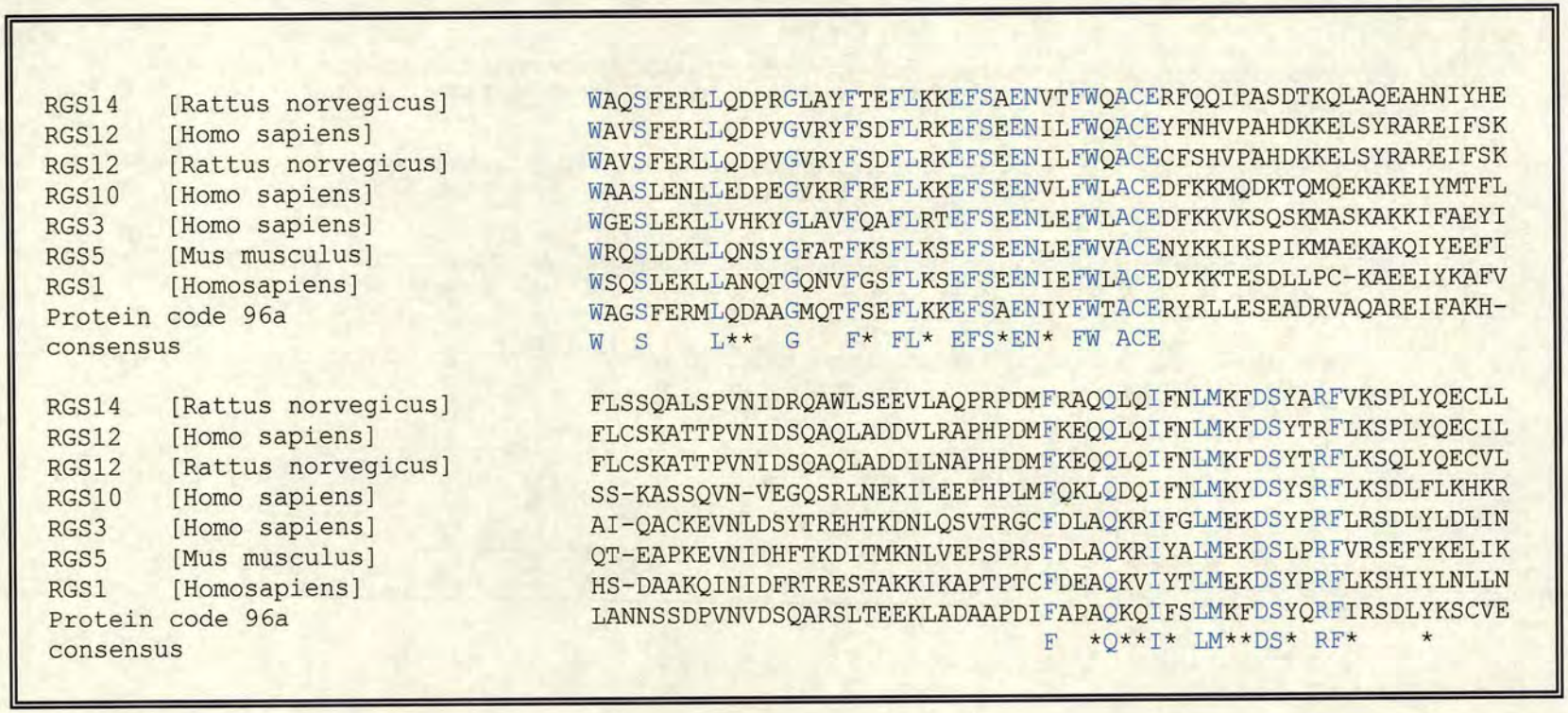




Legend 4.2

This is the complete sequence of cDNA 96a, with reading frame 1 below it. The first possible start methionine is at base 655. There is no stop codon in this reading frame suggesting that the 3′ end of the cDNA is incomplete. Triangles mark the sites of introns. The region that came up with a high degree of homology to RGS proteins is marked in bold.

Fig 4.3 **Regulator of G-Protein Signal Protein line up.**



Legend

This figure shows a protein line up between cDNA 96a and several mammalian RGS genes over the RGS functional domain. cDNA 96a exhibits a 51% identity to Rat RGS 12 at a protein level but, more importantly, there are key conserved amino acids (blue) across this domain, which cDNA 96a shares with other RGS Proteins.

seen that there are certain key amino acids shared by all RGS Proteins. These key amino acids are also present in the translation of cDNA 96a.

4.2.2 Subcloning and sequencing of cDNA 118a

118a is a 0.9Kb cDNA. It did not have suitable restriction sites for subcloning. Therefore Exo-mung deletion was used to generate small fragments. In this process, Exo III nuclease will attack the ends of DNA with a 3′ overhang. This can be done over a time course to generate the desired length of DNA fragment. The Mung bean nuclease was then used to blunt both ends of the DNA prior to ligation (Fig 4.4).

cDNA 118a was first reversed so that the same enzymes could be used in both directions. Both the cDNA 118a and 118aRev were subjected to a digest with SacI generating 3′ overhang and SpeI that generates 5′ overhang. The cDNAs were then digested with ExoIII nuclease over a time course. This was done for the construct in both orientations, resulting in two 550bp overlapping cDNA fragments 118aRev34 and 118a26, that were ready for sequencing (Fig 4.4).

The cDNAs generated were sequenced in the same manner as 96a, and the sequence compiled on *Gene-Jockey*. The sequence obtained was 895bp long and had a potential ORF in frame 2, that appeared to read through from the start (Fig 4.5). There was no start codon but a stop codon was present in reading frame 2 at nucleotide 701. At the time, this indicated that the 5′ end of the gene was probably missing, as well as the 3′UTR end (as there was no poly A tail to indicate the end). A database search was carried out but no sequence similarity to cDNA 118a was identified. Therefore there was no evidence to indicate that cDNA 118a coded for a protein.

Legend 4.4

Part A

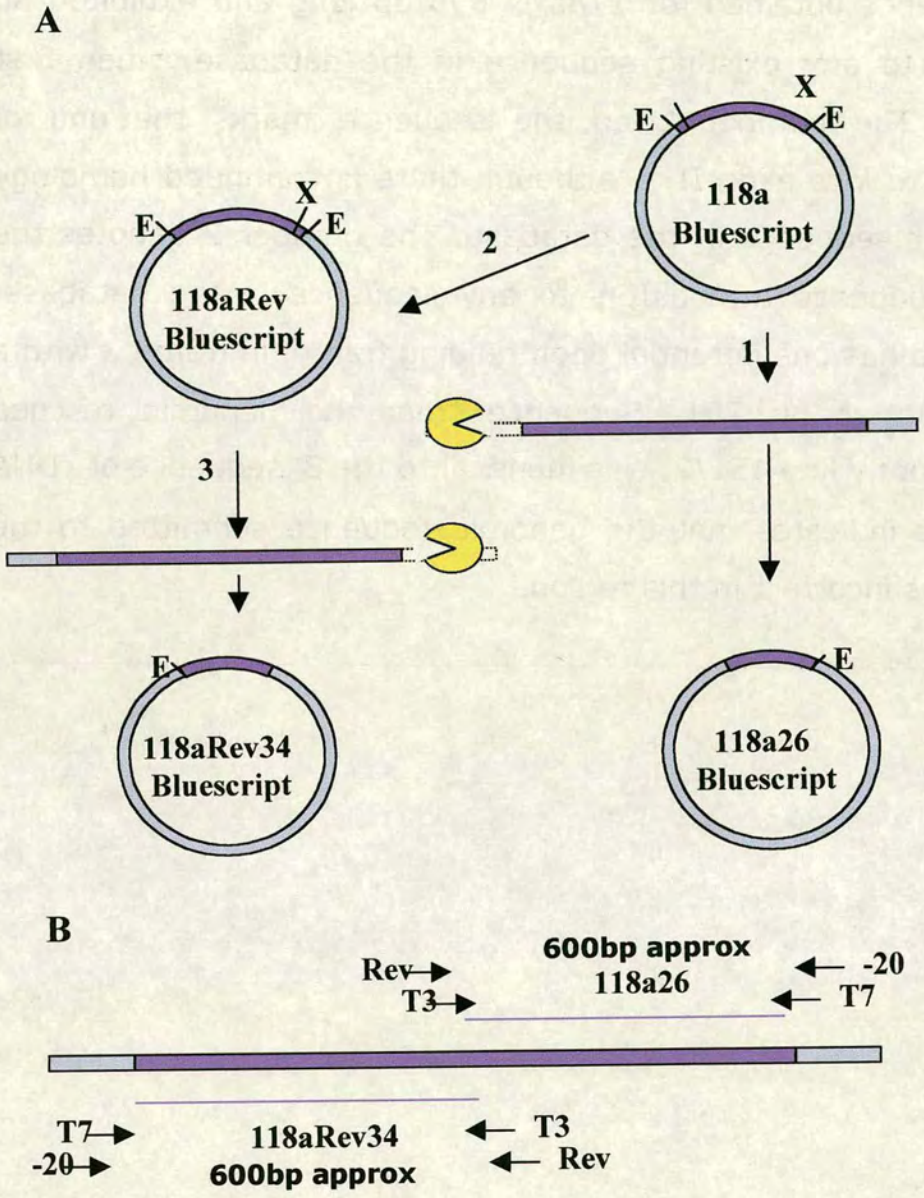
In 1, cDNA 118a was cut with SacI and SpeI and it was then digested with ExoIII Nuclease over a time course. The fractions from the time course were run on a gel and the appropriate size fragments were blunt ended with Mungbean nuclease and ligated. This was named 118a26, with a size of 600bp.

In 2 cDNA 118a was cut out with EcoRI and religated back into the same vector. It was then transformed into XL1 bluescript. The colonies obtained were screened by restriction digest for the insert in the reverse orientation. This construct was named 118Rev. In 3, Exo Mung Deletion was carried out in the same manner on 118Rev, to generate 118Rev34 which was approximately 600bp.

Part B

Part B shows how all the subcloned fragments fit together. They were all sequenced using Bluescript primer T3, T7, Reverse and -20. The sequence was then compiled in *Gene-Jockey* and checked for errors.

Figure 4.4 **Subcloneing and Sequencing of cDNA 118a**



Legend 4.5

The Sequence obtained for 118a is 895 bp long and exhibited no homology to any existing sequence in the database, when first obtained. The number 1 on the sequence marks the end of homology to *loco* exon II-1, although there is continued homology to genomic sequence in the database. The number 2 denotes the start of sequence that differs to any sequence in the database. cDNA 118a has one potential open reading frame, in frame 2 with a stop codon at bp 701. Sequence from the genomic rescued fragment of fly line 151/2, was identical to the 3′ sequence of cDNA 118a. This indicates that the genomic sequence submitted to the database is incorrect in this region.

Fig 4.5

cDNA 118a Complete Sequence

```
    1 CCGACGCAGTGCCTCACCGCAAATAACAATTGCACCGGAGACCGTGCAGGAGGAGAAAGTGTTGGTAAATCCACTGCCTAGGAAATGCGCAGAGGCAGAGGACCT  105
      R  R  S  A  S  P  Q  I  T  I  A  P  E  T  V  Q  E  E  K  V  L  V  N  P  L  P  R  K  C  A  E  A  E  D  L

  106 GATGCCACCACCCATGGCCATGGCACGTGTCCAGGCGGAACGACGCAGTTGCCGCAGTGCCTCCCGCGTGTTGCAGTTCTTCAGTTCGGCCACCAAAAGACGGAG  210
      M  P  P  P  M  A  M  A  R  V  Q  A  E  R  R  S  C  R  S  A  S  R  V  L  Q  F  F  S  S  A  T  K  R  R  S

  211 TGGCAGCCGGAAACTCAGTGAGCCAGAGATGGAGGATAATCGCGCCGATTCCCCAGTGCTGATCAGAGTGACCAGGGATCGGGTGGAGGAGCAACTGGGCTGTAG  315
      G  S  R  K  L  S  E  P  E  M  E  D  N  R  A  D  S  P  V  L  I  R  V  T  R  D  R  V  E  E  Q  L  G  C  S

  316 TCCACTGGCTGCATCTGGCGGAGAAGTACCCACCAGCTCCGAGGACGAGGAGGAGCACGACGACGGCATCAGCTCGGCCAGCTCCAATCTTACCTCGCAGTCGGG  420
      P  L  A  A  S  G  G  E  V  P  T  S  S  E  D  E  E  E  H  D  D  G  I  S  S  A  S  S  N  L  T  S  Q  S  G

  421 CAGCTCCAATAGCCGCAATCTCTCTCCGGACTCCTCCTTCGAGATGCACGCTCCACTCCTGCCCAGCTTCAAGGTCACGCCTCCACGGGCAGTTTGTCGTGTGGG  525
      S  S  N  S  R  N  L  S  P  D  S  S  F  E  M  H  A  P  L  L  P  S  F  K  V  T  P  P  R  A  V  C  R  V  G

  526 AAAGAATGCCGCCTGCGAGTTCGCTCGTTTCCTGCGCGGCTCCTTTCACTCCAAGCGCGCATCGGTGACCACGTTGCGCAGATCCCTCAGCGATCCGGATGCAGT  630
      K  N  A  A  C  E  F  A  R  F  L  R  G  S  F  H  S  K  R  A  S  V  T  T  L  R  R  S  L  S  D  P  D  A  V
                                                                                1
  631 GCAGCAGATGGACTTCAGCAAGCCGCCTCCGCTGCGCGACGCCACCAACGTCATGCGAGTGAGTCACTTCTAGATTCCATTCACAACCCAAGTGGGATTAATCGC  735
      Q  Q  M  D  F  S  K  P  P  P  L  R  D  A  T  N  V  M  R  V  S  H  F  *  I  P  F  T  T  Q  V  G  L  I  A

  736 GCCTGGGAGTACACTCAACAATATTTACGAAATTATTAAAATCTATCATCTAAAGCGTCAATAATAAGACTGCAATGCAAGTAATAGGTCAAGACTGAATAGATA  840
      P  G  S  T  L  N  N  I  Y  E  I  I  K  I  Y  H  L  K  R  Q  *  *  D  C  N  A  S  N  R  S  R  L  N  R  *
  2
  841 AGATAAATATAATCTTGAATGCTTATTTTAATACACTTAAGTTTTGAATATATGG  895
      D  K  Y  N  L  E  C  L  F  *  Y  T  *  V  L  N  I  W
```

4.3 ESTs and a Full Length Transcript

The poor quality of the library from which the cDNAs were isolated, posed a problem, as neither of the cDNAs were complete. Both lacked their 3′ ends (as no poly A tail was present) and there was no indication how complete the 5′ ends were. A full length cDNA should start with a guanine nucleotide, which is part of the post transcriptional modification a transcript undergoes; therefore this guanine nucleotide, should not be present in the genomic sequence. Although cDNA 96a starts with a guanine nucleotide there was no genomic sequence available to confirm that this represented the very 5′ of the cDNA.

To obtain full-length cDNAs, several approaches were adopted. A Northern was carried out, to try and establish the number and size of the transcripts present. Despite several attempts at detecting transcripts none were observed. Controls carried out with RP49 indicated that the method was correct, so it was deduced that the transcripts were very rare. Both 3′ and 5′Race were attempted, both of which failed. This could have been due to the rarity of the transcripts, or to the absence of a lot of the cDNA, making the RACE difficult. One of the few remaining options left would be to screen a different library. Unfortunately, another good ovarian cDNA library was not available and to screen one specific to another tissue or developmental stage would not have ensured isolation of the correct transcripts.

The solution lay in the *Drosophila* Genome Project, which had generated random Expressed Sequence Tags (ESTs) from several different tissues and developmental stages in *Drosophila*. When a screen was carried out with both cDNAs, one EST LD27006 (Acc No AA941661) was isolated from the *Drosophila* embryo library. This contained 719 bp of sequence that was 100% identical to cDNA 96a, starting at nucleotide 896. Thus, despite being from a different tissue, this clone was obtained and sequenced. RT-PCR could be done to establish whether it was present in the ovary. EST LD27006 was 2.6Kb, to help reduce time taken to sequence this cDNA, internal primers were designed to sequence inward from the ends of the EST. The EST LD27006 had its polyA tail intact, indicating that the 3′ end was complete. This

sequence was compiled with 96a to make a full-length cDNA (Fig 4.7), comprising a 5'UTR of 655bp followed by an ORF of 2618bp coding for a protein of 872 aa. The 3'UTR is 214 bp with the poly-adenylation site at nucleotide 3487. The complete cDNA 96a was later renamed *loco-c3* to fit nomenclature assigned to *loco* transcripts by Granderath *et al* (1999).

4.4 *loco* and its cDNAs

4.4.1 Details of transcript loco-c1

loco-c1 (Acc no AF130745) is a 2912 bp cDNA (Fig 4.8). I have subsequently identified another 117bp of 5'UTR sequence from EST SD01027 (Acc No AI534801) in the database. This suggests that there is a 5'UTR of at least 324bp, followed by an ORF of 2487bp, starting at an ATG in frame 1. The resultant Loco-c1 protein is 829aa long with an expected size of 91.2kDa. The 3'UTR is identical to the 3'UTR of cDNA 96a. Using genomic sequence submitted to the database, I have established the exact locations of the exon-intron boundaries of *loco-c1*. Its first exon I-1 is 327bp, with a start codon consisting of the last three nucleotides. Exon 2 is 680 nucleotides and contains the RGS domain, while exon 3 is 1142 nucleotides and exon 4 is 779 nucleotides.

4.4.2 Details of transcript loco-c2

loco-c2 (Acc no AF130744) is 3995 bp with a short 5'UTR of 142bp (Fig 4.9). A coding region of 3525bp results in a Loco-c2 protein of 1175aa, with an expected size of 129.3kDa. *Loco-c2* shares the same 3'UTR of *loco-c3* and *loco-c1*. There are 5 exons in *loco-c2*, exon II-0 being the first, which is 136 nucleotides. The second exon is exon II-1 which is 1046 nucleotides and contains the start codon at the very 5' of it. The remaining 3 exons (exon 2,3,and 4) are common to *loco-c1*.

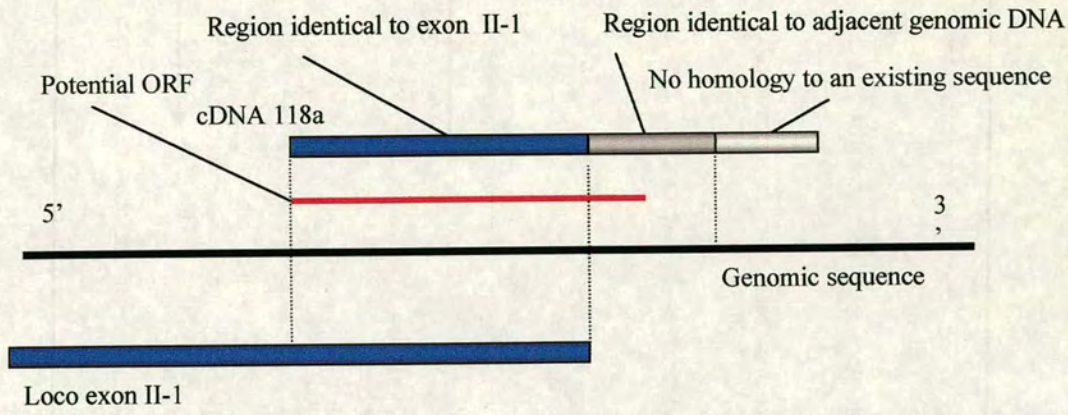
4.4.3 Details of transcript loco-c3

cDNA 96a or *loco-c3* (Fig 4.7) appears to share common exons 2,3,4, with *loco-c1* and *loco-c2*. It has a different 5′ exon of 786bp. There are 2 potential start methionine codons towards the end of exon III-1. The first of these potential start methionines (at nucleotide 655) is the correct one, as it has an adenine in the DNA sequence 4bp 5′ of the ATG which is followed immediately by a guanine (5′ A***ATGG 3′). Mutational analysis has shown that the position of the adenine and guanine in relation to the ATG are important (Kozak, 1996). Therefore *loco-c3* codes for a protein of 872aa with a predicted size of 95.9kDa. I submitted this sequence on 15/03/00 (Acc No AF245455).

4.4.4 Details of cDNA 118a

cDNA 118a was confusing as it lined up with *loco-c2* from nucleotide 492 to 1182, where exon II-1 ends, after which the sequence is different. When compared to genomic sequence in the database, the following 152bp line up with the genomic sequence, while the remaining 53bp was not similar to any existing sequence in the database, genomic or otherwise. This might indicate that cDNA 118a is co-ligated with another gene. However, when the Dundee fly line 151/2 was plasmid rescued and sequenced (Chapter 3), the sequence obtained from the genomic plasmid rescued fragment was identical to the latter half of cDNA 118a. As this is genomic sequence from an independent source to cDNA 118a, it confirms that this cDNA is not a co-ligation and is real. Therefore cDNA 118a is an independent cDNA to *loco-c2* and would definitely make a different transcript (Fig 4.6). However both the 3′ and 5′ end of this transcript are still missing.

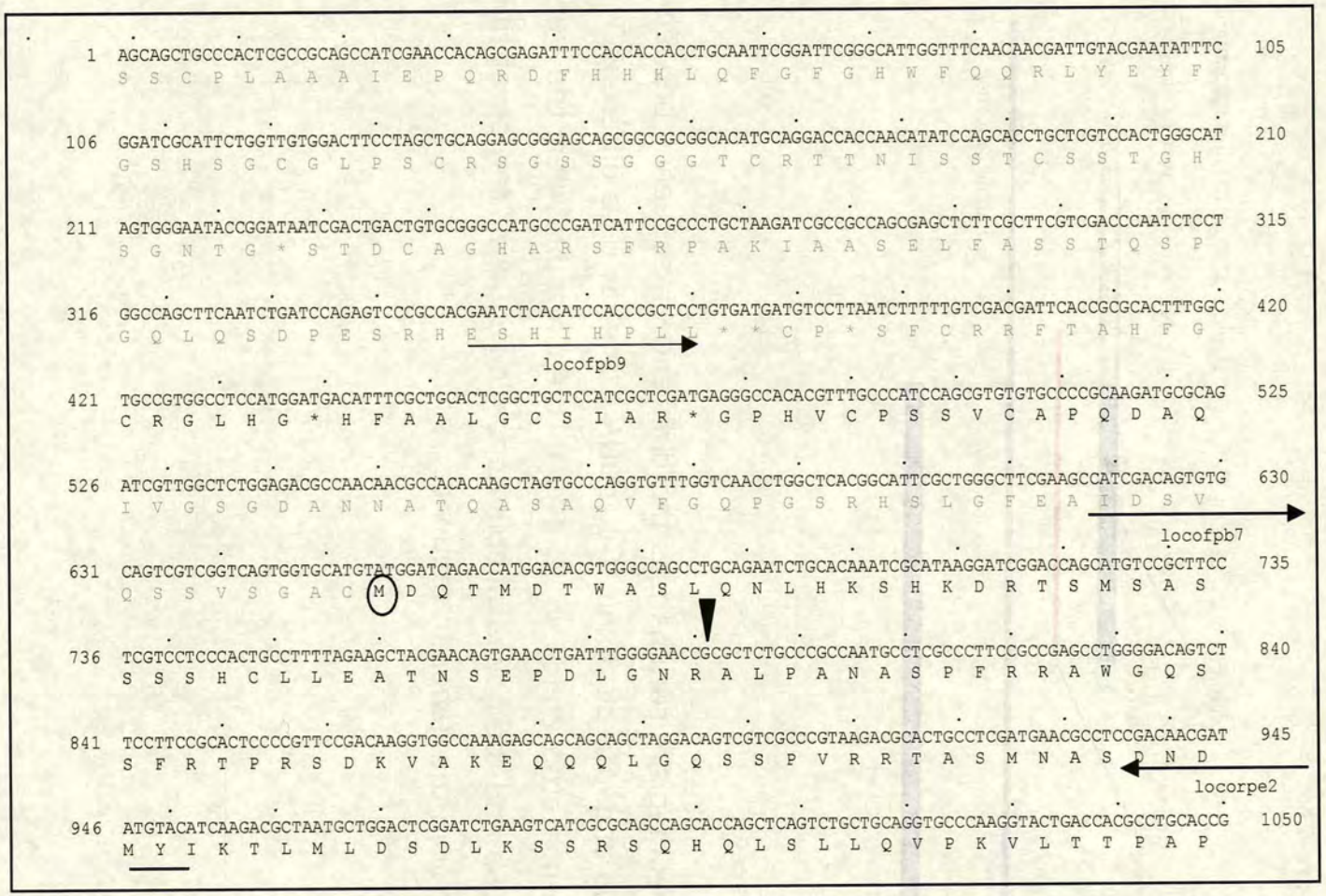
Figure 4.6

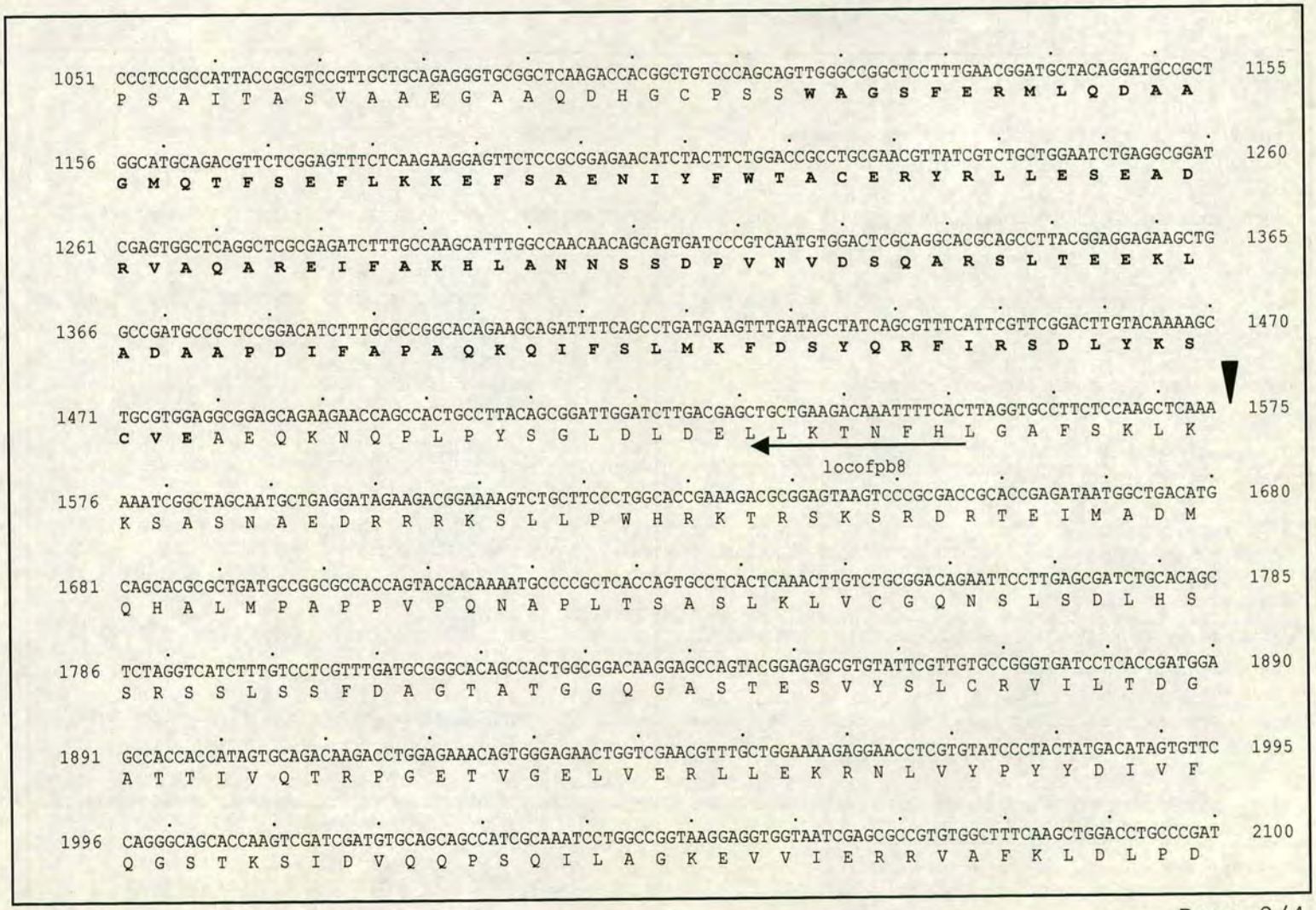


Legend

This diagram summarises the difference in sequence between cDNA 96a and *loco-c2,* Exon II-1. cDNA 118a having a different 3′ region, with a stop codon in it. The discrepancy between the very 3′ of 118a and the now available genomic sequence can be attributed to error in the genomic sequence.

