Characterisation of Two Genes Expressed During *Drosophila* Oogenesis and Embryogenesis

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Declaration

I declare that this thesis was composed by myself and that the work described is my own, unless otherwise stated.

Shenyin Lin

2003

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Abstract

The interaction between somatic follicle cells and germline cells during oogenesis establishes the anterior/posterior and dorsal/ventral axes of the oocyte and future embryo. Identification of novel gene expression in groups of follicle cells at specific stages of oogenesis may therefore help us to understand pattern formation in *Drosophila* oogenesis. The novel gene *Z14/Dmbves* was identified as part of a GAL4/UAS enhancer trap screen. The *Z14/Dmbves* gene is similar to a gene encoding blood vessel/ epicardial substance (bves) in Chicken and humans. We found that *Z14/Dmbves* is expressed in the nurse cell and all the follicle cells surrounding the oocyte **except** those in the anterior-dorsal and posterior regions, which receive the *gurken* (Grk) signal from the oocyte during polarity establishment. Further analysis indicated that the expression of *Z14/Dmbves* is repressed by the Grk signal. P-element mobilisation to generate mutants was undertaken but no mutants of *Z14/Dmbves* were obtained. Investigation of transgenic flies revealed that disruption of the function of *Z14/Dmbves* caused lethality to both embryos and larvae. Our data suggest that *Z14/Dmbves* might still be an essential gene and is crucial for embryonic development.

Many developmental events rely on signal transduction pathways that result in the selective nuclear import of transcriptional regulatory proteins. One of them is the Toll signalling pathway which ultimately cause the translocation of the Dorsal protein, a transcription factor, from the cytoplasm to the nucleus during the *Drosophila* early embryogenesis, thus enabling it to initially establish the dorsal-ventral polarity in the embryo. Although it has been demonstrated that the Toll signalling pathway leads to dissociation of Dorsal protein from its inhibitor Cactus

protein, little is known about the factor which mediates the translocation of Dorsal protein into the nucleus. The *ntf-2* gene was identified as part of a P-element mobilization screen for generating mutants of *Z14/Dmbves*. Mammalian Ntf-2 is a homodimer of approximately 14kDa subunits which stimulates the efficient nuclear import of a RanGDP. In *Drosophila* it was found that *ntf-2* transcripts are present during oogenesis and embryogenesis. *ntf-2* is provided maternally to the embryo since its mutants only die as late embryos and during larval stages. Germline clones were generated to knock out the maternal *ntf-2* mRNA. Homozygous *ntf-2* null embryos generated from germline clones are found to be dead in the early stages of embyrogenesis and show a dorsalised phenotype. The result of *in situ* hybridisation and antibody staining of *ntf-2* null embryos revealed that *ntf-2* is involved in the Toll signalling pathway. Therefore, we propose that *ntf-2* is essential for the nuclear translocation of the Dorsal protein, which is a crucial step in the establishment of dorsal-ventral patterning during *Drosophila* embryogenesis.

Abbreviations

General Abbreviations:

λ	lambda
Δ	delta
♂	male
β	beta
μ	micro- (1.10^{-6})
♀	female
[x]	concentration X
^{32}p	β- emitting isotope of phosphorus
aa	amino acids
A_{xnm}	Absorption at Xnm
bp	base pair(s)
C-	Carboxy – (terminal)
cDNA	complementary DNA
Cm	centimetre(s)
C°	degrees centigrade
g	gram
g	G centrifugal force
kb	kilobase(s)
krpm	kilo (1000) revolutions per minute
l	litre
M	Molar
ml	millilitre(1.10^{-3})

mM	millimolar(1.10^{-3})
mol	-moles
n	nano- (1.10^{-9})
N-	Amino – (terminal)
nm	nanometres(1.10^{-9}m)
nt	nucleotides
O/N	overnight
OD_{xnm}	optical Density at X nm
ORF	open reading frame
P	pico – (1.10^{-12})
PCR	polymerase Chain Reaction
Pers.comm.	personal communications
pH	-log10 [hydrogen ion]
RT-PCR	Reverse Transcription PCR
U	Units
UTR	Untranslated region
UV	Ultraviolet
v/v	volume to volume ratio
w/v	weight to volume ratio
w/w	weight to weight ratio

Chemicals:

Amp	Ampicillin
AP	Alkaline phosphatase
$Cacl_2$	Calcium chloride

$CHCL_3$	Chloroform
CLAP	Calf intestinal alkaline phosphatase
dATP	2'deoxyadenosine-5'-triphosphate
dCTP	2'deoxycytosine-5'-triphosphate
ddH2O	double distilled water
dGTP	2'deoxyguanosine-5'-triphosphate
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonclease
dNTP	deoxynucleotide-5'-triphosphate
dTTP	2'deoxythymidine-5'-triphosphdte
dUTP	2'deoxyuridine-5'-triphosphate
EST	expressed sequence tag
EtBr	Ethidium Bromide
EtOH	Ethanol
GAP	GTPase-activating protein
HCl	Hydrochloric acid
Hepes	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid]
Kan	kanamycin
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
KOAc	Potassium Acetate
KOH	Potassium hydroxide
MeOH	Methanol

MEK	mitogen-activated protein kinase kinase
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
$MnCl_2$	Manganese chloride
mRNA	messenger RNA
Na_2HPO_4	Disodium hydrogen phosphate
NaAc	Sodium acetate
NaCl	Sodium chloride
$Na_2H_2PO_4$	Sodium dihydrogen phosphate
NaOH	Sodium hydroxide
NH_4OAc	Ammonium acetate
PBS	Phosphate buffered saline
PIPES	Piperazine-N,N'-bis[2-ethanesulphonic acid]
Ran	ras-related nuclear protein
RCC1	regulator of chromosome condensation 1
RbCl	Rubidium chloride
RNA	Ribonucleic acid
rRNA	ribosomal RNA
SDS	Sodium dodecyl sulphate
Tris	Tris(hydroxymethyl)-amino-methane
Triton X-100	Octylphenoxypolyethoxyethanol
tRNA	transfer RNA
Tween-20	Polyoxyethylene sorbitan monolaurate
UAS	upstream activation sequence

Single letter amino acid code:

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

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Chapter One: Introduction

Drosophila oogenesis is an extremely valuable system to understand development. The egg is probably the most complex cell in the body. It contains all of the nutrients needed to nourish the embryo and has the ability to develop to a complete organism. Many events start during oogenesis such as cell differentiation, polarity determination and pattern formation that are crucial for subsequent embryogenesis. Investigation of how oogenesis produces an egg capable of generation of adult will provide insights into many aspects of developmental biology.

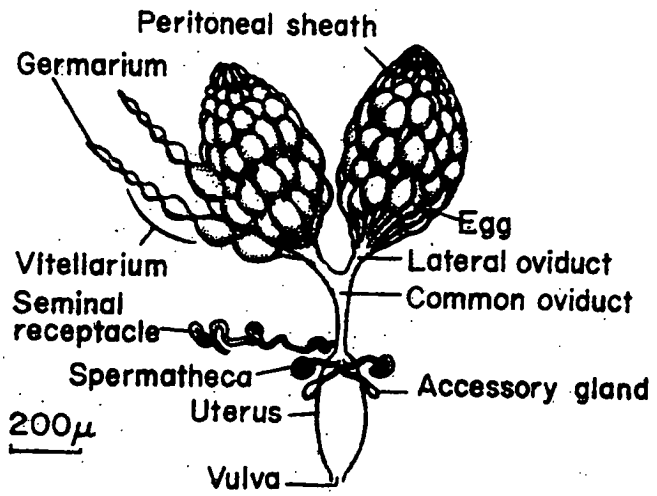
1.1 *Drosophila* Oogenesis

1.1.1 Morphology of the *Drosophila* oogenesis

The *Drosophila melanogaster* female has a pair of ovaries each containing 13 to 17 ovarioles (Fig.1.1). At the tip of each ovariole is the germarium. The germarium contains 1-2 germline stem cells as well as stem cells for the follicle cells. Oogenesis starts within the anterior compartment of the germarium (Spradling, 1993). In response to an unknown signal a germline stem cell can produce a daughter stem cell and a cystoblast. The cystoblast undergoes four consecutive mitotic divisions to form 16 cells (de Cuevas et al., 1997). The 16 germline cells are connected to each other through specialized cytoplasmic junctions called ring canals. Meiosis occurs in one of the these 16 cells and it is always one of the two cells which has four cytoplasmic connections. This cell is determined to become the oocyte, the others become nurse cells which provide materials to the developing oocyte and embryo.

The germarium has been divided into four regions (Grieder et al., 2000). The stem cells and the cystoblast lie within germarial region 1. Region 2a contains newly

Figure1.1 Adult Female Ovaries



From King, 1970

Legend

The figure illustrates that the *Drosophila melanogaster* female has a pair of ovaries each containing 13 to 17 ovarioles. At the tip of each ovariole is the germarium which contains the germline stem cells.The germarium is followed by a string of progressively more developed egg chambers, leading up to mature egg. The two ovaries connected to the uterus by a common oviduct. The mature eggs are fertilised in the uterus from the Sperm stored in the seminal receptacle and spermathecae.

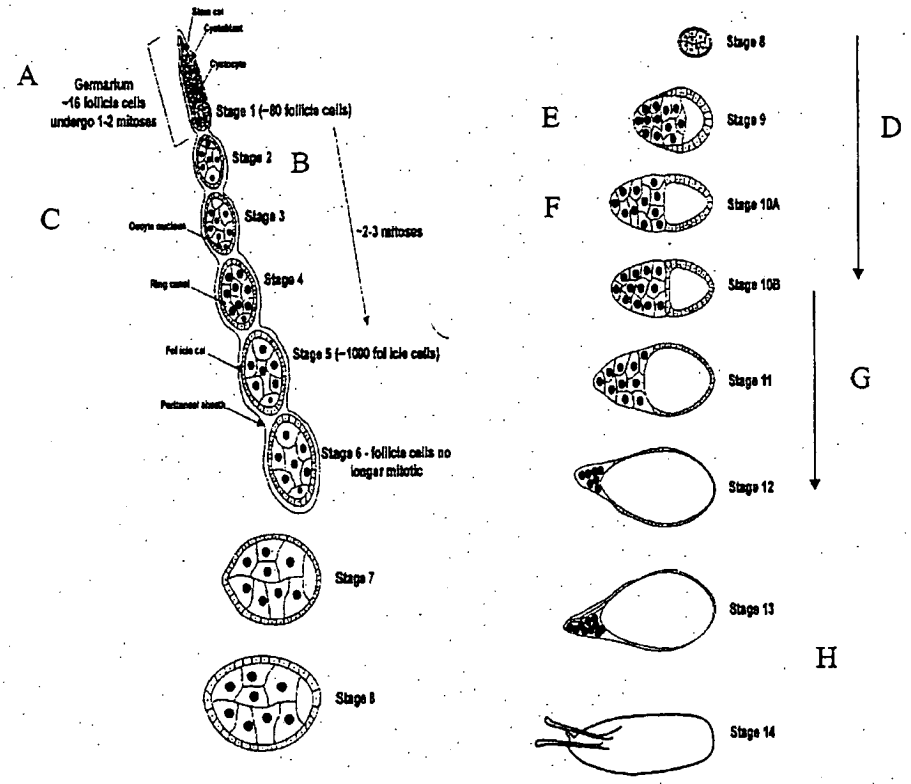
formed 16-cell cysts, whereas in region 2b the cysts become lens shaped and span the width of the germarium. The pro-oocyte is positioned in the centre of the cyst. In region 3 the 16-cell cyst acquires a monolayer of follicle cells around it and is referred to as stage 1 egg chamber. The oocyte is located at the posterior pole of the cyst and remains in this position throughout the completion of oogenesis.

The developmental stages of oogenesis have been numbered from stage 1 to stage 14 (King, 1970, Fig.1.2). Stages 1-6 comprise the developmental period before the uptake of yolk by the oocyte. During this previtellogenic period the oocyte and the nurse cells are similar in size. The developing egg chambers are connected by stacks of interfollicular stalk cells.

During stages 7-10 the oocyte begins more rapid growth as a consequence of the uptake of yolk proteins which are synthesised in the fat body and follicle cells (Hames and Bownes 1978; Isaac and Bownes 1982). Yolk proteins synthesised in the fat body are subsequently taken up by the oocyte via the haemolymph. The yolk from the follicle cells is directly transported to the oocyte.

At stage 9 a series of follicle cell migrations take place. About six to ten follicle cells called border cells, located at the anterior end of the egg chamber migrate posteriorly between the nurse cells towards the oocyte. The role of the border cells is limited to creating the micropylar pore on the eggshell (Montell et al., 1992; Edwards and Kiehart, 1996). Other follicle cells, which overlay the nurse cells, migrate posteriorly to cover the oocyte, so that by stage 10A the oocyte is covered by a sheet of thick columnar follicle cells, while the nurse cells have a thin layer of stretched cells associated with them. At stage 10B, the oocyte is 50% of the size of the egg chamber. At this stage follicle cells begin secretion of the chorion . In addition to

Figure 1.2 Drosophila oogenesis



Modified from Clyde

Legend

The figure illustrates the developmental sequence of oogenesis

A. Oogenesis starts in the germarium.
B. From stage2, egg chambers leave the germarium.
C. The nurse cells and oocyte are appoximately the same size from stages1 to 6.
D. From stages 8 to stage10B, the egg chamber grows quickly.
E. A series of follicle cells migrations take place at stages 9-10.
F. The oocyte is covered by a sheet of thick columnar follicle cells in stage10.
G. From stages10B to 12 nurse cell cytoplasm is rapidly transferred into oocyte.
H. At stages 13 and 14, the remaining nurse cells and follicle cells degenerate and die, the mature egg is wrapped with the complete eggshell and a pair of dorsal appendages is produced.

these events, the first RNAs and proteins become asymmetrically localized within the oocyte at stage 7. These localized molecules are essential for establishing anterior-posterior and dorsal-ventral positional information within the oocyte and embryo which will develop from it after fertilisation.

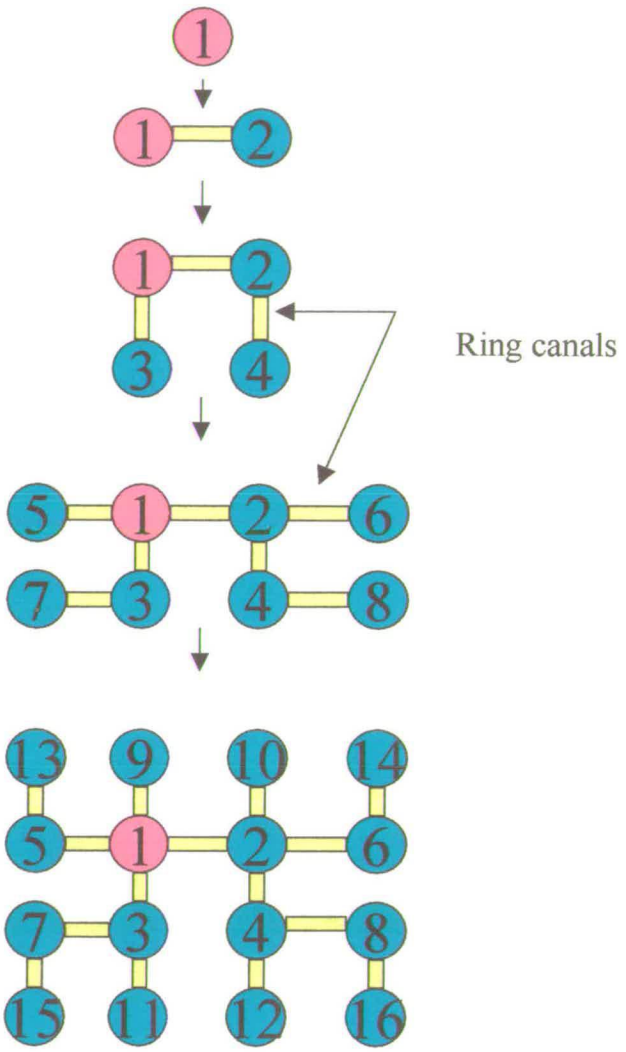
During stages10B to 12 the nurse cells transfer their cytoplasm rapidly through the ring canal into the oocyte. In the final stages of oogenesis (stage 13-14), the nurse cells and follicle cells degenerate and die, leaving behind the mature egg which is wrapped with a complete egg shell (chorion) along with its specialised structures: the micropyle for sperm entry; the operculum for larvae exit and two dorsal appendages (filaments) for facilitating embryonic respiration.

1.1.2 The development of the ovary

1.1.2.1 oocyte specification

The cystoblast undergoes four consecutive mitotic divisions to form a 16-cell cyst. Due to incomplete cytokinesis the 16 germ line cells are connected to each other through specialised cytoplasmic junctions called ring canals. The first two cells retain four canals (de Cuevas et al., 1997, Fig.1.3), the next two retain three retain three canals, the next four retain two canals, and the last eight cells have one canal. It is always one of the four-ring-canal cells that becomes the oocyte. Two gene, *Bicaudal-D* (*BicD*) and *egalitarian* (*egl*) have been identified as specifically required for oocyte differentiation. Their mRNAs accumulate selectively in the pro-oocyte in region 2A of the germarium (Suter and Steward 1991; Schupbach and Wieschaus, 1991). *BicD* and *egl* are believed to be required for oocyte determination because *BicD* and *egl* mutant cysts contain no oocyte and 16 nurse cells, and all oocyte-

Figure 1.3 oocyte specification



Legend

The cystoblast undergoes four consecutive mitotic divisions to form a 16-cell cyst. Due to incomplete cytokinesis the 16 germ line cells are connected to each other through specialized cytoplasmic junctions called ring canals. It is always one of the four-ring-canal cells with a fusome that becomes the oocyte. The other 15 cells differentiate into nurse cells.

specific markers fail to accumulate in one cell. *egl* protein colocalizes with *BicD* protein at all stages of oogenesis (Mach and Lehmann, 1997). Both proteins are part of a protein complex which is required to transport factors promoting oocyte differentiation. Another factor involved in oocyte determination is the fusome, a vesicular organelle which is associated with one of the poles of the mitotic spindle and is asymmetrically distributed in one of the daughter cells (de Cuevas and Spradling, 1998). The daughter cell with four ring canals and a fusome always become the oocyte, and thus the fusome is thought to be an oocyte determining factor.

1.1.2.2 Nurse cell and oocyte development

The oocyte is transcriptionally active in germarial cysts but it becomes repressed shortly after the egg chamber leaves the germarium. Oocyte chromosomes condense into a karysome by stage 3 (Spradling, 1993).

Nurse cells also undergo changes in their nuclear organization during this stage. The nucleus condenses and becomes transcriptionally inactive until just before the oocyte matures. Large scale organization of nurse cell chromatin can not be seen. Instead of the chromatin, the nurse cell chromosomes become polytene and these banded polytene chromosomes progressively disperse to form large nuclei. Satellite DNA, becomes underrepresented as the nurse cell DNA content increases (Hammond and Laird, 1985). These changes are thought to facilitate high levels of ribosome synthesis and other components required for oocyte growth.

Mechanisms must exist to coordinate the growth of the nurse cells and oocyte since their volumes increase at a similar rate till stage 7. After that, the oocyte grows faster than nurse cell because it takes up yolk protein (Spradling, 1993).

1.1.2.3 Follicle cell migrations

The first migration of follicle cells is from the wall of the germarium to surround the germinal cyst. This is a significant step in the formation of the egg chamber. Egg chamber encapsulation is dependent upon Notch function in the polar follicle cells and Delta function in the germ line (Lopez-Schier and St Johnston, 2001). There are only two cytoskeletal protein, myosin II regulatory light chain (Edwards and Kiehart, 1996) and filamin have been identified that are involved in early follicle cell morphogenesis (Sokol and Cooley, 2003). Later in oogenesis, at the start of stage 9, the majority of the follicle cells move posteriorly toward the oocyte to form a columnar epithelium covering the oocyte. Only about 50 cells remain over the nurse cells. These follicle cells stretch so that they still cover all the nurse cells (Gonzalez-Reyes and St Johnston, 1998). At the same stage a group of 6-10 follicle cells at the anterior tip of the egg chamber migrate through the nurse cell cluster to the oocyte border. These cells are called the border cells. Their role is to produce an anterior egg shell structure, the micropyle (Montell et al., 1992). Recently studies reveal that Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway is required for border cell migration (Silver and Montell, 2001; Beccari et al., 2002).

During stages 10B and 11, anterior cells from the columnar layer migrate in between the oocyte and adjacent nurse cells to cover the anterior of the oocyte. These cells are called centripetal cells (Denef and Schupbach, 2003). At stage 12 the anterior dorsal follicle cells migrate anteriorly to produce a pair of dorsal appendages (Spradling, 1993). Recent work have shown that cells of dorsal

appendage are determined not only by Epidermal Growth Factor Receptor (EGFR) signalling pathway and Decapentaplegic (Dpp) pathway (Deng and Bownes, 1997), but also by another signalling pathway, the Drosophila Jun-N-terminal kinase (JNK) cascade, which is essential for the correct morphogenesis of the dorsal appendages and the micropyle during oogenesis (Suzanne et al., 2001).

1.1.2.4 Vitellogenesis

Vitellogenesis occurs during stages 8 to 10. It is the process by which *Drosophila* eggs accumulate large amounts of yolk which provides nutrients to the developing embryo. There are three major yolk constituents: protein-containing particles, glycogen-rich particles and lipid droplets. The yolk proteins are produced outside the ovary in the fat body, secreted into the haemolymph and taken up by the oocyte (Dimario and Mahowald 1986). Yolk proteins are also produced by the follicle cells during stages 9 and 10 (Brennan et al, 1982). Lipid droplets appear during stage 8 and are probably synthesised by the nurse cells and transported into the oocyte. Glycogen-rich yolk particles do not appear until stage 13 and are probably synthesized in the oocyte in association with the mitochondria that surround them (Spradling, 1993).

There are three genes which encode yolk protein call YP1, YP2 and YP3 (Bownes, 1990). The transcription of yolk protein genes was believed to be regulated by juvenile hormone (JH) and 20-Hydroxyecdysone, although a direct action has not been demonstrated (Bownes, 1994; 1996).

1.1.2.5 Nurse cell-oocyte transportation

Nurse cells provide the oocyte with the vast majority of its cytoplasmic constituents. Proteins, mRNA, ribosomes and mitochondria are transferred into the oocyte through the ring canals (Spradling, 1993).

Two distinct phases of transport were suggested by studies of histone mRNA within the egg chamber (Ruddell and Jacobs-Lorena, 1985; Ambrosio and Schedl,1985). Prior to stage 10, synthesis during the early phase may be primarily for use within the nurse cells. From stage 10B synthesis may be primarily for storage in the egg.

Several genes, *chickadee*, *kelch* and *singed* have been identified as genes involved in cytoplasmic transfer (Cooley et al., 1992; Xue and Cooley, 1993; Paterson and O'Hare, 1991). The oocyte in these mutants develops to one-half the normal size, nurse cells remain large and attached at the anterior end.

At stages 11-12, most of the cytoplasm of the nurse cells streams through the ring canal to the oocyte at a rapid speed. This process is called dumping. It induces the oocyte to double its volume and the nurse cells degenerate, leaving behind the mature egg.

1.1.2.6 Egg shell formation

During stages 8-14 the follicle cells do a precise job to build the egg shell. This process includes vitelline membrane synthesis, chorion production, micropyle and dorsal appendage formation.

The constituents of the vitelline membrane are synthesised by the follicle cells (Fargnoli and Waring, 1982). First they are secreted and accumulated in vitelline bodies at stage 9. During stage 10 they form a uniform layer surrounding the egg.

The vitelline membrane is a rigid structure that can maintain the shape of the egg even after removal of the chorion.

The chorion proteins are all synthesised by the follicle cells after vitelline membrane formation. At their expression peak the major chorion genes are among the most abundantly transcribed genes known in *Drosophila.* This is called chorion gene amplification and it is closely tied to follicle cell cycles (Calvi et al., 1998). The amplification of the eggshell protein genes lead to rapid synthesis of the eggshell later in oogenesis.

A group of 40-50 follicle cells that derive from two distinct subpopulations produce the micropyle (Margaitis 1985; Montell et al., 1992). The cone-shape structure functions as a sperm receptor. Two groups of columnar cells at the dorsal-anterior region migrate anteriorly to produce a pair of dorsal appendages which are part of the anterior eggshell structures. Staining with a dorsal appendage marker, the Broad complex (BR-C) shows that there are 55-65 follicle cells in each group (Deng and Bownes, 1997). Filament follicle cells begin to secrete the filament base 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 (Spradling, 1993). The dorsal appendages complete their elongation at stage 14.

1.2 Polarity Determination and Pattern Formation

1.2.1 Origin of polarity

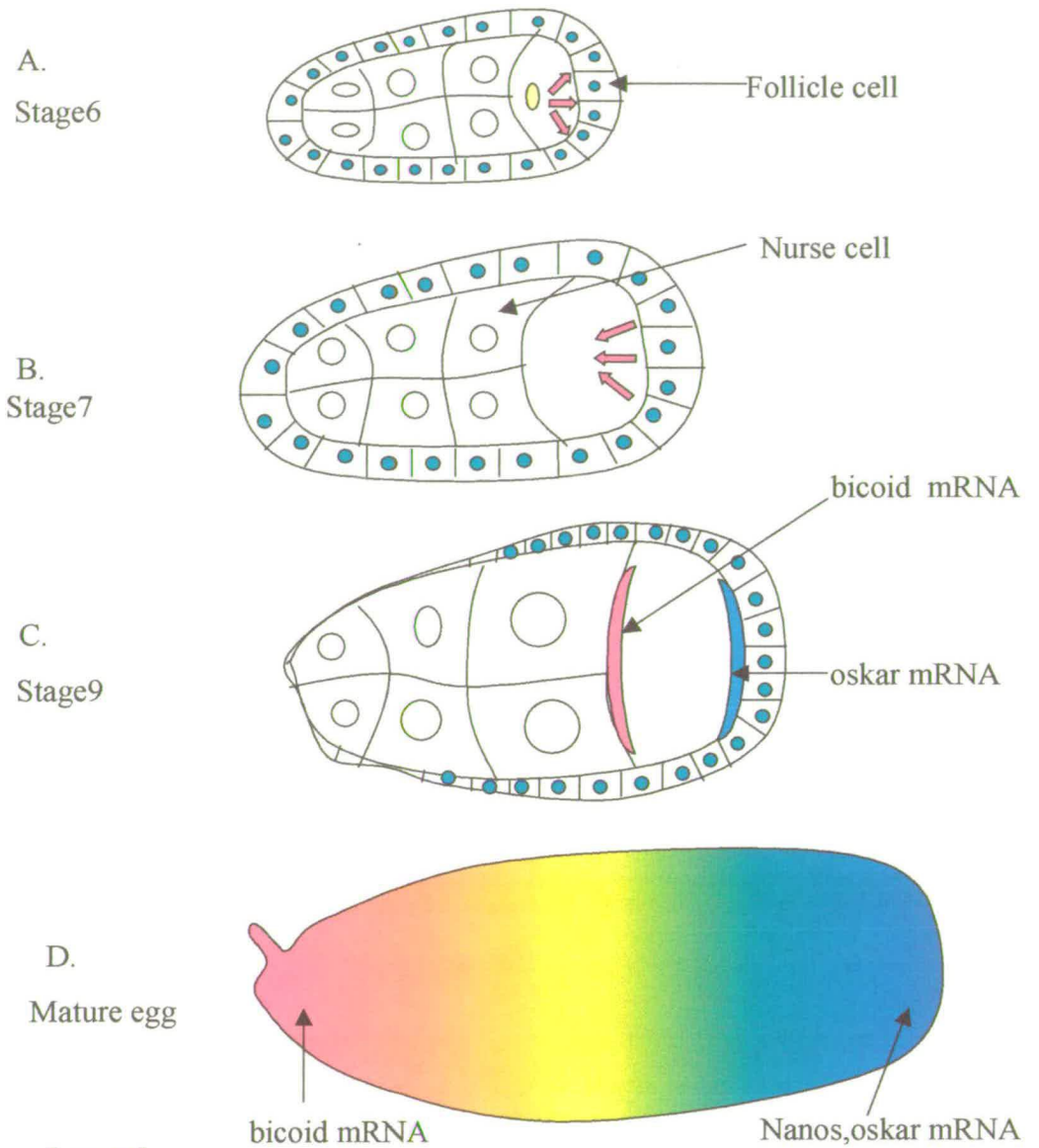
Anterior-posterior asymmetry is first evident in the germarium, when the oocyte moves towards the posterior to make contact with the somatic follicle cells. The

movement of the oocyte to the posterior requires the functions of several genes, including *armadillo* (Peifer et al., 1993), *dicephalic* (Lohs-Schardin, M.1982), and five *spindle* genes (spindle A to E) (Gonzalez-Reyes and St. Johnston, 1994; Gonzalez-Reyes et al., 1997). *spindle* genes are required for the correct positioning of the oocyte early in oogenesis. In *spindle* mutant egg chambers, the oocyte fails to move to the posterior and lies either at the anterior of the germline cyst or in the middle, with nurse cells on either side. This phenotype has also been described for *dicephalic* mutants. *armadillo* (*arm*) is a segment polarity gene and encodes a *Drosophila* homologue of the adhesion junction components plakoglobin and β-catenin (Peifer, et al., 1993). In the germarium *arm* is distributed at the posterior pole of the earliest egg chamber. This suggests that its function is to hold the oocyte at the posterior of the egg chamber. It was observed that the follicle epithelium forms a symmetrical pattern along the A/P axes prior to the oocyte movement, with the polar cells located at both anterior and posterior ends (Margolis and Spradling, 1995). It was suggested that the polar cells send signals to regulate the movement of the oocyte, and maintain its posterior position (Peifer et al., 1993).

1.2.2 Anterior-Posterior axis formation

The *Drosophila* Epidermal Growth Factor Receptor (EGFR) signalling pathway is involved in anterior-posterior patterning of the follicle cell epithelium and polarization of the anterior-posterior axis of the oocyte (Gonzalez-Reyes et al.,1995, Fig.1.4). At stage 6 of oogenesis, the *gurken* (*grk*) gene is expressed in the oocyte and encodes a signalling molecule which has been identified as the Transforming Growth Factor (TGF)-α homologue (Neuman-Silberberg and Schupbach, 1993). The

Figure 1.4 Anterior-Posterior axis formation



Legend

A.At stage 6 of oogenesis, the *gurken* gene is expressed in the oocyte and it signals to adjacent terminal follicle cells. These cells adopt a posterior follicle cell fate.

B. At stage 7 of oogenesis, the posterior follicle cells that have received the *gurken* signal from oocyte then signal back to the oocyte that induces a reorganization of the microtubules in the ooplasm.

C. The microtubule reorganization leads to the localisation of *bicoid* mRNA to the anterior pole and *oskar* mRNA to the posterior pole.

D. The localisation of *bicoid* mRNA and *oskar* mRNA establishes the anterior-posterior polarity in the egg.

torpedo (*top*) gene (EGFR), is expressed in the follicle cells. It is known that the Grk protein is the ligand of Top. Grk is cleaved in the germline and then it can trigger EGFR signalling. Two transmembrane proteins Star and Brho collaborate to promote an activating proteolytic cleavage and release of Grk (Ghiglione et al., 2002). Once the oocyte is determined, *grk* mRNA is located to the posterior end and signals to adjacent terminal follicle cells via EGFR and activates the *Ras* pathway (Lee and Montell, 1997). These cells adopt a posterior follicle cell fate. Thus, EGFR signalling is required to specify posterior follicle cell fate, and in the absence of this signal, the posterior terminal follicle cells adopt the default anterior fate (Gonzalez-Reyes et al.,1995).

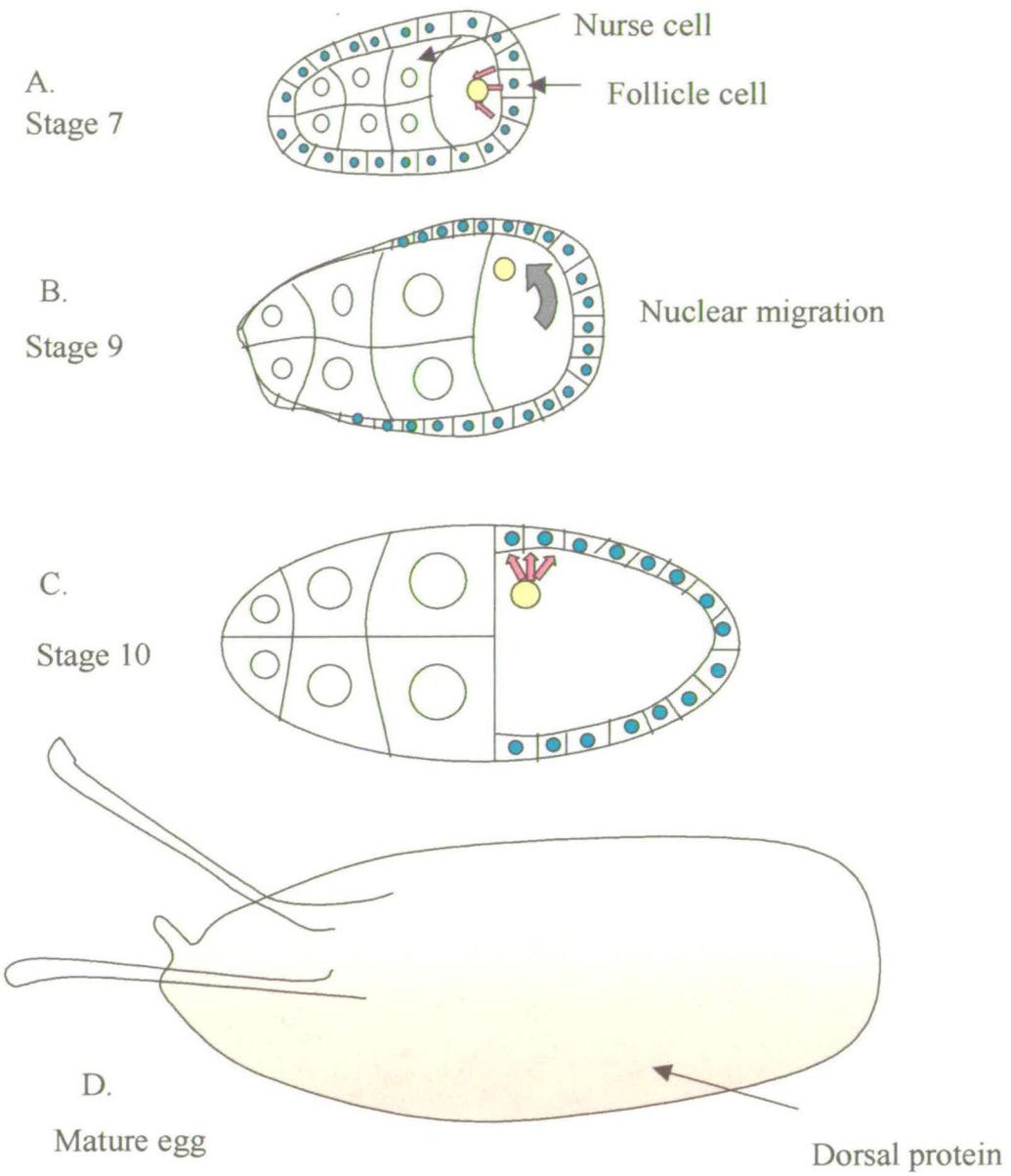
The effect of EGFR signalling on the anterior-posterior polarity in the germline is evident from establishing the anterior-posterior polarity of the microtubule in the oocyte. Because EGFR is required only in the soma. The activation of the EGFR system in the follicle cells must lead to the production of a second signal back to the oocyte that mediates the effect on microtubule polarity. Thus, the correct specification of anterior-posterior polarity in the egg chamber depends on bidirectional signalling between germline and soma. At stage 7 of oogenesis, the posterior follicle cells that have received the *grk* signal from oocyte then signal back to the oocyte, mediated by the *Protein Kinase A* (PKA) gene, that induces a reorganization of the microtubules in the ooplasm (Lane and Kalderon, 1994; 1995). The nature of the reverse signal is not clear but two genes have been identified that are required in the follicle cells for transduction of this signal. One of these genes, *merlin*, encodes a member of the ERM family of proteins, act in intracellular targeting of the signal to the apical membrane of the posterior follicle cells

(Macdougall et al., 2001). The other gene, *laminin A*, encodes a component of the extracellular matrix and its function is to transduct the signal to the oocyte (Deng and Ruohola-Baker, 2000). Additionally, *mago nashi* also appears to be required in the germline for the reorganization of the microtubules (Micklem et al., 1997) This microtubule reorganization leads to localization of *bicoid* mRNA to the anterior pole and *oskar* mRNA to the posterior pole. Because the transport and anchoring of *bicoid* and *oskar* mRNA is microtubule dependent, when the microtubule polarity is re-oriented, the maternal determinants *bicoid* and *oskar* mRNA are re-distributed. It was found that *swallow* is required for *bicoid* mRNA localisation within the oocyte (Berleth et al., 1988). The Swallow protein co-localizes with *bicoid* RNA and binds dynein light chain (Schnorrer et al., 2000). Therefore, it enables Dynein to transport *bicoid* RNA along microtubules to their minus ends at the anterior pole of the oocyte. Staufen is required for localisaiton and translation of *oskar* mRNA (St Johnston et al., 1991; Kim-Ha et al., 1995). The *staufen-oskar* mRNA is transported by Kinesin to microtubule plus ends, near the posterior pole (Brendza et al., 2000). The localization of these cytoplasmic determinants establishes the anterior-posterior polarity in the oocyte.

1.2.3 Dorsal-Ventral axis formation

The reorganization of the microtubule network also directs the movement of the oocyte nucleus to a region of the anterior cortex (Theurkauf et al., 1992). This is the earliest visible dorsal-ventral asymmetry in the egg chamber (Fig.1.5). As the oocyte nucleus moves to the new location, the *grk* mRNA remains associated with it. The *grk* mRNA is also localized to the region of the anterior cortex and this induces the

Figure 1.5 Dorsal-Ventral axis formation



follicle cells facing the oocyte nucleus to adopt a dorsal fate (Neuman-Sillberberg and Schupbach, 1994). The genes *fs(1) K10*, *squid*, *orb*, *cappuccino*, and *spire* are required for localising *grk* mRNA (Neuman-Sillberberg and Schupbach, 1993). Mutations in all of these genes affect the localizaion of *grk* mRNA to the nucleus. *fs(1) K10* encodes a novel protein that contains a small domain with homology to a prokaryotic helix-turn-helix motif, suggesting that the gene product maybe a nucleic acid-binding protein (Prost et al., 1988;). The *squid* locus encodes several isoforms of a heterogeneous nuclear ribonucleoprotein, hrp40, that is similar to the hnRNPA/B proteins of vertebrates, suggesting a role for *squid* in *grk* mRNA metabolism (Kelley, 1993) . Mutations in *orb* , *capu* and *spir* have more general effects on RNA localization in the ovary . The *orb* gene encodes a protein with similarity to the RNA recognition motif (RRM) family of RNA or single stranded nucleic acid-binding protein (Lantz et al., 1992). *orb* is also required to promote the synthesis of *fs(1) K10* and negatively regulated by it (Chang et al., 2001). The gene product of *orb* may interact directly with *grk* mRNAs in the oocyte. This is comparable to the effect of *capu* and *spir* function to establish or maintain the localization of *grk* mRNA. In addition, the mutant egg chambers show abnormal distributions of microtubules and are defective in a number of microtubule based processes in the oocyte (Emmons et al., 1995; Manseau and Schupbach, 1989; Theurkauf, 1994). Thus, these genes may affect mRNA localization by regulating microtubule dynamics.

In addition to the *grk* gene, production of the signal requires the activities of *cornichon* in the germline. *Cornichon* encodes a hydrophobic protein that is thought to be involved in the membrane localization or proper activation of the Grk protein

(Roth, et al, 1995). The oocyte signals to the follicle cell a second time again via the EGFR and the Ras pathway (Lee and Montell, 1997). The GTPase-activating protein Gap1, the serine/threonine kinase Raf (Brand and Perrimon, 1994), and the threonine/tyrosine kinase Mek (Brunner et al., 1994) are required for EGFR signalling pathway. For instance, the *Drosophila* homologue of the GTPase activating protein GAP appears to antagonize EGFR signalling in the follicle cells. Females mutant for amorphic alleles of GAP lay weakly dorsalised eggs (Gaul et al.,1992), which is consistent with ectopic activation of the EGFR pathway, and analysis of this phenotype in mosaic females indicates that GAP is required in the soma for dorsal-ventral patterning (Chou et al. 1993). Two other Ras pathway genes, *Raf* and *Mek*, have been shown to play a positive role in dorsal-ventral patterning in the follicle cells. Certain loss-of-function alleles of Raf cause weak ventralisation of the chorion, while a synthetic dominant activated Raf allele can dorsalise the eggshell when expressed in the follicle cell epithelium(Brand and Perrimon, 1994). A synthetic temperature-sensitive allele of *Drosophila* Mek also ventralises the chorion when females are shifted to the restrictive temperature (Hsu and Perrimon, 1994). The fact that genes at the beginning (Gap),middle(Raf), and end (Mek) of the Ras pathway appear to play a role in dorsal-ventral patterning in the ovary suggests strongly that the entire pathway is acting downstream of EGFR in the follicle cells. The activation of Top also induces the expression of *rhomboid* (*rho*) (Ruohola-Baker et al., 1993). The Rho protein then appears to enhance the interaction between Grk and Top. The EGFR signalling pathway leads to dorsal follicle cell differentiation and eventually the establishment of dorsal-ventral polarity in the egg chamber and embryo. The principal effect of the EGFR signal on embryonic polarity is to repress

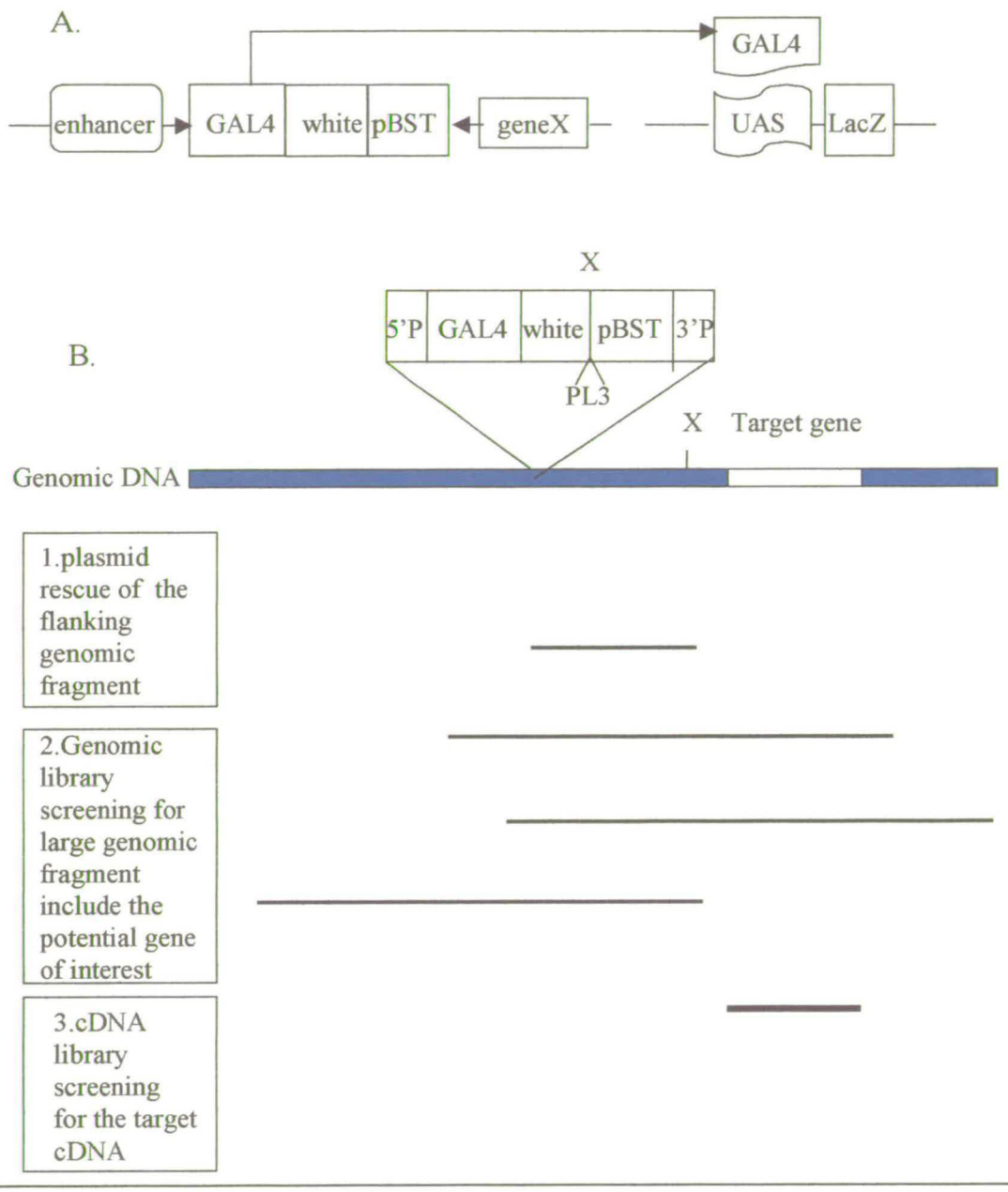
the production of active Toll ligand on the dorsal side. *Pipe* which encodes a putative glycosaminoglycan-modifying enzyme functions together with genes *nudel* and *windbeutel* to initiate an extracellular cascade for activation of the Toll receptor in the embryonic plasma membrane. They are both necessary and sufficient to induce embryonic ventral cell fates (Nilson and Schupbach, 1998; Sen et al., 2000). It is proposed that EGFR activity in dorsal follicle cells of the egg chamber repressed transcription of *pipe*, which is only expressed in the ventral-most third of the follicular epithlium (Sen et al., 1998). One hypothesis, EGFR induced *mirror* expression that represses *pipe* at a distance (Jordan, et al., 2000). The other hypothesis is Ras, which is downstream of EGFR signalling pathway, represses *pipe* transcription (James, et al., 2002). Thus, the *grk* signal restricts and orients the dorsal-ventral patterning events in egg chamber and embryo.

1.3 Approaches Used to Isolate Genes Involved in Oogenesis

1.3.1 Enhancer trap systems

The GAL4/UAS enhancer trap system (Brand and Perrimon, 1993; Deng et al, 1997) is a good method to find new genes expressed in subsets of follicle cells in *Drosophila* oogenesis (Fig.1.6). The GAL4/UAS system allows the rapid generation of individual strains in which ectopic expression of the gene of interest (the target gene) can be directed to different tissues or cell type. Second, the method separates the target gene from its transcriptional activator in two distinct transgenic lines. Only when the two lines are crossed is the target gene turned on in the progeny, and the phenotypic consequences of misexpression can be conveniently studied. The *P[GAL4]* element contains a reporter gene, the yeast *GAL4* gene, a marker gene,

Figure 1.6 GAL4/UAS enhancer trap system



Legend

A.The *P[GAL4]* element contains a reporter gene, the yeast *GAL4* gene, a marker gene, *white* (Red eye colour), and the sequence from the *E. coli* plasmid, pBluescript, has been inserted randomly into the fly genome. The expression of the *GAL4* gene is regulated by the enhancer of a gene which is close to the P- insertion. The *GAL4* gene encodes a specific transcriptional activator which binds to a UAS target sequence. Crossing a *GAL4* line with UAS-lacZ fly line triggers the expression of a secondary reporter gene *lacZ*. After dissection of the female progeny and staining for β-galactosidase, expression can be dectected as a blue stain.
B. cloning of the target gene by using the "plasmid rescue" technique. The genomic DNA of the enhancer line is digested with a restriction enzyme X. The restriction enzyme has one recognition site in the *P[GAL4]* element and the other site in the flanking genomic sequence. This genomic DNA can be recovered to a plasmid containing vector pBluescript after ligation and transformation into *E.coli*. The plasmid rescue of the genomic fragment is followed by screening a *Drosophila* genomic library with the rescued genomic fragment as a probe. Following this process target cDNA is isolated by screening an ovarian cDNA library with genomic DNA as a probe.

white (Red eye colour), and the sequence from the *E. coli* plasmid, pBluescript, has been inserted randomly into the fly genome (Fig.1.6). The Upstream region of the *GAL4* gene contains only the TATA box and the transcription initiation site of hsp70. The expression of the *GAL4* gene is therefore regulated by the enhancer of a gene which is close to the P- insertion. The *GAL4* gene encodes a specific transcriptional activator which binds to a UAS target sequence. Crossing a *P[GAL4]* line with UAS-lacZ fly line triggers the expression of a secondary reporter gene *lacZ*. β-galactosidase staining was observed in subsets of follicle cells in 112 fly lines (Deng, et al, 1997). The cloning of the target gene was achieved by using the "plasmid rescue" technique. The genomic DNA of the enhancer line is digested with a restriction enzyme. The restriction enzyme has one recognition site in the *P[GAL4]* element and the other site in the flanking genomic sequence. The digested fragment containing pBluescript can be recovered by ligation and transformation into *E. coli*. The plasmid rescue of the genomic fragment is followed by screening a *Drosophila* genomic library with the rescued genomic fragment as a probe and enables us to clone genomic DNA covering the region of interest. Following this process a cDNA was isolated by screening an ovarian cDNA library with genomic DNA as a probe.

The P-element mediated enhancer trap system is so useful that it has led to the identification of a number of important genes.

1.3.2 Molecular Genetics Methodology

There are other methods for isolating and analysing genes involved in oogenesis. One approach is to screen female sterile mutations. Female sterility means either that no eggs are produced or that the eggs are defective and can not develop normally.

In order to isolate recessive female sterile mutations, flies are usually mutagenised with ethyl methane sulfonate (EMS) or using X-ray radiation.

There are also a number of maternal effect lethal genes which have functions at more than one time during development. Many mutations of these genes are lethal during one or more developmental stage. Thus multiple functions of these genes become difficult to detect. Consequently, additional methods are needed to identify this class of genes. The dominant female sterile technique (Wieschaus 1980, Perrimon and Gans 1983) can be used to generate mosaic clones. Testing the phenotype of a large number of lethals in germline clones by the dominant female sterile technique has provided the best information on the number of vital genes required for oogenesis (Perrimon et al, 1984, 1989). Another useful method is generating heatshock inducible transgenic flies by P-element transformation. The heatshock treatment can tell more about the function of the gene at different times in oogenesis.

Besides the enhancer trap system, P-element mobilisation is another molecular method to find genes expressed in oogenesis (Zhang and Spradling, 1993; Golic, 1994). If a P-element insertion is known for its location, the P-element can be mobilised by crossing with a fly strain which provides a source of transposase. Thus the P-element can jump to a new position which may be a exon of a near-by gene. The insertion of the P-element is likely to disrupt the function of the gene, therefore causing a mutant phenotype.

1.4 Project Aim

A fertilised egg has the ability to give rise to all the cell types of an organism. To achieve this, many maternal products are assembled in the oocyte. The highly organised oocyte is established during oogenesis. Research at the molecular and cell level on mammalian oogenesis lags behind some other species because of its small egg, difficult operation of the experiment and the reason of ethic. Thus, some other systems are used to gain entry into understanding the mechanism of mammalian oogenesis.

The Drosophila oocyte has been considered as a model system for understanding the ovarian development. The development of the polarized *Drosophila* egg requires intercellular communication between germline cells and somatic follicle cells. The determination of anterior-posterior (AP) and dorsal-ventral (DV) polarity has been investigated extensively and the Gurken/Epidermal Growth Factor Receptor (Grk/EGFR) is believed to be essential for axis determination of the egg chamber and subsequent embryo. Many components involved in Grk/EGFR signalling pathway, such as rhomboid, argos, pointed and CF2 have been studied. however, the epistasis of these components and many of the transcription factors downstream of Grk/EGFR signalling pathway remain to be identified.

Two novel genes, *Z14/Dmbves* and *ntf-2* which are expressed during oogenesis and embryogenesis, were investigated by detailed molecular and genetic approach in this thesis. The *Z14/Dmbves* gene was identified as part of a GAL4/UAS enhancer trap screen. To further study biological function of *Z14/Dmbves* is able to bring insights of Grk/EGFR signalling pathway during *Drosophila* oogenesis. The *ntf-2* gene was identified as part of a P-element mobilization screen for generating mutants

of *Z14/Dmbves*. In mammals, Ntf-2 was demonstrated to stimulate the efficient nuclear import of a RanGDP. In *Drosophila*, the function of Ntf-2 is not clear. Functional analysis of the *ntf-2* gene should provide insights into key aspects of trafficking in the cell.

Chapter Two: Materials and Methods

2.1 Materials

2.1.1 Chemicals and radioactive isotopes

Chemicals were supplied by SIGMA, BDH, and Aldrich. Radioactive isotopes were obtained from Amersham.

2.1.2 Restriction and modifying enzymes

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

2.1.3 Buffers and solutions

All buffers and solutions were prepared using double distilled water (ddH_2O), and pH adjusted appropriately. Sterilisation, when required, was achieved by autoclaving (15psi, 15minutes) or by passing through a 0.22μm pore size filter. Solutions required to be RNAase free were prepared by supplementing with 0.05%(v/v) Diethyl pyrocarbonate (DEPC), incubated overnight at 37^0C and then autoclaved. Unless stated otherwise, all buffers and solutions were stored and used at room temperature.