Fig 4.7 **Complete cDNA 96a (*loco-c3*)**




1051 CCCTCCGCCATTACCGCGTCCGTTGCTGCAGAGGGTGCGGCTCAAGACCACGGCTGTCCCAGCAGTTGGGCCGGCTCCTTTGAACGGATGCTACAGGATGCCGCT 1155
P S A I T A S V A A E G A A Q D H G C P S S W A G S F E R M L Q D A A
1156 GGCATGCAGACGTTCTCGGAGTTTCTCAAGAAGGAGTTCTCCGCGGAGAACATCTACTTCTGGACCGCCTGCGAACGTTATCGTCTGCTGGAATCTGAGGCGGAT 1260
G M Q T F S E F L K K E F S A E N I Y F W T A C E R Y R L L E S E A D
1261 CGAGTGGCTCAGGCTCGCGAGATCTTTGCCAAGCATTTGGCCAACAACAGCAGTGATCCCGTCAATGTGGACTCGCAGGCACGCAGCCTTACGGAGGAGAAGCTG 1365
R V A Q A R E I F A K H L A N N S S D P V N V D S Q A R S L T E E K L
1366 GCCGATGCCGCTCCGGACATCTTTGCGCCGGCACAGAAGCAGATTTTCAGCCTGATGAAGTTTGATAGCTATCAGCGTTTCATTCGTTCGGACTTGTACAAAAGC 1470
A D A A P D I F A P A Q K Q I F S L M K F D S Y Q R F I R S D L Y K S
1471 TGCGTGGAGGCGGAGCAGAAGAACCAGCCACTGCCTTACAGCGGATTGGATCTTGACGAGCTGCTGAAGACAAATTTTCACTTAGGTGCCTTCTCCAAGCTCAAA 1575
C V E A E Q K N Q P L P Y S G L D L D E L L K T N F H L G A F S K L K
locofpb8
1576 AAATCGGCTAGCAATGCTGAGGATAGAAGACGGAAAAGTCTGCTTCCCTGGCACCGAAAGACGCGGAGTAAGTCCCGCGACCGCACCGAGATAATGGCTGACATG 1680
K S A S N A E D R R R K S L L P W H R K T R S K S R D R T E I M A D M
1681 CAGCACGCGCTGATGCCGGCGCCACCAGTACCACAAAATGCCCCGCTCACCAGTGCCTCACTCAAACTTGTCTGCGGACAGAATTCCTTGAGCGATCTGCACAGC 1785
Q H A L M P A P P V P Q N A P L T S A S L K L V C G Q N S L S D L H S
1786 TCTAGGTCATCTTTGTCCTCGTTTGATGCGGGCACAGCCACTGGCGGACAAGGAGCCAGTACGGAGAGCGTGTATTCGTTGTGCCGGGTGATCCTCACCGATGGA 1890
S R S S L S S F D A G T A T G G Q G A S T E S V Y S L C R V I L T D G
1891 GCCACCACCATAGTGCAGACAAGACCTGGAGAAACAGTGGGAGAACTGGTCGAACGTTTGCTGGAAAAGAGGAACCTCGTGTATCCCTACTATGACATAGTGTTC 1995
A T T I V Q T R P G E T V G E L V E R L L E K R N L V Y P Y Y D I V F
1996 CAGGGCAGCACCAAGTCGATCGATGTGCAGCAGCCATCGCAAATCCTGGCCGGTAAGGAGGTGGTAATCGAGCGCCGTGTGGCTTTCAAGCTGGACCTGCCCGAT 2100
Q G S T K S I D V Q Q P S Q I L A G K E V V I E R R V A F K L D L P D

```
2101  CCGAAGGTGATCTCGGTGAAGAGTAAACCCAAGAAGCAACTGCACGAGGTGATCCGACCAATACTCAGCAAGTACAACTACAAGATGGAGCAGGTGCAGGTGATC  2205
      P  K  V  I  S  V  K  S  K  P  K  K  Q  L  H  E  V  I  R  P  I  L  S  K  Y  N  Y  K  M  E  Q  V  Q  V  I

2206  ATGAGGGATACTCAAGTGCCAATCGACCTCAATCAGCCGGTTACCATGGCCGATGGCCAGCGACTGCGCATCGTGATGGTGAATTCGGATTTTCAGGTAGGCGGC  2310
      M  R  D  T  Q  V  P  I  D  L  N  Q  P  V  T  M  A  D  G  Q  R  L  R  I  V  M  V  N  S  D  F  Q  V  G  G

2311  GGCAGTAGCATGCCGCCGAAGCAAAGCAAACCTATGAAGCCACTGCCGCAGGGTCATTTGGATGAGCTAACGAACAAGGTGTTCAACGAGCTGCTGGCCAGCAAG  2415
      G  S  S  M  P  P  K  Q  S  K  P  M  K  P  L  P  Q  G  H  L  D  E  L  T  N  K  V  F  N  E  L  L  A  S  K

2416  GCTGATGCAGCGGCCAGCGAGAAGTCGCGGCCTGTCGATCTGTGCTCCATGAAGTCCAACGAGGCGCCCTCGGAGACATCATCGCTCTTTGAACGCATGCGGCGT  2520
      A  D  A  A  A  S  E  K  S  R  P  V  D  L  C  S  M  K  S  N  E  A  P  S  E  T  S  S  L  F  E  R  M  R  R

2521  CAGCAGCGCGATGGCGGCAACATTCCGGCTAGCAAGCTACCTAAGCTCAAGAAGAAGTCCACTAGCAGCAGCCAACAATCCGAGGAGGCAGCGACAACTCAAGCA  2625
      Q  Q  R  D  G  G  N  I  P  A  S  K  L  P  K  L  K  K  K  S  T  S  S  S  Q  Q  S  E  E  A  A  T  T  Q  A

2626  GTCGCGGATCCCAAGAAGCCAATTATAGCCAAGCTAAAAGCGGGTGTGAAGCTGCAGGTGACGGAGCGAGTAGCCGAGCACCAAGATGAACTACTCGAGGGCCTA  2730
      V  A  D  P  K  K  P  I  I  A  K  L  K  A  G  V  K  L  Q  V  T  E  R  V  A  E  H  Q  D  E  L  L  E  G  L

2731  AAGCGAGCACAGCTGGCACGACTGGAGGATCAGCGTGGCACCGAAATCAACTTCGACTTGCCTGATTTTCTGAAAAACAAGGAGAATCTCAGCGCAGCTGTATCA  2835
      K  R  A  Q  L  A  R  L  E  D  Q  R  G  T  E  I  N  F  D  L  P  D  F  L  K  N  K  E  N  L  S  A  A  V  S

2836  AAGCTGCGCAAGGTGCGCGCCAGCTTGAGTCCCGTGAGCAAGGTACCCGCCACGCCCACCGAAATTCCACAGCCGGCGCCACGTCTCTCCATCACACGTAGCCAA  2940
      K  L  R  K  V  R  A  S  L  S  P  V  S  K  V  P  A  T  P  T  E  I  P  Q  P  A  P  R  L  S  I  T  R  S  Q

2941  CAGCCGGTGTCGCCCATGAAGGTGGATCAGGAGCCAGAGACTGACTTGCCTGCCGCGACGCAGGATCAAACGGAATTCGCAAAAGCGCCGCCACCGCTGCCGCCA  3045
      Q  P  V  S  P  M  K  V  D  Q  E  P  E  T  D  L  P  A  A  T  Q  D  Q  T  E  F  A  K  A  P  P  P  L  P  P

3046  AAGCCAAAGGTGCTGCCCATCAAGCCTTCCAATTGGGGCGTGGCTCAGCCGACGGGCAACTATTGCAACAAGTACTCCCCCAGCAAACAGGTGCCAACATCACCC  3150
      K  P  K  V  L  P  I  K  P  S  N  W  G  V  A  Q  P  T  G  N  Y  C  N  K  Y  S  P  S  K  Q  V  P  T  S  P
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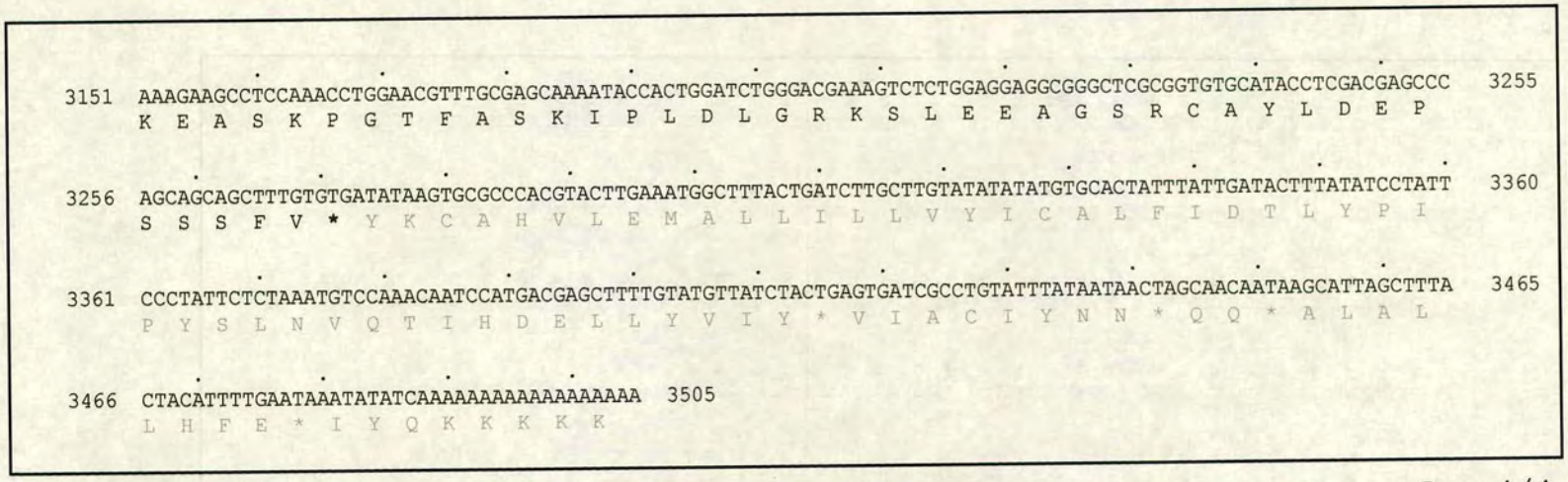
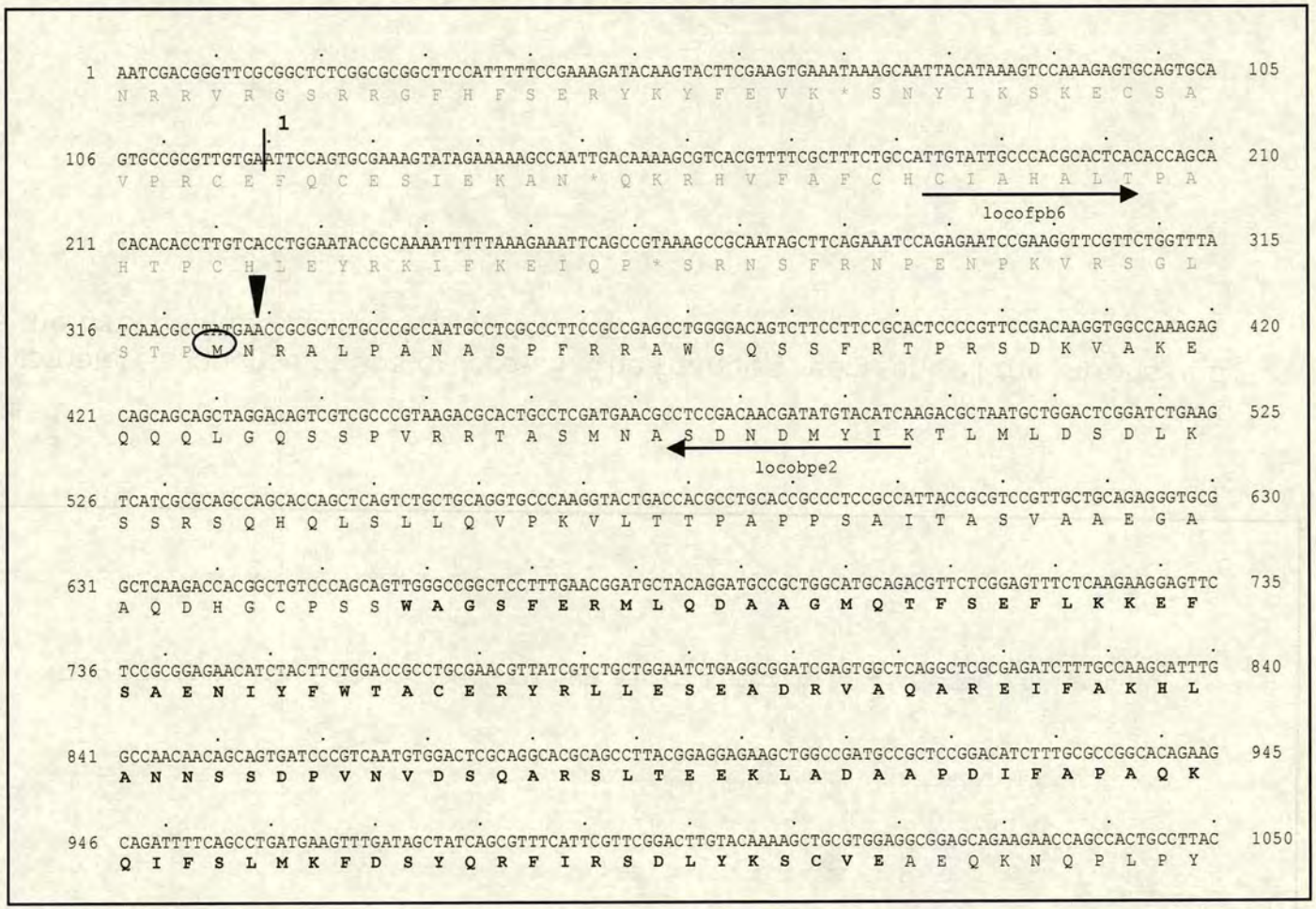
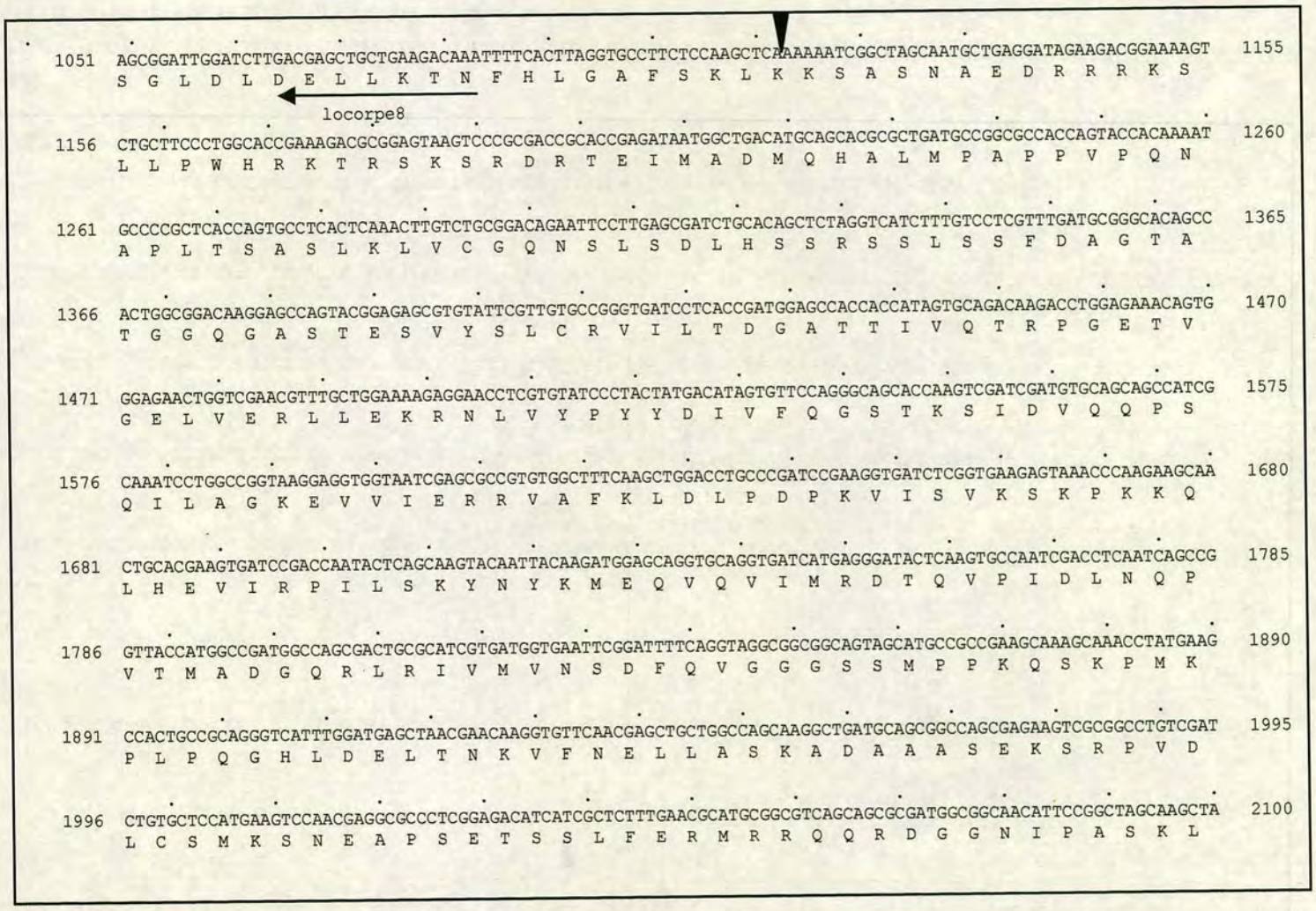


Legend 4.7
This is the complete sequence of 96a or *loco-c3*. The triangles represent all the introns and the arrows the direction and position of primers used in RT-PCR.

Fig 4.8 ***loco-c1* sequence**

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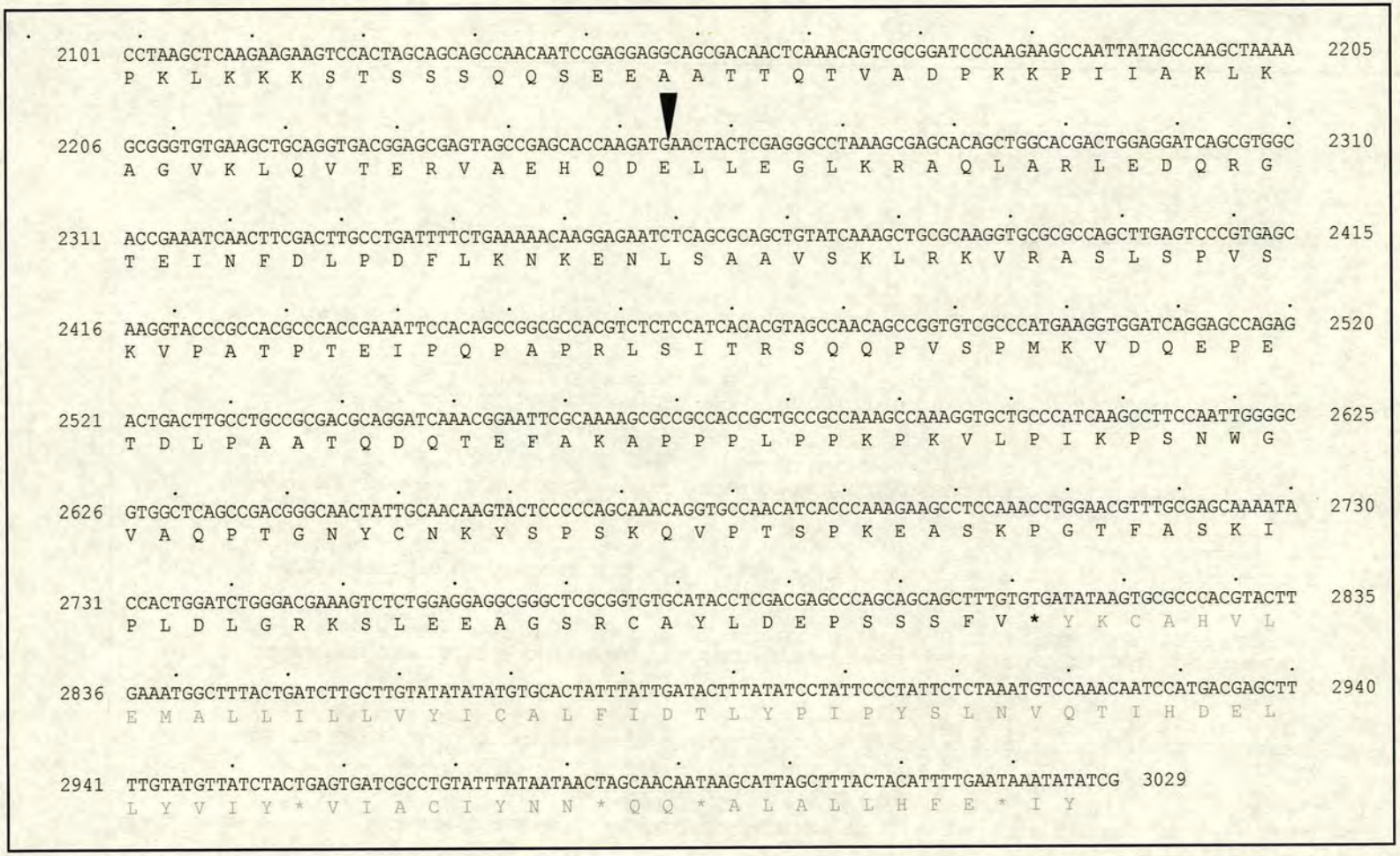
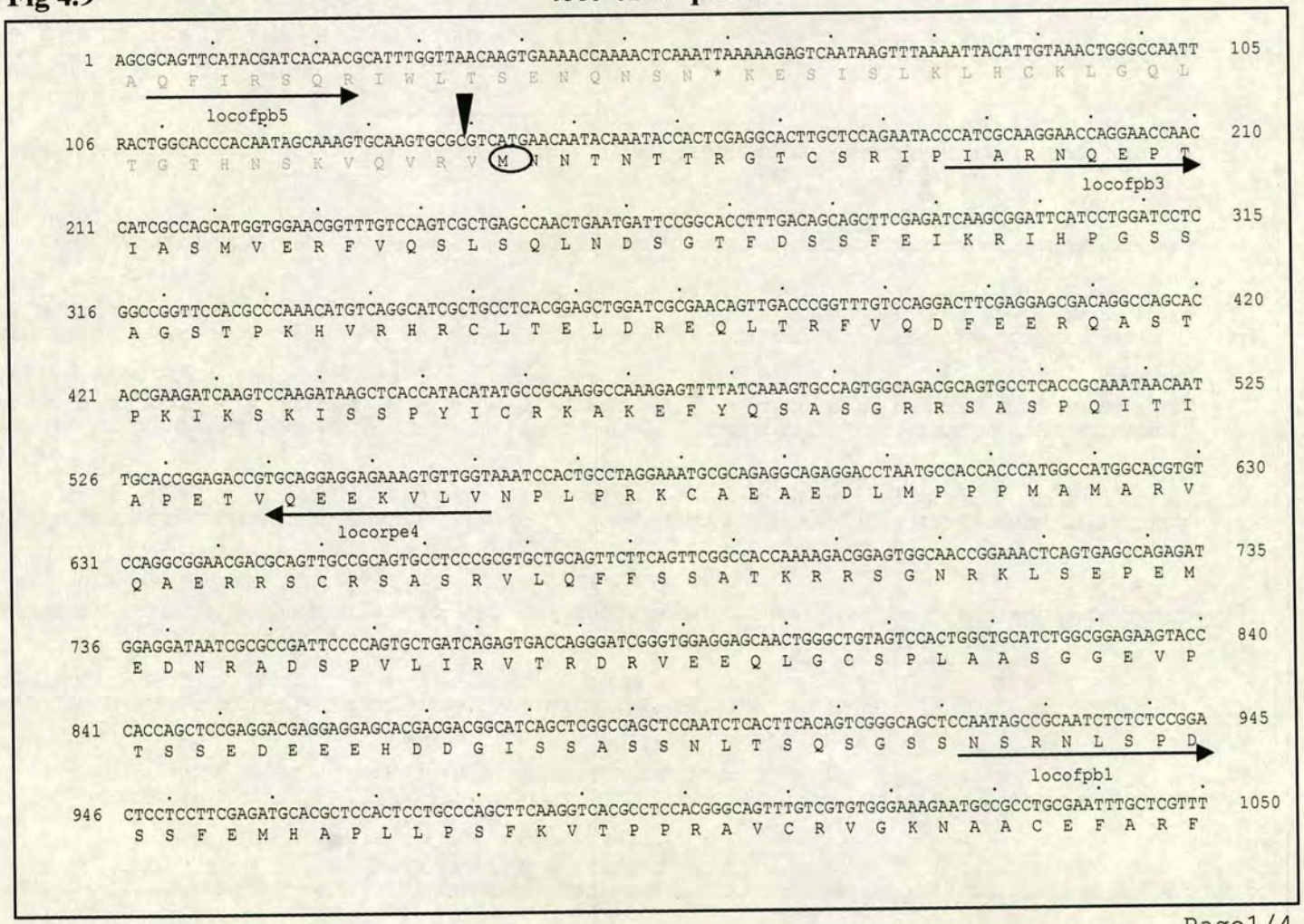
S G L D L D E L L K T N F H L G A F S K L K K S A S N A E D R R R K S
locorpe8
1156 CTGCTTCCCTGGCACCGAAAGACGCGGAGTAAGTCCCGCGACCGCACCGAGATAATGGCTGACATGCAGCACGCGCTGATGCCGGCGCCACCAGTACCACAAAAT 1260
L L P W H R K T R S K S R D R T E I M A D M Q H A L M P A P P V P Q N
1261 GCCCCGCTCACCAGTGCCTCACTCAAACTTGTCTGCGGACAGAATTCCTTGAGCGATCTGCACAGCTCTAGGTCATCTTTGTCCTCGTTTGATGCGGGCACAGCC 1365
A P L T S A S L K L V C G Q N S L S D L H S S R S S L S S F D A G T A
1366 ACTGGCGGACAAGGAGCCAGTACGGAGAGCGTGTATTCGTTGTGCCGGGTGATCCTCACCGATGGAGCCACCACCATAGTGCAGACAAGACCTGGAGAAACAGTG 1470
T G G Q G A S T E S V Y S L C R V I L T D G A T T I V Q T R P G E T V
1471 GGAGAACTGGTCGAACGTTTGCTGGAAAAGAGGAACCTCGTGTATCCCTACTATGACATAGTGTTCCAGGGCAGCACCAAGTCGATCGATGTGCAGCAGCCATCG 1575
G E L V E R L L E K R N L V Y P Y Y D I V F Q G S T K S I D V Q Q P S
1576 CAAATCCTGGCCGGTAAGGAGGTGGTAATCGAGCGCCGTGTGGCTTTCAAGCTGGACCTGCCCGATCCGAAGGTGATCTCGGTGAAGAGTAAACCCAAGAAGCAA 1680
Q I L A G K E V V I E R R V A F K L D L P D P K V I S V K S K P K K Q
1681 CTGCACGAAGTGATCCGACCAATACTCAGCAAGTACAATTACAAGATGGAGCAGGTGCAGGTGATCATGAGGGATACTCAAGTGCCAATCGACCTCAATCAGCCG 1785
L H E V I R P I L S K Y N Y K M E Q V Q V I M R D T Q V P I D L N Q P
1786 GTTACCATGGCCGATGGCCAGCGACTGCGCATCGTGATGGTGAATTCGGATTTTCAGGTAGGCGGCGGCAGTAGCATGCCGCCGAAGCAAAGCAAACCTATGAAG 1890
V T M A D G Q R L R I V M V N S D F Q V G G G S S M P P K Q S K P M K
1891 CCACTGCCGCAGGGTCATTTGGATGAGCTAACGAACAAGGTGTTCAACGAGCTGCTGGCCAGCAAGGCTGATGCAGCGGCCAGCGAGAAGTCGCGGCCTGTCGAT 1995
P L P Q G H L D E L T N K V F N E L L A S K A D A A A S E K S R P V D
1996 CTGTGCTCCATGAAGTCCAACGAGGCGCCCTCGGAGACATCATCGCTCTTTGAACGCATGCGGCGTCAGCAGCGCGATGGCGGCAACATTCCGGCTAGCAAGCTA 2100
L C S M K S N E A P S E T S S L F E R M R R Q Q R D G G N I P A S K L

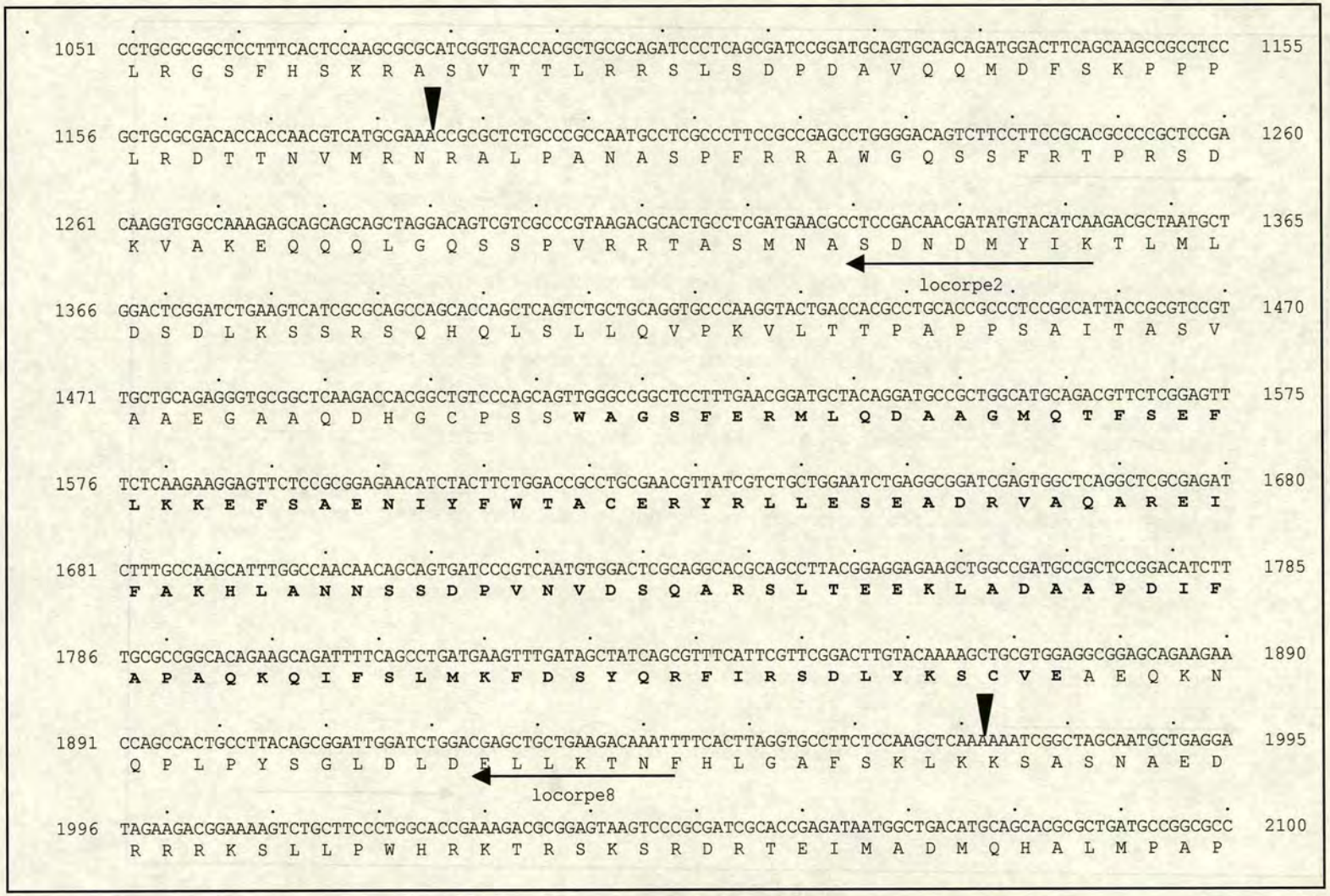


Legend 4.8

This is the complete sequence of *loco-c1* Acc no AF130745. The triangles represent all the introns and the arrows the direction and position of primers used in RT-PCR.

Fig 4.9 ***loco-c2* Sequence**

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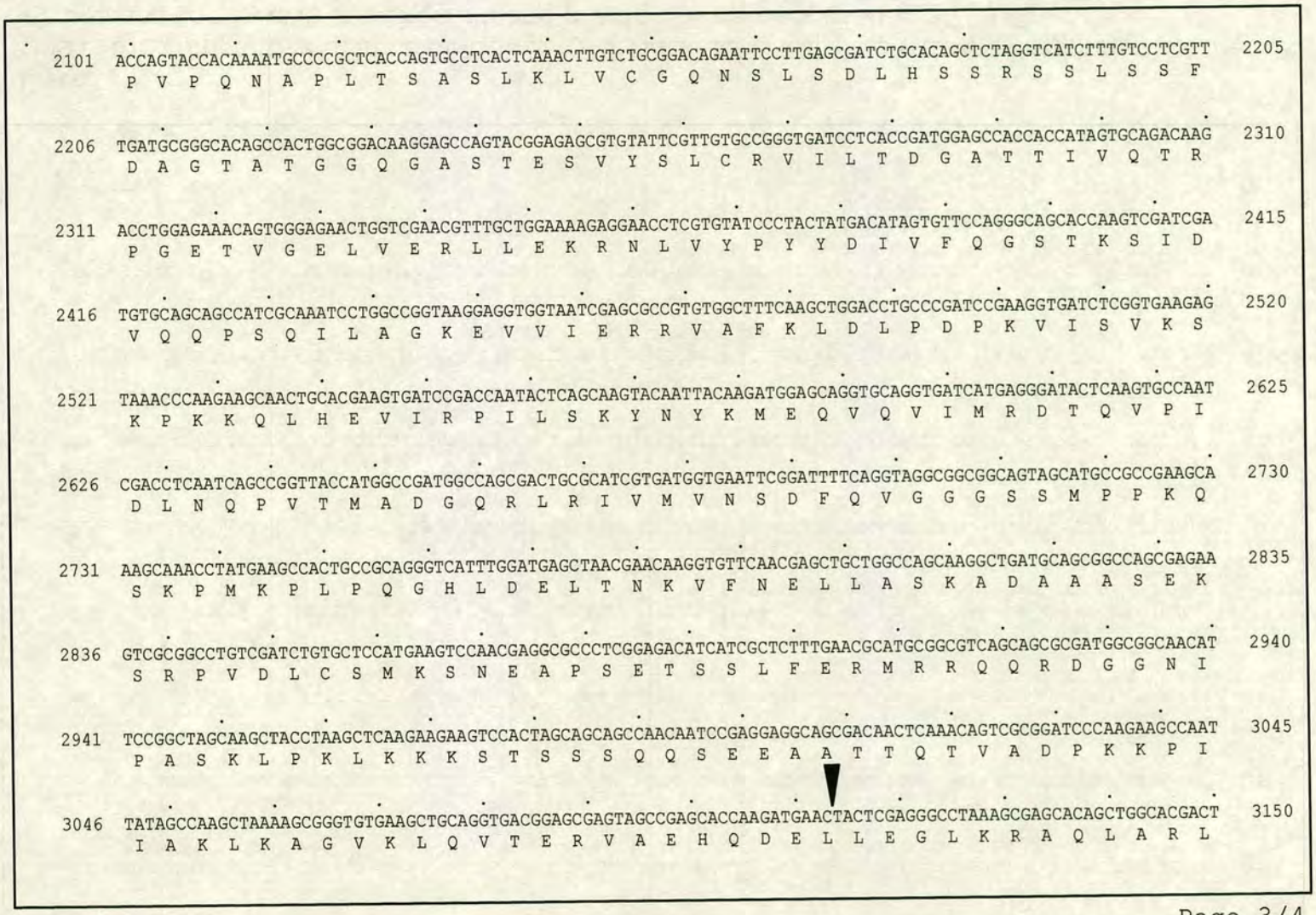
1156 GCTGCGCGACACCACCAACGTCATGCGAAACCGCGCTCTGCCCGCCAATGCCTCGCCCTTCCGCCGAGCCTGGGGACAGTCTTCCTTCCGCACGCCCCGCTCCGA 1260
L R D T T N V M R N R A L P A N A S P F R R A W G Q S S F R T P R S D
1261 CAAGGTGGCCAAAGAGCAGCAGCAGCTAGGACAGTCGTCGCCCGTAAGACGCACTGCCTCGATGAACGCCTCCGACAACGATATGTACATCAAGACGCTAATGCT 1365
K V A K E Q Q Q L G Q S S P V R R T A S M N A S D N D M Y I K T L M L
locorpe2
1366 GGACTCGGATCTGAAGTCATCGCGCAGCCAGCACCAGCTCAGTCTGCTGCAGGTGCCCAAGGTACTGACCACGCCTGCACCGCCCTCCGCCATTACCGCGTCCGT 1470
D S D L K S S R S Q H Q L S L L Q V P K V L T T P A P P S A I T A S V
1471 TGCTGCAGAGGGTGCGGCTCAAGACCACGGCTGTCCCAGCAGTTGGGCCGGCTCCTTTGAACGGATGCTACAGGATGCCGCTGGCATGCAGACGTTCTCGGAGTT 1575
A A E G A A Q D H G C P S S W A G S F E R M L Q D A A G M Q T F S E F
1576 TCTCAAGAAGGAGTTCTCCGCGGAGAACATCTACTTCTGGACCGCCTGCGAACGTTATCGTCTGCTGGAATCTGAGGCGGATCGAGTGGCTCAGGCTCGCGAGAT 1680
L K K E F S A E N I Y F W T A C E R Y R L L E S E A D R V A Q A R E I
1681 CTTTGCCAAGCATTTGGCCAACAACAGCAGTGATCCCGTCAATGTGGACTCGCAGGCACGCAGCCTTACGGAGGAGAAGCTGGCCGATGCCGCTCCGGACATCTT 1785
F A K H L A N N S S D P V N V D S Q A R S L T E E K L A D A A P D I F
1786 TGCGCCGGCACAGAAGCAGATTTTCAGCCTGATGAAGTTTGATAGCTATCAGCGTTTCATTCGTTCGGACTTGTACAAAAGCTGCGTGGAGGCGGAGCAGAAGAA 1890
A P A Q K Q I F S L M K F D S Y Q R F I R S D L Y K S C V E A E Q K N
1891 CCAGCCACTGCCTTACAGCGGATTGGATCTGGACGAGCTGCTGAAGACAAATTTTCACTTAGGTGCCTTCTCCAAGCTCAAAAAATCGGCTAGCAATGCTGAGGA 1995
Q P L P Y S G L D L D E L L K T N F H L G A F S K L K K S A S N A E D
locorpe8
1996 TAGAAGACGGAAAAGTCTGCTTCCCTGGCACCGAAAGACGCGGAGTAAGTCCCGCGATCGCACCGAGATAATGGCTGACATGCAGCACGCGCTGATGCCGGCGCC 2100
R R R K S L L P W H R K T R S K S R D R T E I M A D M Q H A L M P A P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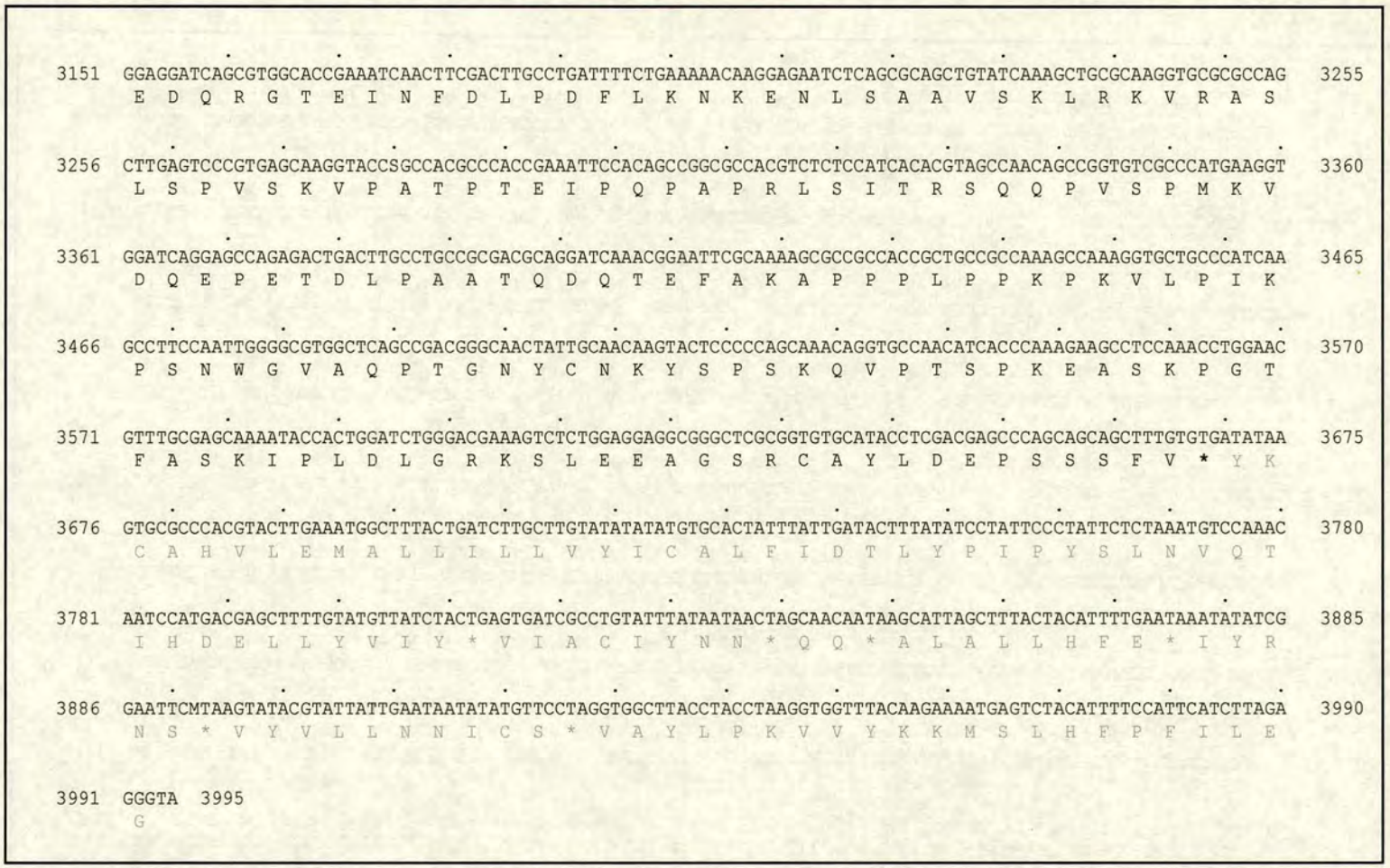
D A G T A T G G Q G A S T E S V Y S L C R V I L T D G A T T I V Q T R
2311 ACCTGGAGAAACAGTGGGAGAACTGGTCGAACGTTTGCTGGAAAAGAGGAACCTCGTGTATCCCTACTATGACATAGTGTTCCAGGGCAGCACCAAGTCGATCGA 2415
P G E T V G E L V E R L L E K R N L V Y P Y Y D I V F Q G S T K S I D
2416 TGTGCAGCAGCCATCGCAAATCCTGGCCGGTAAGGAGGTGGTAATCGAGCGCCGTGTGGCTTTCAAGCTGGACCTGCCCGATCCGAAGGTGATCTCGGTGAAGAG 2520
V Q Q P S Q I L A G K E V V I E R R V A F K L D L P D P K V I S V K S
2521 TAAACCCAAGAAGCAACTGCACGAAGTGATCCGACCAATACTCAGCAAGTACAATTACAAGATGGAGCAGGTGCAGGTGATCATGAGGGATACTCAAGTGCCAAT 2625
K P K K Q L H E V I R P I L S K Y N Y K M E Q V Q V I M R D T Q V P I
2626 CGACCTCAATCAGCCGGTTACCATGGCCGATGGCCAGCGACTGCGCATCGTGATGGTGAATTCGGATTTTCAGGTAGGCGGCGGCAGTAGCATGCCGCCGAAGCA 2730
D L N Q P V T M A D G Q R L R I V M V N S D F Q V G G G S S M P P K Q
2731 AAGCAAACCTATGAAGCCACTGCCGCAGGGTCATTTGGATGAGCTAACGAACAAGGTGTTCAACGAGCTGCTGGCCAGCAAGGCTGATGCAGCGGCCAGCGAGAA 2835
S K P M K P L P Q G H L D E L T N K V F N E L L A S K A D A A A S E K
2836 GTCGCGGCCTGTCGATCTGTGCTCCATGAAGTCCAACGAGGCGCCCTCGGAGACATCATCGCTCTTTGAACGCATGCGGCGTCAGCAGCGCGATGGCGGCAACAT 2940
S R P V D L C S M K S N E A P S E T S S L F E R M R R Q Q R D G G N I
2941 TCCGGCTAGCAAGCTACCTAAGCTCAAGAAGAAGTCCACTAGCAGCAGCCAACAATCCGAGGAGGCAGCGACAACTCAAACAGTCGCGGATCCCAAGAAGCCAAT 3045
P A S K L P K L K K K S T S S S Q Q S E E A A T T Q T V A D P K K P I
3046 TATAGCCAAGCTAAAAGCGGGTGTGAAGCTGCAGGTGACGGAGCGAGTAGCCGAGCACCAAGATGAACTACTCGAGGGCCTAAAGCGAGCACAGCTGGCACGACT 3150
I A K L K A G V K L Q V T E R V A E H Q D E L L E G L K R A Q L A R L



Legend 4.9

This is the complete sequence of *loco-c2* (Acc no AF130744). The triangles represent all the introns and the arrows the direction and position of primers used in RT-PCR.

4.5 RT-PCR and ovarian transcripts

4.5.1 loco-c3

Having constructed *loco-c3* from EST 27006 and cDNA 96a, it was important to establish whether *loco-c3,* was an ovarian transcript, as EST LD27006 was from an embryonic library. At the time of doing this work the complete genomic sequence for the region was not all available and was limited to sequence spanning exons 2,3 and 4, indicating that the size of the two introns separating them was 70bp each. I therefore also aimed to identify the size of the intron between exons III-1 and 2 by genomic PCR.

The RT-PCR between exon III-1 and 2(Primers 7-2) was successful and produced a band of approximately 300bp, confirming that 96a was a real ovarian transcript (Fig 4.10A lane1). The genomic PCR initially failed, as I had assumed that exon III-1 was situated in close proximity to exon 2, based on mapping of cDNAs carried out in Chapter 3. It was only after much deliberation and re-analysis of the mapping data, that I realised that exon III-1 was 5′ of exon II-0, which implied a minimum intron size of 12Kb. A genomic PCR was conducted with primers 9 and 4, resulting in a PCR product of 7kb (Fig 4.10,A lane 2), confirming that the intron between the first and second exon of transcript *loco-c3* was 15Kb.

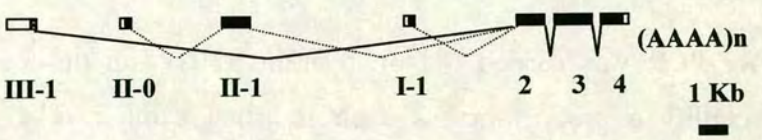
4.5.2 loco-c1

RT-PCR was carried out with primers 6 and 2, which should amplify across exon I-1 and exon 2. The RT-PCR produced a product of approximately 300bp (Fig 4.10,B lane 1), indicating that *loco-c1* was present in the ovaries. The genomic PCR with the same primers produced a product of approximately 3.5kb (Fig 4.10,B lane 2) positioning exon I-1 3.5Kb 5′ of exon 2. The genomic PCR also confirmed that the RT-PCR was genuine and not due to genomic contamination.

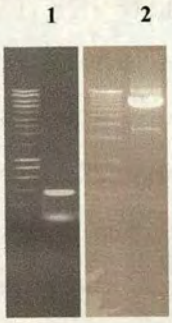
Legend 4.10

This figure summarises the RT-PCR and PCR carried out to establish the transcripts present in oogenesis and the sizes of the major introns. The transcripts are drawn with their exons as bars and introns as v shaped lines. The primer names (1-9)(full names for these primers are in Chapter2), positions and direction, are marked by arrows above the appropriate exons. The figure is divided into parts A, B and C for the three different transcripts tested, the data in the gel pictures being interpreted in the adjacent table. The expected size of product is from the sequence now available in the database (http://www3.ncbi.nlm.nih.gov/)(this was not available when these RT-PCRs and genomic PCR were conducted).

Figure 4.10 **Ovarian RT-PCR and Genomic PCR**

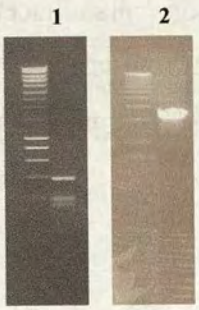


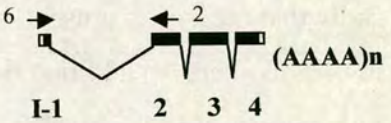
A ***loco-c3***



LANE No	RT-PCR/ G-PCR	EXPECTED SIZE Bp	OBSERVED SIZE	COMMENT
1	RT 7-2	340	400	Correct
2	G-PCR 9-4	7063	7000	Correct

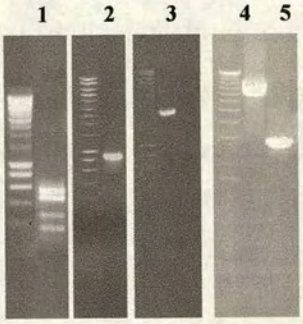
B ***loco-c1***





LANE No	RT-PCR/ G-PCR	EXPECTED SIZE Bp	OBSERVED SIZE	COMMENT
1	RT 6-2	310	300	Extra Bands
2	G-PCR 6-2	3707	3500	Correct

C ***loco-c2***



LANE No	RT-PCR/ G-PCR	EXPECTED SIZE Bp	OBSERVED SIZE	COMMENT