In situ hybridisation Solutions:

RNA-HybriX:	50% formamide, 5 x SSC, 500 ug/ml Torula RNA or tRNA (RNase-free !!), 50 ug/ml

Heparin , 0.1% Tween -20

DNA-HybriX: 50% formamide, 5 x SSC, 100 ug/ml sonicated DNA, 50 ug/ml Heparin, 0.1% Tween -20

TLMNT: 100mM Tris, pH 9.5, 50mM $MgCl_2$ 100mM NaCl, 0.1% Tween-20, 1mM Levamisole (stock: 100 mM =24mg/ml).

PBT: 1 x PBS + 0.1% Tween-20

PBS: 0.137M NaCl 8.01g, 0.0027M KCl 0.20g , 0.00115M KH_2PO_4 0.156 g, 0.0065M Na_2HPO_4 $2H_2O$ 1.157 g, pH 7.0 –7.4, ddH_2O add to 1000 ml

Proteinase K : 20mg proteinase K in 1ml ddH2O

Glycine: 100 mg glycine in 1ml ddH2O

Fixation Solution: 4% paraformaldehyde in PBS (dissolve with 5 ul 5 N NaOH at 68^0C)

Staining Solution:	100 mM NaCl , 2 ml 5 M 50 mM $MgCl_2$ 5 ml 1 M 100 mM Tris, pH9.5 10 ml 1 M 0.01% Tween 20 100 ul 10 %
Staining Buffer	10mM Phosphate Buffer(PH7-7.6), 150 MM NaCl, 1 mM $MgCl_2$, 7 mM K.Ferro, 7 mM K.Ferri
0.1M K.Ferrocyanide (Mw:422.4)	4.224g in 100 ml
0.1 M K.Ferricyanide (Mw:329.26)	3.293g in 100 ml
8% x-gal	40 mg x-gal in 0.5 ml N-N-Dimethylformamide (stored in -20^0C)
β-gal Staining Solution	25 ul 8% x-gal in 1 ml staining Buffer
DAPI stock	0.1 mg/ml in 0.1 M Tris HCL, pH-7.4

Antibody staining solution

10% Triton X-100	1 ml Triton X-100 in 9 ml H2O
1xPBT (0.5% Triton X-100)	0.25 ml Triton X-100, 5 ml 10xPBS (PH6.8)

	ddH2O add to 50ml
Formaldyhyde mix	3.25 ml H2O, 0.5ml 10xPBS, 1.25ml Formaldehyde 37%
Ethanol 80%	40 ml Ethanol, 1xPBS 10 ml
Block solution	1% NGS (Normal Goat Serum) in 0.5% PBT

Western blot solution:

Separating gel	7.9ml ddH_2O, 5ml 1.5M Tris-HCl, pH8.8, 200μl 10% SDS, 6.7ml 30% protogel, 200μl 10%(w/v) $(NH_4)_2S_2O_8$, 20μl TEMED
Stacking gel	3.44ml ddH_2O, 630μl 1M Tris-HCl, pH6.8, 50μl 10% SDS, 830μl 30% protogel, 50μl 10%(w/v)$(NH_4)_2S_2O_8$, 10μl TEMED
Running buffer(10x)	30.3g Tris, 144g Glycine, 10g SDS in 1 litre.
Sample buffer(2x)	0.75ml ddH_2O, 1.25ml 1M Tris-HCl, pH6.8, 2ml Glycerol, 4ml 10% SDS, 1ml β-ME, 1ml 0.1% Br-PhenolBlue
Stripping buffer	3.47ml 14.4M β-Mercapto Ethanol, 100ml 10% SDS 31.25ml 1M Tris-HCl, pH6.7, total 500ml

Molecular hybridisation

Solution:

20XSSC	175.3g NaCl, 88.2g NaCitrate, 800ml ddH_2O pH7.0 (NaOH) 1000ml
20XSSPE	175.3g NaCl, 27.6 gms NaH_2PO_4 7.4 gms EDTA, 800ml ddH_2O pH7.0 (NaOH) 1000ml
RNA Loading Buffer	50% (V/V) glycer 0.1 mg/ml bromophenol blue 0.4 mg/ml EB
10XMOPS Buffer	(0.2 M MOPS, 0.5 M NaAc, 0.01 M EDTA, pH7.0) 20.6 gms MOPS, 10.25 gms NaAC, 0.93 gms EDTA, Add H2O to 250 ml
Prehybrix Buffer-0.5% SDS	125ml 20XSSPE 25ml 100Xdenhards 25ml 1M phosphate buffer, pH7.0 50ml 0.1M pyrophosphate, 1ml 10mg /ml salman sperm DNA, 25ml 10% SDS, 269ml ddH_2O

100X Denhardts	5g Ficoll, 5g polyvinylpyrrolidone 5g BSA, 250 ml ddH_2O
1 M Phosphate, pH7.0	288.5g 1M Na_2HPO_4, 11.5ml 1M NaH_2PO_4

General solutions:

TAE	40mM Tris-acetate; 1mM EDTA; pH7.7
TBE	89mM Tris-HCl; 89mM Boric acid; 2.5mM EDTA; pH8.3
TE	10mM Tris-HCl; 1mM EDTA; pH8.0
0.5M EDTA	0.5M Diaminoethanetetra-acetic acid, pH8.0
Ringer's solution	6.5g NaCl, 0.14g KCl, 0.2g $NaHCO_3$, 0.12g $CaCl_2$, 0.0lg NaH_2PO_4 per litre, sterilised by autoclaving.
TFB1 buffer	30mM KOAc, 10mM $CaCl_2$, 50mM $MnCl_2$, 100mM RbCl, 15% glycerol, pH5.8, sterilise through 22μ filter, store at 4°C.

TFB2 buffer	10mM Pipes, 75mM $CaCl_2$, 10mM RbCl, 15% Glycerol, pH6.5, sterilise through 22μ filter, store at 4°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. store at 4°C.
70% (v/v) Ethanol	70 ml Ethanol made up to 100 ml with sterile ddH_2O Ethidium Bromide 10mg/ml in sterile ddH_2O
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 4^0C.
Ribonuclease A	20mg/ml in 50% glycerol, boiled for 5 min to inactive Contaminating DNAases, and stored at -20^0C.
Fly buffer	5 ml 1M Tris-HCl, pH 8.5, 800μl 5M NaCl, 2.5g Sucrose, 2.5ml 10% SDS, 5ml 0.5M EDTA,

pH 8.0, up to 50ml H_2O.

2.1.4 Media

Apple juice plates	boil 9g agar and 10g sugar in 300ml water, Add 100ml apple juice, boil again, add 10ml 10% Nipagin (w/v) pour into petri dish at 60^0C.
Tomato juice plates	melt 4g agar in 160ml H_2O, add 40ml tomato juice, boil Again, add 5ml 10% Nipagin pour into dish after cooling to 60^0C.
Grape juice plates	melt 15g agar in 1litre (minimal 200ml), add 50ml grape Juice, add 3ml 10% Nipagin.
Staffan Fly food	Agar 10gms, Sugar 10 gms, Corn Flour 25 gms, Live Yeast 17.5 gms, Polenta 10 gms, Nipagin 15ml, in 1litre H_2O
Luria Broth (LB)	1% (w/v) Bacto-Tryptone; 0.5% (w/v) yeast Extract; 1% (w/v)

2.1.5 Bacterial strains

Table 2.1

Strain	Genotype	Comments
XL-1 Blue (Stratagene)	*A1, gyrA96, thi, hsdR17, supE44, {F', proAB, lacI^q ZΔM15 Tn10 (Tet^r)}*	Used to propagate recombinant pBluescript plasmids
XL10-Gold Supercompetent Cells (Stratagene)	*Tet^r Δ(mcrA)183, Δ(mcrCBhsd-SMR-mrr)173, endA1, supE44, Thi, recA1, lac The, {F' proAB lacI^q ZΔM15 Tn10 (Tet^r)}*	Used to propagate rescued Plasmid from *Drosophila* P-insertion fly lines

2.1.6 Plasmids

The following plasmids were used as detailed in table

Table 2.2

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 mis-expression experiments
pCasPer	Place 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 mis-expression experiments
pΔ2-3	Helper plasmid for P-element transformation, provides transposase but it can not transpose itself.	Helping microinjection constructs integrate into the genome.

2.1.7 Antibiotics

Stock solutions of antibiotics were prepared according to the table below, and stored at -20^{0}C in 1ml aliquots.

Table 2.3

Antibiotic	Stock solution concentration	Working concentration
Ampicillin	50mg/ml in ddH_2O	50-100μg/ml
Tetracylin	50mg/ml	50μg/ml
Chloramphenicol	10mg/ml in ethanol	10μg/ml

2.1.8 Oligonucleotides

Primers were designed by using GCG and Primer 3 software. Oligonucleotides were obtained from TAGC and Sigma. All oligonucleotides used for PCR or automated sequencing are tabulated below. All stocks were at 50pmol/μl.

Table 2.4

Name	Sequence (5'—3')	T_m(°C)	Comments
SL1	cagttgtgggggcaatacaaa	60.3	Z14 specific primers for Sequencing experiments
SL2	ctaggatatgaaccgcaa	57.3	
SL3	aatacgactcactatagg	49.1	
SL4	attggctgcctgatgatggg	59.4	
SL5	attcgagcaggaaatcgaagc	57.9	
SL6	ccccacaactgcaaacaatta	58.4	
SL7	aatttcctgtaccatctggacg	58.4	
SL8	atatgctgaggatgcggagg	59.4	
SL9	ggtgctctgatttctgttcg	57.3	
NT1	cggatttcccataatctctg	60.8	Ntf-2 specific primers for RT-PCR
NT2	ttgctgataatcgtgagacc	60.2	
NT3	ttccgcctatccttcaagtg	63.9	

NT4	ggcatctttggatgtcgatt	63.7	
RNT1	taatacgactcactatagggag acggatttcccataatctctg	76.0	Ntf-2 specific primers containing T7 promoter for producing double strands RNA
RNT2	taatacgactcactatagggag attgctgataatcgtgagacc	75.8	

2.2 *Drosophila* Methods

2.2.1 Maintenance of *Drosophila* stocks

Drosophila melanogaster stocks were maintained on Staffan food at 18^0C or 25^0C. 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

Table 2.5

Drosophila stocks used in this project

Stock	Feature	Comments	Reference
Oregon-R	Wild type		Lab stock
Δ2-3	*w*; *Dr, Δ2-3/TM6*	Used for P element mobilisation, Provide transposase.	Lab stock

C648	w^-/pGawB	*Z14/Dmbves* gene cloned from this fly line	Lab stock
W^K	w^-	Used for P element transformation	Lab stock
UAS-lacZ	None		Lab stock
4648	*L(1)2Ad*^{1}*/FM6*	Flies have Bar eye, used for X chromosone balancer	Bloominton Centre
P6a+1045C^{22}	w^-/pUAST	GFP vector inserted into X chromosome on W^K line	Lab stock
M 228	*grk[HK] cn bw sp/CyO, 10(2) 100[DTS]*		Lab stock
M 214	*grk[WG] b bw sp/CyO, 1(2) 100 [DTS]*		Lab stock
M103	*fs(1) K10 wf wal/CIB*		Lab stock
2062	*dpp*^{d6}*/CyO*		Bloominton Centre
2073	*dpp*^{e87} *cn*^{1} *bw*^{1}*/CyO*		Bloominton Centre

10880	$y^1 w^*$; $P\{w^{+mc}=lacW\}$ $mirr^{cre2}/TM3, Sb^1$		Bloominton Centre
1309	ovo^{D1} $v^{24}/C(1)$ *DX*, y^1 w^1 f^1	Dominant sterile flies used for generating germ-line clone	Bloominton Centre

2.2.3 Collection of embryos

Embryos were collected on apple juice plates that were smeared with a little wet yeast. Unless otherwise stated, the collection period was 18 hours. Embryos were washed off from the petri dish with water, dechorionated in bleach and washed with water again. Embryos were then put in the glass tube, 1ml heptane was added for 30 seconds, followed by adding 1ml 1x PBS (pH6.8) containing 125μl formaldehyde. The tube was shaken and agitated for 20 minutes at room temperature. After removal 0.5ml upper solution and all the low solutions, 1ml methanol was added. The solutions were vortexed to devitellinise the embryos. The embryos from the bottom of the tube were collected and washed twice in methanol and stored at -20^{0}C.

2.2.4 Collection of first, second, third instar larvae

Egg laying flies were placed in apple juice plates cage for 4 hours and then removed. Wet yeast was smeared in the centre of the plate. The plate was incubated at 25^{0}C for 24hours, 48hours and 72hours. The larvae were picked out with a paintbrush into a microfuge tube and frozen in liquid nitrogen.

2.2.5 Collection of virgin flies

Virgin flies were collected by either of the following two ways: (1) New hatched flies were collected in the morning and in the evening each day. The flies were anaesthetised and examined with a microscope. Virgin females could be distinguished by their pale colour and a dark spot in the abdomen. (2) Dark pupae were picked out with a paintbrush and placed into vials individually.

2.3 Bacteria and Bacteriophage Methods

2.3.1 Short-term storage of bacterial cultures: agar plates

Bacterial cultures were maintained as individual colonies for up to 1 month on LB plates supplemented with the appropriate antibiotic. Plates were stored at 4^0C.

2.3.2 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 37^0C, 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 -70^0C.

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.

2.3.3 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 37^0C, with shaking at approximately 180rpm. O/N cultures were subcultured the next morning by diluting them 1: 100 in fresh LB. The cells were when grown as before until the appropriate O.D. at A_{600nm} was attained.

2.3.4 Plating bacteria

Bacterial cells were grown and subcultured as described in section 2.3.2. When an O.D. (A_{600nm}) 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 4^0C.

2.3.5 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^{0}C. Next day an individual plaque, and the bacterial cells surrounding it, was used to inoculate 10ml LB supplemented with 10mM $MgSO_4$. This culture was grown O/N at 37^{0}C, with shaking at 180rpm.

The following morning 0.1ml $CHCl_3$ was added to the culture, which was then shaken for a further 10minutes. Recombinant bacteriophage DNA was then prepared as described in 2.4.1.4.

2.4 DNA Methods

2.4.1 Preparation of DNA

2.4.1.1 Mini-preparation of plasmid DNA

Qiagen Spin Miniprep Kit was used to prepare up to 20μg plasmid DNA, following the manufacturer's instructions.

2.4.1.2 Midi-preparation of plasmid DNA

Qiagen filter Midi Kit was used to prepare up to 100μg plasmid DNA, following the manufacturer's instructions.

2.4.1.3 Preparation of *Drosophila* genomic DNA

Approximately 60mg of flies were ground in a 1.5ml eppendorf tube containing 500μl fly buffer, using a plastic pestle. 4μl RNase (10mg/ml) was added and incubated for 30 minutes at 37^{0}C, then 30μl Proteinase K (10mg/ml) was added and

incubated for 30 minutes at 50^{0}C. Cell debris was pelleted by centrifugation (12krpm, 15minutes, 4^{0}C). The supernatant was transferred to a clean eppendorf tube. This was then extracted once with an equal amount of phenol/chloroform for 5 minutes with rotating, then extracted once with an equal amount of chloroform for 5minutes with rotating. The DNA was precipitated in the supernatant with 0.6 volumes of isopropanol, mixed well, left for 5 minutes at RT. The DNA was pelleted at 15krpm for 20 minutes at 4^{0}C, and washed with 70% ethanol, this was then spun and all liquid traces removed with a tip. The pellet was dried completely at 37^{0}C for 10minutes and dissolved in 50μl 10mM Tris-HCl, pH8.0 for 1hour at RT. The DNA was frozen and stored at -20^{0}C.

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, 1 μl DNaseI added, and incubated at RT for 30minutes. 200μl TES was then added, and the mixture incubated at 70^{0}C for 15 minutes. 135μl KOAc was added, the solution mixed thorough, and incubated on ice for 15minutes. 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 2.6 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 to be free of contamination if this ratio was larger or equal to 2.0.

Table 2.6 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
Double stranded RNA	1.0	50
Single stranded RNA	1.0	40
Oligonucleotides	1.0	40

The concentration of the sample was calculated using the following formula.

$$\text{Concentration (ng/}\mu\text{l)} = \frac{A_{260} \times DF \times C}{1000}$$

Where: DF=dilution factor, and C =50 (double strand) or 40 (single strand)

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 isopropanol or ethanol

DNA was precipitated from solution using 0.6 volume of isopropanol or 2.5 volumes of 95% ethanol. In the latter case, 0.1volume of 3M NaOAc, pH5.2, was also added, where no salt had been used in the previous step of the protocol. The solution was centrifuged at maximum speed in a refrigerated benchtop centrifuge for 15 minutes at 4^0C. The resulting DNA pellet was washed in 1ml of 70% ethanol, and centrifuged for 5 minutes at maximum speed. The pellet was then put in a 37^0C incubator for 10 minutes before being dissolved 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, $1/10^{th}$ 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 37^0C), for a minimum of 2 hours.

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 (Boehringer Mannheim) added to a final concentration of 1x. Reactions were incubated at 18^0C overnight. The DNA was then ethanol precipitated and resuspended in ddH_2O, to a final concetration of 1ng/μl.

2.4.6 Transformation of plasmid DNA into *Escherichia coli*

2.4.6.1 Preparation of competent cells

An O/N culture was diluted 1:100 into fresh LB supplemented with 20mM $MgSO_4$ and grown at 37^0C, 218rpm, until an A_{600} of 0.4-0.6 was reached. The cells were pelleted by centrifugation at 4,500xg for 5 minutes at 4°C, and then resuspended in 0.4 volume of ice-cold TFB1. After incubating on ice for 5 minutes, the cells were again pelleted at 4,500xg for 5 minutes at 4^0C. The cells were gently resuspended in 10ml of ice-cold TBF2 and incubated on ice for 15-60 minutes. The cells were then divided in to 200μl aliquots, quick-frozen in a dry ice/isopropanol bath and stored at -80^0C.

2.4.6.2 Transformation

Up to 10ng of plasmid DNA, in a volume not exceeding 10μl, was added to an aliquot of competent cells, previously thawed on ice. The mixture was incubated on ice for 30minutes, and then heat-shocked at 42^0C for 60 seconds. After a further 2-minute incubation on ice, the cells were incubated at 37^0C, for 45 minutes, shaking at 218rpm. The cells were then pelleted, resuspended in 50μl LB, plated on appropriate selective LB agar plates, and incubated at 37^0C overnight.

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 oven. EtBr was added to a final concentration of 0.1mg/ml. DNA loading buffer was added to the samples to a final concentration of 1x, the samples loaded on the gel, and electrophoresed 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 was used to determine the size of electrophoresed DNA fragments, as directed by the manufacturer.

2.4.7.3 Visualisation and imaging

Nucleic acids on EtBr-stained gels were visualised by UV illumination, and a digital imager used to obtain permanent records.

2.4.7.4 Recovery of DNA fragments

Where necessary, DNA fragments were purified from agarose gels using the QIAquick kit from Qiagen, as directed in the accompanying manual.

2.4.8 DNA sequencing and sequence analysis

2.4.8.1 Automated DNA sequencing

Approximately 400ng high quality plasmid DNA was used as a template in sequencing reactions. 3.2pmoles of the appropriate primer and 8μl of reaction mix (Big dye) and ddH_2O were added to a final volume of 20μl. The mixture was subjected to thermal cycling (Touchdown thermal cycler, Hybaid), as recommended by the Perkin-Elmer instruction manual. The program used is as follow: 25 cycles; 30seconds denaturation at 96^{0}C, 15 seconds at the appropriate primer annealing temperature; and a 4 minute extension period at 60^{0}C. The products were analysed using an Robot sequencer (Corporation) by onsite sequencing service.

2.4.8.2 Sequence analysis

DNA sequences were analysed using GenejockeyII (Biosoft) and the Genetics Computer Group (GCG) program package, version 10.

2.4.9 Polymerase chain reaction (PCR)

A typical 50μl PCR reaction consisted of the following: 25 pmole each of primer 1and 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.

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^{0}C. Subsequent steps were: 95^{0}C denaturation, 15 seconds; 50^{0}C-60^{0}C annealing, 15seconds; 72^{0}C elongation, 30 seconds to 3 minutes. 30 cycles

were used in all reactions. This was completed by a final 72^{0}C elongation step of 7 minutes.

The annealing temperature was adjusted according to the primer 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 Methods

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 RT for 3 minutes. The aqueous and organic phases were separated by centrifugation at 12,000xg, for 15 minutes at 4^{0}C. The aqueous phase was transferred to a fresh eppendorf tube and 0.7 volumes of isopropanol were added and incubated at RT for 1 minutes. The RNA was precipitated by centrifugation at 12,000xg for 15 minutes at 4^{0}C. The supernatant was removed and the RNA pellet washed with 1 ml 70% (v/v) ethanol (in DEPC water). The pellet was incubated at 37^{0}C for 10 minutes then dissolved in DEPC sterile water at 55-65^{0}C for 10 minutes.

2.5.2 Preparation of double stranded RNA

2.5.2.1 Preparation of Template DNA

Each primer contained a T7 promoter sequence sequence on its 5' end (described in Table 4) such that sense and antisense RNAs could be synthesized simultaneously from a single PCR-derived template.

2.5.2.2 Preparation of double stranded RNA

Ambion MEGAscript T7 Transcription of RNA Kit was used to prepare up to 100μg of RNA, following the manufacturer's instructions. The RNA products were boiled for 1 minute and allowed to cool to room temperature for 18 hours, then stored frozen at -20^{0}C.

2.5.3 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 10ml 10x MOPS and 73ml of water and cooled to about 55^{0}C, then 17ml 37% (v/v) formaldehyde was added, the solution mixed and poured immediately into a gel tray. Gels were run in a 1x MOPS buffer.

RNA samples (15-20μg of total RNA or 1μg of mRNA) were incubated at 65^{0}C for 5 minutes with RNA sample buffer in a ratio 1:4, to remove any secondary structures from the RNA, and cooled on ice.

2.5.4 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 70^0C 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 42^0C for 60 minutes, followed by a 10 minute heatshock at 70^0C to deactivate any remaining Superscript.

$1/20^{th}$ 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 2.4.9.

2.6 Hybridisation techniques

2.6.1 Southern blotting

Both alkaline and salt transfer was used in this work. For alkaline transfer, Hybond N+ (Amersham) membrane was used and the DNA agarose gel was used directly for transfer after electrophoresis. For salt transfer (using Hybond-N), the gel

was washed for 30 minutes in denaturing solution (1.5M NaCl, 0.5M NaOH) and 30 minutes in neutralising solution (1.5M NaCl, 0.5M Tris-HCl pH7.2, 0.001M EDTA).

Transfer buffer (0.4M NaOH for Hybond N+ and 20 x SSC for Hybond N) was poured into a tray. The gel containing the DNA was placed on two layers of Whatman filter paper which were supported by a plastic plate. Two ends of the filter paper were rested in the transfer buffer. The selected membrane of appropriate size was placed carefully over the gel (bubbles should be prevented), followed by overlaying two sheets of Whatman filter paper (cut to size), 6 cm depth of absorbent paper and a small weight on the top. Saran wrap was placed around the gel to prevent shortcircuiting. The transfer was allowed to proceed for a minimum of 3 hours.

After transfer, the membrane was rinsed briefly in 6 x SSC before drying. Hybond-N was exposed to UV light (λ254 and λ365) for 10 minutes to fix the DNA.

2.6.2 Northern blotting

2.6.2.1 Gel preparation

Table 2.7

Tray size	180cm^2	150cm^2	65cm^2
Gel volume	120ml	100ml	43ml
H_2O	105ml	87ml	38ml
10xMOPS	12ml	10ml	4.3ml
Agarose	1.4g	1.2g	0.5g
37%Formaldehyde	6.1ml	5.1ml	2.2ml

2.6.2.2 Electrophoretic separation of RNA

One volume RNA probes were added to five volumes sample buffer (15-20μg of total RNA), the probes were denatured for 10minutes at 65^{0}C and chilled on ice, then spun and loaded on the gel. The gel was run at 20V overnight.

2.6.2.3 Blotting

The transfer of RNA to the membrane is similar to the transfer of DNA from the agarose gel. Hybond-N membrane was chosen and 20 x SSC was used as the transfer buffer, the Formaldehyde denaturing gel was washed gently in RNAase free ddH_2O (to remove formaldehyde) for 45 minutes prior to blotting. The membrane was exposed to UV light (λ254 and λ365) to fix the RNA.

2.6.3 Pre-hybridisation and hybridisation

Pre-hybridisation and hybridisation were carried out inside the hybridisation bottles in the hybaid ovens. Membranes were pre-hybridised at 42^{0}C for 1-2 hours in 25-50ml of pre-hybridising solution (6 x SSC, 5 x Denhardt's, 0.5% [w/v] SDS, 1mg/ml sonicated salmon sperm DNA). The radio-labelled probe (2.) was denatured in a heated block at 100^{0}C for 2 minutes and then plunged into ice. The denatured probe was added to an appropriate volume of fresh pre-hybridisation solution after the old solution was removed. Hybridisation was allowed to proceed at 42^{0}C for overnight.

2.6.4 Washing of hybridised membranes

The hybridisation solution was transferred into a fresh 50ml conical flask and retained for further hybridisation. Approximately 100ml washing solution (2 x SSPE) was added to the hybridisation bottle and gently shaken for 15 minutes at 45^0C.

2.6.5 Autoradiography

The membranes having been washed were wrapped in SaranwrapTM, placed into a cassette, overlaid with X-ray film and allowed to expose at -80^0C. The exposure time was dependent on the signal emanating from the membrane. The films were developed in an X-OGRAPH compact X2 automated film processor.

2.6.6 Stripping of membranes

The membranes were boiled in ddH_2O for 10 minutes to remove the old probe in order to be hybridised with a new probe. The membranes were checked for absence of probe by exposing to X-ray film overnight.

2.7 *in situ* hybridisation to mRNA

2.7.1 Preparation of digoxygenin-labelled probes

Digoxygenin (DIG)-DNA labelled probes were prepared using Boehringer Mannheim's Expand PCR DIG-labelling kit, as directed by the manufacturer's

protocol. Dig RNA was prepared using Boehringer Mannheim's DIG RNA-labelling kit, as directed by the manufacturer's protocol.

2.7.2 *in situ* hybridisation to whole-mount ovaries Dig-DNA method

Ovaries were dissected in Ringers, and fixed for 20minutes in 4% paraformaldehyde in PBS, with agitation. After rinsing 3 times for 10 minutes in PBT, the ovaries were washed in 1:1 methanol/PBT for 10 minutes followed by a 9:1 methanol/EGTA wash for 10 minutes and a wash in 100% methanol for 10 minutes, they can then be stored at -20^{0}C for several months.

Next, the ovaries were washed in 1:1 PBT/methanol for 10 minutes, then rinsed 3x for 10 minutes in PBT to remove all methanol. The ovaries were then incubated in 100μg/ml proteinase K in PBT for 1hour at RT. The reaction was stopped by adding 2mg/ml glycine in PBT, followed by 3x5minutes rinses in PBT. The ovaries were then re-fixed for 20 minutes in 4% paraformaldehyde in PBS. This was followed by 6x5 minutes washes in PBT, a 10 minute wash in 1:1 PBT/DNA Hybrix buffer. The ovaries were then pre-hybridised for at least 1 hour for 45^{0}C. Denatured DIG-labelled DNA probes were added, and the ovaries hybridised over night at 45^{0}C.