1	RT 5-4	552	500	Extra bands
2	RT 1-8	1010	1000	Correct
3	RT 5-8	1935	2000	Correct
4	G-PCR 1-2	7072	7000	Correct
5	G-PCR 5-4	2837	3000	Correct

4.5.3 loco-c2

RT-PCR was carried out on ovarian RNA with primers 1 and 8, which should amplify a product across exon II-1 and exon 2. The correct size product was obtained: approximately 1Kb (Fig 4.10,C lane 2). A comparable genomic PCR was carried out with primers 1 and 2, generating a product of approximately 7Kb (Fig 4.10,C lane 4). This confirms the position of exon II-1 relative to exon 2 and indicates that a transcript containing exon II-1 and exon 2 in succession, is present in the ovaries. An RT-PCR was carried out with primers 5 and 4, which should amplify across exons II-0 and II-1. This was successful producing a product of 500 bp (Fig 4.10,C lane 1). The same PCR was conducted with genomic DNA and produced a product of 2.8Kb, positioning exon 11-0 2.8Kb 5′ of exon II-1 (Fig 4.10,C lane 4). The RT-PCR reactions carried out with primer pairs 5-4 and 1-8, do not conclusively show that *loco-c2* is present, only that transcripts exist with these exon pairs adjacent to one another. An additional RT-PCR was carried out to ensure that transcript *loco-c2* was present in the ovaries, with primers 5 and 8 amplifying a product from across exon II-0 to exon 2. This produced a product of 2kb (Fig 4.10,C lane3), showing conclusively that *loco-c2* is indeed present in the ovaries.

4.5.4 cDNA 118a

It was difficult to test for the presence of cDNA 118a, as it is only a partial cDNA. The 3′ end of cDNA 118a has a stop codon in the same reading frame as *loco-c2*. This indicates that the 3′ coding region is complete, but there is no indication how much 3′UTR is missing. RT-PCR with primer 1 and 2 did not produce bands other than the band expected for *loco-c2*. Although it is not possible to establish what other exons are part of cDNA 118a, it does represent part of an ovarian cDNA, having been isolated from an ovarian library.

4.6 Is there more to *loco* than four transcripts?

The initial cDNA screen by Dr D Zhao, produced 2 transcripts of *loco*. Work done by Granderath *et al* (1999) produced 2 further transcripts. The presence of all 4 transcripts has been confirmed within the ovaries. Genomic PCR and mapping has shown that the gene is large spanning 20+Kb. The size and current complexity indicates that there are probably further transcripts to be identified. LacZ staining on the enhancer trapped gal^4 line C139 shows that in addition to an ovarian expression pattern, staining is also observed in the eye imaginal disc and the testis. This all indicate that *loco* plays an important developmental role being expressed in several tissues, and increases the likelihood of other as yet unidentified transcripts being present.

With the availability of Genomic sequence for the region surrounding *loco*, all intron sequence and sequence 5′ and 3′ of the existing transcripts could be used to search the EST database for further *loco* transcripts.

Six ESTs were identified that had 5′ sequence identical to *loco-c1*, LP04511 (Acc no AI260586), GH08607 (Acc no AI109417), SD08637 (Acc no AI542348), SD07479 (Acc no AI534578), SD01027 (Acc no AI534801) and SD07817 (Acc no AI541867) (Fig 4.11). These ESTs were identified in Drosophila Schneider cell culture (SD), Larval tissue (LP) and Drosophila adult head (GH). The sequence was identical to Exons I-1 and 2, indicating that these ESTs probably represent *loco-c1*.

A cDNA clone bs07a01.y1 (Acc no AI 944917), was identified that aligned with genomic sequence in the intron between exon II-1 and I-1 (Fig 4.11). This was isolated from a testis library and may represent part of a testis specific transcript.

Five ESTs were identified, LD24778 (Acc no AA820862), LD39937 (Acc no AI520016), SD01202 (Acc no AI534916), LD16240 (Acc no AA441343) and LD38168 (Acc no AI518578), all with identical sequence (Fig 4.11). These 5 ESTs mapped to a region 3.0Kb 5′ of exon III-1. When sequence from these ESTs was used in a blast search for protein homology, they were found to exhibit a 54%

Figure 4.11 Legend

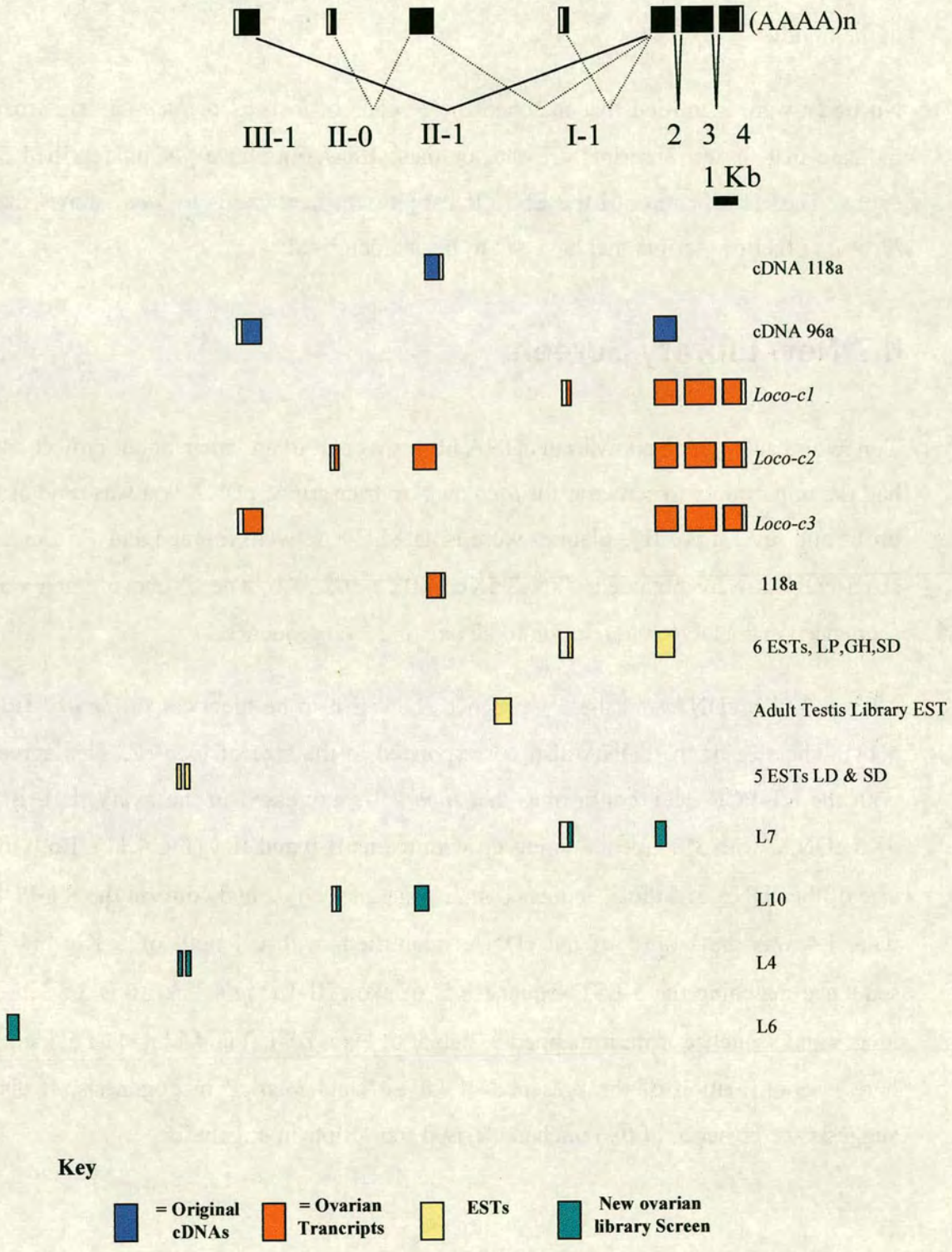
This figure summarises what is known about *loco* and its transcripts. At the top of figure are the established exons and introns (with exons as bars and the introns as lines, and ORF shaded in black), with which all information below is aligned.

The two original cDNAs, 96a and 118a, are marked in blue. Below are the 4 transcripts marked in red: *loco-c1*, *loco-c2*, *loco-c3* and 118a.

Marked in yellow are the ESTs identified in the region. 6 ESTs are identical to *loco-c1* and are found in larval (LP) tissues, *Drosophila* head (GH) and *Drosophila* cell culture (SD). One EST was identified in a *Drosophila* adult testis library and is in the intron between exon II-1 and I-1. 5 ESTs with identical sequence were positioned 3 kb 5′ of exon II-1. These were found in embryonic tissue (LD) and *Drosophila* cell culture (SD).

The new ovarian library screen resulted in the identification of 4 different sized transcripts. The 5′ end of each different sized transcript was sequenced and is marked in green. L7 was a 3Kb cDNA with 5′ sequence identical to *loco-c1*. L10 was a 4Kb cDNA with sequence identical to *loco-c2*. L4 was a 5Kb transcript with sequence identical to 5 ESTs positioned 5′ of exon II-1. L6 is new and the sequence maps 10Kb 5′ of exon III-1.

Figure 4.11 Summary of *loco* transcripts ESTs and Library Screens



identity to Rat and Human RGS proteins. This is clear evidence that these ESTs are part of the *loco* gene. The EST sequence obtained also appeared to have a splice site in the middle.

No ESTs were identified that matched the 5′ ends of *loco-c2* or *loco-c3*. This may indicate that these transcripts are rare or these transcripts have yet unidentified 5' exons. The identification of the ESTs that represent new exons in *loco*, shows that *loco* has other transcripts that have yet to be characterised.

4.7 New Library Screen

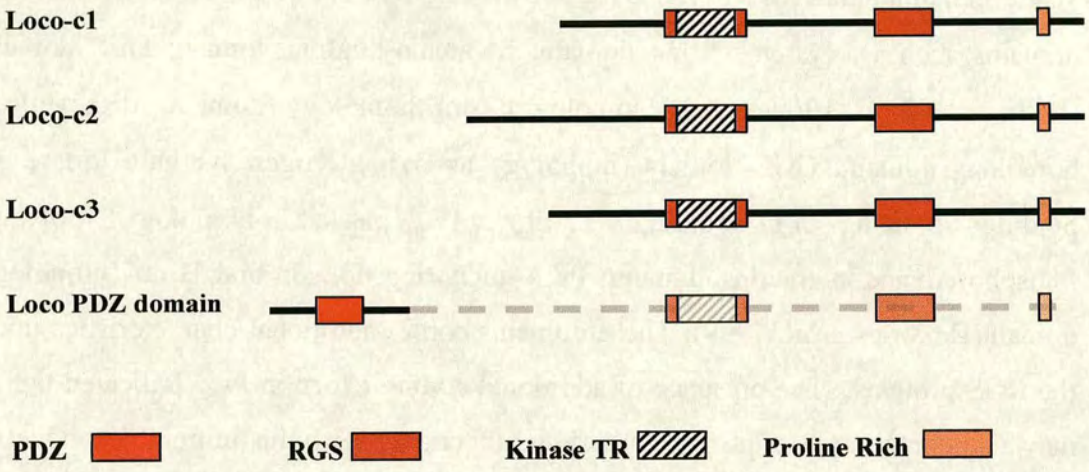
Tim Wood and I made an ovarian cDNA library as part of an independent project and had the opportunity to screen it for *loco* ovarian transcripts. cDNA 96a was used as a probe and several positive plaques were isolated. These were rescued and 4 different sized cDNAs were obtained: 3kb, 3.5Kb, 4.0Kb and 5Kb. The 5′ end of each was sequenced and analysed in relation to all existing *loco* sequence.

L7 is a 3.0Kb cDNA and the 5′ sequence showed it to be identical to *loco-c1* (Fig 4.11). The size of the cDNA also corresponded to the size of *loco-c1*. This agrees with the RT-PCR data, confirming that *loco-c1* is expressed in the ovary. L10 is a 4Kb cDNA, with 5′ sequence lining up with exons II-0 and II-1 (Fig 4.11). Both the size of the cDNA and the 5′ sequence match that of *loco-c2* and confirm the RT-PCR data. L4 was the largest of the cDNAs identified, with a length of 5 Kb and 5′ sequence matching the 5 EST sequences 5′ of exon III-1 (Fig 4.11). L6 is 3,5 Kb in length and sequence from it mapped 9.7Kb 5′ of exon III-1 (Fig 4.11). The evidence here is confirmation of the presence of *loco-c1* and *loco-c2* in oogenesis. It also suggests the presence of two uncharacterised transcripts in oogenesis.

4.8 The function of *loco* beyond GTPase activity

Work on mammalian RGS proteins has shown that several of them contain additional domains such as, cysteine-string domain; β-catenin-binding domain; DEP domain (Dishevelled/EGL-10/pleckstrin homology); dbl homology domain; dishevelled-homology domain; GGL domain (homology to Gγ); glycogen synthase kinase 3b binding domain, PDZ-binding motif; PH, pleckstrin-homology domain; Phosphotyrosine-interacting domain; PKA-anchoring domain and B-raf- homology domain(De Vries *et al.* 1999). These domains confer additional characteristics upon the RGS proteins. The presence of additional coding exons in *loco* indicated that it may also contain transcripts with additional functions. With this in mind I conducted a protein domain search using Pedros biomolecular tools. Prodom proved the most productive search engine. This search identified a proline rich region at the 3′ end of all the *loco* transcripts, such C-terminal serine/proline-rich coiled-coil could interact with cytoskeletal proteins (De Vries *et al.* 1999). Interestingly RAT RGS also contains a proline rich region. No other domains were identified in any of the three complete transcripts. cDNA 118a did not contain anything of interest in its additional 3′ sequence and the 5′ sequence from the testis transcript appears to be non-coding. The sequence 5′ of exon III-1 however did show homology to PDZ domain. PDZ domains are involved in the clustering of signalling molecules and play an important role in organising protein networks on membranes. This is accomplished by binding consensus C-terminal motifs in target proteins (De Vries *et al.* 1999). A PDZ domain has also been identified in RAT RGS 12. Another interesting observation was that the RGS domain appeared to be split into two regions and that the first of these two regions contained a Kinase Receptor Transferase domain. This suggests a possible link with kinase signalling pathways.

Figure 4.12 **Loco Domains**



Legend

This figure illustrates the domains present in the three loco proteins characterised and the predicted Loco protein containing a PDZ domain. The dotted region represents predicted protein. This data was obtained from a ProDom search at- (http://protein.toulouse.inra.fr/prodom/cgi-bin/).

4.9 Other RGS genes in *Drosophila*

There are several different G-Proteins that are involved in different signalling pathways. The specificity of the G-Protein is dependent on the Gα subunit, with different Gα subunits having different downstream effectors. In mammals, several RGS genes have been identified and it has been shown that these different RGS genes have specificity for particular Gα subunits (Bohm *et al.*1997). Other domains have also been identified within mammalian RGS genes and are thought to confer other functions to particular RGS genes, perhaps linking them with other pathways (De Vries and Gist Farquhar, 1999).

Drosophila G-Protein signalling also encompasses all the different Gα subunits present in mammals. RNA *in situ* hybridisation and antibody staining, against Giα, Gsα and GOα, show that these G-Proteins are widely distributed throughout *Drosophila* development (Wolfgang *et al.* 1991)(Chapter 1). This raises the question, does *Drosophila* have other RGS genes responsible for the regulation of particular Gα subunits? As the *Drosophila* Genome sequence had recently been completed, there was an opportunity to screen the *Drosophila* genome for any other RGS genes. No other RGS genes were found. This implies that *loco* is the only *Drosophila* RGS gene.

4.10 Summary and Discussion

I have completely sequenced both cDNAs 96a and 118a. cDNA 96a exhibits strong sequence similarity to the RGS gene family, which are responsible for the negative regulation of G-Protein signalling pathways, while cDNA 118a showed no sequence similarity to any sequence in the database. Both cDNAs were incomplete, but sequence from cDNA 96a was identical to EST LD27006. This led to the complete sequencing of EST LD27006 and this sequence was then combined with that of cDNA 96a to produce a full length transcript. Granderath *et al* (1999) identified 2

transcripts in the same gene and named them *loco-c1* and *loco-c2*. These were different transcripts to cDNA 96a, which was renamed *loco-c3*. RT-PCR has established that all three of these transcripts are present in oogenesis. Genomic PCR was also carried out to establish the size of the introns and structure of the gene.

cDNA 118a is also part of *loco,* with its 5′ sequence being identical to exon II-1 from transcript *loco-c2*. However, sequence analysis suggests that it represents part of another *loco* transcript present in the ovary. The presents of a stop codon at the 3′ end, indicates that cDNA 118a would not contain the core exons 2,3 and 4. These exons contain the domains that exhibit high homology to RGS genes. Therefore cDNA 118a would not have the protein function of the other *loco* transcripts. This is interesting, as it may represent a non-functional transcript.

Further transcripts of *loco* were searched for, in the ESTs in the genomic sequence surrounding the *loco* transcripts. A high quality ovarian cDNA library was also screened to identify further ovarian transcripts. These two approaches showed that there were other as yet uncharacterised transcripts of *loco*.

The size of the region that *loco* spans and the indication that there are more than the four transcripts already identified implies complex regulation and/or role. Three of the transcripts so far identified appear to contain the same 3 exons or core domain. This core part of the cDNA contains the region of strong RGS identity and is the functional domain associated with its role. The presence of different 5′ exons can probably be attributed to different regulation of the transcripts. This is especially true for *loco-c1* and *loco-c3,* as their proteins hardly differ and, therefore, their different 5′ exons are probably associated with the regulation of transcription. This may be different for *loco-c2*, which contains a different coding exon. This exon could confer different specificity to a particular Gα sub-unit, or it could confer other characteristics upon the protein produced.