After the probes were removed, the ovaries were washed in preheated DNA Hybrix buffer at 45^{0}C for 20 minutes, followed by 2x20 minutes wash in 1:1 DNA hybrix buffer/PBT, 20 minutes wash in PBT all at 45^{0}C, wash 5x5 minutes in PBT at RT. They were then incubated with 1:1000 pre-absorbed anti-DIG-AP for 1 hour at RT, followed by 3x20 minutes washes in PBT.

Next, the ovaries where washed 3x5 minutes in detection solution TLMNT, before staining in NBT+X-phosphate solution. The colour reaction was stopped by washing the ovaries in PBS, which were then mounted in 80% glycerol in PBS.

2.7.3 *in situ* hybridisation to whole-mount embryos Dig-DNA method

Embryos were collected and fixed as described in section 2.2.3. The embryos were then washed in 1:1 PBT/methanol for 10 minutes, and rinsed 3x 5 minutes in PBT to remove all methanol. The embryos were then incubated in 100μg/ml proteinase K in PBT for 5 minutes at RT. The reaction was stopped by adding 2mg/ml glycine in PBT for 5 minutes, followed by 3x5minutes rinses in PBT. The embryos were then re-fixed for 20 minutes in 4% paraformaldehyde in PBS. This was followed by 6x5 minutes washes in PBT, a 10 minute wash in 1:1 PBT/DNA Hybrix buffer. The embryos were then pre-hybridised for at least 1 hour at 45^{0}C. Denatured DIG-labelled DNA probes were added, and the embryos hybridised over night at 45^{0}C.

After the probes were removed, the ovaries were washed in preheated DNA Hybrix buffer at 45^{0}C for 20 minutes, followed by 2x20 minutes wash in 1:1 DNA hybrix buffer/PBT, 20 minutes wash in PBT at 45^{0}C, then washed 5x5 minutes in PBT at RT. They were then incubated with 1:1000 pre-absorbed anti-DIG-AP for 1 hour at RT, followed by 3x20 minutes washes in PBT.

The embryos were then washed 3x5 minutes in detection solution TLMNT, before staining in NBT+X-phosphate solution. The colour reaction was stopped by washing the embryos in PBS. The embryos were then mounted in 80% glycerol in PBS.

2.7.4 *in situ* hybridisation to whole-mount ovaries Dig-RNA method

Ovaries were dissected in Ringers, and fixed for 20minutes in 4% paraformaldehyde in PBS, with agitation.After rinsing 3 times for 10 minutes in PBT, the ovaries were washed in 1:1 methanol/PBT for 10 minutes followed by a 9:1 methanol/EGTA wash for 10 minutes and a wash in 100% methanol for 10 minutes, they can be stored at -20^{0}C for several months.

The ovaries were then washed in 1:1 PBT/methanol for 10 minutes, then rinsed 3x for 10 minutes in PBT to remove all methanol. The ovaries were re-fixed for at least 1 hour in 4% paraformaldehyde in PBS. This was followed by 5x5 minutes washes in PBT, a 5 minute wash in 1:1 PBT/RNA Hybrix buffer. The ovaries were then pre-hybridised for at least 2 hours at 70^{0}C. DIG-labelled RNA probes were added, and the ovaries hybridised O/N at 70^{0}C.

After the probes were removed, the ovaries were washed in preheated Hybrix buffer at 70^{0}C for 20 minutes, followed by 1 x 20 minute washes in 1:1 Hybrix buffer/PBT, 3 x 20 minute washes in PBT all at 70^{0}C. They were then incubated with 1:500 pre-absorbed anti-DIG-AP for 1 hour at RT, followed by four 20 minute washes in PBT.

Next, the ovaries were washed twice for 5 minutes in staining solution before staining in NBT+X phosphate solution. The colour reaction was stopped by washing the ovaries in PBS. Ovaries were then mounted in 80% glycerol in PBS.

2.7.5 *in situ* hybridisation to whole-mount embryos Dig-RNA method

Embryos were collected and fixed as described in section 2.2.3. After this the embryos were washed in 1:1 PBT/methanol for 10 minutes, then rinsed 3x 10 minutes in PBT to remove all methanol. The embryos were then re-fixed for at least 1 hour in 4% paraformaldehyde in PBS. This was followed by 5x5 minutes washes in PBT, a 5 minute wash in 1:1 PBT/RNA Hybrix buffer. The embryos were then pre-hybridised for at least 2 hours at 70^0C. DIG-labelled RNA probes were added, and the ovaries hybridised O/N at 70^0C.

After the probes were removed, the embryos were washed in preheated Hybrix buffer at 70^0C for 20 minutes, followed by 1 x 20 minute washes in 1:1 Hybrix buffer/PBT, 3 x 20 minute washes in PBT all at 70^0C. They were then incubated with 1:500 pre-absorbed anti-DIG-AP for 1 hour at RT, followed by four 20 minute washes in PBT.

After this the embryos were washed twice for 5 minutes in staining solution before staining in NBT+X phosphate solution. The colour reaction was stopped by washing the embryos in PBS. The embryos were then mounted in 80% glycerol in PBS.

2.8 *In situ* Immunohistochemical Detection

2.8.1 Whole-mount ovaries antibody staining

Ovaries were dissected in Ringer's solution. The anterior parts of the ovaries were torn apart to facilitate antibody penetration. The ovaries were fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature. The fixative was carefully removed and ovaries were washed in 1ml of PBT for 5 minutes. Then the

ovaries were incubated in PBT(1%[w/v] Bovine serum albumin) for 1 hour. Blocking was accomplished by incubation of the ovaries in PBTG(5%[v/v] donkey serum) for 2 hours at room temperature.

The primary antibody in PBTG at an appropriate dilution was added and incubated overnight at 4^0C. The antibody solution was removed and stored at -20^0C to be reused. Residual antibody was washed away with three changes of PBT with 30 minutes incubation per change. The HRP-conjugated secondary antibody was then added to the ovaries and incubated for 2 hours at room temperature or overnight at 4^0C. Excess secondary antibody was removed with three PBT washes at 30 minutes intervals. DAB staining solution was added and the staining was allowed to proceed for 10-30 minutes before washing with several changes of PBS to stop the reaction. Stained ovaries were mounted in 80% glycerol in PBS.

2.8.2 Whole-mount embryos antibody staining

Embryos were collected and dechorionated as described in section 2.2.3. The embryos were washed with 100% Ethanol twice and 80% Ethanol once. The embryos were then washed 0.5% PBT 15 minutes for three times. Blocking was accomplished by incubation of the embryos in 1% NGS (Normal Goat Serum) for 1 hours at room temperature. Afterwards the embryos were incubated with primary antibody in 0.5% PBT at 4^0C overnight and washed with 0.5% PBT 15 minutes for three times. The embryos were then incubated in 1% NGS for 30 minutes. Secondary antibody (FITC) was added in 0.5% PBT and incubated for 2 hours. Excess secondary antibody was removed with twice 0.5% PBT washes at 30 minutes

intervals. The embryos were then washed with 1xPBS for 10 minutes. Embryos were mounted in mounting solution (Vectashield).

2.8.3 DAPI staining

Ovaries were dissected in PBS and fixed in 4% paraformaldehyde (w/v in 1xPBS) for 20 minutes. This was followed by washing in 1xPBS containing Triton –X100 (1%[v/v]) for 30 minutes. The ovaries were then washed in 3x PBS and stained in 0.5μg/ml DAPI (dissolved in 3 x PBS) in the dark for 1 hour. After washing in 3 x PBS for overnight, the ovaries were mounted in 80% glycerol in PBS and examined under a fluorescent microscope.

2.8.4 β-Galactosidase staining

The ovaries were dissected in PBS and stained at room temperature for a few hours to overnight in β-gal staining solution (see section2.1.3). The staining of the ovaries was stopped by simply washing with PBS a few times and mounted in 80% glycerol in PBS.

2.8.5 Preparation of the eggshell for dark-field microscopy

Freshly laid eggs were collected from an apple juice plate, then mounted in a drop of Hoyer's mount solution[Hoyer's medium:Lactic acid, (1:1)] on a glass slide. The slide was incubated overnight at 65^{0}C and was then ready for dark-field microscopy.

2.9 Protein Methods

2.9.1 Preparation of protein from *Drosophila* tissues

Protein samples from different developmental stages of *Drosophila* were prepared in the following way (Table 8). Tissues were dissected in Ringer's solution and homogenised in the appropriate volume of protein sample buffer. Samples were boiled for 5 minutes and cooled on ice. The cell debris and body particles were removed by centrifugation at 22,000 x g for 5 minutes and cleared supernatant transferred to a clean microcentrifuge tube. The samples were stored at -80^{0}C.

Table 2.8

Developmental stage	Protein sample buffer(2x)
3 ovaries	110 μl
30 testes	100 μl
40 embyros	60 μl
2 third instar larvae	150 μl
2 light pupae (LP)	150 μl
2 dark pupae (DP)	150 μl
3 male flies (carcass)	110 μl
2 female flies (carcass)	150 μl

2.9.2 Estimation of protein concentration

Protein concentrations were estimated using the Bradford method. The Bradford method depends on quantitating the binding of a dye, Coomassie Brilliant Blue, to an

unknown protein and comparing this binding to that of different amounts of standard protein, usually bovine serum albumin (BSA).

Duplicate amounts of 0.5mg/ml BSA (5, 10, 15 and 20 μl) were measured into microfuge tube and made up to 100 μl with 0.15M NaCl. Two blank tubes contained 100 μl 0.15M NaCl. 1 ml Coomassie Brilliant Blue solution was added to each tube and vortexed, then allowed to stand for 2 minutes at room temperature. The A_{595nm} was measured using a 1cm pathlength cuvette and a standard curve constructed by plotting absorbance at 595nm versus protein concentration. The standard curve can then be used to determine the protein concentration of the unknown sample, by determining the unknown absorbance. This method can be used to quantify 1 to 10 μg of protein.

2.9.3 SDS-Polyacrylamide gel electrophoresis of protein

Protein gel plates were washed and set up according to manufacturer's instructions. The separating mix was poured between the plates, overlaid with 1-2ml H_2O and left to sent for approximately 30 minutes. The H_2O was poured off and the stacking gel mix was poured between the plates. The comb was inserted in the top of the gel and the gel was left to set for 15 minutes. The gel plates were fixed into the gel tank and 1 x running buffer was poured into the reservoirs of the tank. Protein samples and *Drosophila* tissue used for electrophoresis were mixed with an equal volume of 2x sample buffer and loaded onto the gel. 12μl protein marker (supplied by BioRad) with 12μl protein sample buffer was boiled for 5 minutes before loading on the gel. Electrophoresis was generally carried out overnight at 35-50V.

Proteins were transferred from gels onto PVDF membrane by electroblotting using a Trans-Blot SD semi-dry transfer cell (Sigma Aldrid). PVDF membrane was pre-wet in 100%(v/v) methanol, washed 1 x 5 minutes in distilled water and then equilibrated in the protein running buffer for at least 10 minutes. Three pieces of Whatman paper, soaked in running buffer, were placed on the bottom electrode plate, one at a time ensuring no air bubbles remained trapped between layers. On top of this was laid the membrane, the gel, and three further pieces of Whatman paper soaked in running buffer, again checking for air bubbles. The other electrode plate was positioned on top to complete the circuit, and proteins were transferred at 150mA for 2 hours. In order to confirm that complete transfer had occurred, the membrane was stained in Ponceau S solution for 10 minutes and the transfer checked, then washed with H_2O to remove the Ponceau S.

2.9.4 ECL method for immunodetection

The membrane was incubated in 50ml Blocking buffer for 1 hour at RT with shaking or overnight at 4^0C (no shaking required). Next, the membrane was incubated with the primary antibody in 16ml TBS for 1 hour at RT with shaking (the membrane was sealed in a nylon bag). The primary antibody was used in a dilution of 1:500-1:1000(serum) or 1:2000-1:40,000 (affinity purified antibody). The membrane was washed 3 x 10 minutes in TBST at RT with shaking. The membrane was then placed in a fresh bag and incubated with 16ml TBS containing the secondary antibody for 1 hour at RT with shaking. The secondary antibody, typically HRP-conjugated anti-rabbit (mouse or sheep) IgG, is used at a dilution recommended by the supplier, normally 1:1000 to 1:4000. The secondary antibody was then

discarded and the membrane washed 3 x 10 minutes in TBST at RT with shaking. The membrane was immersed in 20ml ECL Reagent with 8μl 30% H_2O_2 for 1 minute and was then sealed in a nylon bag. The membrane was placed in a film cassette and exposed to film for 5 seconds to 1 hour.

2.10 P-element mediated germline transformation

2.10.1 Preparation of DNA

Plasmid DNA to be injected was prepared and purified by using Qiagen plasmid kit. The DNA pellet was dissolved in injection buffer (5mM KCl, 0.1 mM sodium phosphate, pH6.8) at a concentration of 400μg/ml for the transforming plasmid and 100μg/ml for the helper plasmid pΔ2-3.

2.10.2 Embryo collection and chorion removal

Embryos were dechorionated manually by rolling the embryos gently on double sided sticky tape. Dechorionated embryos were transferred to a 22 x 22 mm cover-slip (attached to a 76 x 26 mm slide) which had a very thin strip of double sided sticky tape on it. Using a dissecting needle the embryos were oriented with their posterior ends over one side of the tape. Normally, around 20 embryos could be lined up on each cover-slip. As soon as the desiccation was complete (about 6-10 minutes), the embryos were covered with oil.

2.10.3 Microinjection

The embryo-laden slide was placed on the stage of the microscope with the posterior of the embryos towards the needle. The embryos were injected under the control of the micromanipulator at 1.4 kpsi, 0.3-0.5 seconds.

2.10.4 Post-injection treatment

After all the embryos had been injected, damaged and improperly aged embryos were removed under the dissecting microscope. The cover-slip containing the injected embryos was placed onto a tomato juice plate and incubated at 25^0C for 1-2 days. The larvae were transferred into a fresh vial of Staffan food (about 10 larvae for each vial) and incubated at 25^0C.

2.10.5 Examination for transformants

When the larvae developed to late pupae stage, individuals were transferred to fresh vials of food. These were crossed with flies of opposite sex when they eclosed from the pupae case. Progeny of the cross were examined for red-eyed transformants.

2.11 RNAi Method

2.11.1 Preparation of double strands RNA

Each primer contain a T7 promoter sequence on its 5 prime end (TAATACGACTCACTATAGGGAGA). Double strands RNA was made as described in section 2.5.2.

2.11.2 Embryo collection and chorion removal

Embryos were collected and dechorionated as described in section 2.10.2.

2.11.3 Microinjection

Embryos were injected as described in section 2.10.3. Each embryo received 0.2 fmol of RNA. Embryos were injected at the preblastoderm stage.

2.11.4 Examination of the results

Antibody staining as described in section 2.8.2 was carried out to check the result of RNAi.

2.12 Germline Clone Method

2.12.1 Preparation of the larvae for mitotic recombination

ovo^{D1} males were mated to *Ntf-2* mutant virgin female for two days and transferred to fresh food vials. The flies were allowed to lay eggs in the vials for 8 hours and removed. The vials then put into 25^0C incubator for 40 hours.

2.12.2 **X-ray exposion**

The larvae (40-48 hours) old in the vials were put into TRX 2800 X-ray machine. The larvae were exposed to 1000 rads of X-ray (shelf 5, 8 min) and allowed to recover at 25^0C.

2.12.3 Collection of germline clone flies

Virgin female were collected and put 10 female flies in each vial. They were mated with 10 OrR male flies. Each vial was checked under microscope everyday. If eggs were found in the vial, all the females were separated to single in every vial. The females laying eggs was picked up and put together. The eggs of these female flies were collected and dechorionated as described in section 2.2.3.

2.12.4 Analysis of germline clone

The embryos were carried out by in situ hybridisation and antibody staining to check their expression pattern.

Chapter Three: Molecular Characterisation of *Z14/Dmbves*

3.1 Introduction

The follicle cells play crucial roles in oogenesis. Some of the functions of follicle cells are well known, for example, they produce chorion proteins, vitelline membrane proteins, the micropyle and dorsal appendages. The interactions between follicle cells and the oocyte which establish dorsal-ventral polarity have been investigated in recent years (Neuman-Silberberg and Schupbach, 1994; Roth et al., 1995). Many genes are involved in this determination event, but precise functions of many of the genes expression in follicle cells are not yet known. We were interested to establish the roles of genes expressed in subsets of follicle cells, which are involved in follicle cell differentiation and their interactions with the oocyte. Using a GAL4/UAS enhancer trap system, 413 lines with *P[GAL4]* insertions on different chromosomes were observed (Deng et al., 1997). Approximately 22% of the lines show β-galactosidase staining in subsets of follicle cells. They show highly dynamic and characteristic expression patterns. Since the pattern of reporter gene expression reflects the spatial and temporal expression of the gene controlled by the enhancer, we use these expression pattern to identify genes that could be important in oogenesis.

3.2 *Z14/Dmbves* cDNA and ESTs

3.2.1 Isolation of *Z14/Dmbves* cDNA

The *Z14/Dmbves* gene was identified as part of a GAL4/UAS enhancer trap scheme. When line C648, a *GAL4* line, is crossed with UAS-lacZ line, staining is detected in a patch of follicle cells at stage 10 of oogenesis. Follicle cells at this stage

are different between dorsal and ventral sides. As we know that there is an interaction between follicle cells and oocyte during oogenesis and that such interaction eventually leads to the dorsal-ventral pattern of egg chamber and future embryo. Thus the gene which is close to the P insertion may have an important role in the follicle cells during oogenesis. Cloning of the target gene was achieved by using the "plasmid rescue" technique (Bellen et al, 1989). To clone genomic DNA covering the region of interest, plasmid rescue of the genomic fragment was followed by screening a *Drosophila* genomic library using the rescued genomic fragment as a probe. Following this process a cDNA was isolated by screening an ovarian cDNA library with genomic DNA as a probe. The cDNA isolated was named *Z14* {later on, we found vertebrate homologues of *Z14* are called blood vessel/epicardial substance (bves). Hence, we named *Z14 Drosophila melanogaster* bves (Dmbves)}. The cDNA has an insert of 819 bp. Whole mount mRNA *in situ* hybridisation using ovaries and the cloned cDNA as a probe helped to establish if the cDNA clone was from the target gene. A similar expression pattern was observed when comparing its expression pattern with the reporter gene expression pattern. Expression was observed in the nurse cells and all follicle cells surrounding the oocyte at stage 10 of oogenesis, except those in the anterior-dorsal and posterior region. The *Z14/Dmbves* cDNA was sequenced and found to encode a putative protein of 149 amino acids (Appendix 1) (Zhao and Bownes, unpublished data).

3.2.2 Database search and chromosome position of *Z14/Dmbves*

To establish what kind to gene *Z14/Dmbves* is, I need to compare the *Z14/Dmbves* sequence to other *Drosophila* DNA sequences. Thanks to Berkeley Drosophila Genome Project (BDGP), it is possible to do such a work with the whole *Drosophila*

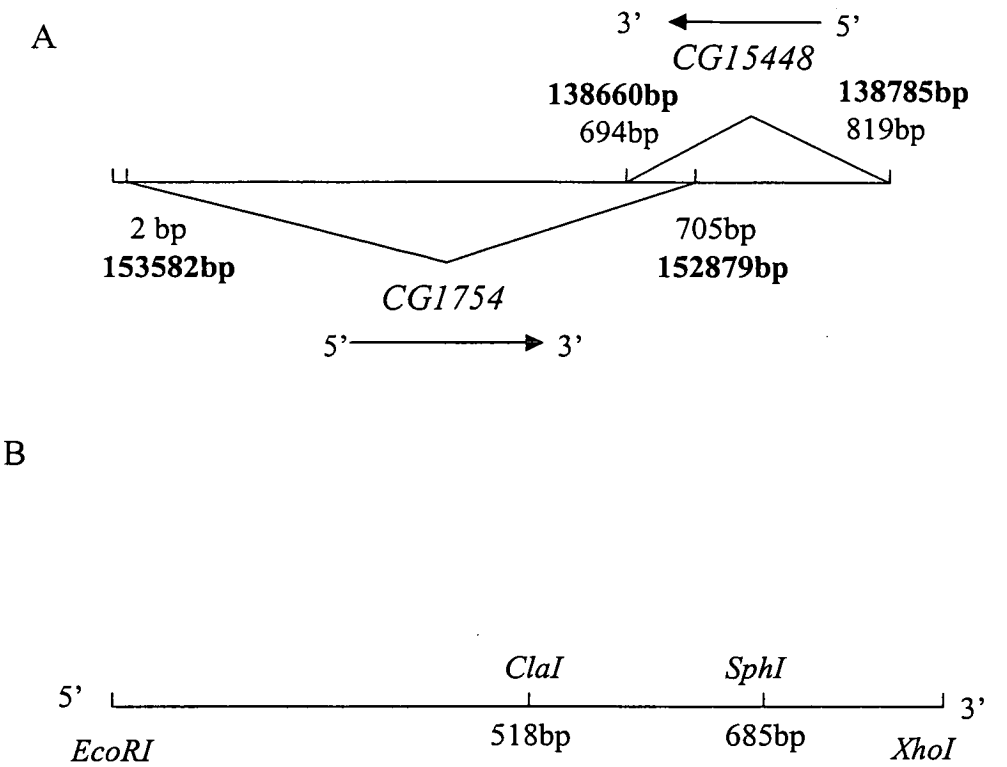
genome available. Using a BLAST search of the NCBI database (National Centre for Biotechnology and Information, http://www.ncbi.nlm.nih.gov/) which contains *Drosophila* genomic sequences from BDGP (Adams et al., 2000), *Z14/Dmbves* cDNA was found to align to two positions in the genome. (Fig.3.1). At the 5' end, from 2bp to 705bp of the *Z14/Dmbves* sequence is 100% identical to the genomic sequence from 153582bp to 152879bp. At the 3' end from 694bp to 819bp is 100% identical to the genomic sequence from 138660bp to 138785bp. It therefore seemed possible that the cDNA insert was a co-ligation of two different genes, especially as these genomic regions were proposed by the genome annotaters to encode genes transcribed in opposite directions. The 703bp sequence at the 5' end of *Z14/Dmbves* cDNA corresponds to a putative gene called *CG1754* (Fban0001754). The 125 bp sequence at the 3' end of *Z14/Dmbves* corresponds to another putative gene called *CG15448* (Fban0015448) .

Both *CG1754* and *CG15448* are located on chromosome X at position 19E-7. The cloned cDNA was checked by *in situ* hybridisation to polytene chromosomes and it hybridises to a single chromosome site (Zhao and Bownes, unpublished data), hence it was not realized at the time that it may be a co-ligation.

3.2.3 Identification of the *Z14/Dmbves* gene

To determine which gene, *CG1754* or *CG15448*, had the expression pattern that we observed with *Z14/Dmbves* (Chapter 5), RNA probes were made for the two fragments of *Z14/Dmbves* representing each gene (Fig.3.2). RNA *in situ* hybridisation on the wild type ovaries demonstrated that *CG1754* has the same

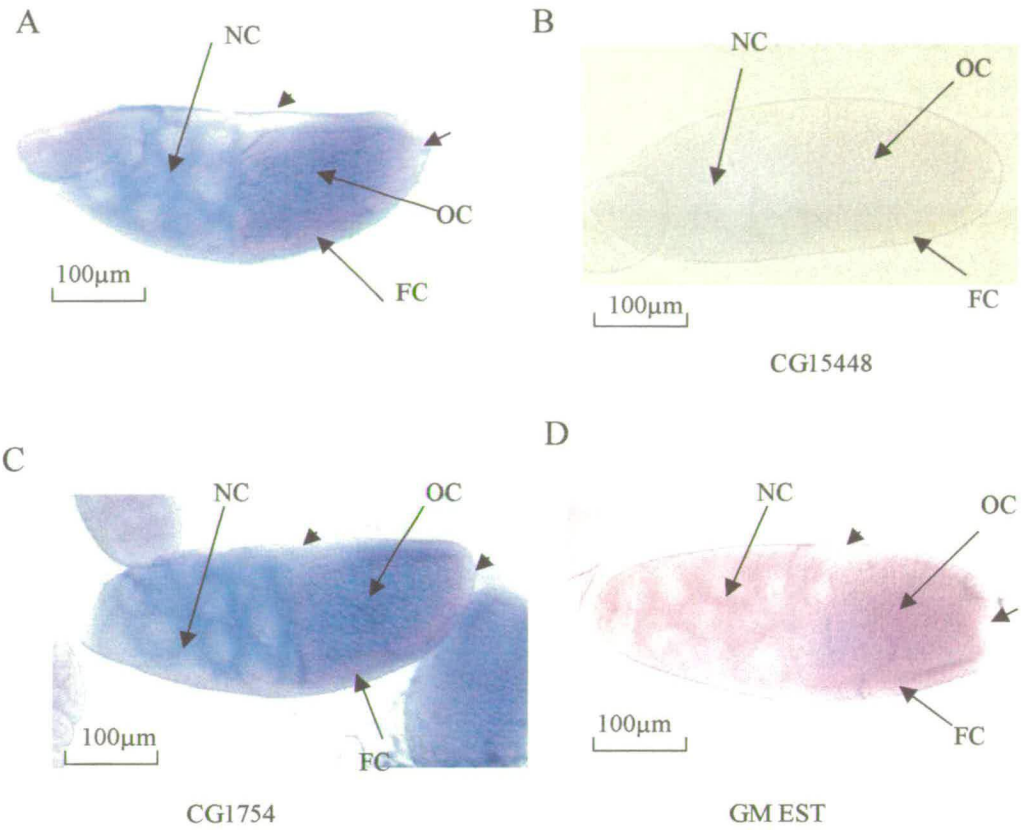
Figure 3.1 Map of original *Z14/Dmbves* cDNA



Legend

A.The original *Z14/Dmbves* cDNA contains two putative genes (from 2bp to 705bp is *CG1754*, from 694bp to 819bp is *CG15448*). These two genes overlap from 694bp to 705bp, and they are connected in reverse direction. The bold numbers in the figure correspond to position of the base pairs in the genome.
B. Restriction map of *Z14/Dmbves* cDNA. *EcoRI* and *XhoI* sites are from the vector sequence.

Figure 3.2. RNA in situ hybridization to the wild type ovaries.



Legend

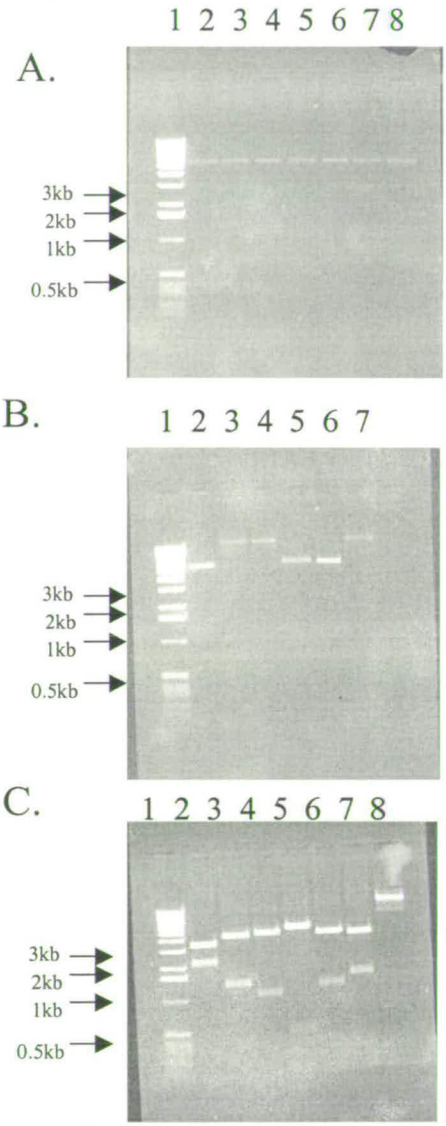
A. Expression of *Z14/Dmbves.* Expression is observed in the nurse cells and all follicle cells except the anterior dorsal an posterior region (the arrows head indicate the regions).
B. With RNA probe made from the fragment of CG15448, it shows that CG15448 is not expressed in the ovary.
C.With RNA probe made from the fragment of CG1754, it shows that CG1754 has the same expression pattern as *Z14/Dmbves*.
D. With RNA probe made from EST GM07524. The arrows indicate that the EST has the same expression pattern as *Z14/Dmbves* .
NC: nurse cell; OC:oocyte; FC: follicle cell; egg chamber in stage10 in oogenesis.
Anterior is to the left.

expression pattern as *Z14/Dmbves* cDNA. *CG15448* showed no detectable expression (Fig.3.2).

3.2.4 Analysis of ESTs

Searching for EST is a good approach that I can use to get full length cDNA of *Z14/Dmbves*. Two EST clones, one from an ovarian library and the other from an embryonic library, were found to be similar to the gene *CG1754*. The EST from the ovarian library is named GM07524 and the EST from the embryonic library is named LD22978. According to the annotation of Berkeley *Drosophila* Genome project (BDGP), these two ESTs are directionally cloned into *EcoRI/XhoI*-digested plasmid. GM07524 was constructed in the pbluescript vector and LD22978 was constructed in the pOT2 vector. The pictures of pbluscript and pOT2 are shown in Appendix 2. Using restriction enzymes *EcoRI* and *XhoI* to digest these two ESTs, it was found that EST GM07524 had an insert of 2.6kb and EST LD22978 had an insert of 5.5kb. To make a restriction map of the EST GM07524, twelve enzymes were used to digest the EST (Fig.3.3). The same method was used for the LD22978 EST (Fig.3.4). The restriction maps of two ESTs were then made (Fig.3.5). Comparing the two ESTs maps, it can be seen they have the same restriction site in the length of 2.6kb sequence from the 5' end (Fig.3.5). Using the GM07524 EST to make a RNA probe and performing mRNA *in situ* hybridization to wild type ovaries, it was found that the EST has the same expression pattern as *Z14/Dmbves* cDNA (Fig.3.2). This means that the GM07524 EST is related to the *Z14Dmbves* gene. Since the LD22978 EST has the same restriction site in the 2.6kb sequence from the 5' end to the GM07524 EST, it could be a different transcript of the *Z14/Dmbves* gene.

Figure 3.3 Restriction analysis of EST GM07524



A.Enzymes used to digest the the GM07524 EST

1.1kb ladder
2.*EcoRV*
3.*KpnI*
4.*NotI*
5. *PstI*
6. *SacI*
7. *SalI*
8. *XbaI*

B. Enzymes used to digest the GM07524 EST.

1.1kb ladder
2.*BamHI*
3.*HindIII*
4. *Cla*
5.*SmaI*
6.*StuI*
7.undigested plasmid cDNA

C. Enzymes used to double digest the GM07524 EST.

1.1kb ladder
2.*EcoRI /EcoRV*
3.*EcoRI/SalI*
4.*EcoRV/SphI*
5.*EcoRv/XhoI*
6.*SalI/XhoI*
7.*SphI/XhoI*
8.undigested plasmid cDNA

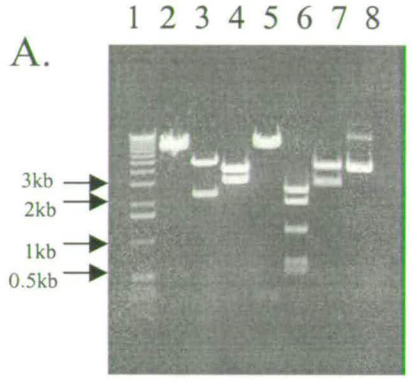
Legend

A.The picture shows that seven of the enzymes used have only one restriction site in the EST.

B.This picture shows that *HindIII* and *Cla* do not digest the EST.

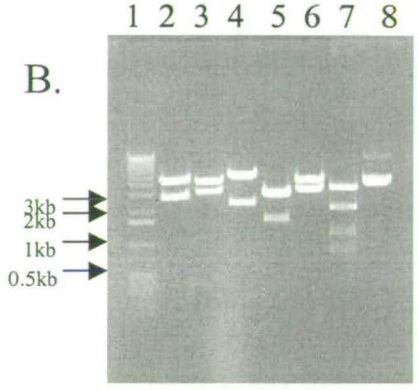
C.Three restriction sites (*SphI*, *SalI* and *EcoRV*) were found in the insert of GM07524 EST. Double digests using one of them together with *EcoRI* or *XhoI* enabled us to calculate the distance between restriction sites.

Figure 3.4 Restriction analysis of EST LD22978



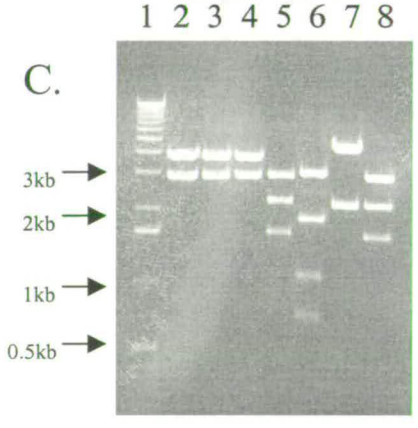
A. Enzymes used to digest LD22978 EST.

1.1kb ladder
2.*BamHI*
3.*BglII*
4.*EcoRI*
5.*PstI*
6.*PvuII*
7.*SmaII*
8.*XbaI*



B. Enzymes used to single or double Digest LD22978 EST.

1.1 kb ladder
2.*SphI*
3.*SalI*
4.*EcoRV*
5.*SphI/XhoI*
6.*SalI/XhoI*
7.*EcoRI/SphI*
8.undigested plasmid cDNA



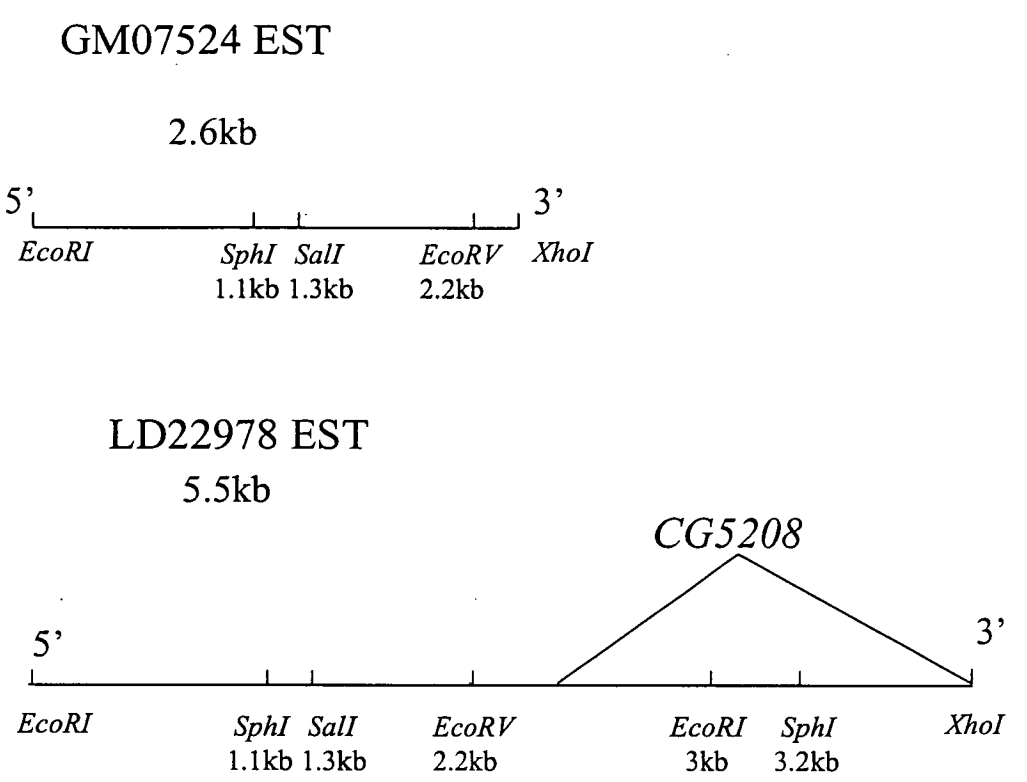
C. Enzymes used to single or double Digest LD22978 EST.

1.1 kb ladder
2.*SalI*
3.*SalI/XhoI*
4.*EcoRI*
5.*EcoRI/XhoI*
6.*EcoRI/SphI*
7.*EcoRV*
8.*EcoRV/XhoI*

Legend

A.The picture shows that *BamHI* and *PstI* have only one restriction site in the EST. The others all have two or more restriction sites. B. And C.Three restriction sites (*SphI*, *SalI* and *EcoRV*) which were found in the insert of both GM and LD EST. Double digests using one of them together with *EcoRI* or *XhoI* enabled us to calculate the distance between restriction sites.

Figure 3.5 Restriction maps of EST GM07524 and LD22978



Legend

GM07524 EST and LD22978 EST have the same restriction sites in 2.6Kb near the 5' end. LD22978 EST has another putative gene Named *CG5208* near the 3' end.

3.3 Sequencing ESTs

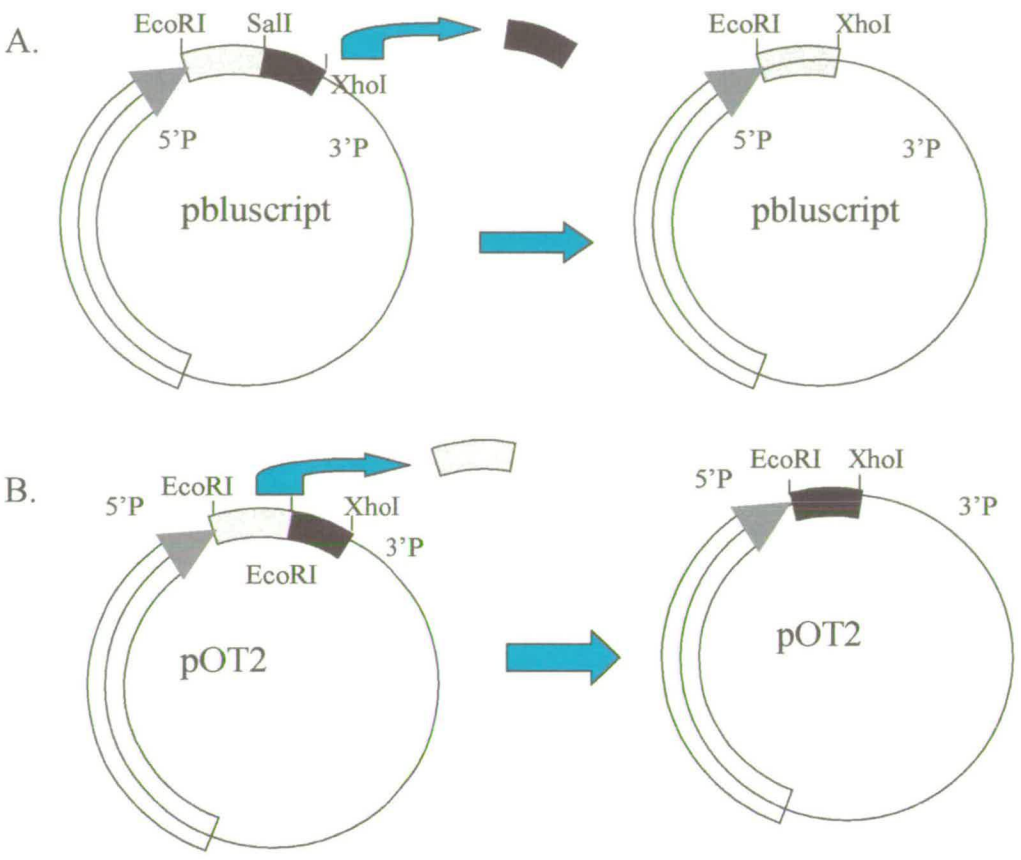
3.3.1 Subcloning EST

ESTs usually have several hundred base pairs of known sequence at the 5' end. To find if both EST GM07524 and LD22978 have the same sequence as *Z14/Dmbves*, these two ESTs must be sequenced completely. The restriction maps of the two ESTs were analysed and the *SalI* restriction site was found in the middle of the insert of the GM07524 EST. *SalI* and *XhoI* were used to digest the GM07524 EST. The fragment between *SalI* and *XhoI* was removed and the rest of vector was re-ligated (Fig.3.6). To subclone the LD22978 EST, *EcoRI* was used to digest the LD22978 EST. The fragment between the two *EcoRI* restriction sites was removed and the rest of the vector was re-ligated (Fig.3.6). Both ligated vectors were transformed into XL-1 blue competent cells. The subclone of the GM07524 EST was isolated by using Ampicillin selection and the subclone of LD22978 EST was selected by using chloramphenicol selection.

3.3.2 Design of primers

GM07524 EST is a 2.6kb cDNA in pbluescript vector. T3 primer can be used to Sequence the 5' end of the EST and T7 primer can be used to sequence both 3' ends of the EST and of the subclone of the EST. Afterwards, SL2,SL4, SL5, SL6 and SL8 primers were designed to sequence the EST from 5' to 3'. SL1 and SL9 primers were designed to sequence from 3' to 5'. For the LD22978 EST, SL3 primer was designed to sequence both 5' end of the EST and subclone of the EST. SL6 and SL7 were designed to sequence the key region of the EST in the opposite direction (Fig.3.7).

Figure 3.6 Subcloning ESTs

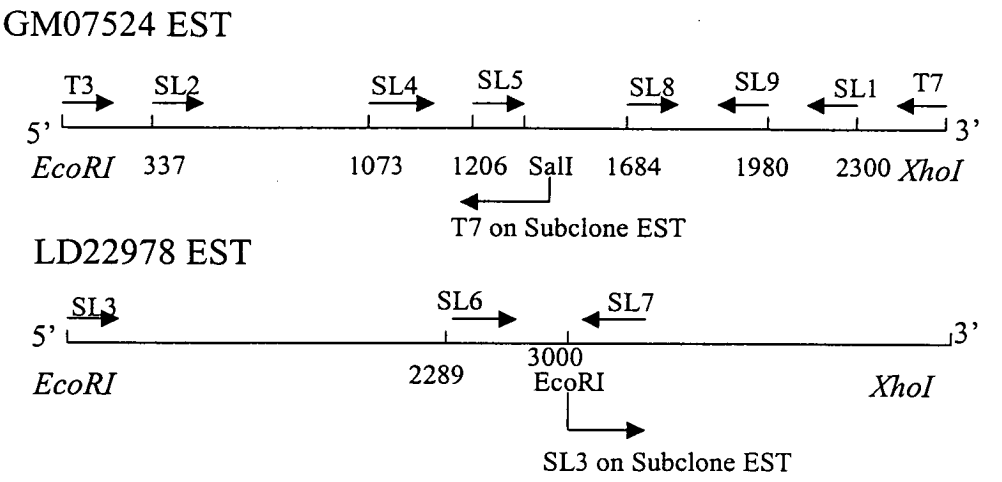


Legend

A. GM07524 was constructed in the pbluescript vector. *SalI* and *XhoI* were used to digest the GM07524 EST. The fragment between *SalI* and *XhoI* was removed and the rest of vector was ligated.

B. LD22978 was constructed in the pOT2 vector. *EcoRI* was used to digest the LD22978 EST. The fragment between two *EcoRI* restriction sites was removed and the rest of vector was ligated.

Figure 3.7 Sequencing map of two ESTs



Legend

The primers used to sequence the two EST clones are shown with arrows.

3.3.3 Full lenth *Z14/Dmbves* cDNA

The result of sequencing EST GM07524 revealed that the putative *CG1754* gene and *CG15448* gene are actually the same gene. Why did one sub-fragment of original *Z14/Dmbves* cDNA (Cloned by Zhao) which represents CG15448 sequence not show ovary expression? It is understandable now, because this fragment was connected with *CG1754* in the reverse direction. When this fragment was used to make an antisense RNA probe , actually a sense probe was made instead. The EST GM07524 has a poly A tail and so appeared to extend to the 3' end of the corresponding RNA. The result of sequencing EST LD22978 revealed that this cDNA also consists of CG1754, CG15448 and another putative gene named *CG5208*. This is a misligation because *CG5208* is located on chromosome 3. The genome annotation is wrong in this region. It is hypothesized by genome annotation (Adams et al., 2000) that *CG1754* is a gene, its mRNA is 1255bp and encodes an Open Reading Frame (ORF). *CG15448* is another gene, its mRNA is 669bp and also encodes an ORF. The cDNA we obtained from our cDNA library and the two ESTs all indicate that *CG1754* is always connected to *CG15448,* and therefore that they are actually one gene. We contacted the genome project and this mistake is corrected by the new version of the *Drosophila* genome using our result. The full length of *Z14/Dmbves* cDNA is 2606bp (Fig.3.8).

Figure 3.8 Sequence of Z14/Dmbves cDNA

```
          10         20         30         40         50
----:----|----:----|----:----|----:----|----:----|
ACAAGAAAACATCGGATGAATCGCCGGGCAGGAAAATCACGGTTTCCACT

          60         70         80         90         100
----:----|----:----|----:----|----:----|----:----|
GAAAAACTACTGATAGTTATCACATATCCGAAATATATGTATTTTCAATC

          110        120        130        140        150
----:----|----:----|----:----|----:----|----:----|
GAAACGAGAACCAAATCCCCGAAAAAACGCGAATCGGAGTAAATACAAGT

          160        170        180        190        200
----:----|----:----|----:----|----:----|----:----|
TGACTTCTGCTGCAGCTGCCTAAGCGATGCAAAATGTGACAGGCGGCAGC

          210        220        230        240        250
----:----|----:----|----:----|----:----|----:----|
AGCAGTAGCCTCATCAAAGGTTAGCTCACCAATAGCCCCAGCTAAATATT

          260        270        280        290        300
----:----|----:----|----:----|----:----|----:----|
AAACAAATACGGAAATAAGAGTCTAAGGCCATTAACTAAACGGCTGACTA

          310        320        330        340        350
----:----|----:----|----:----|----:----|----:----|
AGCTGTAAAATGCAAAGTAATTCAAATTTCAACTAGCTAGGATATGAACC

          360        370        380        390        400
----:----|----:----|----:----|----:----|----:----|
GCAAGCAAAAAATACATTCAAGACACCAACCGCAAAGCACAAACATTTTG

          410        420        430        440        450
----:----|----:----|----:----|----:----|----:----|
AAAAGGGCAGCAGCAATAACAAAACAAACCACCACCAACAAAGGTTGAGG
```

```
          460        470        480        490        500
----:----|----:----|----:----|----:----|----:----|
AAAACCGCGCGTAAGCAACAACAAACGAATTAGGCGAAGGAGAGAAAGGT

          510        520        530        540        550
----:----|----:----|----:----|----:----|----:----|
GGAAGATAGAAACAAAAGGCAGATTTTTGGCATTCGCCTAGCGTGGACAA

          560        570        580        590        600
----:----|----:----|----:----|----:----|----:----|
CAACCAACCAGTACATCAACCAATCAATTTGGTAATTAGGCAAAATACGT

          610        620        630        640        650
----:----|----:----|----:----|----:----|----:----|
GCTGCAAAAAATAGCTCAACGATCAGGCTGAGAGGAGAGTGACAGACTGA

          660        670        680        690        700
----:----|----:----|----:----|----:----|----:----|
AACGAAGGCAGTTCGCCTGGATCAACGGAATGCCCAGCACGGCGGGCAGT

                                 M  P  S  T  A  G  S

          710        720        730        740        750
----:----|----:----|----:----|----:----|----:----|
GCAGCTGGCGTTGGTATGGGCGCCCTGATCAACAGTGCCGGCAGCAGCGC

A  A  G  V  G  M  G  A  L  I  N  S  A  G  S  S  A

          760        770        780        790        800
----:----|----:----|----:----|----:----|----:----|
CAGTAGTGTCATGGGCATTGGCATGGGCAGAGCAGCTGCTACGGGAGCAG

 S  S  V  M  G  I  G  M  G  R  A  A  A  T  G  A  G

          810        820        830        840        850
----:----|----:----|----:----|----:----|----:----|
GAGCACCAGGCAGTTCGGGATCTGGAGCAGATGCCTCCGCCGCCGGCACT

  A  P  G  S  S  G  S  G  A  D  A  S  A  A  G  T

          860        870        880        890        900
----:----|----:----|----:----|----:----|----:----|
TTAATCGCCCAGAGCACGGCGGGAACGAGCGCCGCCAGCAGTGGAACGAT

L  I  A  Q  S  T  A  G  T  S  A  A  S  S  G  T  I

          910        920        930        940        950
----:----|----:----|----:----|----:----|----:----|
```

```
CACCTGGGACAACAATGGAACCCTGCGATCGATCAATCCCGGCGATTGGT

 T  W  D  N  N  G  T  L  R  S  I  N  P  G  D  W  S

         960       970       980       990       1000
----:----|----:----|----:----|----:----|----:----|
CCATCGAACAGTGCCTCGGGCCACATCACCTGTACTTTCAGCTCGGCTGG

  I  E  Q  C  L  G  P  H  H  L  Y  F  Q  L  G  W

         1010      1020      1030      1040      1050
----:----|----:----|----:----|----:----|----:----|
GCCTTTCTCTTCCTAGCCTTTTTGGCTCCACACGGTCCCTACGGCGCCCT

A  F  L  F  L  A  F  L  A  P  H  G  P  Y  G  A  L

         1060      1070      1080      1090      1100
----:----|----:----|----:----|----:----|----:----|
GTGGATGCGTGCCATGCTGCTCATTGGCTGCCTGATGATGGGCATGCATG

 W  M  R  A  M  L  L  I  G  C  L  M  M  G  M  H  G

         1110      1120      1130      1140      1150
----:----|----:----|----:----|----:----|----:----|
GCTACTTGGTGGCCTTTGCACCGGACGTCATCCTGTGGTCGGGCATGGGA

  Y  L  V  A  F  A  P  D  V  I  L  W  S  G  M  G

         1160      1170      1180      1190      1200
----:----|----:----|----:----|----:----|----:----|
CTTTTTGTCAACTTCATCTATCTCGTCGTGGTGCTATGCCGGCTGAGGCC

L  F  V  N  F  I  Y  L  V  V  V  L  C  R  L  R  P

         1210      1220      1230      1240      1250
----:----|----:----|----:----|----:----|----:----|
TGTGCGATTCGAGCAGGAAATCGAAGCGGTCTACCTGGCACTTTTCCAGC

 V  R  F  E  Q  E  I  E  A  V  Y  L  A  L  F  Q  P

         1260      1270      1280      1290      1300
----:----|----:----|----:----|----:----|----:----|
CGCTGCACGTGACGCGCCACCAGTTCAAGAAGGTGCTCAACTGCATGAAG

  L  H  V  T  R  H  Q  F  K  K  V  L  N  C  M  K

         1310      1320      1330      1340      1350
----:----|----:----|----:----|----:----|----:----|
GTGATACGTGCCCTGAAGTACCAGGAGGTCTACGCCCAGGAGAAGGTCAC

V  I  R  A  L  K  Y  Q  E  V  Y  A  Q  E  K  V  T

         1360      1370      1380      1390      1400
----:----|----:----|----:----|----:----|----:----|
CAAGGTCGACAGCCTGTCGCTGGTGCTGAGCGGCAAACTGGTGGTGTCGC

 K  V  D  S  L  S  L  V  L  S  G  K  L  V  V  S  Q

         1410      1420      1430      1440      1450
```

```
----:----|----:----|----:----|----:----|----:----|
AGCATCAGCGCGCCCTGCACATTGTGTTTCCCCATCAGTTCCTAGACTCG

  H  Q  R  A  L  H  I  V  F  P  H  Q  F  L  D  S

          1460       1470       1480       1490       1500
----:----|----:----|----:----|----:----|----:----|
CCAGAATGGTTTGGCGTCTCGACCGATGACTACTTTCAGGTCTCCATCAT

P  E  W  F  G  V  S  T  D  D  Y  F  Q  V  S  I  M

          1510       1520       1530       1540       1550
----:----|----:----|----:----|----:----|----:----|
GGCCATGGAGGAGTCGCGGGTGCTGATCTGGCATCGCGACAAGCTCAAAT

 A  M  E  E  S  R  V  L  I  W  H  R  D  K  L  K  L

          1560       1570       1580       1590       1600
----:----|----:----|----:----|----:----|----:----|
TGTCAATTATGGCCGAGCCCTTCTTGCAGACCGTCTTCGATCACATTCTG

  S  I  M  A  E  P  F  L  Q  T  V  F  D  H  I  L

          1610       1620       1630       1640       1650
----:----|----:----|----:----|----:----|----:----|
GGCCGGGATGTGGTCAAGAAGCTGATGCAGGTCACCCAGGTGAGCGAGTC

G  R  D  V  V  K  K  L  M  Q  V  T  Q  V  S  E  S

          1660       1670       1680       1690       1700
----:----|----:----|----:----|----:----|----:----|
GATAGCCAGCAATGGCTTCCTGCCCTCTGGTGGATATGCTGAGGATGCGG

 I  A  S  N  G  F  L  P  S  G  G  Y  A  E  D  A  E

          1710       1720       1730       1740       1750
----:----|----:----|----:----|----:----|----:----|
AGGACAAGCCCATGTTGATACTAAAGAAGAGTGTGGATGTGGGACACGGA

  D  K  P  M  L  I  L  K  K  S  V  D  V  G  H  G

          1760       1770       1780       1790       1800
----:----|----:----|----:----|----:----|----:----|
CTGACGGCCCTGATCAACCGGCAGCTACAGGACGAGCATGTTCCTTTACT

L  T  A  L  I  N  R  Q  L  Q  D  E  H  V  P  L  L

          1810       1820       1830       1840       1850
----:----|----:----|----:----|----:----|----:----|
CGGTCGCACGTACAAACAACAACAACAACAACAACTACTGCTACTGC

 G  R  T  Y  K  Q  Q  Q  Q  Q  Q  Q  L  L  L  L  Q

          1860       1870       1880       1890       1900
----:----|----:----|----:----|----:----|----:----|
AACAACAACTAGAACAACAACGCGTACAAGAAGCAACAACCAGCGCCAAC

  Q  Q  L  E  Q  Q  R  V  Q  E  A  T  T  S  A  N
```

```
          1910       1920       1930       1940       1950
----:----|----:----|----:----|----:----|----:----|
AACATCGAGCAAAGTGCAATCTGAATTTATGCAACCGCCCGCTGCTGCTC

N  I  E  Q  S  A  I  *

          1960       1970       1980       1990       2000
----:----|----:----|----:----|----:----|----:----|
AACTTGCCACGAACAGAAATCAGAGCACCCATCCGTCCCTTCACCTTTGA


          2010       2020       2030       2040       2050
----:----|----:----|----:----|----:----|----:----|
CCTGCACAAGAACTACCAGAAAAACAACCCCACCCACACATGCAAACAGC


          2060       2070       2080       2090       2100
----:----|----:----|----:----|----:----|----:----|
CATCAGCCATCAGTCATCAGTCAGTCAGTCATCAATCATGCGCAGCCACA


          2110       2120       2130       2140       2150
----:----|----:----|----:----|----:----|----:----|
CCAACAGCAACATCGATCACGATCATCAGAAAAGAGCGAAAAGAGCTGTC


          2160       2170       2180       2190       2200
----:----|----:----|----:----|----:----|----:----|
GAAGATATCTGGAAGCTATCCATCTAAATCCGCATCCTAAGTTCCATATA


          2210       2220       2230       2240       2250
----:----|----:----|----:----|----:----|----:----|
TAATAATCTCATCTTTAGCATAGCAACTAGCACTAGCTAAACAAGCATCT


          2260       2270       2280       2290       2300
----:----|----:----|----:----|----:----|----:----|
AACACATCTAATCGTTCTAATAGCAAACTTTGTATTGCCCCCACAACTGC


          2310       2320       2330       2340       2350
----:----|----:----|----:----|----:----|----:----|
AAACAATTAGCCAGAGACTTGAGTTTTTGTGTAAAGTAATTGACTTTGGG


          2360       2370       2380       2390       2400
----:----|----:----|----:----|----:----|----:----|
AAACATAAACTTTTCGTCTCAGCGGAAAAGTGCTAAAACACCGTGTTACC
```

```
          2410       2420       2430       2440       2450
----:----|----:----|----:----|----:----|----:----|
ATTTAGAAGAAACTTGGAGAAGTTACTAAAGAAGTTTACAGGCGTGAAAA

          2460       2470       2480       2490       2500
----:----|----:----|----:----|----:----|----:----|
AAAAAATGATTCGCCATAAAACATAAGTAGTCTTTTATCAAACGTACACT

          2510       2520       2530       2540       2550
----:----|----:----|----:----|----:----|----:----|
GACTTAAATCTAGGAAACTGGGAGAACTGCCTAAAATGATTTGTAGTTTC

          2560       2570       2580       2590       2600
----:----|----:----|----:----|----:----|----:----|
ACCTGACAACTCTATTTAGCAGTGTAGAGAAAAACGTAACATCGTAGACC

          2610       2620       2630       2640       2650
----:----|----:----|----:----|----:----|----:----|
GTTATT
```

Legend

Z14/Dmbves cDNA is 2606bp long. It is predicted to be translated from 680bp to 1924bp, producing a peptide of 414 amino acids.