Mammals have several RGS proteins that have specificity to different Gα subunits and have been implicated in other roles, aside from regulating G-Protein signalling. Genomic sequence available indicates that *loco* is the only RGS gene in *Drosophila*.

However there are still all the different Gα subunits that need regulating, perhaps by *loco*. The apparent complexity of *loco* and presence of several transcripts, may mean that *loco* is able to compensate for lack of other RGS genes, by the complex regulation and splicing of transcripts.

Chapter Five: Expression Analysis

5.1 Introduction

To learn more about the gene *loco* and its roles in oogenesis, I needed to establish where the different transcripts were expressed. RT-PCR and sequence analysis (Chapter 4) had shown that at least 4 transcripts of *loco* were present in oogenesis. *in-situ* hybridisation with transcript-specific probes, would show in which subsets of cells these transcripts are expressed.

In the mutant analysis described in Chapter 3, complex phenotypes were observed with both dorsal anterior defects and nurse cell dumping defects seen in mutant eggs. These phenotypes needed to be resolved to establish how loco was functioning in oogenesis. An approach used to learn more about *loco* was antisense analysis. Fly lines were generated, carrying either the construct pCaSpeR*HS* or pCaSpeR*UAS*, containing *loco* in an antisense orientation. This enabled antisense *loco* to be driven at specific stages of oogenesis, disrupting normal *loco* function.

The mRNA expression patterns would indicate when and where *loco* may have a role, but determining where the protein is expressed would be more informative. Producing an antibody against Loco would also allow identification of the different isoforms of Loco, both in the ovaries and at other developmental stages. I therefore raised two antibodies against parts of the Loco core domain. These antibodies would also enable further analysis of the mutant lines described in Chapter 3, as absence of Loco protein in the mutant fly lines would indicate a genuine mutation in the *loco* gene.

5.2 Expression of *loco* mRNA in oogenesis

5.2.1 Initial expression pattern of loco in oogenesis

The two cDNAs, 96a and 118a, isolated from the library screen were used to carry out RNA in-situ hybridisation on OrR wild type ovaries, using DIG RNA probes.

Several attempts had to be made before reproducible results were achieved and, even then, very long incubation times were necessary for a signal to appear. Long incubation times led us to believe that there were very low levels of transcript present. In contrast, abundant transcripts, such as *gurken* needed short incubation times, a signal being detected in less that a tenth of the time needed for either cDNA 96a or 118a.

The expression patterns observed for cDNAs 96a and 118a, appeared to be similar but not identical. Figure 5.1 illustrates RNA expression pattern of cDNA 96a. Transcripts are first detected in the anterior-dorsal follicle cells at stage 10a of oogenesis and persist in these follicle cells until the end of oogenesis. Furthermore the expression in the dorsal midline follicle cells appeared stronger than in the dorsal-lateral cells (Fig 5.1B). Expression is also detected in the nurse cells from stage 10 onward. The anterior-most follicle cells bordering the nurse cells also showed higher levels of expression. The varying levels of expression and dynamic pattern are illustrative of a gene with a complex regulation. The expression pattern of cDNA 118a differed in lacking expression in the nurse cells.

At the time these *in-situs* were carried out, there was no direct evidence proving that cDNA 96a and 118a were part of the same gene. When more information was established about the structure of *loco* (Chapter 4), it became apparent that cDNA 96a contained exons common to all 3 transcripts so far identified, while 118a consisted of sequence specific to transcript *loco-c2*. In light of this knowledge the *in-situs* were repeated with transcript specific exons.

5.2.2 Expression analysis of loco-c2

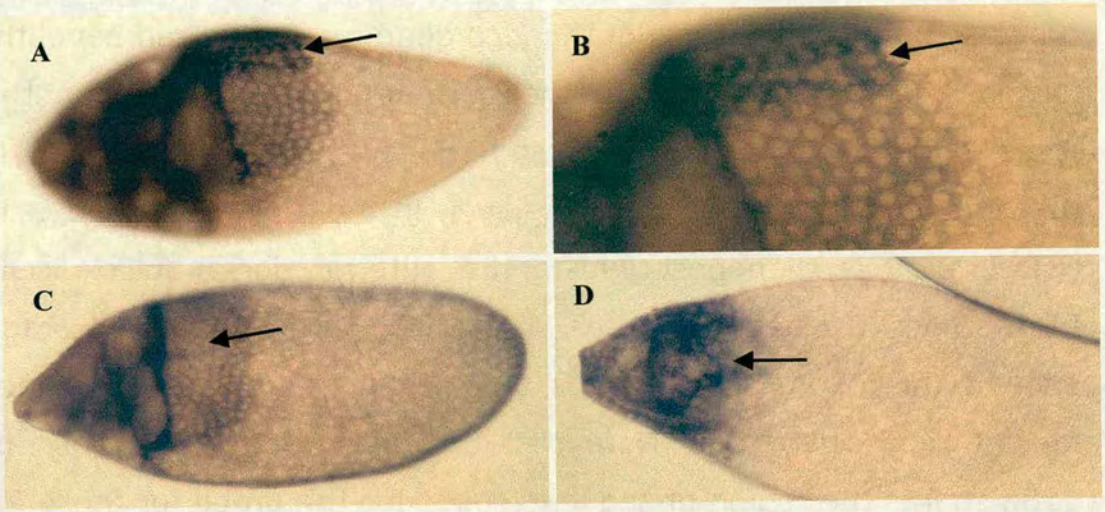
To detect *loco-c2*, I used a probe consisting of exon II-1 (Fig 5.2B). Expression is first seen in the germarium at stage 2, in the prefollicular mesoderm surrounding the cystocytes (Fig 5.2A). The prefollicular mesoderm goes on to form the follicular epithelium (King 1970)(Fig5.2A) and staining is then observed in the follicle cells at stage 8 (Fig 5.2B). The expression at this stage appears patchy, without clear localisation to a subset of cells. From stage 10 onwards, the *loco-c2* transcript is

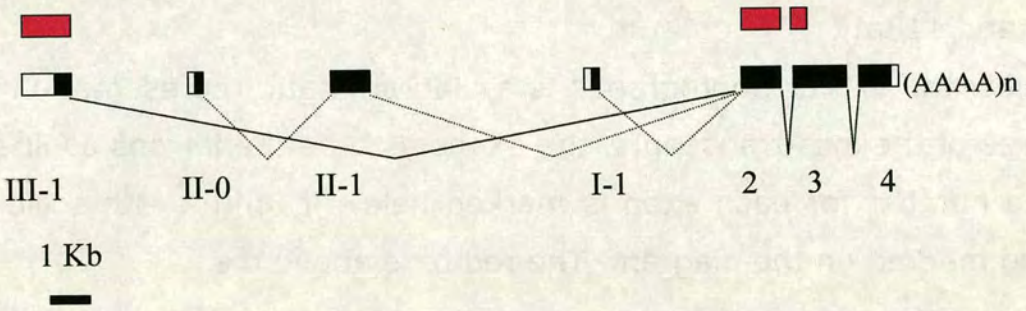
Legend 5.1

In-situ hybridisation using Dig labelled RNA probes made from the *loco* cDNA 96a. The probe spanned the parts of the *loco* transcript marked in red on the diagram of the gene structure. A shows the expression pattern obtained in a wild type (OrR), stage 10 egg chamber. Staining is observed in the nurse cells and in the anterior dorsal follicle cells. B is an enlargment of the dorsal anterior follicle cell region of A. It can clearly be seen that there are higher levels of *loco* expression in the dorsal midline cells, and the anterior most follicle cells. C shows the *loco* expression pattern at stage 11-12; expression persists in the nurse cells and the anterior dorsal follicle cells. D shows stage 13-14 where *loco* expression is now only present in the most anterior follicle cells, where the dorsal appendages are made, and in the nurse cells. In A-D the arrows mark the dorsal mid line.

Below the *in-situ* photographs is a diagrammatic representation of three of the loco transcripts, the exons as bars the introns as lines. The number for each exon is marked below it, and a 1Kb scale is also marked on the diagram. The red bars above the transcripts, represent the region of transcript used in the *in-situs* in this figure.

Figure 5.1 **Initial *in-situ* hybridisation data**



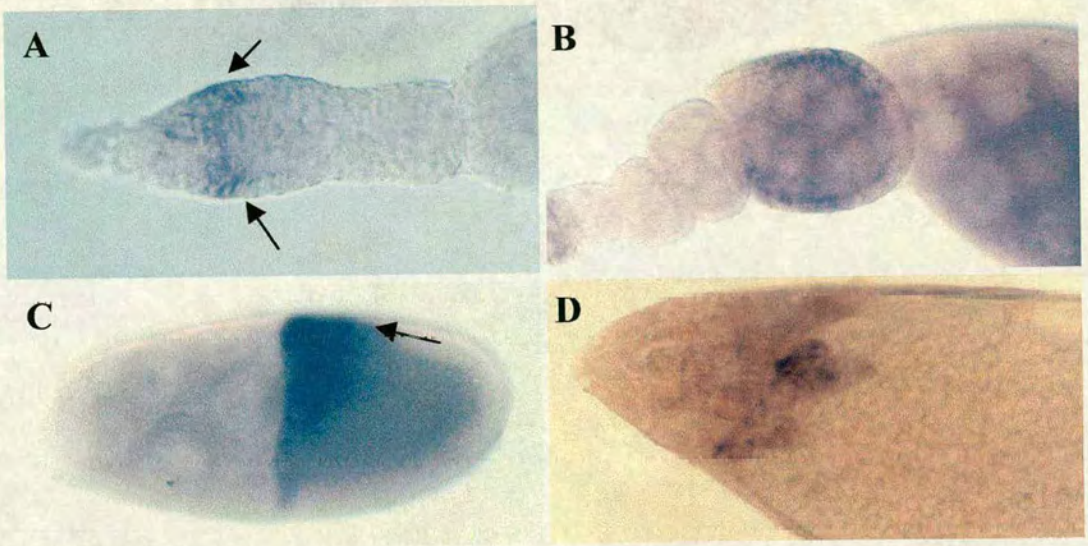


Legend 5.2

Detailed examination of the expression of the *loco c-2* transcript in oogenesis. *In-situ* hybridisation with Dig-labelled RNA probes of exon II-1, specific to transcript *loco c-2*, is illustrated in A-D. The probe spanned the regions of the *loco* gene marked in red beneath. *loco c-2* is first observed in the germarium at stage 2, in the prefollicular mesoderm surrounding the cystocytes (A), and it is then present at stage 8 at a low level, in most of the follicle cells (B). *loco-c2* is then observed in the anterior dorsal follicle cells from stage 10 onwards (C), as shown in Figure 5.1 A-D. At stage 13 –14 this expression reduces to a small group of the anterior most follicle cells, (D). Note that the uncharacterised transcript 118a shares the region of sequence used for the probe for these *in-situ* hybridisations. This indicates that these expression patterns could represent a combination of expression patterns of both *loco-c2* and 118a.

Below the *in-situ* photographs is a diagrammatic representation of three of the loco transcripts, the exons as bars the introns as lines. The number for each exon is marked below it, and a 1Kb scale is also marked on the diagram. The red bars above the transcripts, represent the region of transcript used in the *in-situs* in this figure.

Figure 5.2 **Expression data for *loco-c2***



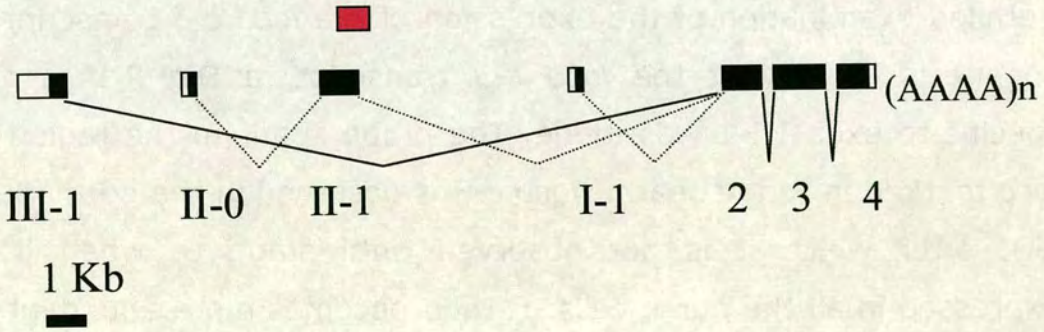
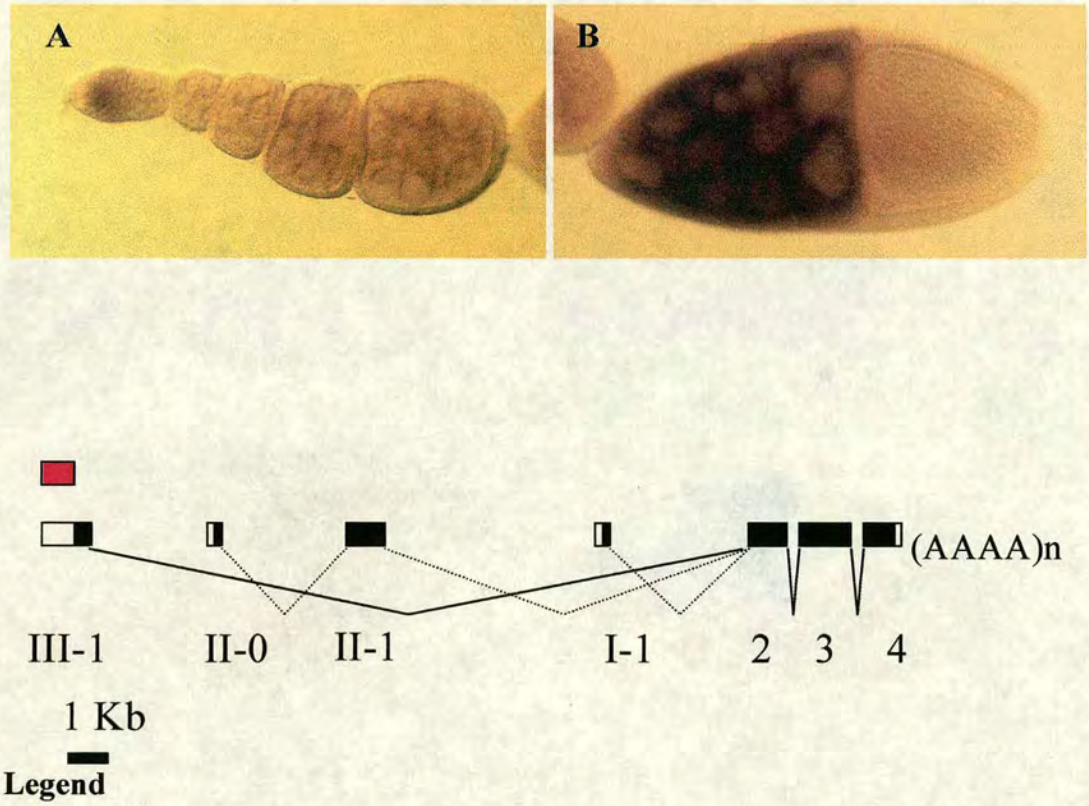


Figure 5.3 **Expression data for *loco-c3***



Detailed examination of the expression of the *loco c-3* transcript in oogenesis. To detect the *loco c-3* transcript, a Dig RNA probe specific to exon III-1 was made. The probe spanned the region of *loco* marked in red beneath. *loco* c-3 is observed in the germarium (A), after which it is not observed until stage 6, when it is expressed in all the nurse cells. It then becomes more abundant at stage 10 (C) and persists in the nurse cell until they degenerate at stage 14.

Below the *in-situ* photographs is a diagrammatic representation of three of the loco transcripts, the exons as bars the introns as lines. The number for each exon is marked below it, and a 1Kb scale is also marked on the diagram. The red bars above the transcripts, represent the region of transcript used in the *in-situs* in this figure.

present in the anterior-dorsal follicle cells and persists there until the follicle cells degenerate (Fig5.2C-D). At stage 13, the cells expressing *loco-c2* appear to reduce to a subset at the very anterior, where the dorsal appendages are made (Fig 5.2D).

5.2.3 Expression analysis of loco-c3

For the *in-situ* hybridisation to *loco-c3* transcript, I used exon III-1 as a probe. Initially the expression pattern is similar to *loco-c2*, as staining is observed in the germarium (Fig 5.3A). *loco-c3* is then undetectable until stage 6, when it is visible in the nurse cells. *loco-c3* becomes more abundant at stage 10 (Fig 5.3B) and persists in the nurse cells until they degenerate at stage 14.

5.2.4 Other loco transcripts

Further understanding of *loco* transcripts indicates that cDNA 118a represents a different transcript to *loco-c2* (Chapter 4) and both transcripts are present in oogenesis. This was not appreciated when these *in-situs* were conducted. It is therefore possible that the expression pattern observed with a probe against exon II-1, represents that of transcripts 118a and *loco-c2*. RT-PCR has also confirmed the presence of *loco-c1* in oogenesis, however time limitations have prevented further analysis of expression of these transcripts. Another consideration is the identification of several more *loco* transcripts through EST analysis (Chapter 4). There may be other ovarian *loco* transcripts that have to be identified; also the exons used for the *in-situs* so far may be common to several transcripts.

5.3 Expression of *loco-c2* is downstream of the *gurken-torpedo* signalling pathway

The presence of *loco-c2* in anterior-dorsal follicle cells indicated that this transcript could be downstream of the *gurken-torpedo,* signalling pathway. To investigate this

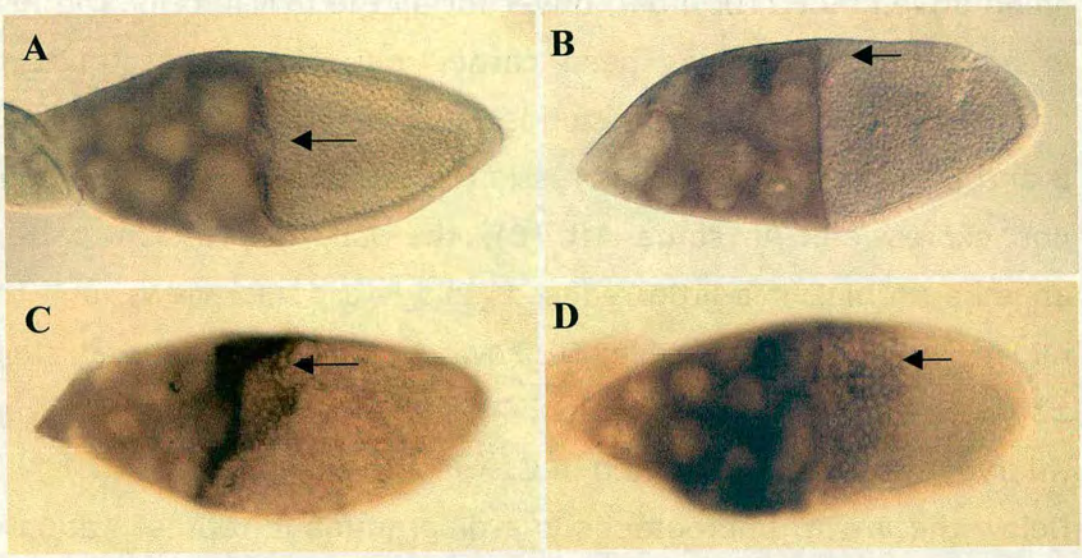
Legend 5.4

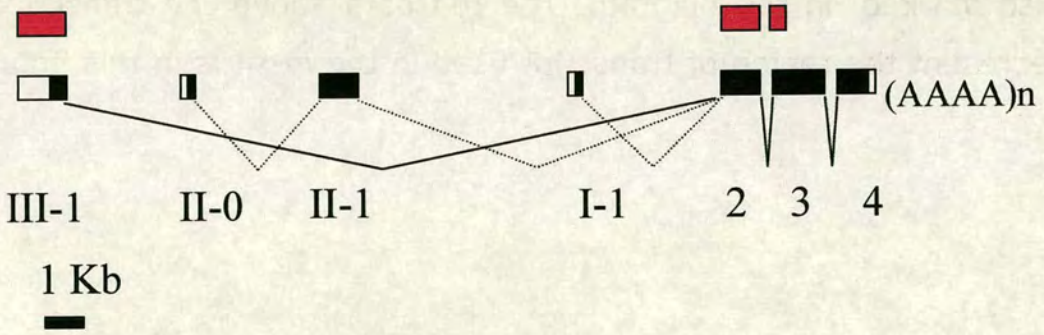
In-situ hybridisation using Dig-labelled RNA probes made from the *loco* cDNA. The probe spanned the parts of *loco* marked in red. A-D are different mutant egg chambers. A shows a stage 10 egg chamber from a grk^{HK} mutant line. In this mutant there is no *loco* expression in anterior dorsal follicle cells. A similar result is observed in a top^{QY1} mutant background, B. C is a stage 10 egg chamber from $fs(1)K10^{-}$ mutant fly line. There is expression of *loco* cDNA in the follicle cells, although it is not confined to the anterior dorsal follicle cells and its expression extends ventrally. D is a stage 10 egg chamber from a fly line which has 4 copies of *gurken*. Here *loco* cDNA is activated in more cells than in wild type.

Below the *in-situ* photographs is a diagrammatic representation of three of the loco transcripts, the exons as bars the introns as lines. The number for each exon is marked below it, and a 1Kb scale is also marked on the diagram. The red bars above the transcripts, represent the region of transcript used in the *in-situs* in this figure.

Figure 5.4

Data proving *loco-c2* is down stream of the *gurken-torpedo* signalling pathway.



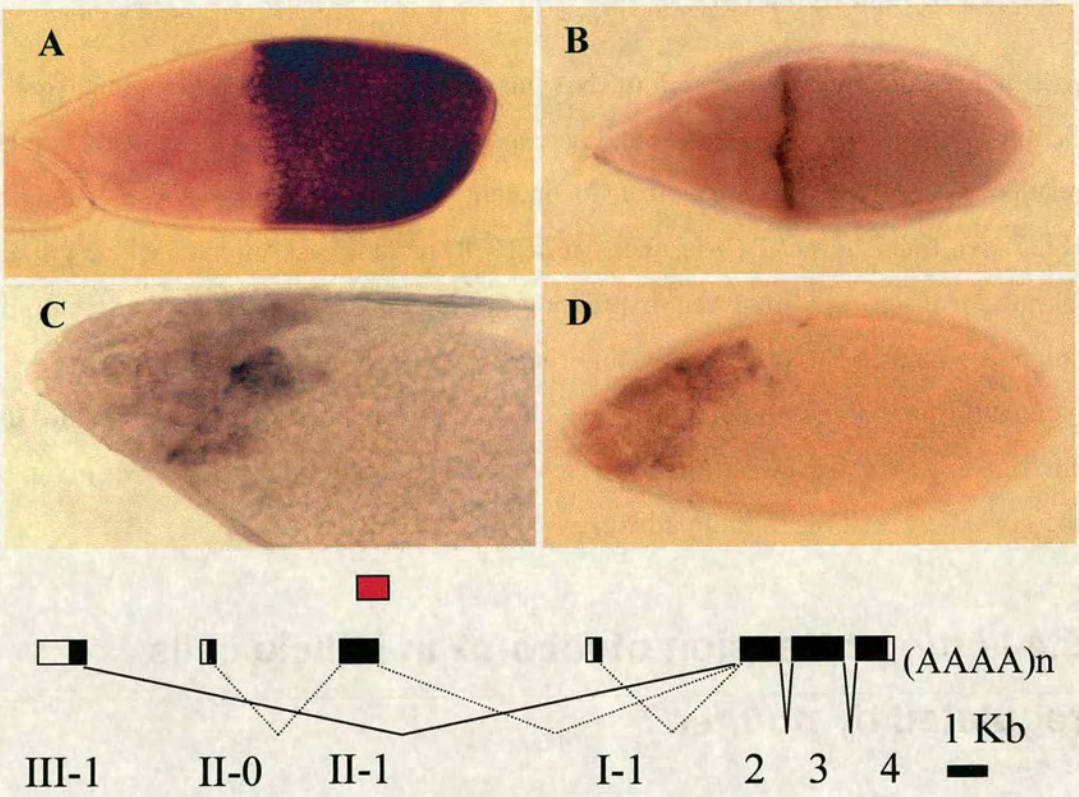


Legend 5.5

A, is a stage 10 egg chamber from a UAS *pnt* line, in which *pnt* is being driven by C710, a gal^4 driver for all the follicle cells. Dig RNA *in-situ* hybridisation has been carried out with a *pnt* probe to demonstrate that *pnt* expression is being driven in the follicle cells. B and D illustrate *loco-c2* expression in egg chambers with ectopic *pnt* expression. At stage 10 (B), the *loco* expression pattern appears normal, if a little reduced (this is a dorsal view), and the midline expression is visible. D shows *loco-c2* expression at stage 13 with ectopic *pnt* expression. This is markedly different from wildtype which is represented in C.

Below the *in-situ* photographs is a diagrammatic representation of three of the loco transcripts, the exons as bars the introns as lines. The number for each exon is marked below it, and a 1Kb scale is also marked on the diagram. The red bars above the transcripts, represent the region of transcript used in the *in-situs* in this figure.

Figure 5.5 **Is *loco-c2* regulated by *pointed* in the follicle cells?**



further, I looked at the expression of *loco-c2* in a range of mutant backgrounds that affect dorsal ventral polarity.

In-situ hybridisation to *loco-c2* mRNA in ovaries from a gurken mutant (grk^{HK} Schupbach 1987) was undertaken and no expression was observed (Fig5.4A). Similar results were obtained using *torpedo* mutant (top^{QY1} Schupbach 1987) ovaries (Fig5.4B). In an *fs(1)k10* (Wieschaus *et al*,1978) mutant background, where *gurken* is not localised, we observed expression of *loco-c2* extending further ventrally (Fig5.4C), and in a transgenic fly line that expresses four copies of *gurken* (grk^{4X} Neuman-Silberberg and Schupbach 1994), *loco-c2* expression is seen in all the anterior follicle cells (Fig5.4D). These results indicate that *loco-c2* is downstream of the *gurken-torpedo* signalling pathway.