3.4 Molecular Structure of *Z14/Dmbves*

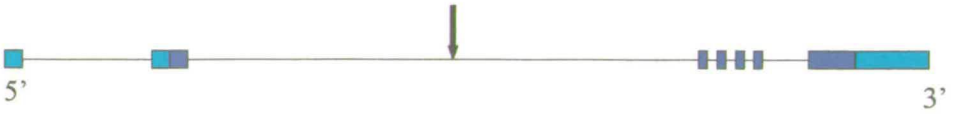
Comparing the *Z14/Dmbves* cDNA with the *Drosophila* genomic sequence established that the *Z14/Dmbves* gene has 7 exons. There is a large intron which is approximately 14kb between exon 2 and exon 3 (Fig.3.9). The *Z14/Dmbves* gene encodes a single ORF, it is translated from 680bp to 1924bp, producing a peptide of 414 amino acids (Fig.3.8).

3.5 *Z14/Dmbves* Related Genes In Other Species

3.5.1 Evolutionary comparison with proteins for other species

The amino acid sequence of *Z14/Dmbves* was used to search the NCBI database. It was found that it is a novel protein, which did not have any known motifs. Using the Simple Modular Architecture Research Tool (SMART), the protein is predicted to have a popeye domain (Fig.3.10). Two vertebrate homologues were found one in chicken and one in humans. The homologues are called blood vessel/epicardial substance (*bves*). There is approximately 28% identity and 50% similarity between these three amino acid sequences. We named our novel gene *Drosophila melanogaster bves* (*Dmbves*) when we submitted the gene sequence to the genebank (Accession No: AF247183, Oct. 3rd, 2000).

Figure 3.9 Molecular structure of *Z14/Dmbves*



Legend

The intron/exon structure of *Z14/Dmbves* gene. The boxes are exons. The box covered by green color represents untranslated regions and blue color represents coding regions. The arrow indicates that the P-element inserted in the middle of large intron between exon 2 and exon 3. Each splice junction correspond to number of base pairs in the genome is shown in table I.

Table I

	Exon 1	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7
Start	132871	138782	153655	153882	154063	154331	155255
End	133032	139847	153812	153984	154212	154472	156115

Figure 3.10 Domain in the *Z14/Dmbves* protein

Legend

The popeye domain starts at position 180 and ends at position 332. The function Of popeye proteins is not well understood. They are predominately expressed in Cardiac and skeletal muscle. This family represents a conserved region which Includes three potential transmembrane domains.

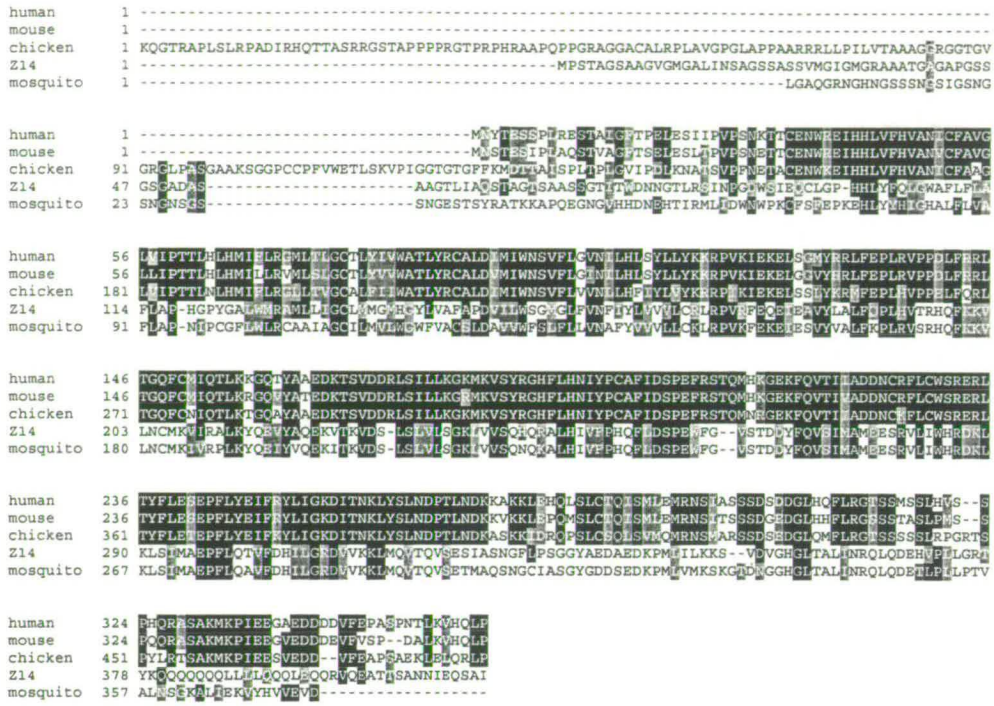
3.5.2 The functions of the homologues of *Z14/Dmbves*

Bves is a novel protein that is highly conserved between chicken and humans. Protein expression analysis showed that Bves is present in cells of the proepicardial organ, migrating epicardium, epicardial-derived mesenchyme and smooth muscle of the developing intracardiac arterial system, including the coronary arteries. (Reese et al., 1999). This suggests that Bves is an early marker of the developing vascular smooth muscle cells. Another homologue was found from a novel gene family called *popeye* (Andree et al., 2000). The *popeye* genes from chicken, mouse, and human share high sequence conservation and more importantly highly conserved expression patterns. *In situ* hybridisation reveals that *popeye* is expressed in skeletal muscle and heart in the vertebrate. Computer-base secondary structure modeling predicted the existence of three transmembrane helices in the N-terminal region of each family member (Sonnhammer et al., 1998). Pop protein was observed to localize to the plasma membrane. Therefore, it is concluded that Pop proteins are associated with membranes. It suggests that the *popeye* gene performs important functions during vertebrate heart development. Recently a new homologue of *Z14/Dmbves* was found in mosquito (Ref. No: XP 311146.1). It shows a high identity (59%) and similarity (71%) to *Z14/Dmbves*. However the function of this mosquito protein is as yet unknown. Protein sequences were aligned by *Z14/Dmbves*, the chicken , mouse, mosquito homologue and the human homologue (Fig.3.11).

3.6 Summary and Discussion

Taking advantage of the GAL4/UAS system, *Z14/Dmbves* cDNA was isolated and identified from the original plasmid rescued fragment. RNA in situ hybridisation to

Figure 3.11 Alignment of homologues of *Z14/Dmbves*



Legend

Alignment of protein sequences encoded by *Z14/Dmbves* gene and the mouse, chicken, mosquito and human(bves)homologues. Amino acids shaded in black are identital and shaded in grey are similar in these five sequences.

wild type ovaries revealed that *Z14/Dmbves* is expressed in the nurse cells and all follicle cells surrounding the oocyte at stage 10 of oogenesis, except those in the anterior-dorsal and posterior region which received the *grk* signal from the oocyte. The cDNA was found that it co-ligated with two different genes named *CG1754* and *CG15448*. Molecular analysis reveals that they are actually a single gene.

Z14/Dmbves encodes a novel protein which has a popeye domain. It is predicted to be a transmembrane protein. Homologues were found in chicken, mouse and human. But *Z14/Dmbves* may not have same function as these homologues since they are low similarities. A homologue which was found in Mosquito is highly similar to *Z14/Dmbves*. Unfortunately it is from the annotation of Anopheles Genome Project. So it is also a novel protein and its function is not known. Further analysis of the function of *Z14/Dmbves* is described in the next chapter.

Chapter Four: Genetic Analysis of *Z14/Dmbves*

4.1 Introduction

Having established the molecular structure of *Z14/Dmbves*, we need to know the function of *Z14/Dmbves* during oogenesis. Generation of mutants in the *Z14/Dmbves* gene will help to do this, as we can investigate the role of *Z14/Dmbves* in oogenesis by studying the phenotype of the mutant.

4.2 Looking for mutations in the *Z14/Dmbves* gene by mobilising the P-element

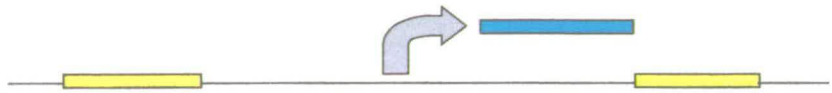
4.2.1 P-element mobilisation

P-element mobilisation was carried out by using the C648 fly line, a *P[GAL4]* line which the *Z14/Dmbves* gene was cloned from, and it was found that the P-element is inserted between exon 2 and exon 3 of the *Z14/Dmbves* gene (Fig.3.9). This fly line was crossed to the *[Δ2-3] Dr* fly line which provides the transposase. This enables the P-element to move to a new position. There are 5 possible outcomes during P-element mobilisation (Fig.4.1). The first is that the P-element remains in its original position with no resulting change in the eye colour of flies. The second outcome is that the P-element excises precisely from its location, with flies reverting to white eyes. The third outcome is that the P-element is excised precisely and re-inserted in a local or distant gene. It may cause lethality if the P-element inserts into a coding region and affects the production of an essential gene. The flies remain red eyed and mutant flies can be generated in this case. The fourth outcome is that the P-element excises imprecisely, either removing the flanking genomic DNA or part of the

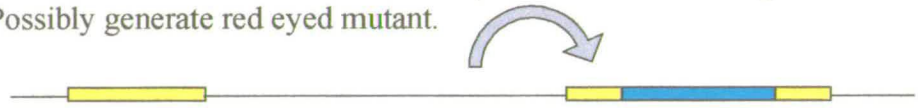
Figure 4.1 Possible outcomes from the mobilisation of a P-element

A. P-element remains in its original position. The flies still have red eye.

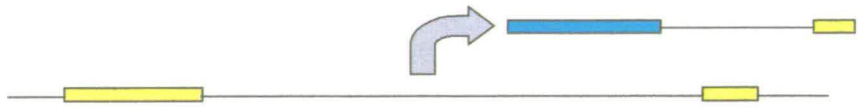
B. Precise excision: revert white eyed flies.



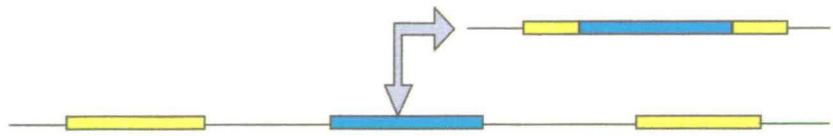
C. Precise excision: P-element can hop to a local or distant gene. Possibly generate red eyed mutant.



D.Imprecise excision: Possibly generates white eyed mutant



E. P-element duplicated. One copy remains in its original position, the other copy hops to a local or distant gene. Possibly generate red eyed mutant.



Legend
Blue boxes represent P-element, yellow boxes represent exon and black lines represent intron.

P-element. Mutant flies can be generated and flies revert to white eye. The final possibility is that a copy of the P-element remains in its original position and an other copy hops to another gene. If it inserted into an exon or key regulating regions of that gene it might cause lethality. This will also result in red eyed flies.

4.2.2 Crossing scheme

Using male flies of C648 line crossed with *[Δ2-3] Dr* female flies which provide transposase, the red eyed *Dr* female flies were selected. These flies were then crossed with male flies carrying an X chromosome balancer to separate the transposase from the P-element in the progeny, thus preventing the P-element moving again. White eyed flies and red eyed flies should appear in the F2 generation. These flies were crossed with the balancer line again. In the F3 generation, the progeny from those female flies of the F2 generation were crossed with their siblings to generate the mutant flies. The progeny from the male flies of the F2 generation were crossed with the balancer line one more time. In the F4 generation, these flies were crossed with their siblings to generate mutant flies. The crossing is scheme detailed in Fig.4.2.

4.3 Analysis of fly lines generated

4.3.1 Result of P-element mobilisation

Table 4.1 Summary of P-hop data

	Lines generated	Homozygous lethal lines
Red eye	156	4
White eye	160	0
Total	316	4

Figure 4.2 Cross scheme of P-hop to generate mutants

Cross 1

♀ $\frac{w^-}{w^-}$; $\frac{\Delta 2\text{-}3\ Dr}{TM6,Tb}$ X ♂ $\frac{w^- PgawB\{w^+\}\ (C648)}{Y}$; $\frac{+}{+}$

F1: Collect red eyed *Dr* virgin female flies: $\frac{w^- PgawB\{w^+\}}{w^-}$; $\frac{\Delta 2\text{-}3\ Dr}{+}$

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

Cross 2

♀ $\frac{w^- PgawB\{w^+\}}{w^-}$; *Δ2-3 Dr* X ♂ $\frac{w^- FM6}{Y}$

F2: Collect wild type eye morphology for male and Bar for virgin female, Keep each fly in a separate vial.

$\frac{w^- PgawB\{w^+\} \text{or P(excision)}}{w^- FM6}$ or $\frac{w^- PgawB\{w^+\} \text{or P(excision)}}{Y}$

Note: All flies containing the *Drop* eye marker were discarded as these flies Contained the source of tansposase (*Δ2-3 Dr*). All other flies—both red eyed (hops) and white eyed (precise excision or deletions) were retained, and kept in separate vials to prevent sib matings.

Cross 3

A. ♀ $\frac{w^- PgawB\{w^+\} \text{or P(excision)}}{w^- FM6}$ X ♂ $\frac{w^- FM6}{Y}$

or

B. ♀ $\frac{w^- l(1)\ 11Ad^1}{w^- FM6}$ X ♂ $\frac{w^- PgawB\{w^+\} \text{or P(excision)}}{Y}$

Collect progeny from A

$\frac{w^- PgawB\{w^+\} \text{or P(excision)}}{w^- FM6}$ or $\frac{w^- PgawB\{w^+\} \text{or P(excision)}}{Y}$

Progeny from B

$\frac{w^- PgawB\{w^+\} \text{or P(excision)}}{w^- FM6}$

Note: Flies selected from Cross 2 were crossed to a X chromosome balancer Line ($w^- FM6/ w^- l(1)\ 11Ad^1$) in order to establish individual fly lines.

Cross 4

Cross progeny from A siblings to obtain homozygous or balance fly lines over FM6.

Progeny from B

♀ $\frac{w^- PgawB\{w^+\} \text{or P(excision)}}{w^- FM6}$ X ♂ $\frac{w^- FM6}{Y}$

Note: Progeny from B need to cross to balancer line one more time to obtain their male siblings.

Cross 5

Cross progeny from cross 4 B siblings to obtain homozygous or balance fly lines over *FM6*

316 balance lines were established. Among them 156 are red eyed lines, 160 are white eyed lines. 4 mutant fly lines were found in the red eyed lines which are homozygous lethal. There are no homozygous lethal lines among the white eyed lines. No other visible mutants were found in either the red-eyed flies and white-eyed flies, such as female steriles, or abnormal phenotypes of the adult flies.

4.3.2 Analysis of mutant flies by using southern hybridisation

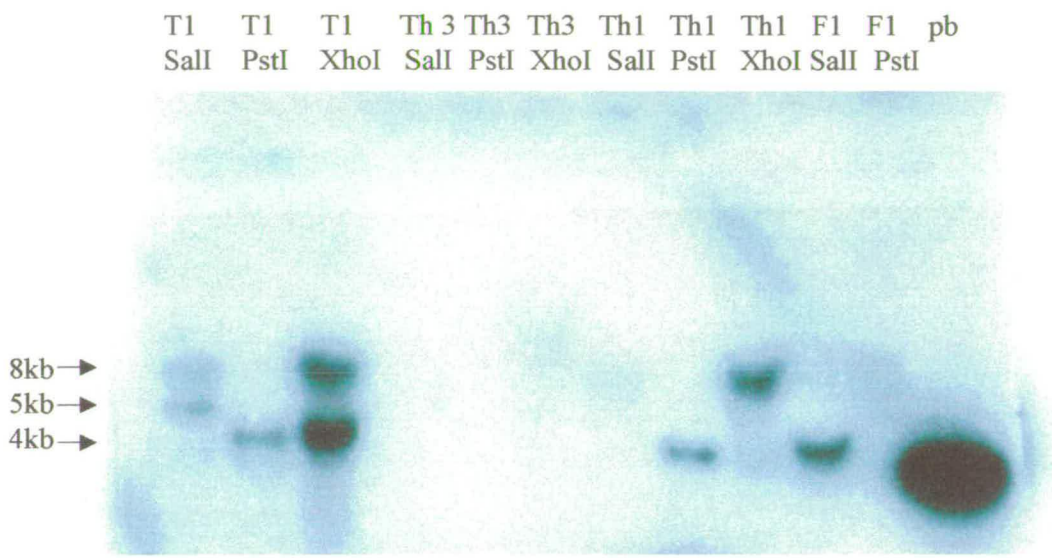
All homozygous lethal flies generated by P-element mobilisation are red eyed flies. It is possible that the P-element has hopped to a local gene or distant gene. Southern hybridisation was carried out to check if the P-element is really inserted in the genome of these mutant flies. The result revealed that the P-element is present in three lines which are T1, Th1 and F1 (Fig.4.3). No band was found in Th3 line. Later on, the genomic DNA flanking a P-insertion of the Th3 line could not be obtained by plasmid rescue. Since pbluescript DNA was used as probe for southern hybridisation, this suggests the P-element in Th3 line excised imprecisely, and the pbluescript was damaged by P-element mobilisation. There are two P-elements in T1 line.

4.3.3 Plasmid rescue and DNA sequencing

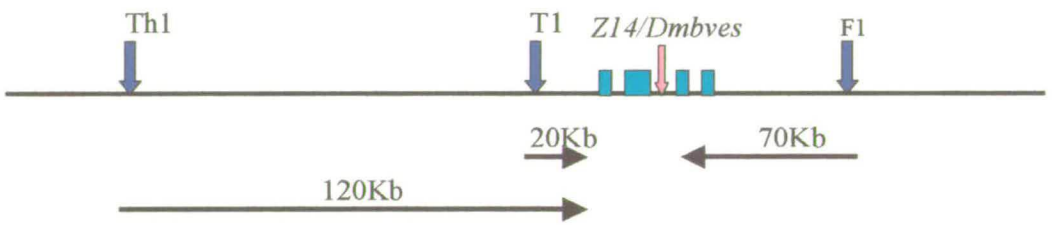
To determine the positions of the P insertions, plasmid rescue was carried out and sequencing undertaking of the rescued DNA fragment (Appendix 3). The result revealed that the P-element had hopped to a new position upstream of *Z14/Dmbves* gene in the Th1 line (Fig.4.3). In the F1 line, the P-element had hopped to a position downstream of *Z14/Dmbves* gene. In the T1 line, one copy of the P-element remains

Figure 4.3 Analysis of mutant flies generated

A.Genomic southern blot



B.The map of P-element insertions resulting in homozygous lethal lines



Legend

A.Southern hybridisation revealed P-element inserts in three fly lines. pBluscript DNA used as Probe. 12µg genomic DNA of each P-insertion line was loaded on the gel.

B.The position of the inserts are shown in relation to the original position They are on either side of the *Z14/Dmbves* gene. Red arrows represent the original P-element and blue arrows represent P-element hops to other positions.Green boxes are exons of *Z14/Dmbves*

in its original position, and the other copy is inserted into a new gene which is *ntf-2*. Unfortunately there are no homozygous lethal mutant resulting for P insertion in the *Z14/Dmbves* gene.

4.4 Summary and Discussion

P-element mobilisation was carried out to generate mutants of the *Z14/Dmbves* gene. 4 homozygous lethal red-eyed fly lines were found. Why was there no mutants in the white eyed lines? We found that there is a large intron of 13kb between exon 2 and exon 3 in the *Z14/Dmbves* gene. The P-element is inserted in the middle of the intron. Perhaps the P-element removes some flanking genomic DNA when it excises imprecisely but it did not remove enough genomic DNA to cause a mutation. Thus, there are no white-eyed mutant flies generated by P-element mobilisation. 4 red-eyed fly lines which are homozygous lethal were obtained. The red eyed mutant flies were analysed by southern hybridisation and rescued plasmids of them were sequenced. They have inserted into genes either side of the *Z14/Dmbves* gene. Unfortunately there are no homozygous lethal mutant resulting for P insertion in the *Z14/Dmbves* gene. Further analysis the function of *Z14/Dmbves* by using transgenic flies is described in next chapter.

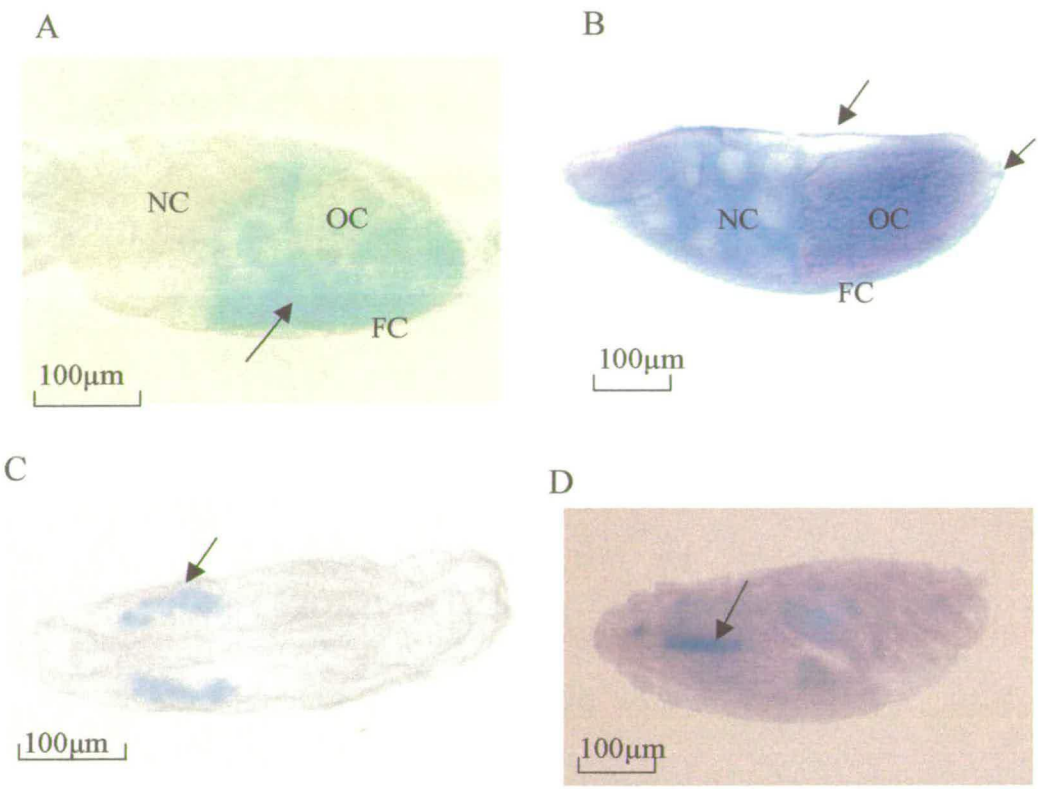
Chapter Five: Expression Analysis of *Z14/Dmbves*

5.1 Expression Pattern of *Z14/Dmbves* in Oogenesis and Embryogenesis

The *Z14/Dmbves* gene was identified as part of a GAL/UAS enhancer trap scheme which was described in chapter 3. When line C648, a *P[GAL4]* line, was crossed with UAS-lacZ line, staining was detected in a patch of follicle cells at stage10 of oogenesis (Fig.5.1A). Since GAL4/UAS system has no expression in the germline cells, we are not able to see staining in the nurse cells by β-galactosidase staining. To learn more about the *Z14/Dmbves* gene and its role in oogenesis, *in situ* hybridisation with *Z14/Dmbves* cDNA as a probe was carried out on wild type ovaries. A similar expression pattern in the follicle cells was observed when comparing the expression pattern of reporter gene driven by line C648. Expression was observed in the nurse cells and all follicle cells surrounding the oocyte at stage10 of oogenesis, except those in the anterior-dorsal and posterior region (Fig.5.1B). There was no expression in any stages before or after stage10 of oogenesis.

Since LD22978 EST was from an embryonic library, *Z14/Dmbves* should be present in the embryo. The *P[GAL4]* line C648 was then used again to crossed with the UAS-lacZ line. The embryos of the progeny were collected and stained for β-galactosidase. Blue staining was found in the embryos at a late developmental stage in salivary glands (Fig.5.1C). *In situ* hybridisation to the wild type embryos was also carried out. The expression was, however, observed in the salivary glands of the embryo (Fig.5.1D). The expression pattern is consistent with that of the reporter

Figure 5.1 Expression pattern of *Z14/Dmbves*



Legend

β-galactosidase staining and RNA *in situ* hybridisaton of the ovary and embryo. RNA probe made from *Z14/Dmbves* cDNA used in B and D.

A.β-galactosidase staining in the ovary. A patch of follicle cells were stained (Arrow).

B. *In situ* bybridisation to the wild type ovary, showing the similar expression pattern in the follicle cells as that of the report gene. (Arrow indicates that *Z14/Dmbves* is not expressed in these regions).

C. β-galactosidase staining in the embryo. only salivary glands were stained (Arrow).

D. *In situ* hybridisation to the embryo. Salivary glands were stained (Arrow).

NC: nurse cell; OC: oocyte; FC: follicle cells. Anterior is to the left.

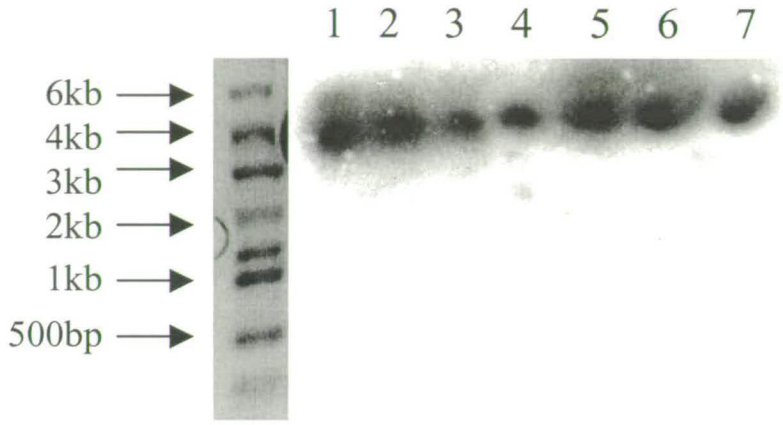
gene drive by line C648. We also found that *Z14/Dmbves* was expressed in the central nerve system in some embryos (Data not shown). But we cannot demonstrate that it is a specific expression.

5.2 Expression Analysis by Developmental Northern Hybridisation

To learn more about the function of the *Z14/Dmbves* gene, we need to know its developmental expression profile and this was established by northern blotting. The result revealed that the *Z14/Dmbves* gene is present at various developmental stages (Fig.5.2). A high level of *Z14/Dmbves* mRNA was found in ovaries and early embryos. But it was present at low level in the late embryos and 1st instar larvae. Then it was present at intermediate level during 2nd and 3rd instar larval and pupal stages.

The transcript of *Z14/Dmbves* was 4kb long, This was not consistent with the *Z14/Dmbves* cDNA (2.6kb), which we predicted from the early EST clones. This problem is solved by the *Drosophila* genome sequence and the latest *Drosophila* genome annotation, which predicts that the *Z14/Dmbves* cDNA is 3.9kb long. When we sequenced the GM07524 EST, there were 24 adenines at the 3'end which was considered as a poly A tail. The length of *Z14/Dmbves* cDNA in GM07524 EST is 2.6kb long and we thought it was the full length cDNA of *Z14/Dmbves*. According to the *Drosophila* genome annotation, *Z14/Dmbves* cDNA is almost 4kb long (Appendix 4). Comparing the sequence of GM07524 EST with the sequence of *Z14/Dmbves* in the genome, an approximately 1.2kb sequence was found right after the 24 base pairs of adenines. The open reading frame of *Z14/Dmbves* is the same in

Figure 5.2 Developmental Northern of *Z14/Dmbves*



Legend

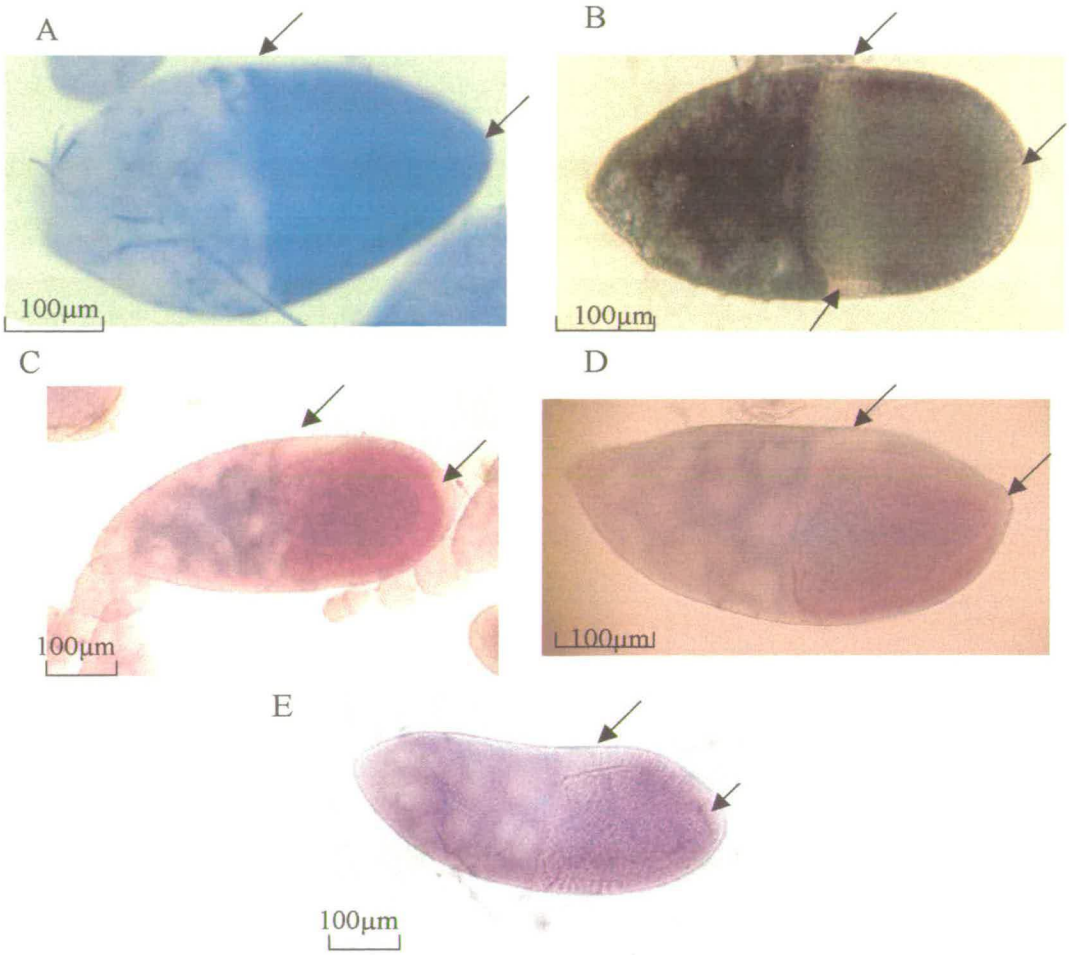
Total RNAs were extracted from different developmental stages. lane 1, ovries; Lane2, 0-4hr embryos; Lane 3, 6-14hr embryos; lane 4, first instarlarvae; Lane 5, second instar larvae; Lane 6, third instar larvae; Lane 7, pupae. *Z14/Dmbves* DNA used as the probe. 20µg total RNA of each sample was loaded on the gel.

either case. It encodes 414 amino acids. So, it seems that the full length *Z14/Dmbves* cDNA is almost 4kb long.

5.3 Expression of *Z14/Dmbves* Is Repressed by the Grk/EGFR Signalling

Whole mount mRNA *in situ* hybridisation to wild type ovaries reveals that *Z14/Dmbves* is expressed in all the follicle cells surrounding the oocyte at stage10 except those in the anterior-dorsal and posterior region which receive the Grk signal from oocyte. *grk* signals to the follicle cells in the posterior region at stage 6 and signals again to the follicle cells in the anterior-dorsal region at stage 10. It is therefore possible that the Grk signal represses *Z14/Dmbves* expression. To test this hypothesis, *in situ* hybridisation to the ovaries of a number of mutants that affect the Grk/EGFR signalling pathway was carried out. In the ovaries of the $grk^{[HK]}$ homozygous female mutant, follicle cells do not receive the Grk signal either from posterior or anterior-dorsal region and as a result, the expression of those genes downstream of Grk/EGFR signalling pathway was affected. Using *Z14/Dmbves* as a probe for mRNA *in situ* hybridisation to the $grk^{[HK]}$ mutant ovaries, we found that *Z14/Dmbves* was expressed in all follicle cells surrounding the oocyte (Fig.5.3A), indicating that *Z14/Dmbves* expression is negatively regulated by Grk/EGFR signalling. In *fs(1) K10* mutant ovaries, *grk* transcripts diffuse from their normal anterior-dorsal location towards anterior-ventral positions within the oocyte, therefore all anterior follicle cells receive the Grk signal. *In situ* hybridisation to *fs(1) K10* mutant ovaries with *Z14/Dmbves* as a probe revealed that *Z14/Dmbves* was not expressed at all in anterior follicle cells (Fig.5.3B). Take together results show that

Figure 5.3 Expression of *Z14/Dmbves* in mutant ovaries



Legend

In situ hybridisation to stage 10 egg chamber. RNA probe made from *Z14/Dmbves* cDNA used in A-E.

A.Expression of *Z14/Dmbves* in *gurken* mutant ovaries. Arrow indictes that *Z14/Dmbves* is expressed in all the follicle cells.
B..Expression of *Z14/Dmbves* in *fs(1) K10* mutant ovaries.
C. Expression of *Z14/Dmbves* in *dpp* mutant ovaries.
D. Expression of *Z14/Dmbves* in *mirror* mutant ovaries.
E. Expression of *Z14/Dmbves* in *fringe* heat shock mutant ovaries.
Arrow indicated that *Z14/Dmbves* is not expressed in these regions
Anterior is to the left and dorsal is to the up.

the Grk/EGFR signalling pathway represses the expression of *Z14/Dmbves* gene in cells that received the Grk signal.

5.4 The Relationship of *Z14/Dmbves* With Other Genes

5.4.1 Does *Z14/Dmbves* respond to the Dpp signalling pathway ?

Having established that *Z14/Dmbves* is repressed by Grk/EGFR signallling, we want to know if *Z14/Dmbves* is regulated by other signalling pathways which act during similar stages of oogenesis and have roles in follicle cell determination. *dpp*, a *Drosophila* transforming growth factor-beta homologue, expressed in centripetal follicle cells, could be a good candidate. Dpp signalling is directly involved in the specification of the anterior-most columnar cells (Twombly et al., 1996) and indirectly controls the appendage producing cells (Deng and Bownes, 1997). It could potentially affect the expression of *Z14/Dmbves* in the anterior follicle cells. To investigate if *Z14/Dmbves* is regulated by *dpp*, we selected dpp^{d6} and dpp^{e87} alleles because they are all involved in the morphology of dorsal appendage and egg operculum. Ovaries of dpp^{d6} and dpp^{e87} mutant lines were collected. *In situ* hybridisation to *dpp* mutant ovaries was carried out by using a *Z14/Dmbves* antisense RNA as the probe. The expression pattern of *Z14/Dmbves* was not changed in *dpp* mutant ovaries (Fig.5.3C). This suggests that *dpp* does not affect the expression of *Z14/Dmbves*.

5.4.2 Is *Z14/Dmbves* regulated by the *mirror* gene?

The establishment of the dorsal-ventral axis of both the egg chamber and embryo of *Drosophila* is initiated by the Grk/EGFR signalling pathway during oogenesis.

The *mirror* gene is one of many genes involved in this signalling pathway. It encodes a putative homeodomain transcription factor. It is expressed in the germarium, in centrally-located follicle cells at stage 6 and the anterior-dorsal and centripetal follicle cells at stage 10 (McNeill et al., 1997; Zhao and Bownes, 2000). Its expression in anterior-dorsal follicle cells is activated by Grk/EGFR signalling. Since *Z14/Dmbves* is downstream of Grk/EGFR signalling pathway and is not expressed in the anterior-dorsal region in a pattern similar to the *fringe* gene we thought that it was possible that its expression would be repressed by *mirror* in a similar way to *fringe* (Zhao and Bownes, 2000). To test this hypothesis, *in situ* hybridisation to the $mirror^{cre2}$ mutant ovaries was carried out with *Z14/Dmbves* as probe. The expression pattern in the $mirror^{cre2}$ mutant ovaries was the same as in the wild type ovaries (Fig.5.3D).

5.4.3 Is there any relation between *Z14/Dmbves* and *fringe*?

The *fringe* gene has been found to be crucial for Notch/Delta signalling pathway. The *fringe* gene encodes a putative secreted protein, and its function is required for the formation of the wing margin (Irvine and wieschaus, 1994). Recently it has been demonstrated that the *fringe* gene is also essential for the morphogenesis of ovarian follicle cells at different stages of oogenesis (Jordan et al., 2000; Zhao and Bownes, 2000). It has a dynamic expression pattern in oogenesis. First it is expressed in the germarium and then expressed in both anterior and posterior follicle cells from stages 2-9. At stage 10 *fringe* is expressed in all follicle cells surrounding the oocyte, except the anterior-dorsal follicle cells. It has been demonstrated that the expression of *fringe* in anterior-dorsal follicle cells is negatively regulated by Grk/EGFR signalling

(Zhao and Bownes, 2000). *Z14/Dmbves* has a similar expression pattern to *fringe* and its expression is also inhibited as a result of *gurken* gene activity. The possible relation between these two genes was investigated. To reveal how these two genes affected by each other, in situ hybridisation was carried out. Since there are no mutant flies of the *fringe* gene available, heat shock antisense fly lines were used as a substitute. The ovaries of *fringe* antisense fly lines were collected 6 hours after heat shock. *Z14/Dmbves* antisense RNA was used as probe to do *in situ* hybridisation. The expression pattern of *Z14/Dmbves* was not affected as shown in Fig.5.3E.

5.5 Investigation of the Biological Function of *Z14/Dmbves*

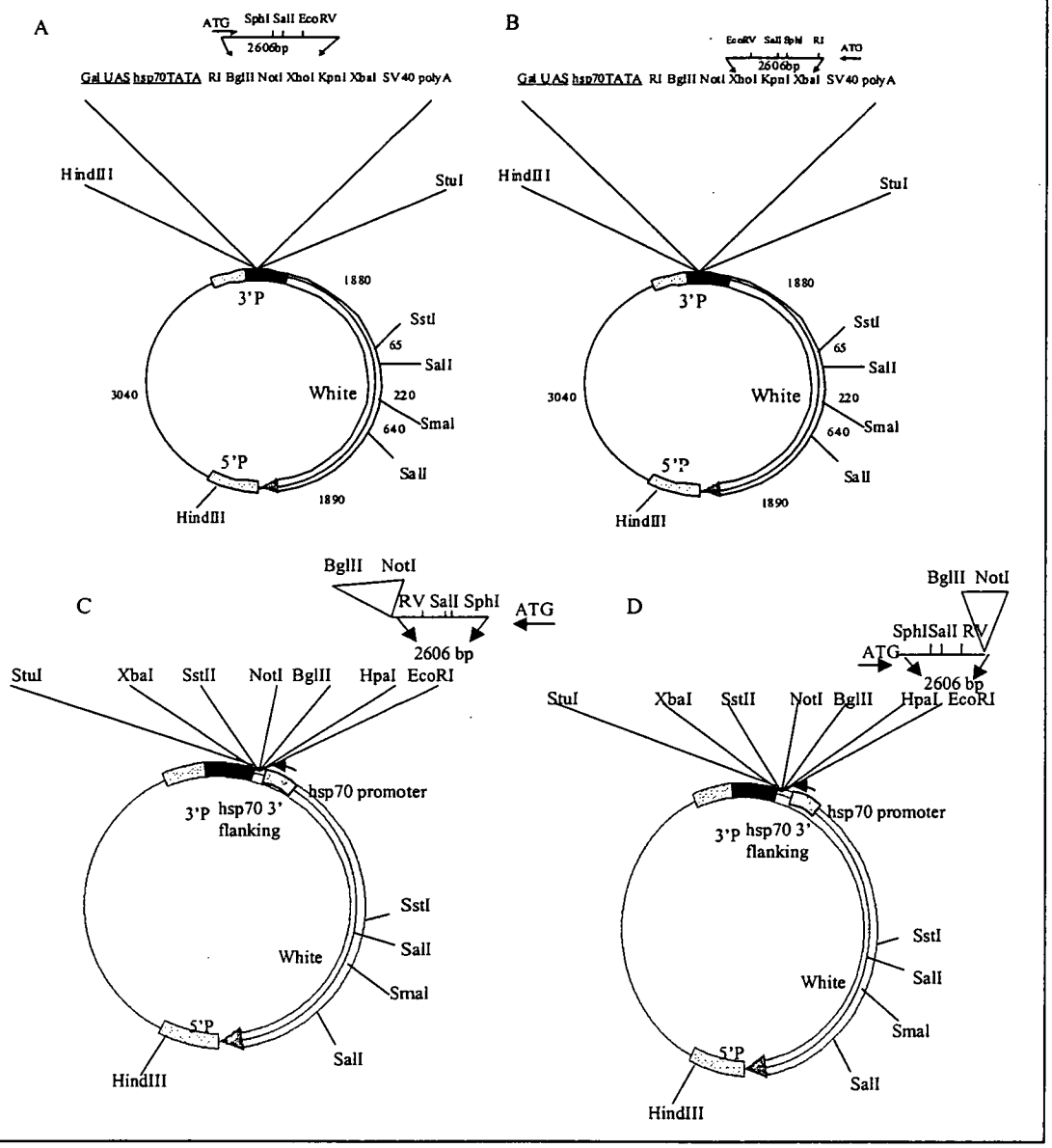
5.5.1 Generation of transgenic fly lines

5.5.1.1 GAL/UAS and heat shock constructs

There are no *Z14/Dmbves* mutants available. We did not generate any lethal *Z14/Dmbves* mutants using P-element mobilisation (Section 4.2). So the generation of transgenic fly lines was used as an alternative approach to analyse the function of *Z14/Dmbves* gene. The GAL/UAS system works by using an enhancer trapped GAL4 element to drive the expression of a cDNA under the control of the yeast UAS promoter. *Z14/Dmbves* cDNA was cloned into pUAST in a sense or antisense orientation (Fig.5.4). We have a number of GAL4 lines which will enable us to misexpress the gene or knock out the gene function in follicle cells at specific stages of oogenesis when crossed with *Z14/Dmbves* sense or antisense lines.

Z14/Dmbves cDNA was also cloned into pCaSpeR which contains the heat shock promoter hsp70 in a sense or antisense orientation (Fig.5.4). The hsp70 is activated

Figure 5.4 Construction of vectors for microinjection



Legend

A.pUAST-*Z14/Dmbves* vector (sense). *Z14/Dmbves* cDNAs (2.6kb) was cut of EST GM07524 with *EcoRI* and *XhoI* and then ligated into *EcoRI/ XhoI*-cut pUAST vector .

B.pUAST- *Z14/Dmbves* vector (antisense). *Z14/Dmbves* cDNAs (2.6kb) was cut of EST GM07524 with *XhoI* and *XbaI* and then ligated into XhoI/ *XbaI*-cut pUAST vector.

C.pCaS-hs-*Z14/Dmbves* vector (sense). *Z14/Dmbves* cDNAs (2.6kb) was cut of EST GM07524 with *EcoRI* and then ligated into *EcoRI*-cut pCaS-hs vector.The ATG of *Z14/Dmbves* is the same orientation of the heat shock promoter.

D.pCaS-hs-*Z14/Dmbves* vector (antisense). *Z14/Dmbves* cDNAs (2.6kb) was cut of EST GM07524 with *EcoRI* and then ligated into *EcoRI*-cut pCaS-hs vector. The ATG of *Z14/Dmbves* is the opposite orientation of the heat shock promoter.

RI: *EcoRI*; RV: *EcoRV*

when the transgenic flies are exposed to temperatures range from 36°C to 39°C. This drives the expression of the *Z14/Dmbves* cDNA downstream of it. The advantage of the heat shock approach is that it can be used to investigate the function of the *Z14/Dmbves* gene at all stages of oogenesis.

5.5.1.2 The result of microinjection

Both pUAST and pCaSpeR constructs contain the *white* gene, So the transgenic flies will have coloured eyes if these constructs are successfully transformed into *wk* flies. The construct and *Δ2-3* helper DNA to supply transposase were injected into 0-30 minute w^k embryos together. The results from the microinjection are summarized in Table 5.1

Table 5.1

Construct	Numbers of embryo microinjected	Hatched larvae	Larval Survival rate	Red-eyed Fly lines	Success rate
pUAST+ *Z14/Dmbves* (antisense)	600	167	27.8%	7/41* (17%)	4.73%
pUAST+ *Z14/Dmbves* (sense)	300	154	51.3%	8/73* (10.95%)	5.62%
pCaSpeR+ *Z14/Dmbves* (antisense)	200	109	54.5%	9/40* (22.5%)	12.26%
pCaSpeR+ *Z14/Dmbves* (sense)	184	83	45.1%	8/26* (30.77%)	13.88%

* Indicates the number of flies checked. Because there were too many red-eyed fly lines for the analysis, those unchecked transgenic flies were all discarded.

5.5.1.3 Transgenic fly lines generated

Transgenic fly lines were established for each of the 4 different constructs made.

Table 5.2

Transgenic fly lines		
pUAST+*Z14/Dmbves* (antisense)	PUAST-Z14-AS (2) *	PUAST-Z14-AS (11)
	PUAST-Z14-AS (15)	PUAST-Z14-AS (23)
	PUAST-Z14-AS (24)	PUAST-Z14-AS (30)
	PUAST-Z14-AS (41)	
pUAST+*Z14/Dmbves* (sense)	PUAST-Z14-S (5)	PUAST-Z14-S (8)
	PUAST-Z14-S (10)	PUAST-Z14-S (11)
	PUAST-Z14-S (15)	PUAST-Z14-S (23)
	PUAST-Z14-S (24)	PUAST-Z14-S (73)
pCaSpeR+*Z14/Dmbves* (antisense)	PCas-hs-Z14-AS (2)	PCas-hs-Z14-AS (7)
	PCas-hs-Z14-AS (13)	PCas-hs-Z14-AS (27)
	PCas-hs-Z14-AS (29)	PCas-hs-Z14-AS (33)
	PCas-hs-Z14-AS (34)	PCas-hs-Z14-AS (35)
	PCas-hs-Z14-AS (40)	
pCaSpeR+*Z14/Dmbves* (sense)	Pcas-hs-Z14-S (5)	Pcas-hs-Z14-S (6)
	Pcas-hs-Z14-S (8)	Pcas-hs-Z14-S (9)
	Pcas-hs-Z14-S (16)	Pcas-hs-Z14-S (18)
	Pcas-hs-Z14-S (22)	Pcas-hs-Z14-S (26)

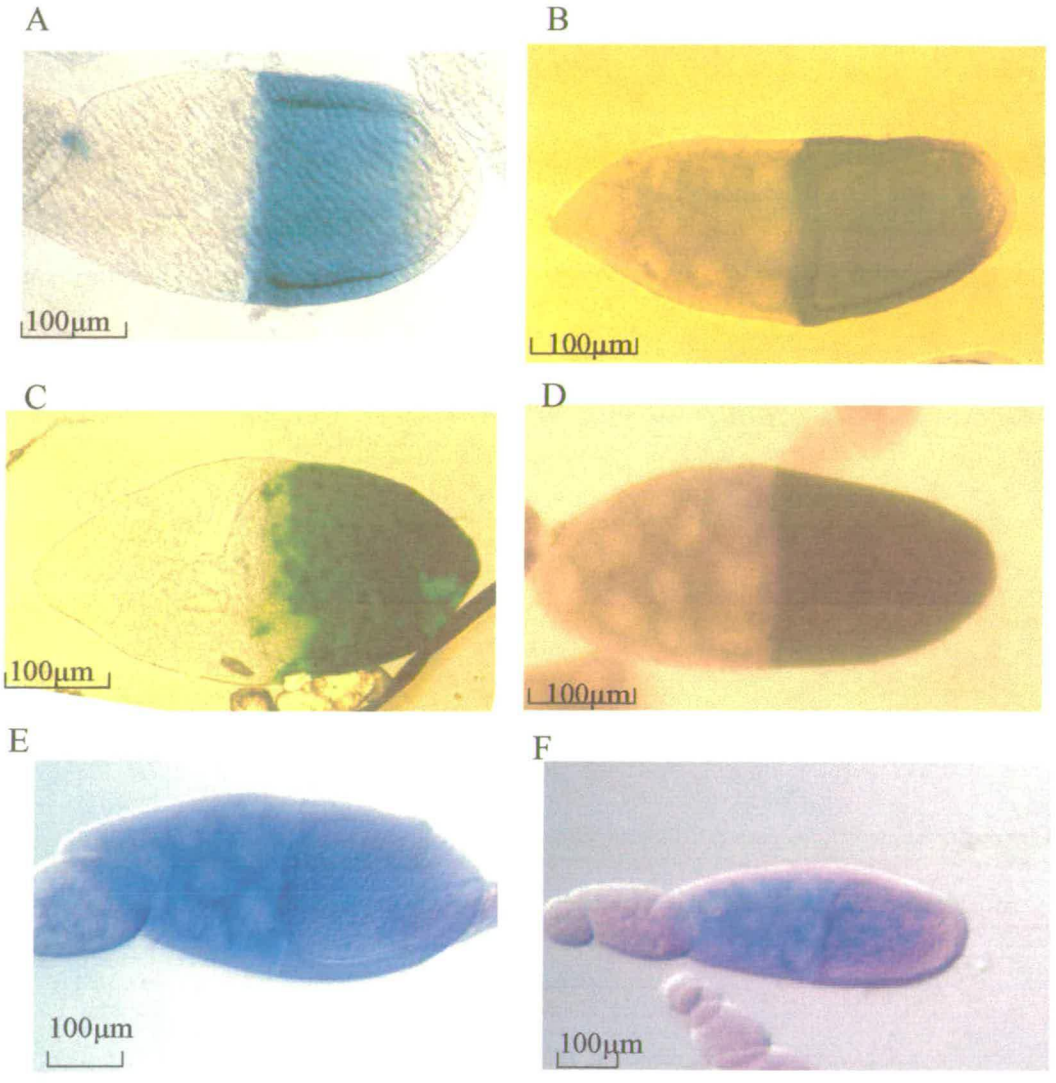
* The number in the bracket is the number of lines generated.