5.4 Is the expression of *loco-c2* in follicle cells regulated by *pointed*?

Granderath *et al* (1999) identified *loco* in a screen for genes downstream of *pointed* in glial cells. As *pointed* is also involved in dorsal ventral patterning within the ovary it was a good candidate for a gene upstream of *loco-c2* in the anterior dorsal follicle cells.

pointed has 2 transcripts, *pointed 1* and *pointed 2*, both of which are expressed in subsets of anterior dorsal follicle cells during oogenesis (Morimoto *et al*, 1996). The expression of *pointed* in oogenesis is very dynamic, expression first being observed in the germarium, then later at stage 8 downstream of *torpedo* in the posterior follicle cells, and again at stage 10 downstream of *torpedo* in the anterior dorsal follicle cells (Gonzalez-Reyes *et al*, 1995). I therefore analysed the relationship between *loco* and *pointed* in these cells. Using a *pointed 1* sense UAS pnt^{P1UAS} (Morimoto *et al*, 1996) fly line, expression of *pointed 1* was driven in all the follicle cells using the GAL4 driver (Fly line T155). Fig 5.5A is an ovarian *in-situ* hybridisation using a *pointed* specific probe and clearly shows *pointed* expression being driven in all the follicle cells. I then followed the expression of *loco-c2* in these egg chambers where *pointed*

1 was being ectopically expressed. There was normal spatial distribution in anterior-dorsal follicle cells at stage 10, although the level of *loco-c2* expression was reduced in all anterior dorsal follicle cells except those on the anterior margin of the oocyte (Fig 5.5B). At stage 13, the expression pattern is slightly different to wild type, as there is a patch of cells expressing *loco* in a more posterior dorsal position (Fig 5.5D), in contrast to wild type expression (Fig 5.5C). Ectopic expression of *pointed* does not drive *loco* in all follicle cells overlying the oocyte. This rules out a simple linear relationship, where *loco* is directly downstream of *pointed*. However, ectopic *pointed* did lead to a reduction in *loco* expression in anterior dorsal follicle cells at stage 10. This may imply that *pointed* does interact with *loco*. It may appear that *loco* is being repressed by *pointed*, but the ectopic expression of one gene may upset a fine balance of expression of several genes and result indirectly in reduced *loco* expression in these cells.

The stage 13 expression of *loco* in cells more posterior than normal can be attributed to oocytes with ectopic *pointed* expression failing to make dorsal appendages. These cells seen expressing *loco* would normally have migrated further anteriorly.

We were unable to repeat the same experiment with *pointed 2,* as our GAL4/UAS cross was lethal. This was because the T155 GAL4 driver used must have had an earlier stage of expression that coincided with *pointed 2* function. However Morimoto *et al*, (1996) characterisation of the role of *pointed* in oogenesis focused on *pointed 1* and little is known about the role of *pointed 2*.

5.5 Investigating the role of *loco* in oogenesis using antisense analysis

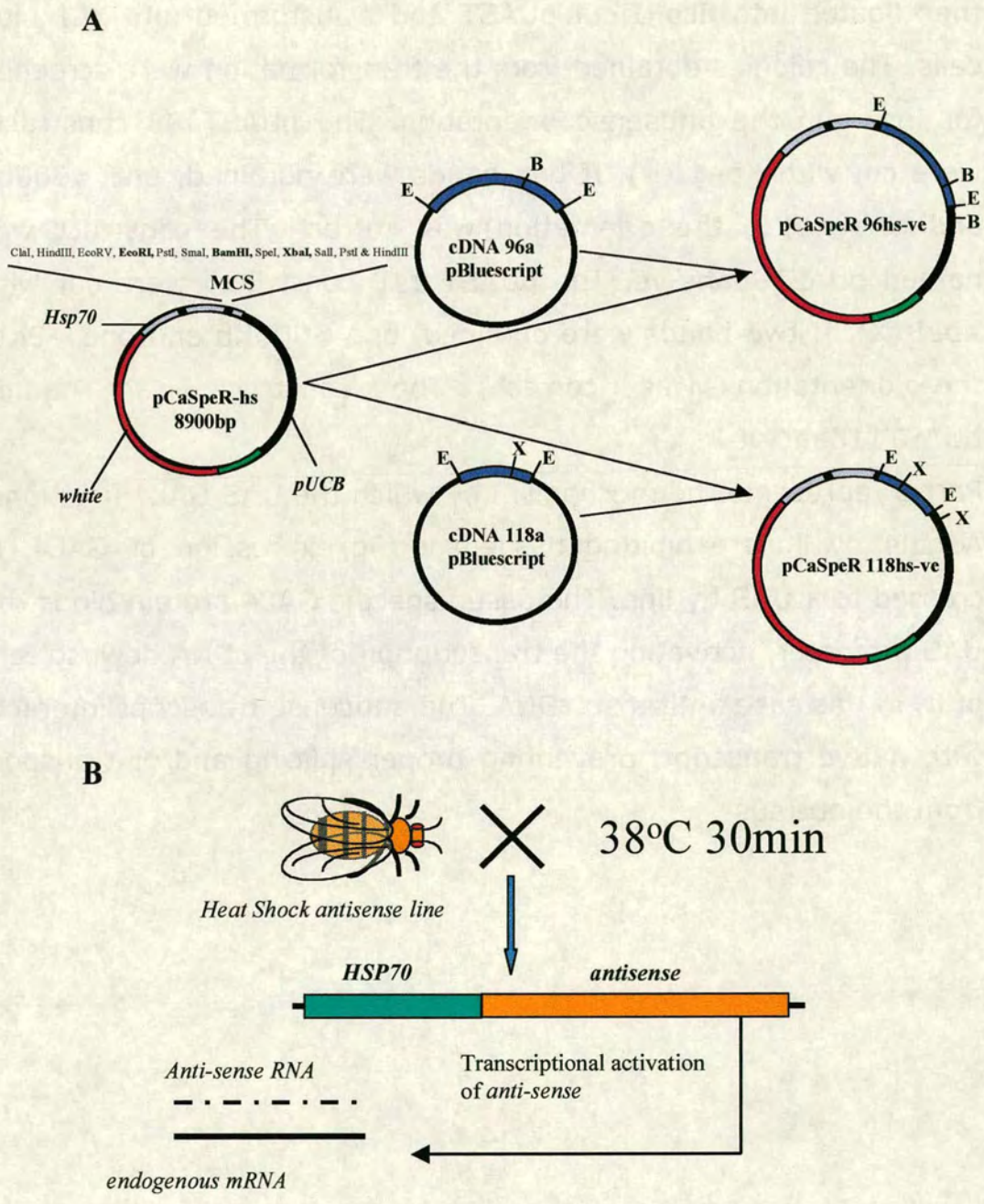
At the time the antisense work was started, I had not generated any mutants in *loco*. It was highly probable that once mutants were generated, they would be lethal at embryonic or a slightly later stage. This would mean obtaining an ovarian phenotype would demand further work, such as mosaic analysis. In parallel to generating

Legend 5.6

Part A represents the cloning of both the cDNAs 96a and 118a into pCasper heatshock vector. Note that only antisense clones were made. EcoRI was used to cut the cDNAs out of Bluescript, they were then ligated into EcoRI (E) cut CaSpeR HS and transformed into XL1 blue cells. The colonies obtained from the transformation were screened for inserts in the antisense orientation. The CaSpeR 96 constructs were cut with BamHI (B). If two bands were obtained, one <300bp and one >9Kb, the orientation was correct. The construct was named pCaSpeR96ahs-ve. The CaSpeR 118 constructs were cut with XbaI (X). If two bands were obtained, one >500Kb and one >9Kb, the orientation was correct. The construct was named pCaSpeR118ahs-ve.

Part B represents the mechanism by which the heat-shock functions. When flies containing the HS construct are exposed to temperature of 38°C, this activated the HS70 promoter. This results in transcription of the cDNA inserted downstream of the promoter, in this case antisense cDNA. The antisense transcript interacts with the native transcript, preventing proper splicing and/or transport from the nucleus.

Figure 5.6 Heat Shock Constructs

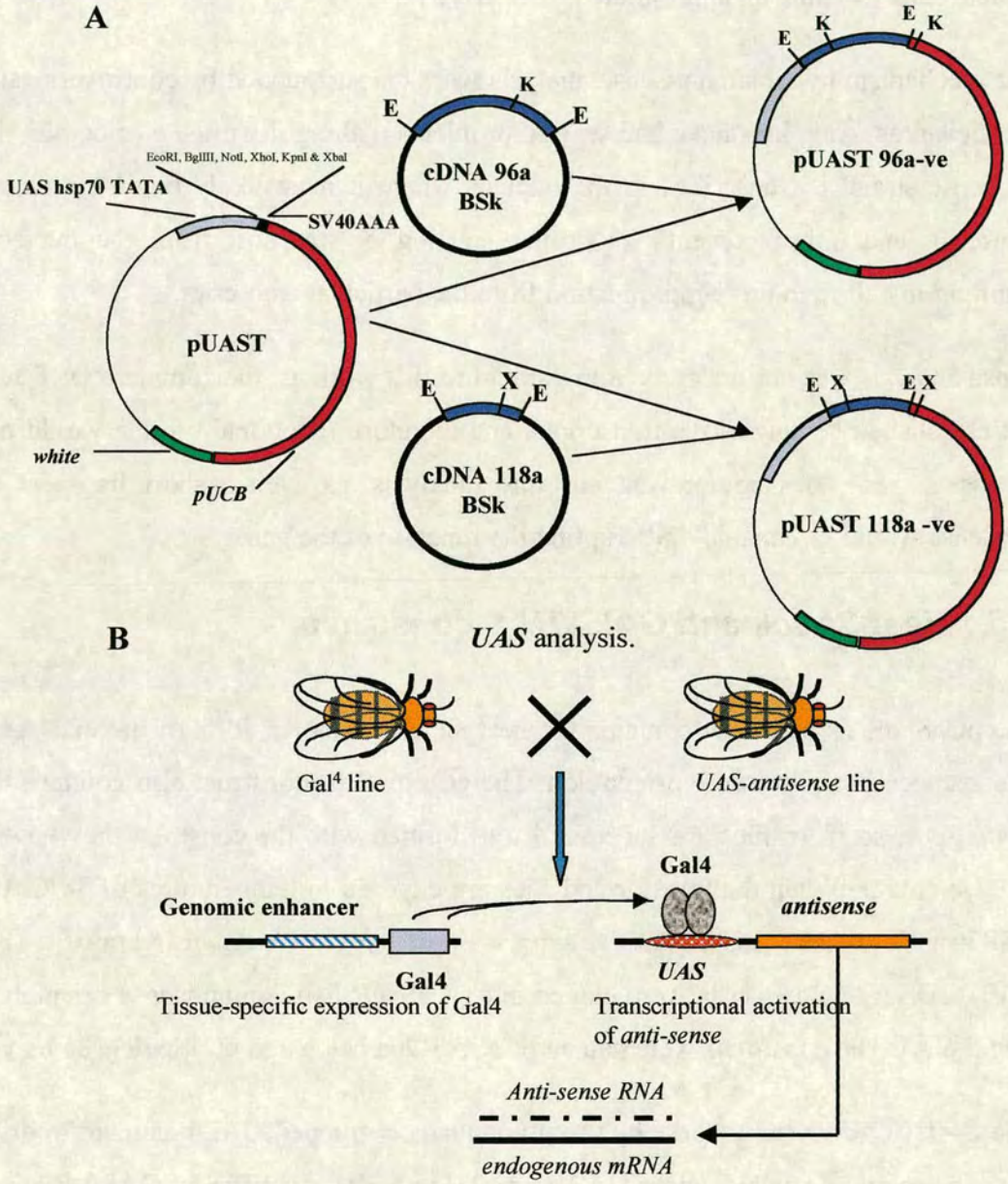


Legend 5.7

Part A represents the cloning of both the cDNAs 96a and 118a into the pUAST vector. Note that only antisense clones were made. EcoRI (E) was used to cut the cDNAs out of Bluescript; they were then ligated into EcoRI cut pUAST and transformed into XL1 blue cells. The colonies obtained from the transformation were screened for insert in the antisense orientation. The pUAST 96 constructs were cut with Kpn1 (K). If two bands were obtained, one <300bp and one >9Kb, the orientation was correct. The construct was named pUAST96ahs-ve. The pUAST 118 constructs were cut with XbaI (X). If two bands were obtained, one >500Kb and one >9Kb, the orientation was correct. The construct was named pUAST118ahs-ve.

Part B represents the mechanism by which the UAS GAL4 functions. A gal4 fly line, exhibiting tissue specific expression of GAL4, is crossed to a UAS fly line. The tissue specific GAL4 protein binds the UAS promoter, activating the transcription of the cDNA downstream of it, in this case antisense cDNA. The antisense transcript interacts with native transcript, preventing proper splicing and/or transport from the nucleus.

Figure 5.7 **UAS Constructs**



mutants, I decided to attempt antisense analysis, using the *GAL4/UAS* system and heat shock to drive expression. I also thought that antisense analysis, when coupled to the *GAL4/UAS* system, would allow disruption of *loco* in specific subsets of follicle cells by using an appropriate *GAL4* driver.

The mechanism by which antisense analysis works is surrounded by controversy and scepticism as many labs have had several problems making it work. In principle, the antisense strand is transcribed in the nucleus where it most likely binds the native transcript and either prevents its proper splicing or transport from the nucleus, resulting in a drop in protein production from the particular transcript.

Sense analysis was not undertaken in parallel to this work as, the complete ORF was not established for any of the transcripts, and therefore functional protein would not be made. This does not prevent antisense analysis, as even a short fragment of antisense would be capable of disrupting the function of the gene.

5.5.1 Heat Shock and GAL4/UAS constructs

The pCaSpeR hs construct contains the heat shock promoter 70 5' of the cDNA, in this instance in an antisense orientation. The pCaSpeR hs construct also contains the *white* gene, so if *wr* flies are successful transformed with the construct they have a red eye colour. When the transformed flies are exposed to temperatures of 38°C, the HSP70 is activated. This drives the expression of the cDNA downstream of it (Fig 5.6B). cDNA 96a and 118a were cloned into pCaSpeR hs in an antisense orientation (Fig 5.6 A). The constructs were named pCaSpeR96a hs-ve and pCaSpeR118a hs-ve.

The GAL4/UAS system works by using an enhancer trapped GAL4 element to drive the expression of a cDNA under UAS control (Fig 5.7B). As different GAL4 fly lines contain GAL4 elements under the control of different enhancers, a specific GAL4 fly line can be chosen to drive expression of cDNA in a specific subset of cells. cDNA 96a and 118a were cloned into pUAST in an antisense orientation (Fig 5.7 A). The constructs were named pUAST96a-ve and pUAST118a -ve. The four constructs, pUAST96a-ve, pUAST118a –ve, pCaSpeR96a hs-ve and pCaSpeR118a hs-ve, were

transformed into *wr* fly lines. Several transformed lines were generated which had the construct inserted at different positions. Preliminary analysis was carried out on all fly lines generated, to see which ones exhibited a phenotype.

5.5.2 Transgenic fly lines generated

Transgenic fly lines were established for each of the 4 different constructs made. The transgenic fly lines marked in red exhibited phenotypes when analysed.

Table 5.1

CaSpeR HS-cDNA 96a

96HS-ve*1A	96HS-ve*1B	96HS-ve*1C
96HS-ve*2A	96HS-ve*3A	96HS-ve*3B
96HS-ve*3C	96HS-ve*4A	96HS-ve*5A
96HS-ve*5B	96HS-ve*6A	96HS-ve*7A

CaSpeR HS-cDNA 118a

118HS-ve*1A	118HS-ve9*2	118HS-ve9*3
118HS-ve15*3	118HS-ve15*4	

CaSpeR UAS-cDNA 96a

96UAS-ve*1B	96UAS-ve*2A	96UAS-ve*5A
96UAS-ve*5B	96UAS-ve*6A	96UAS-ve*6B

96UAS-ve*6C	96UAS-ve*7A	96UAS-ve*7B
96UAS-ve*8A	96UAS-ve*9A	96UAS-ve*11A
96UAS-ve*11B	96UAS-ve*13A	96UAS-ve*13B

CaSpeR UAS-cDNA 118a

118UAS-ve*1B	118UAS-ve*2A	118UAS-ve*2B
118UAS-ve*3A	118UAS-ve*3B	118UAS-ve*4A
118UAS-ve*4B	118UAS-ve*7A	118UAS-ve*9A
118UAS-ve*10A	118UAS-ve*1A	

5.5.3 Heat Shock analysis

Preliminary heat shock analysis was carried out on all heat shock fly lines. This consisted of one 40 minute heat shock at 38°C, followed by egg collections from the heat shocked flies at four hour intervals for the following 3 days. Observations taken over this period of time ensure that all ovarian stages exposed to the antisense RNA would have time to reach maturity.

From the 12 fly lines containing the CaSpeR HS-cDNA 96a construct, only 3 exhibited an egg phenotype, 96HS-ve*1A, 96HS-ve*2A and 96HS-ve*5A. 96HS-ve*1A exhibited a weak phenotype with some eggs being slightly shorter than wild type or having the appendages fused to some degree. 96HS-ve*2A laid eggs which were significantly shorter than wild type, while 96HS-ve*5A laid eggs with fused appendages and some were ventralized.

From the 5 lines containing CaSpeR HS-cDNA 118a construct, only fly line 118HS-ve15*3 exhibited a weak egg phenotype, with some eggs showing fused appendages. It is difficult to quantify the extent of the heatshock phenotype as the phenotypes are only observed at a time interval after heatshock (See figures 5.8 and 5.9). For this reason the percentage phenotypes quoted in table 5.2 represent the percentage phenotype observed at an individual time point, spanning the duration of 4 hours.

It was interesting that a range of phenotypes was observed from the same construct, and that these different phenotypes were from different lines: 96HS-ve*2A and 96HS-ve*5A. This required further analysis.

Table 5.2 Percentage heatshock phenotypes observed

	Phenotype observed	**%**
96HS-ve*1A	Short eggs/ Fused appendages	6%
96HS-ve*2A	Short eggs	9%
96HS-ve*5A	Fused appendages	14%
	Ventralised eggs	4%
118HS-ve15*3	Fused appendages	5%

*5.5.4 Detailed analysis of Heat Shock fly lines 96HS-ve*2A and 96HS-ve*5A*

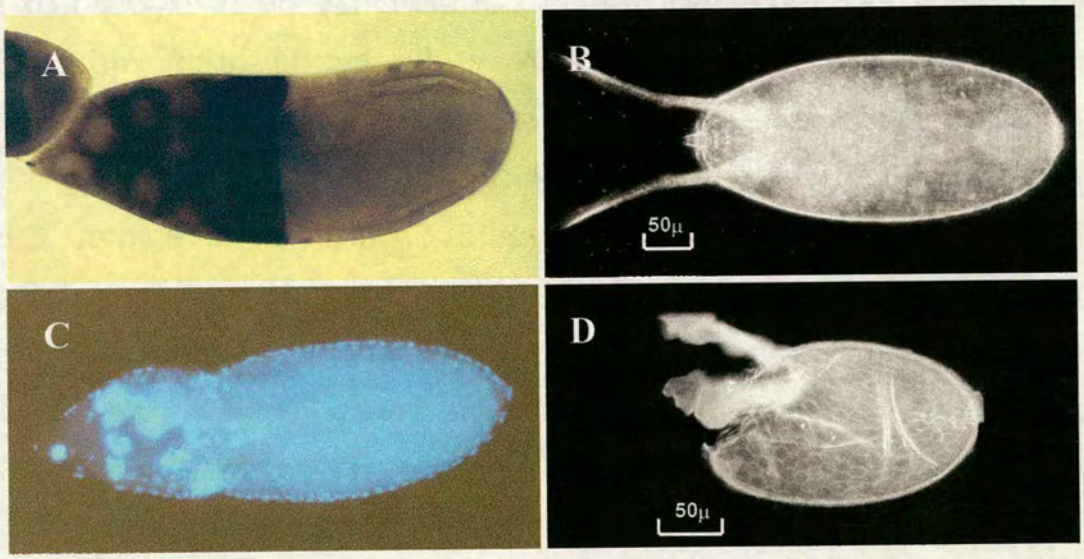
One line 96HS-ve*2A (2A) laid eggs that were shorter than wild type and the phenotype varied from slightly shorter than normal to approximately a quarter the length of wild type eggs (Fig 5.8D). A short egg phenotype is often associated with failure of the nurse cells to dump their contents at the end of oogenesis. The ovary of

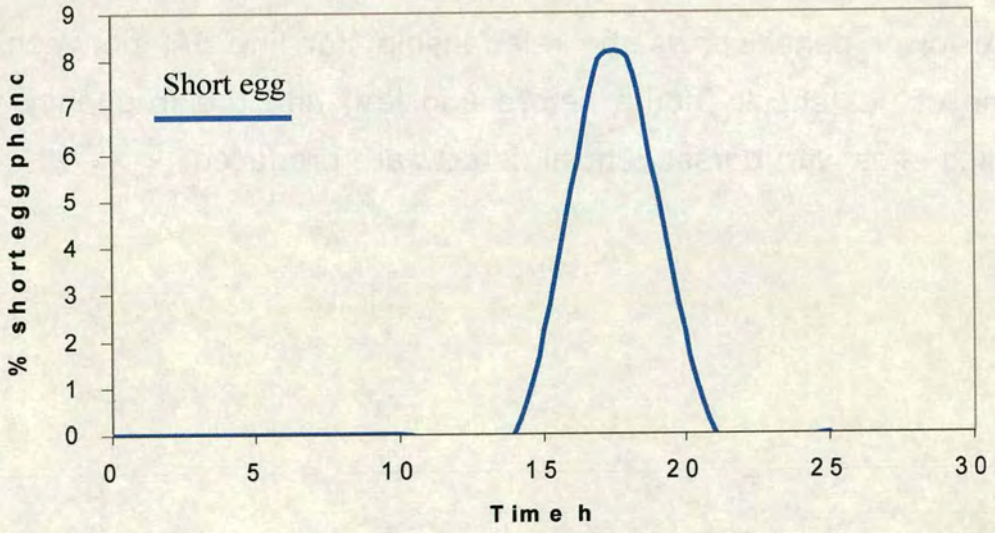
Legend 5.8

Figure A is a stage 10 egg chamber from fly line 96hs-ve2a (Antisense *loco* heatshock fly line) after it had been exposed to a heatshock (HS) regime. Dig RNA *in-situ* hybridisation with RNA probe against the anti-sense strand of *loco*. Antisense *loco* expression is driven only in the germ line. C shows the dumpless phenotype exhibited later at stage 13, and D shows the resulting small egg. For comparison, a wildtype egg is shown in B (at lower magnification).

The lower panel shows the relationship, for line 2A, between the time of heatshock (hours before egg lay) and the frequency with which short eggs are produced.

Figure 5.8 **2A Heat Shock Phenotype**





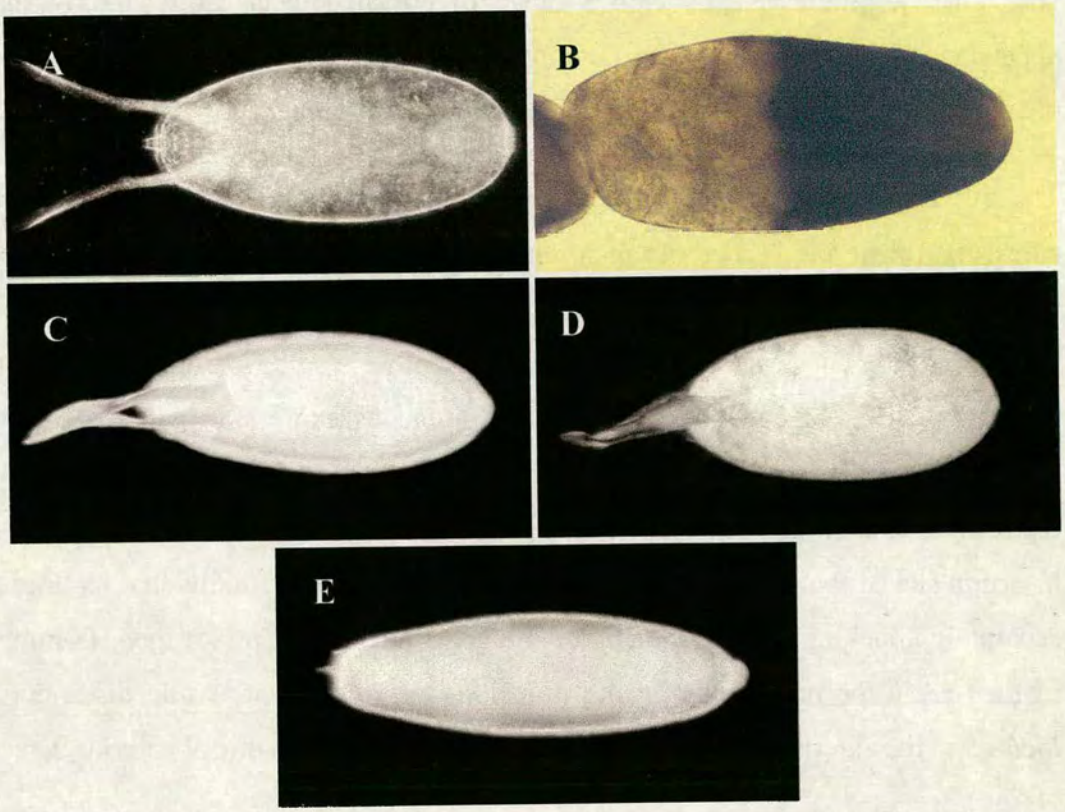
Legend 5.9

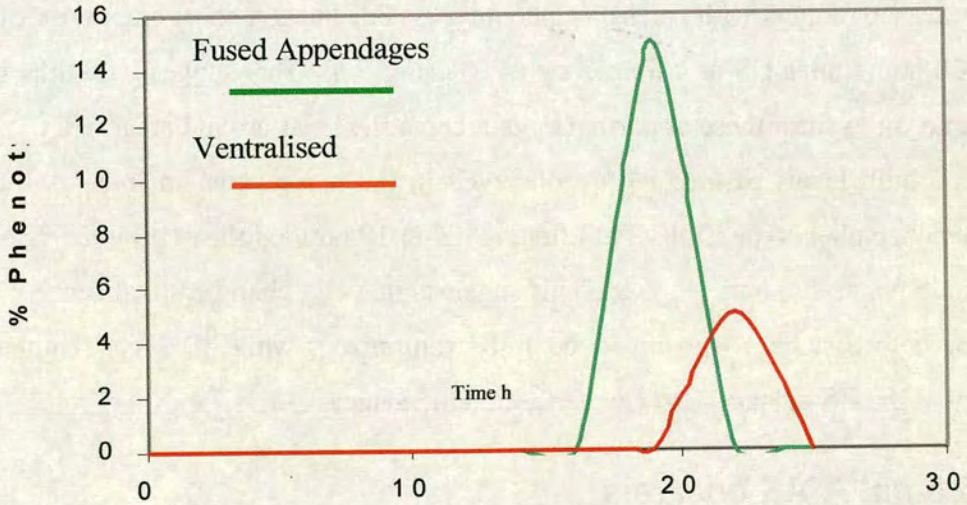
Figure B is a stage 10 egg chamber from fly line 96hs-ve5a (Antisense *loco* heatshock fly line) after it had been exposed to a heatshock (HS) regime. Dig RNA *in-situ* hybridisation with RNA probe against the anti-sense strand of *loco.* Antisense *loco* expression is driven only in the follicle cells.

Phenotypes C, D and E are observed in 5A after a HS regime. C is the weakest phenotype, with the dorsal appendages fused at the base, while in D there is a single fused appendage. Both C and D are representative of a reduction in the dorsal region of the egg. E shows the most severe phenotype observed with 5A, which is a complete loss of dorsal appendage and also a change in shape of egg, which is longer and thinner, this is associated with complete loss of dorsal identity. A represents wild type egg and is not same scale as E.

The lower panel shows the relationship, for line 5A, between the time of heatshock (hours before egg lay) and the frequency with which eggs with dorsal ventral defects are produced.

Figure 5.9 **5A Heat Shock Phenotypes**





this hs line did indeed exhibit a dumpless phenotype (Fig5.8C). The other line 96HS-ve*5A (5A), produces eggs with a range of dorsal defects, from slightly fused appendages to eggs which are completely ventralized (Fig 5.9C,D,E). These phenotypes were similar to those seen in the mutants (Chapter 3).

The difference between the fly lines was unexpected as both contained the same construct. I therefore carried out *in-situ* hybridisation with an RNA probe specific to the anti-sense *loco* being generated under heat shock. This showed that hs lines did not express the construct ubiquitously, as originally thought, and that the two lines generating different egg phenotypes had different hs expression patterns. 2A had germ line specific expression in the ovaries (Fig5.8A), while 5A had expression in the follicle cells (Fig5.9A). This suggests that there is a positional effect, due to the insertion site of the P-element. This proves very useful as the individual hs lines are separately knocking out the somatic or the germ line transcripts of *loco*. Disruption of *loco-c3* in the nurse cells results in a dumpless phenotype, while disruption of *loco-c2* in the anterior dorsal follicle cells results in eggs with dorsal anterior defects.

In the hs lines, there was a relationship between the time elapsed after hs and the appearance of eggs with particular phenotypes. For line 2A short eggs were observed 15-20 hours after HS at a frequency of 9% (Fig 5.8). This suggests that the oocytes that go on to form these abnormal eggs receive the hs at around stage 10, the time at which high levels of *loco-c3* are observed in the nurse cells. In line 5A the fused appendage phenotype is observed first, at about 19 hours, followed by the ventralized phenotype, at 23 hours (Fig5.9). This suggests that egg chambers that receive the HS at or before stage 10 go on to be fully ventralized, while the egg chambers that receive the HS at stage 10-11 have fused appendages.

5.5.5 gal^4/UAS analysis

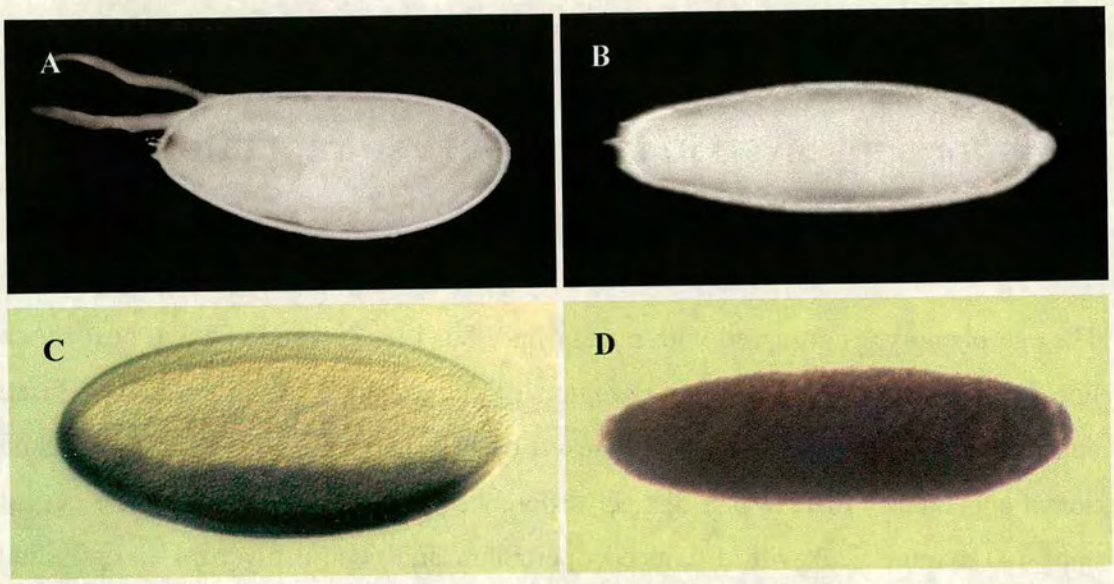
All CaSpeR UAS fly lines were crossed to the gal^4 follicle cell drivers Cu1 and T155. These gal^4 drivers both have gal^4 expression in all columnar follicle cells at stage 10 and should be able to drive antisense *loco* in these cells, disrupting native *loco* expression in the anterior dorsal follicle cells.

Two lines 96aUAS-ve*6A and118UAS-ve*9A, exhibited a weak phenotype, occasionally forming eggs with fused appendages and ventralized eggs. Further analysis of these two lines was not pursued, as the heat shock fly lines had exhibited a stronger phenotype and provided the necessary data.

5.6 Is *loco* involved in the dorsal ventral patterning of the egg as well as the embryo?

The egg phenotypes observed with mutant lines 80, 138, 230 and 455 and heat shock line 5A showed loss of the dorsal region of the eggshell, and range from fused appendages to eggs that completely lacked dorsal appendages. In eggs that lacked dorsal appendages there was a distinct difference in the overall shape of the egg, it appeared symmetrical with a reduced operculum and central mycropyle. Eggs that were completely ventralized always failed to hatch, and these embryos arrested early in development, prior to gastrulation. Most of the eggs with fused appendages hatched. To ascertain if the embryos that failed to develop were also ventralized, we investigated the expression of *twist*. The *twist* gene can act as a marker for ventral cells, in early embryogenesis, as soon as the epithelium is formed. It persists in the ventral cells until late in development. In the mutant S94FE230 *twist* is activated in all embryonic cells (Fig 5.10D), indicating that the embryo is ventralised. This confirms that *loco* is involved in establishing the dorsal-ventral axis of the embryo, as well as the dorsal ventral axis of the egg.

Figure 5.10 ***twist*** **Expression in Mutant Embryos**



Legend

Figure C shows a wild type embryo (mid stage 5), on which *in-situ* hybridisation has been carried out with *twist* DIG RNA probe. *twist* expression can clearly be see along the ventral surface of the embryo. D is a ventralized egg from fly line S94FE230, which has been hybridised with the same probe. *twist* is active around the entire embryo, suggesting that the embryo is ventralised. A represents a wild type egg and B a ventralised egg from fly line S94FE230.

5.7 Antibody production and use

5.7.1 pGEX constructs

The GST gene fusion system (Pharmacia Biotech) was used to make two GST fusion proteins against subdomains of the *loco* gene. EST LD 27006 (Acc No AA941661) contains the core region of *loco* and had suitable Eco R1 restriction sites, producing 862bp and 525bp fragments that were cloned directly into GEX-3X at the EcoR1 restriction site (Fig 5.11). As both inserts 525 and 862 could be cloned in either orientation, the transformants were checked by restriction digest, for correct orientation. The ones with the correct orientation were sequenced to ensure that the ORF was maintained. Correct frame constructs for both 525 and 862 were obtained and named GST-525 and GST-862, respectively.

5.7.2 Protein Expression and Purification

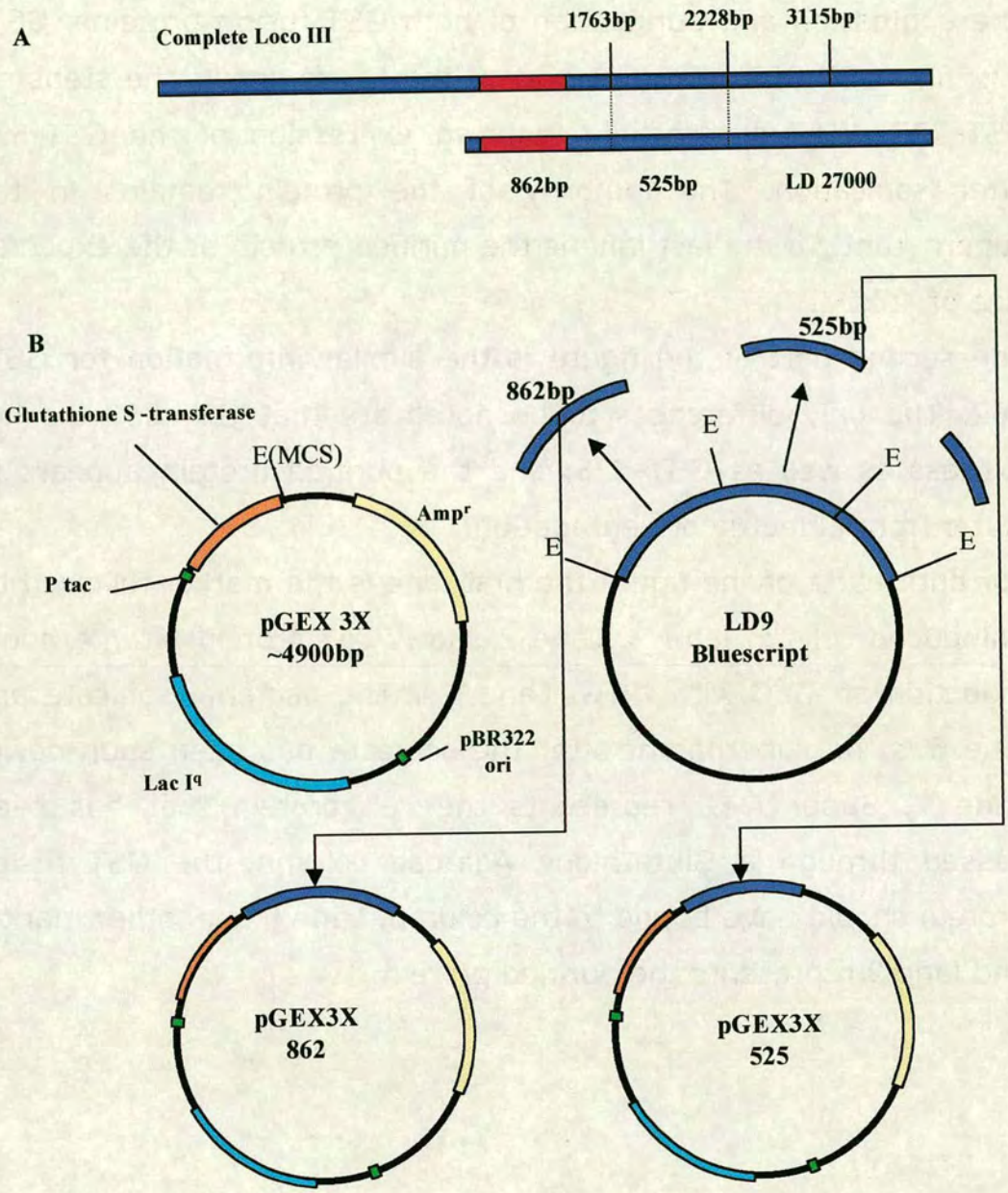
Constructs GST-525 and GST-862 were cloned into BL 21 cells. Conditions were optimised for expression of each fusion protein by varying the levels of IPTG (0.1mM-10mM) for induction, and of temperature for growth (20°C-37°C). GST-525 expressed well under all conditions of temperature and concentration of IPTG (Fig 5.12 A). GST-862 had much lower levels of expression, regardless of concentrations of IPTG or temperature (Fig 5.12 B). Therefore 0.1mM IPTG and a temperature of 37°C were used to raise both fusion proteins. To determine whether the protein produced was soluble, the cells were sonicated, the debris pelleted and the supernatant run on a gel. After sonication both fusion proteins appeared to be primarily in the supernatant (Fig 5.12), so purification was straightforward.

The GST-525 fusion protein was estimated to have an approximate molecular weight of 45.3kDa. This was calculated by adding together 26kDa for the GST protein and 19.3kDa for the 525bp region of *loco* (calculated from average amino acid molecular weight values). The GST-525 was purified from the bacterial cell extract, using

Legend 5.11

The cloning of two fragments (525bp and 862bp) of *loco* in frame into pGEX 3X. Part A shows EST LD27000 lining up with cDNA loco-c3. LD27000 was restriction digested with EcoRI (E), resulting in a 525bp fragment and an 862bp fragment.
In part B the pGEX 3X vector was digested with EcoRI (E). Both fragments 525 and 862 were ligated into the pGEX 3X and transformed into XL1 blue cells. The orientation of the insert was determined by restriction digest. Clones with inserts in correct orientation were sequenced to ensure that the correct reading frame was maintained. Ptac, $LacI^q$, pBR322 ori, Amp^r and Glutathione S-transferase comprise the components of the vector pGEX 3X.

Figure 5.11 **Cloning *loco* into pGEX vector**



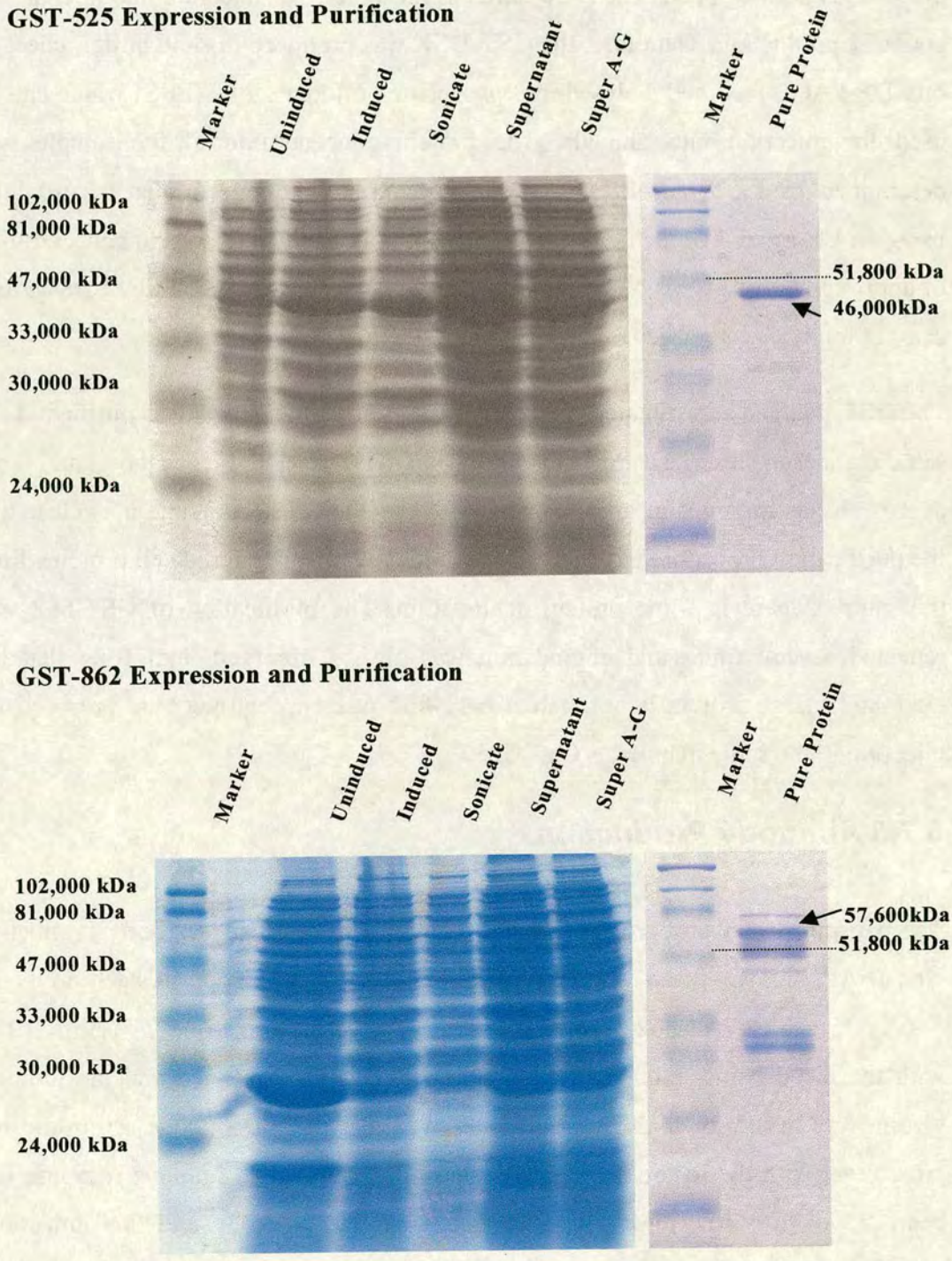
Legend 5.12

The expression and purification of both GST fusion proteins, GST-525 and GST-862. The first part of the figure shows the steps for GST-525, IPTG successfully inducing expression of the GST-525 after sonication. The majority of the protein remains in the supernatant. In the last lane is the purified protein at the expected size of 46kDa.

The second part of the figure is the similar information for GST-862. The only differences to be noted are that GST-862 did not express as well as GST-525, and the purified protein appears to suffer from a degree of degradation.

For both parts of the figure the first lane is the marker followed by uninduced cells in lane 2. Lane 3 shows cell protein after 4 hours induction at 37°C with IPTG. Lane 5 is the bacterial sonicate and lane 6 is the supernatant after the sonicate has been spun down. Lane 7, Super A-G, represents the cell protein that has been passed through a Glutathione Agarose column, the GST fusion protein should have bound to the column. Lane 8 is another marker and lane 9 represents the purified protein.

Figure 5.12 **Expression and purification of GST fusion proteins**



glutathione-agarose beads to bind the GST-525, which was then eluted from the beads in Z-50 buffer. Samples were taken during the purification stages and analysed by SDS-PAGE. The GST-525 eluted from the glutathione-agarose beads migrates at approximately 46kDa (Fig 5.12 A). It is clear that the purification procedure has successfully removed other bacterial cell proteins from the sample and that the protein has not degraded. The Z-50 buffer is unsuitable for injection into animals for antibody production. Therefore, the GST-DSX was produced in Z-50 buffer, checked on SDS-PAGE and then dialysed into phosphate buffered saline (PBS) which can be used for injection into animals. The protein concentration of the sample was determined by the Bradford assay and the sample was concentrated accordingly, using an Ultrafree-4 centrifugal filter unit (Millipore), to give a protein concentration of approximately 1μg/μl. A sample of the protein was checked again by SDS-PAGE prior to injection into sheep, to ensure the protein had not degraded.

The GST-862 had an estimated molecular weight of 57.6 kDa and was purified in the same manner as described for GST-525. The GST-862 eluted from the glutathione-agarose beads migrates at approximately 60kDa (Fig 5.12 B). While it is clear that the purification procedure has successfully removed other bacterial cell proteins from the sample, there is some protein degradation. The purification of GST-862 was repeated several times and degradation was always observed, indicating that the GST-862 fusion protein is not stable. GST-862 was concentrated and prepared for injection in the same manner as GST-525.

5.7.3 Antibody Production

The two purified fusion proteins were sent off to the Scottish Antibody Production Unit (SAPU). Two sheep were assigned, one for the production of each antibody, S46A-525 and S47A –862. The first injections were performed on 21st April 1999, with the bleed being conducted 7 days later. The primary Bleed and pre-immune serum were tested for both sheep. This showed that the pre-immune serum did not cross react with the fusion proteins and that in both sheep an immune response had been provoked by the injection of the fusion proteins. The 3rd and final injections

were conducted on the 21st June 1999 followed by a bleed on the 30th June 1999. Approximately 400ml of serum was obtained from the bleed for each animal, it was this final bleed that was used to conduct all antibody experiments.

The two antibodies received, Anti-525 and Anti-862 were both IgG purified to help reduce background staining (Fig 5.13). They were both tested to ensure that they were binding their appropriate GST fusion protein. Bacterial protein sample expressing GST fusion proteins were run on a SDS-PAGE gel, along with bacterial control protein (BL21 cells) and marker. The gel was used for a Western blot and immunostaining. Immunostaining was initially conducted with the preimmune serum, and this showed that the sheep used did not contain antibodies that cross reacted with the fusion protein. The Western was then immunostained in turn with the Anti-525 and Anti-862 antibodies, which did hybridise to the appropriate GST fusion protein, generating only a little background (Data not shown).

Having established that both antibodies bind the GST fusion protein that they were raised against, the antibodies were tested on an SDS-PAGE gel with fly ovary protein. We were aware that 3 transcripts of loco were expressed in oogenesis, with expected sizes of *loco-c1* 91.2kDa, *loco-c2* 129.3kDa and *loco-c3* 95.9kDa. Preliminary Western analysis showed that antibody staining was observed, at approximately 90kDA, this could correspond to Loco-c1 or Loco-c3. However this band was very faint and is not obvious in figure 5.14, however there is a band at approximately 70kDa that is more abundant and must represent an unidentified transcript.

5.7.4 Developmental profile of loco RGS in Drosophila

A developmental Western was carried out with Anti-525 on ovaries, testis, embryo, larvae, light pupae, dark pupae, female carcass and male carcass (Fig 5.14). The largest isoform observed on the Western is slightly larger than the marker, at 116kDa, and could represent a Loco-c2 isoform of 129.3kDa. This isoform is abundant in the late pupae and is not visible at other stages of development, perhaps due to low levels of expression. An isoform is also visible at just below the 97kDa

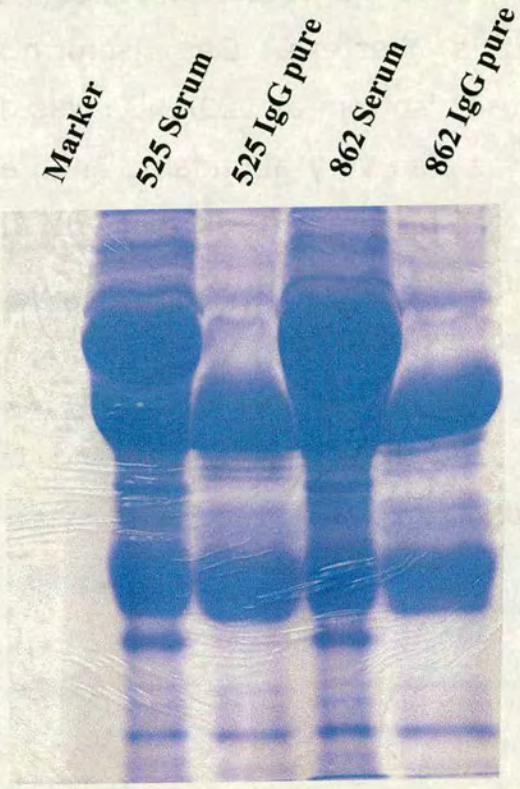
marker. This could represent either Loco-c1 or Loco-c3, or both as they produce isoforms of approximately 91.2kDa and 95.9kDa, respectively. This appears to be in the male and female carcass, although is probably present at undetectable levels in the other tissues. Interestingly, there is also an isoform at about 70kDa that appears in most, if not all, of the tissues.

This antibody data shows that there are at least three isoforms of the Loco protein present in *Drosophila* development. Analysis of transcripts suggests that we are not detecting all Loco isoforms. This can probably be attributed to the levels of expression of individual transcripts, which may not be in more than a few cells. It is also probable that non-specific binding of the antibody is generating background obscuring low levels of transcript. With the antibody affinity purified it should be possible to determine more about the different Loco isoforms.

5.7.5 Western Analysis of Mutant Fly lines

Complementation analysis of mutants generated in the P-Hop (Chapter 3) gives evidence that these are indeed mutants in *loco*. To clarify this, a Western was carried out containing mutant embryos that are embryonic lethal. In the mutant (fly line 94SFP16), there is a loss of Loco protein compared to the equivalently loaded wild type samples (Fig 5.15).

Figure 5.13 **Antibody purification**



Legend

In an attempt to reduce background on westerns both antibodies Anti-525 and Anti-862 were IgG purified using a Caprillic acid purification protocol (see Methods). This successfully isolated the IgG constituent of the serum, as can be seen from the pre and post purification's above. The two westerns shown in this chapter are both done with IgG purified Anti-525 and show reduced background over the initial Westerns (Not shown).

Legend 5.14

This figure represents a developmental Western. The panel on the left is the ponsaeu S staining (Pink) of the Western on the right (blue). Above each lane the tissue from which the protein originated is marked. On the right there are arrows and sizes marked for the protein isoforms detected. Three protein isoforms were detected on this Western. One isoform which could correspond to the Loco-c2 isoform at 129.3kDa was detected in the Dark pupae tract, it was also very abundant. Another isoform was detected in the male and female carcass that could correspond to either Loco-c1 or Loco-c3 isoform at 91.2kDa and 95.9kDa respectively. The third isoform was detected in ovaries, testis, embryos and male and female carcass, this had a size of approximately 70kDa and does not correspond to any as yet characterised transcripts.

Fig 5.14 Developmental profile of RGS -Protein loco

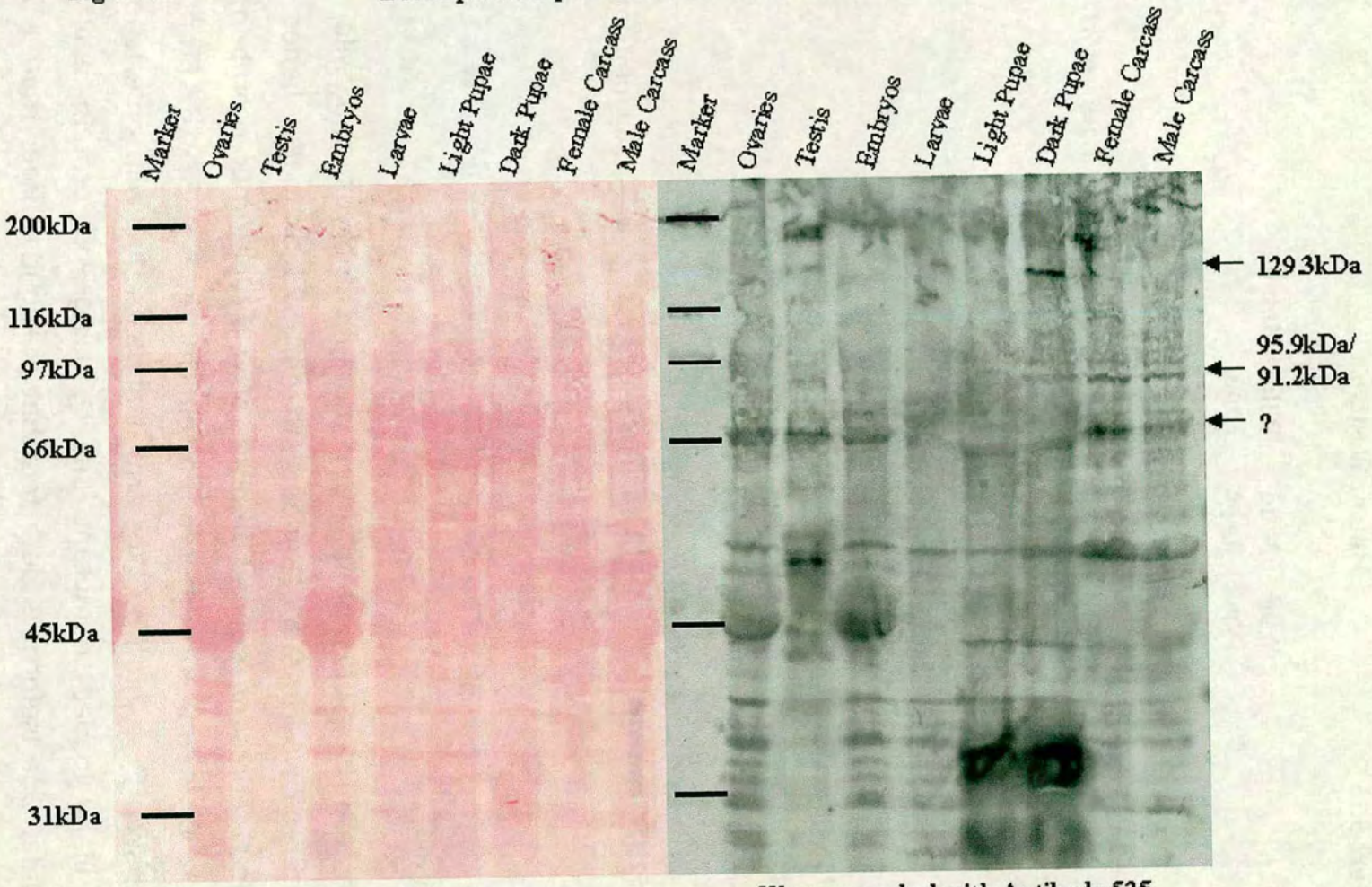
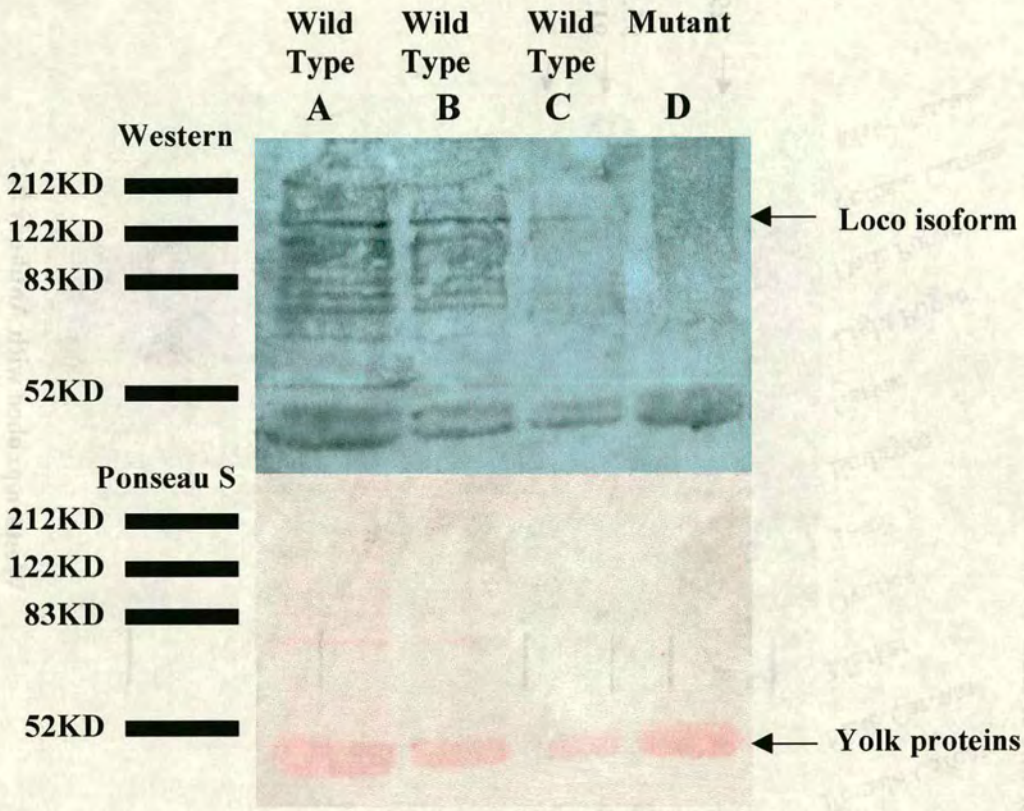


Figure 5.15 **Mutant Western**



Legend

Western blot of protein from mutant embryos and wild type embryos was immunostained with Anti-525 antibody. Lanes A, B and C represent reduced amounts of wild type embryo protein, and lane D is mutant embryo protein for line 94SFP16. From the Ponseau S staining on Western, it appears that wild type lane B is similarly loaded to mutant lane D, using yolk protein level as a guide. One isoform is detected that is slightly larger than 122kDa and could correspond to Loco-c2, which has a predicted size of 129kDa. This is clearly present in lane B and absent in lane D, indicating that the *loco* mutants do indeed lack the Loco protein.

5.8 Summary and Discussion

5.8.1 mRNA expression analysis

The initial *in-situ* analysis shows clearly that *loco* is expressed in oogenesis, in a dynamic expression pattern. Further work using exons specific to transcripts *loco-c2* and *loco-c3* showed that both transcripts were present in the germarium. *loco-c3* then appeared at low level at stage 6 in the nurse cells, becoming more abundant at around stage 10 and persisting in the nurse cells until they degenerate at stage 13-14. More interestingly, the expression of *loco-c2* in the anterior dorsal follicle cells first appears at around stage 10 and remains in these follicle cells until they degenerate at stage 14. The expression of *loco c2* in the anterior dorsal follicle cells is not uniform being expressed at a higher level in the dorsal midline than in dorsal lateral follicle cells. The anterior most columnar follicle cells bordering the nurse cells also express *loco*. This variation in level of expression could mean that *loco* is regulated by different components over the anterior dorsal region. In this chapter, *in-situs* of two of the transcripts were observed. *loco-c1* is also expressed (see the RT-PCR results in Chapter 4 and the library screen) but this transcript was not examined, due to time constraints. cDNA 118a shares the exon II-1 with *loco-c2*, this is the exon used to conduct the *in-situ* hybridisation for this transcript. This means that the probe used would show the combined expression pattern of 118a and *loco-c2,* this needs resolving. The second library screen (Chapter 4) showed that there are other as yet unidentified *loco* transcripts in oogenesis. The expression patterns of the new transcripts still have to be determined.

5.8.2 loco is downstream of the gurken-torpedo signalling pathway in oogenesis.

The *in-situ* analysis of *loco-c2* expression pattern in the *fs(1)K10*$^{-}$, *torpedo*$^{-}$ and *gurken*$^{-}$ mutant backgrounds, clearly shows that *loco* is downstream of the *gurken-torpedo* signalling pathway. The expression pattern of *loco-c2* in anterior dorsal

follicle cells is not uniform and subdomains of its expression pattern may be controlled differently. This observation is further backed up by results of experiments with *pointed,* in which ectopic *pointed* expression results in a reduction in *loco* expression in anterior dorsal follicle cells, except the ones at the margin.

5.8.3 Antisense analysis

The antisense analysis has shown that disruption of *loco* in follicle cells affects D/V polarity and reduces dorsal identity. This phenotype was observed in the heat shock fly line 5A, when antisense *loco* has been expressed in the follicle cells only. These effects were not seen in wild type flies exposed to hs and hence must be attributed to the antisense. The UAS antisense also produced the same phenotype although at a much lower frequency. This phenotype was consistent with the phenotypes observed in the mutants described in Chapter 3. As both the mutant fly lines and the heat shock fly lines exhibited the same severe phenotype, both producing eggs that were completely ventralized, *twist* expression was analysed to determine whether the ventralisation extended to the embryos. It was found these embryos were completely ventralized, expressing *twist* in all cells. This indicates that failure of *loco* expression disrupts the dorsal signal.

When *loco* is disrupted in nurse cells, it results in a short egg phenotype, indicating that dumping is affected. This was confirmed by observation of the ovarian phenotype, where nurse cells fail to dump properly. As far as can be determined, *loco-c3* is the only transcript detected in the nurse cells, and could be involved in either of the two processes that affect dumping. *loco-c3* could be involved in growth of ring canals needed to maintain flow rate to the oocyte, or in cytoskeleton organisation needed to maintain the nurse cell nuclei in the centre of the cells and prevent them blocking the ring canals during dumping.

5.8.4 Antibody

Two antibodies were made against small sections of the core region of loco encompassing the RGS domain. Both antibodies generated, Anti-525 and Anti-862,

bind the fusion protein they were raised against. The production of antibody has enabled detection of Loco protein on Westerns. The developmental Western has shown that at least 3 isoforms of loco are present in *Drosophila* development and that loco is present thought development, being especially abundant during metamorphosis. Although the data obtained is preliminary, it is promising and with affinity purification of the antibody clearer results should be possible, as well as antibody *in-situ* hybridisation.

The anti-525 was also used to show that Loco protein is lost in *loco* mutants. This is clear evidence to confirm that the mutants generated are mutants in the *loco* gene.

Chapter 6 Final Discussion

6.1 Introduction

The data in this thesis clearly illustrates that the RGS gene *loco* is expressed in oogenesis, with at least four different transcripts being present. *loco-c1*, *loco-c2* and *loco-c3* were demonstrated by RT-PCR. The remaining transcript, cDNA 118a, is an incomplete cDNA, but is definitely part of an ovarian transcript as it was isolated from an ovarian library. The ESTs identified and the second cDNA library screened, suggest that there are additional *loco* transcripts, yet to be identified.

loco-c3 is expressed in the germarium, after which the transcript is next seen at stage six, in the nurse cells. Expression levels in the nurse cells increase at stage ten and it persists in these cells until the end of oogenesis. *loco-c2* is expressed in the germarium and reappears in the anterior dorsal follicle cells at stage ten. Its expression in these follicle cells is not uniform, the dorsal mid line cells exhibiting a higher level of expression than the dorsal lateral cells. Having now determined that cDNA 118 and *loco-c2* represent different transcripts, this expression may, in fact, represent transcript 118a as these *in-situs* were done with an exon common to both transcripts.

The *in-situ* hybridisation data obtained so far indicates that transcripts of *loco* are expressed in different subsets of cells at different stages and are involved in different developmental processes. Analysis of UAS and heatshock induced antisense expression and *loco* mutants, along with molecular studies, implicate different components of *loco* expression in different developmental mechanisms.

Several of the mutant lines generated were homozygous lethal. This is expected for a gene that is involved in several developmental processes. The screen for female infertility and partial lethality yielded several weaker alleles that exhibited a complex range of abnormal egg phenotypes. Eggs were observed that were significantly shorter than wild type, corresponding to an ovarian dumpless phenotype. Eggs were also observed with anterior dorsal defects, varying from dorsal appendages fused at the base, to appendages fused along the entire length, to completely absent

appendages. A mutant resulting from a deletion will probably disrupt more than one transcript, possibly by affecting splicing or disruption of one of the core exons. Therefore observing several phenotypes in one fly line is expected.

The heatshock antisense results were crucial for interpreting how the different mutant phenotypes were generated and which *loco* transcripts and cell types are involved. The different heatshock lines exhibit antisense transcript expression in different groups of cells. This has allowed us to disrupt specific *loco* transcripts separately in the germline(line 2A) and follicle cells(Line 5A). *loco–c3* is expressed in the nurse cells and, when disrupted, leads to a dumpless phenotype and smaller-than-normal eggs are laid. When *loco–c2* (and or 118a) is disrupted in the anterior dorsal follicle cells, this results in a range of dorsal defects in the egg. The phenotypes observed, varying from dorsal appendage fused at the base, to appendages fused along the whole length, to completely absent appendages.

loco is expressed in anterior dorsal follicle cells and the disruption of *loco* in these follicle cells leads to dorsal anterior defects in the egg and embryo, implying that loco is involved in dorsal ventral patterning. As *loco* is a G-protein regulator, these observations suggest a role for G-Protein signalling in DV pattern formation in oogenesis and link G-Protein signalling to the EGFR signalling pathway. Granderath, *et al*(1999) have carried out a two-hybrid screen and using Giα, they isolated *loco* from a two-hybrid library. This is direct evidence for a conserved role between *loco* and mammalian RGS genes.

During the course of this study, two antibodies were raised against Loco. Experiments with these antibodies have shown that several isoforms of Loco are present during *Drosophila* development, and that there is at least one unidentified Loco isoform of approximately 68kDa, which is much smaller than any of the predicted isoforms from transcripts 1, 2, and 3. The antibody "525" has also been used to demonstrate that embryos from one of the homozygous lethal mutants do lack Loco protein. This is strong evidence that the mutants are a genuine disruption of the *loco* gene.

6.2 *loco* and the EGFR signalling pathway in anterior dorsal follicle cells

In recent years, the study of the EGFR signalling pathway has highlighted the complexity of dorsal-ventral patterning of the eggshell. Rhomboid (Ruohola-Baker et al., 1993) is known to upregulate the initial activation of the EGFR signal by Gurken. Rhomboid is thought to act by cleaving Spitz, releasing activated Spitz (Stevens 1998; Wasserman and Freeman, 1998) which binds to Torpedo(EGFR), upregulating it. Another ligand of EGFR, Vein, is also thought to be involved in this process. In this way, the initial paracrine signal from *gurken* in the oocyte becomes an autocrine signal within the follicle cells.

The secreted protein, Argos, is produced following high levels of EGFR activation (Wasserman and Freeman, 1998; Zhao and Bownes, 1999). This secreted protein represses the EGFR signal, possibly by interacting with the external domain of EGFR, preventing any further ligands binding. Because Argos is a secreted protein, it has been suggested that it diffuses away from the site of its production, resulting in a gradient around the dorsal midline follicle cells. It is postulated that Argos separates the single EGFR signal into two peaks, resulting in two dorsal appendages (Wasserman JD and Freeman M, 1998). The regulation of the EGFR signal does not stop here, as Kekkon I, which is a transmembrane protein is thought to associate with the external domain, thereby modulating the signal (Ghiglione C et al., 1999). The product of another novel gene, *sprouty*, has also been shown to negatively regulate the EGFR signal (Reich A et al., 1999).

Why is there such an elaborate process to specify cell fates along this axis and how does *loco* fit into the existing pathway? *loco* is downstream of Torpedo in the follicle cells and appears to be activated both at high and moderate levels of Torpedo activation, although *loco* expression is not uniform (Figure 2B). The levels of expression are higher in the dorsal midline follicle cells and the follicle cells at the nurse cell/follicle cell junction. This may imply part of *loco* expression pattern, are controlled by different genes, expressed in the anterior-dorsal follicle cells. For

example the high levels of *loco* in the anterior most follicle cells correlate with the region in which *dpp* is expressed. Recent work by Peri and Roth (2000) has shown that *dpp* needs to interact with the *gurken* signal to produce dorsal appendages. *loco* could be playing a role in these more localised interactions. Alternatively the variation in levels of *loco* expression over the dorsal anterior region could be a response to the levels of activated EGFR.

The role of RGS proteins is to negatively regulate G-Protein signalling. Granderath *et al* (1999) have shown with a two hybrid screen that *loco* binds Giα and Wolfgang *et al* (1991) showed Giα is present in the anterior dorsal follicle cells. Thus it is probable that G-Protein signalling has a role in DV axis formation. In the most severe *loco* phenotypes there is a loss of DV polarity in the egg and embryo (ventralised eggs). This phenotype correlates with the complete loss of *loco* in these cells and is similar to *gurken* mutants, suggesting that in the absence of *loco,* repression of the EGFR signalling occurs. Since RGS genes negatively regulate G-Protein signalling, one can assume that in egg chambers G-Protein signalling inhibits EGFR signalling, but the expression of *loco* prevents or regulates this inhibition in the anterior-dorsal follicle cells. This adds to the complexity of regulation of EGFR and suggests that the tight regulation or modulation of EGFR is critical for the correct sequence of morphological events to occur in the process of specification of follicle cell fates along the DV axis. This helps explain the range of *loco* phenotypes observed in the egg, as the resulting phenotype will depend on the precise time and location *loco* is affected. It also explains the weak phenotypes observed, as there are several feedback loops, regulating the EGFR signal that could compensate for the disruption of *loco* activity.

Granderath et al. (2000) show that the relationship between *pointed* and *loco-c1* transcript is also dependent on the gene *gcm*, and that *pointed* and *gcm* act synergistically to activate *loco-c1*. In the follicle cells it is *loco-c2* that is present. The absence of a simple link between *pointed* and *loco* in the follicle cells is illustrative of many developmental situations, where the control mechanisms vary and are adapted to a new situation and a different set of developmental decisions. Another

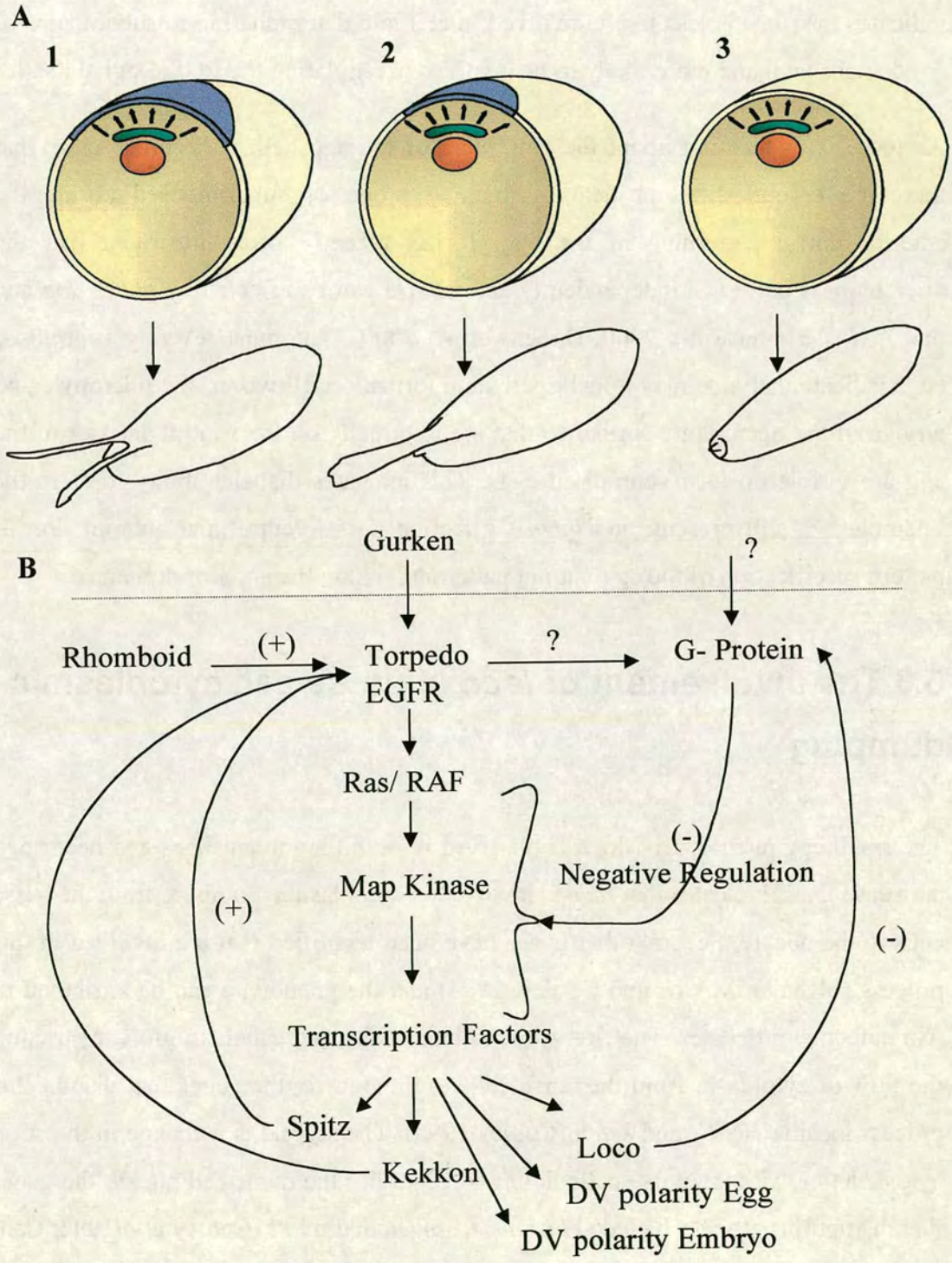
Legend 6.1

Part A suggests a possible role of *loco* in the follicle cells, 1 is the normal wild type egg. The nucleus (orange) is positioned dorsally with the *gurken* transcript (green) localised to the dorsal side of the nucleus, resulting (after translation) in the activation of Torpedo in the anterior dorsal follicle cells. This will result in the activation of *loco* in all the dorsal-anterior follicle cells (blue) and normal EGFR signalling. When *loco* is activated normally, a normal wild type egg results. 2 illustrates what happens when *loco* is disrupted to some degree, resulting in reduced response to the EGFR signal. This results in an egg with a reduced dorsal area and a fused dorsal appendage. 3 shows complete disruption of *loco,* resulting in a complete lack of response to the EGFR signal and a ventralised egg phenotype.

Part B represents a genetic flow diagram of events in the follicle cells. Gurken protein activates Torpedo (EGFR) in the anterior dorsal cells, this in turn leads to a Ras/Raf activating MAPK, which results in specific transcription factors being activated. Among the genes switched on are *rhomboid*, *argos* and *spitz* which are all involved in regulating the dorsal signal or subdomains of it. The involvement of *loco* suggests that G-Protein signalling is part of DV patterning. This figure illustrates one possible way in which G-Protein signalling could be linked to our current understanding of the EGFR signalling pathway. If the G-Protein is only present in anterior dorsal cells, as shown in work by Wolfgang *et al*(1991), it must either be activated by *gurken* or a parallel pathway. *loco* is activated downstream of EGFR and negatively regulates Giα and this, in turn, negatively regulates Ras/Raf MAPK signalling. Thereby *loco* helps modulate the level of EGFR signal.

Figure 5.1

Regulation of the EGFR signal in anterior dorsal follicle cells.



indication that *loco* is not downstream of *pointed* is the different phenotypes observed in mutants for these two genes. In *pointed* mutants there is a more extensive loss of dorsal midline fate, while in *loco* mutants there is a loss of dorsal fate. This indicates that *loco* is less likely involved in cell fate determination in subdomains of the dorsal signal and more likely to be involved in regulating the EGFR signal itself.

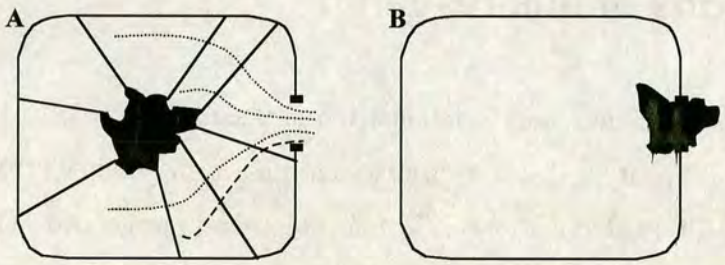
As more is understood about the patterning of the eggshell, it becomes clear that there are several different decision making processes superimposed during the anterior dorsal patterning of the egg. It has recently been illustrated that the operculum is patterned independently and its size can be affected by genes *dpp* and *bunched* (Peri and Roth, 2000; Dobens et al., 2000). The most severely ventralised eggs indicate that *loco* may affect operculum formation. However, the micropyle and a ridge of the operculum, similar to that seen normally on the ventral anterior of the egg are visible on *loco* ventralised eggs. This indicates that the ability to form the operculum is still present, and *loco* is affecting dorsal-ventral and anterior dorsal-pattern specification of the egg but not patterning within the anterior domain.

6.3 The involvement of *loco* in nurse cell cytoplasmic dumping

The small egg phenotype, which is observed in both the mutant lines and heatshock antisense lines, indicates that *loco* is involved in cytoplasmic dumping from the nurse cells to the oocyte. Several other genes have been identified that are involved in this process (Mahajan-Miklos and Cooley, 1994) and the phenotype can be attributed to two defective processes. The first is the failure of the ring canals to grow, restricting the flow of cytoplasm from the nurse cells to the oocyte, the genes that disrupt this process include *HtsRC* and *kelch* (Cooley 1998). The second is a change in the actin cytoskeleton, with the nurse cells failing to centralise the nurse cell nuclei, the genes that disrupt this process include *chickadee*, *singed* and *quail* (Cooley *et al*1992; Cant *et al*1994; Mahajan-Miklos and Cooley 1994; Guild *et al*1997). When this happens, the nuclei block the ring canals and prevent dumping (Fig 6.2).

G-Proteins are responsible for the transduction of a signal from a cell surface receptor to specific downstream effectors. This implies that *loco* is involved in communication between cells. Therefore it is unlikely that *loco* is directly involved in either of the two processes that cause nurse cell dumping to fail, ring canal growth and cytoskeletal structure. If disruption of *loco* leads to failure of the nurse cells to dump, which cells are failing to communicate? The nurse cells may have to communicate with one another to co-ordinate dumping, ensuring that the dumping happens in the correct sequence. The nurse cells may receive a signal from either the oocyte or follicle cells, indicating that certain developmental processes are complete and dumping is the next process that needs to happen. These questions remain to be answered. Although a function has been assigned to *loco* in the nurse cells, it does not imply that there is not a maternal *loco* transcript or protein that is present in the formed egg, necessary for early embryonic development.

Figure 6.2 **Nurse cell dumping**



A Is the wildtype the condensed nucleus is held in position during dumping, by actin cytoskeleton.

B In a mutant with disrupted cytoskeleton, the nucleus blocks the ring canal.

6.4 *loco* in *Drosophila* development

The work described by Wolfgang *et al*(1991), show that GOα, Gsα and Giα are expressed several times during *Drosophila* development. This suggests that *loco* as an RGS gene will have multiple roles in development. Lac Z staining on discs from fly line C139 show that *loco* is present in the eye disc, lac Z staining was also

observed in the testis. Granderath *et al* (1999) have identified a role for *loco* in glial cell formation in embryogenesis. A developmental Western has shown that at least one Loco isoform is present at every developmental stage (Chapter 5). An interesting observation made when comparing loco to mammalian RGS genes was that mammals had numerous RGS genes responsible for the regulation of specific Gα subunits and that several of these RGS proteins also had other domains conferring additional functions to the proteins. Searches on the *Drosophila* genome have not identified any other RGS genes suggesting that *loco* is the only RGS gene. In light of the size of the gene and its many transcripts it could be that different transcripts are responsible for the regulation of specific Gα subunits. In addition protein domain searches have identified the presence of a DEP domain in one of the transcripts. The DEP domain is also found in some of the Mammalian RGS Proteins and may regulate the membrane localisation of RGS. The presence of other domains within Loco isoforms suggests that they could have a role extending beyond being GAPs for heterotrimeric G-Proteins.

6.5 Future directions of this research

The work described in this thesis has only scratched the surface of the role of the *loco* gene in oogenesis. It is apparent that transcripts identified in the library screen need to be sequenced, and RT-PCR done to verify their presence in oogenesis. This may shed more light on cDNA 118a, which appears to have a stop codon before the RGS domain, indicating that it may not be making a functional G-Protein. However it may be involved in the regulation of *loco* transcript via alternative splicing, or the cDNA 118a protein isoform could have another functional role, depending on the domains present within it.

With the transcripts resolved, more informative *in-situ* hybridisations may be conducted, with transcript specific probes. This may resolve the varying levels of expression seen in the anterior dorsal follicle cells, showing this to result from the presence of several different transcripts.

The antibodies generated in this research need affinity purifying as this may make them suitable for *in-situ* detection in ovaries. The protein expression pattern will be a better indicator of where *loco* is active, and it may also be possible to identify the subcellular localisation of Loco, which may lead to an insight into how Loco is functioning in the nurse cells.

The mutants generated have not been analysed completely and further complementation analysis is needed to establish how many complementation groups there are and relate them to specific transcripts that are affected. A male sterile test could be conducted, to see if *loco* has an important role in spermatogenesis, as there is a testis transcript (Testis library EST Chapter 4).

One of the big questions that remains to be answered is, which Gα subunits does *loco* interact with and whether the interaction is isoform specific. This could be done through a two- hybrid screen, using the different isoforms of the Loco –protein. It would also help to determine which Gα subunits are expressed in oogenesis and where.

6.6 Conclusion

loco is an RGS gene that has an important regulatory role in oogenesis. Disruption of this gene leads to defects in dorsal-ventral polarity of the egg and embryo, and in the process of nurse cell dumping. This gene is probably under complex regulation, due to its central role in several developmental processes, both within and beyond oogenesis. The role of *loco* in EGFR signalling links G-Protein signalling to dorsal-ventral pattern formation in oogenesis, illustrating how different pathways can interact to specify the correct developmental outcome.

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