5.5.2 GAL4/UAS analysis of the transgenic fly lines

To investigate the function of the *Z14/Dmbves* gene, antisense analysis with the GAL4/UAS system was carried out. The antisense strand is transcribed in the nucleus and its RNA product probably binds to the native transcript which fail to produce the protein possibly due to RNA degradation. Using different *P[GAL4]* drive lines to cross with *Z14/Dmbves* pUAST antisense line, it should disrupt the function of *Z14/Dmbves* in subsets of follicle cells.

When *P[GAL4]* line C673 was crossed with UAS-lacZ line, staining was observed in all follicle cells except the posterior region (Fig.5.5A). Since *Z14/Dmbves* is expressed in the all follicle cells surrounding the oocyte except the anterior dorsal and posterior region, C673 line was used to cross with pUAST *Z14/Dmbves* antisense line, to investigate ectopic expression of *Z14/Dmbves* in all anterior follicle cells. The ovaries of the progeny were used for RNA *in situ* hybridisation with *Z14/Dmbves* sense RNA as probe. It was found that *Z14/Dmbves* antisense RNAs were expressed in all anterior follicle cells as expected (Fig.5.5C). This indicated that GAL4/UAS system worked well. The egg chambers at different stages of development and deposited eggs were checked. Unfortunately there was no abnormal phenotype observed. It is therefore possible that the levels of *Z14/Dmbves* antisense expression were not sufficient to entirely block *Z14/Dmbves* function or that it has no role at this stage despite its limited expression pattern. The *P[GAL4]* line C710 was also used since it drives UAS-lacZ reporter gene expression in all the follicle cells surrounding the oocyte at stage 10 (Fig.5.5B). So it should be able to drive antisense *Z14/Dmbves* in these cells and disrupt native *Z14/Dmbves* function in all the follicle cells. After the C710 line was crossed with pUAST *Z14/Dmbves* antisense line, the ovaries of the progeny were investigated using the *Z14/Dmbves* sense RNA as probe. Strong expression was found in all the follicle cells surrounding the oocyte (Fig.5.5D). This suggests *Z14/Dmbves* antisense RNA was located in these cells as expected, but still no abnormal phenotype was found in the ovaries or embryos. pUAST *Z14/Dmbves* sense lines have also been tested in the similar way and no abnormal phenotype was found either (data not presented). Further analysis of GAL4/UAS transgenic fly lines

Figure 5.5 Expression in the transgenic fly lines



Legend

A,C β-galactosidase staining; B,D,E,F in situ hybridisation to stage 10 egg chambers, *Z14/Dmbves* DNA used as the probe.

A. β-galactosidase expression pattern driven by the GAL4 line C673.
B.Ectopic expression of *Z14/Dmbves* by using C673 line crossed with pUAST *Z14/Dmbves* antisense line.
C. β- galactosidase expression pattern driven by the GAL4 line C710.
D. Ectopic expression of *Z14/Dmbves* by using C710 line crossed with pUAST *Z14/Dmbves* antisense line.
E. Expression of *Z14/Dmbves* antisense in thePCas-hs-Z14-AS (2) ovaries.
F. Expression of *Z14/Dmbves* antisense in thePCas-hs-Z14-AS (40) ovaries

were not pursued since all the lines tested had failed and the heat shock approach were then used to investigate the function of the *Z14/Dmbves* gene.

5.5.3 Analysis of the transgenic fly lines by Heat shock

To test the function of heat shock constructs, preliminary heat shock analysis was carried out on all *Z14/Dmbves* antisense heat shock fly lines. These fly lines were kept in the 39°C incubator for 45 minutes, then put in the 18°C incubator for 30 minutes and finally the flies were heat shock again at 39°C for 45 minutes. The ovaries of the heat shock flies were collected 6 hours after the last heat shock. *In situ* hybridisation to these ovaries was carried out using the *Z14/Dmbves* sense RNA as a probe. Strong expression of *Z14/Dmbves* antisense RNA were observed in two fly lines. The level of expression has been shown to depend upon chromosomal position of the insert. In the PCas-hs-Z14-AS (40) line, *Z14/Dmbves* antisense RNA was expressed in the nurse cells and oocyte (Fig.5.5F). In the PCas-hs-Z14-AS (2) line, *Z14/Dmbves* antisense RNA was expressed in the nurse cells and the follicle cells (Fig.5.5E). The different expression pattern of these two lines might be related to the position of the P-element insert in the genome. These two lines were used for further analysis.

To investigate the function of *Z14/Dmbves* in oogenesis, PCas-hs-Z14-AS (40) line and PCas-hs-Z14-AS (2) line were heat shocked and the ovaries were collected. Unfortunately again, there were no defects detectable in the ovaries. The larvae of these two lines were also heat shocked. After the flies from these heat shock larvae hatched, they were checked under the microscope. No abnormal phenotype were found in these flies. The eggs laid by heat shock flies were also checked. They all

had normal egg shells and dorsal appendages. But it was found that many eggs were died. The embryos could be killed either by the heat shock treatment or the expression of *Z14/Dmbves* antisense RNA. To investigate this further, wild type flies were heat shocked together with *Z14/Dmbves* antisense heat shock lines over a three day period. Each day they were heat shocked twice. Embryos were collected after the heat shock and it was found that embryos of wild type had much higher survival rate than that of *Z14/Dmbves* antisense lines (Table 5.3). This suggests most dead embryos are not killed by heat shock, but by *Z14/Dmbves* antisense RNA which knocked out the native *Z14/Dmbves* RNA during oogenesis.

Table 5.3

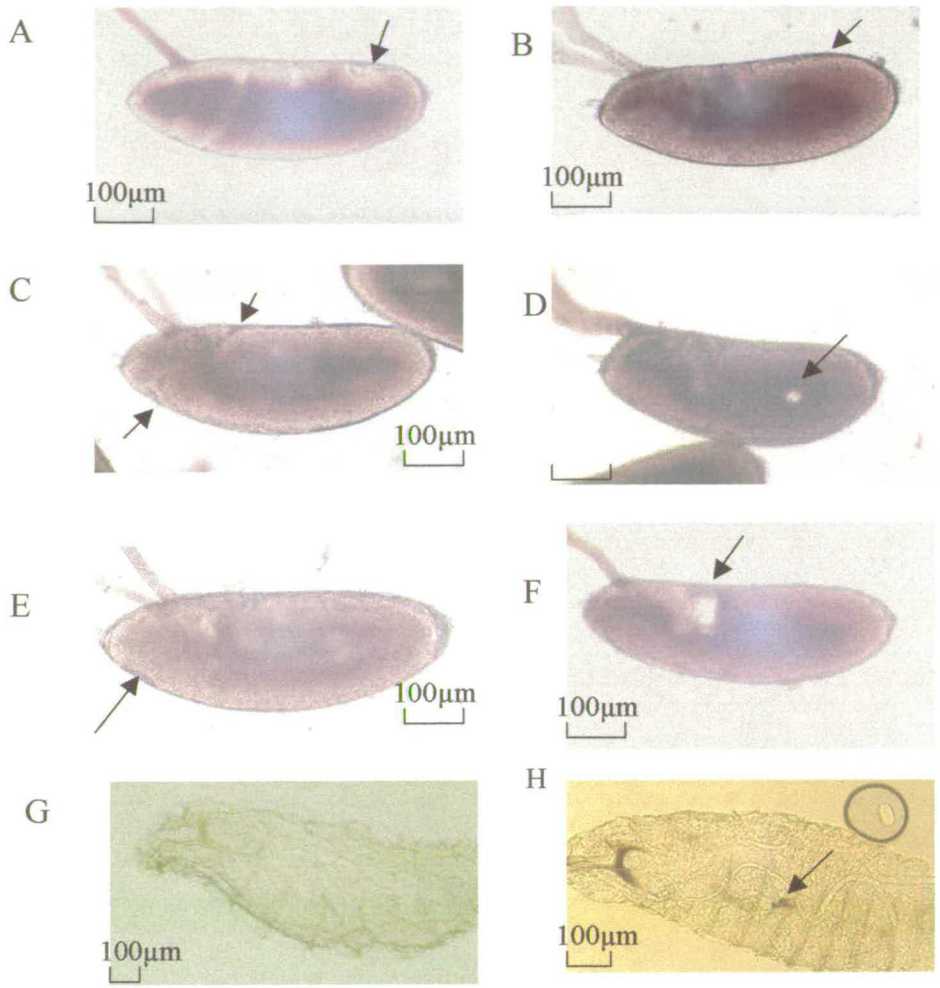
Time	Fly lines	Total embryos	Dead embryos	Larvae hatched	Survival rate
First day	OrR	361	38	323	89.47%
	PCas-hs-Z14-AS (2)	309	160	149	48.22%
	PCas-hs-Z14-AS(40)	135	72	63	46.67%
Second day	OrR	150	15	135	90%
	PCas-hs-Z14-AS (2)	164	61	103	62.8%
	PCas-hs-Z14-AS(40)	53	22	31	58.49%
Third day	OrR	69	3	66	95.65%
	PCas-hs-Z14-AS (2)	123	49	74	60.16%
	PCas-hs-Z14-AS(40)	76	47	29	38.15%

To know why the embryos laid by antisense heat shock lines after heat shock were dead, living embryos, either heat shock lines or wild type, were observed in KELF oil. Since the embryos laid by heat shock flies were found to be dead early in embryogenesis and soon degeneration, this allowed us to observe the embryos while they are continuing to develop.

Embryos laid by heat shock flies (hs embryos) develop normally from stage 1 to 6. (The number of stages are the same as those suggested by Campos-ortega and

Hartenstein, 1985; Roberts, 1998). At stage 7, in the wild type embryos the cephalic furrow has deepened and the posterior midgut plate is parallel to the long axis of the egg and thus has shifted the pole cells to the dorsal side of the embryo, but posterior midgut invagination did not occur properly in some hs embryos (Fig.5.6). At stage 8, in wild type embryos the posterior midgut invagination reaches the head region along the dorsal side. On the ventral side of the embryo, the germ band buckles into the interior of the embryo at the level of the cephalic furrow producing a transient gap between it and the vitelline membrane. But in the hs embryos, the germ band buckle can not be seen in the ventral side and there is a hole in the posterior region in the yolk. At stage 9, in wild type embryos the future stomodeal invagination is identifiable as a shallow gap at the level of the earlier anterior midgut invagination. But in the hs embryos the future stomodeal invagination was not visible. The gut opening has failed to reach the head region, resulting in a hole in the middle of the embryo. (Fig.5.6). These embryos failed to develop further and a three-layered germ band was never formed. There are almost 40-50% hs embryos dead at the embryo stage and another 2-3% hs embryos can survive to larva stage. The phenotype of these larvae was that they all had a dark spot inside body (Fig.5.6). But eventually they died. The rest of hs embryos can survive to adult flies with normal development. The lethality of the hs embryos reached the peak between 18 –24 hours after heat shock.

Figure 5.6 Phenotype of *Z14/Dmbves* antisense flies



Legend

A,C,E wild type embryos, B,D,F embryos laid by heat shock female, G wild type larva, H larva which hatched from eggs laid by heat shock female.

A. Arrow indicated that posterior midgut invagination at stage 7.

B. Arrow indicated that midgut invagination not occur at posterior region.

C. Arrow indicated the posterior midgut invagination reached the head region and germ band buckle at ventral side at stage 8.

D. Arrow indicated that there is a hole in the posterior region in the yolk .

E. Arrow indicated that stomodeal plate formation at stage 9.

F. Arrow indicated that stomodeal invagination is not visible and a hole was found in the middle of the embryo.

G. Wild type larva as control.

H. Larva which hatched from eggs laid by the heat shock female has a dark spot inside body (arrow).

5.6 Summary and Discussion

Z14/Dmbves is an important gene which is required for embryonic development of *Drosophila*. In the oogenesis, *Z14/Dmbves* is expressed in the all follicle cells surrounding the oocyte at stage 10 except the anterior-dorsal and posterior region. It was found that *Z14/Dmbves* is repressed by Grk/EGFR signallling, but not affected by Dpp signalling.

We also investigated if *Z14/Dmbves* relates to *mirror* and *fringe*. Both *Z14/Dmbves* and *fringe* genes are expressed in all follicle cells surrounding the oocyte except those in anterior-dorsal region. *mirror* repressed *fringe* expression in anterior-dorsal follicle cells, but no abnormal expression pattern of *Z14/Dmbves* was observed in $mirror^{cre2}$ mutant ovaries. We can not be sure if this means that *mirror* does not affect *Z14/Dmbves* expression or *mirror* may have had an effect. *mirror* P1 was demonstrated to affect epithelial morphogenesis and $mirror^{cre2}$ has shown abnormalities in the eye (Yang et al., 1999). *mirror* P1 was used to investigate the relation of *mirror* with *fringe* (Zhao and Bownes, 2000), but the fly strain is lost in our lab. We unable to obtain *mirror* P1 from Bloomington Stock Centre. Thus, it is possible that *mirror* P1 might affect egg chamber development.

There is no reported female sterile mutant of *fringe* available. Ovaries of a *fringe* heat shock inducible antisense line were used to look at the expression pattern of *Z14/Dmbves,* but it is not changed in these ovaries. The reason could be the low level of *fringe* antisense RNAs produced after heat shock. Therefore, it may not affect expression of *Z14/Dmbves*. The other possibility is that *fringe* does not affect the expression of *Z14/Dmbves* at all.

To investigate the role of *Z14/Dmbves*, transgenic fly lines were generated. PUAST- *Z14/Dmbves* antisense lines were crossed with GAL4 lines. No any aberrant phenotype was found in the egg chambers, eggshells or adult flies. Both GAL/4 lines were shown to induce *Z14/Dmbves* antisense RNA expression in the follicle cells. But the progeny of the GAL4/pUAST *Z14/Dmbves* flies are heterozygous. Therefore, the expression level may not be high enough to block *Z14/Dmbves* function.

A heat shock inducible antisense approach was therefore used to study the function of *Z14/Dmbves*. There was no abnormal morphology of egg chambers, egg shells and adult flies. However, there were lots of eggs (40-50%) and some larvae (2-3%) found dead. Since the heat shock is transient, only the egg chamber at proper stage can express *Z14/Dmbves* antisense RNA, it is impossible that all the hs embryos will dead after heat shock. Thus, we proposed that *Z14/Dmbves* is essential for normal embryogenesis and post embryogenesis. The dead embryos had a common defect during the midgut invagination. These embryos gradually became more abnormal and failed to form a complete germ band. This suggests that *Z14/Dmbves* is important for the normal embryonic development. Disruption of function of *Z14/Dmbves* probably affects normal embryonic development.

Chapter Six: Functional Analysis of the *ntf-2* Gene

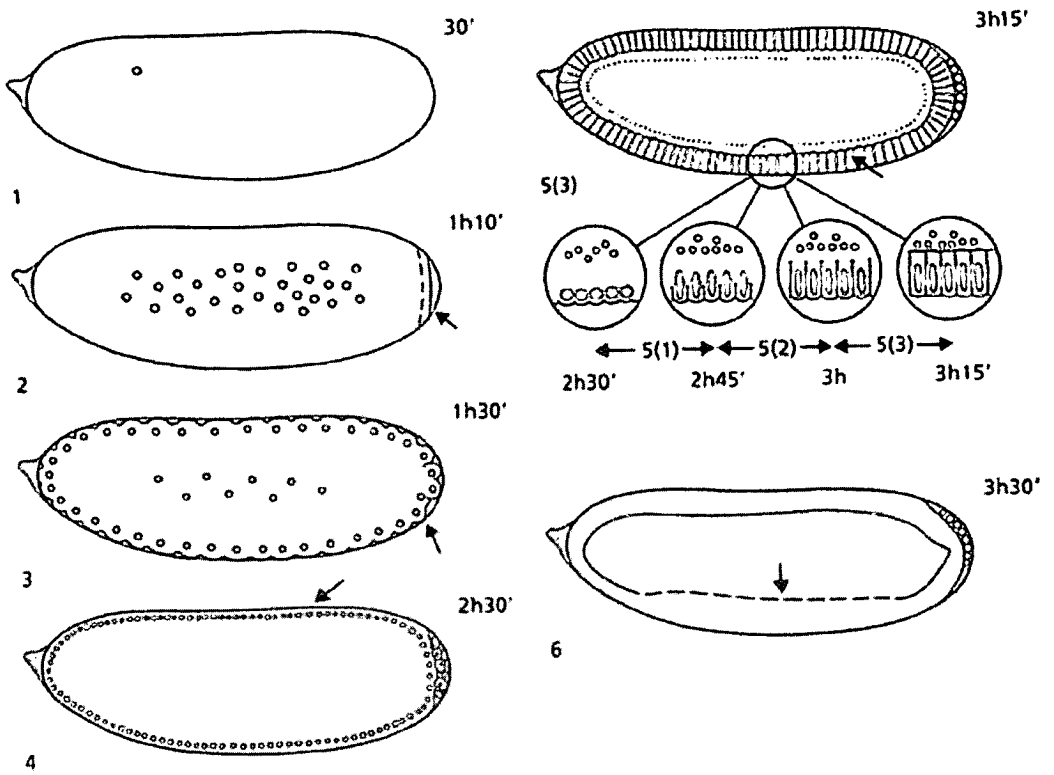
6.1 Introduction

Most of the cytoplasm of the nurse cells, which are rich in RNA and protein, is transferred into oocyte in the later stages of oogenesis. Embryonic development depends on RNAs and proteins which are localized within the oocyte during oogenesis.

6.1.1 Early stage of embryogenesis

During embryogenesis the mitotic divisions which occur after fertilization are not coupled with cell division. The nuclei divide but there are no cells, and the nuclei remain in a syncitium (Fig.6.1). About 1 hour after egg laying, there are 128 nuclei in the central region of the egg. Most of the nuclei move toward the periphery of the egg as they continue to divide. Some nuclei are left behind. These nuclei will become yolk nuclei. At stage 3 about 15 nuclei migrate to the posterior pole of the egg, these nuclei then become separated into pole cells (Lawrence, 1992). At stage 4 the nuclei are near the surface of the egg. The embryo at this stage is called a syncytial blastoderm. During stage 5 the plasma membrane extends centripetally into the egg between adjacent nuclei, partitioning them into individual cells. The embryo at this stage is called the cellular blastoderm. Gastrulation begins as soon as the cells on the ventral side of the embryo have completed cellularization (Roberts, 1998). The embryonic cells then reorganize and form the germ layers.

Figure 6.1 Early stages of Drosophila embryogenesis



From Lawrence,1992

Legend

Early development of *Drosophila* embryo from stages1 to 6.
Stage 1. Freshly laid egg.
Stage 2. The nuclei divide but there are no cells, and the nuclei remain in a syncitium
Stage 3. Pole cell formation.
Stage 4. The nuclei are near the surface of the egg.
Stage 5. Cell formation.
Stage 6. Early gastrulation.
Anterior is to left, and ventral down.

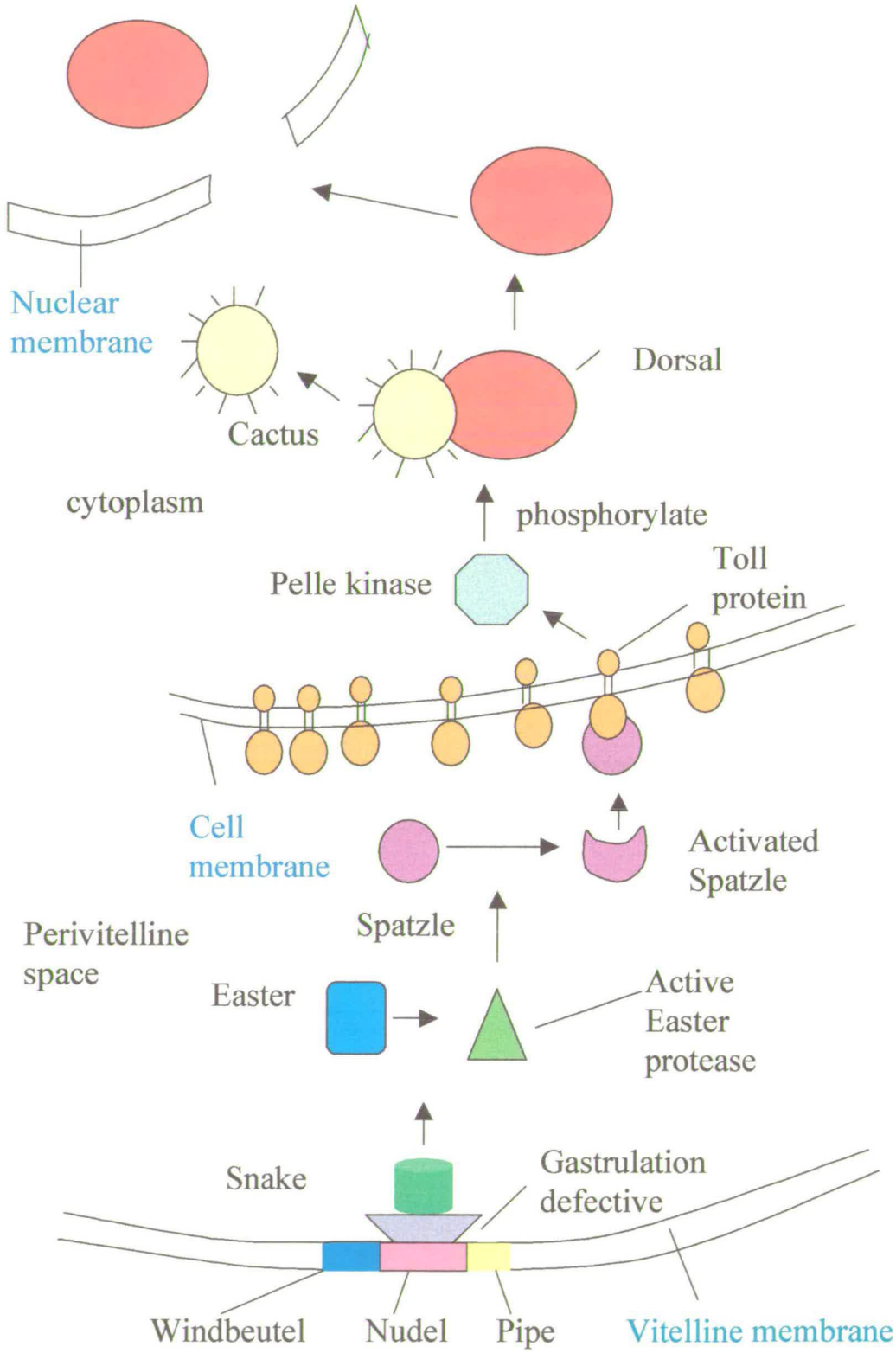
6.1.2 Signalling pathways that establish the Dorsal-Ventral pattern of the embryo

There are three signalling pathways that establish the dorsal-ventral pattern of the *Drosophila* embryo. One of them is encoded by a group of 12 maternal effect genes which are known as the "dorsal group" genes and *cactus*. In contrast to the dorsalising signal produced by the oocyte nucleus during oogenesis, the polarity of the embryo is defined by a signal which is apparently controlled by the ventral follicle cells. These cells fail to receive the Gurken signal during oogenesis which activates Torpedo to inhibit the synthesis of Windbeutel, Nudel and Pipe proteins (Stein et al, 1991). Therefore, these proteins can be made only by the ventral follicle cells.

The three ventral follicle cells produced proteins which are thought to become incorporated into the vitelline membrane, but only on the ventral side of the embryo (Fig.6.2). The complex formed by the Nudel, Pipe and Windbeutel proteins split the products of the *snake* and *gastrulation defective* genes to create an active enzyme which will split the Easter protein into an active Easter protease. The Easter protease cleaves the Spatzle protein (Chasan et al, 1992; Morisato and Anderson, 1994).

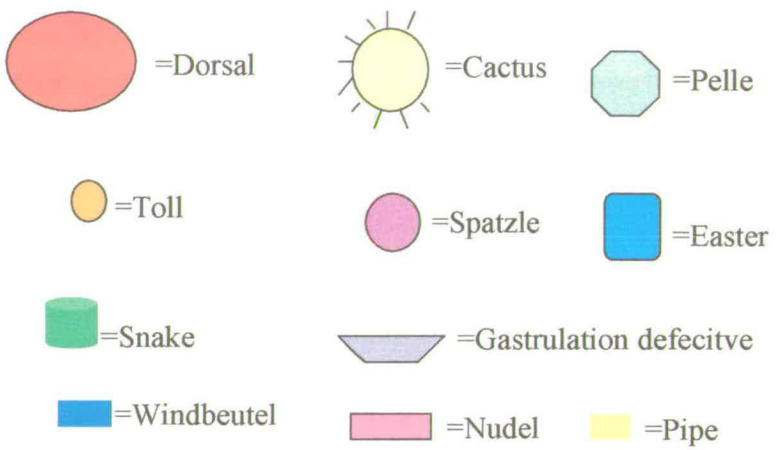
Cleaved Spatzle protein can bind to the Toll receptor which is in the cell membrane of the egg (Hashimoto et al, 1988, 1991). The Toll protein can activate the Pelle protein kinase. This process requires the *tube* gene. The Tube protein is probably necessary for bringing Pelle up to the cell membrane, where it can be activated (Galindo et al, 1995). The Pelle protein kinase can then phosphorylate the Cactus protein, disrupting the complex of Dorsal and Cactus. Once phosphorylated,

Figure 6.2 Toll signalling pathway



Legend

the Nudel, Pipe and Windbeutel proteins were synthesized in the ventral follicle cells. They split Snake and Gastrulation-defective proteins to create an active enzyme which will split the Easter protein into an active Easter protease. The Easter protease cleaves the Spatzle protein which can bind to Toll receptor protein. The Toll signal causes phosphorylation and disrupt the complex of Dorsal and Cactus. The Cactus protein is degraded, and the Dorsal protein can enter the nucleus.



the Cactus protein is degraded, and the Dorsal protein can enter the nucleus (Shelton and Wasserman, 1993; Whalen and Steward, 1993).

The Dorsal protein activates *twist* and *snail* expression to establish the mesoderm and control the formation of dorsal ectoderm and amnioserosa by repressing *decapentaplegic* and *zerknullt* (Morisato and Anderson, 1995). This process eventually establishes the dorsal-ventral pattern of the embryo.

6.1.3 Project background

P-element mobilisation was carried out to generate mutants of the *Z14/Dmbves* gene (Section 4.2). Four mutant red eyed fly lines were obtained and analysed by using plasmid rescue. The results reveal that in one of those lines the P-element has re-inserted into a new gene. Due to the lethality of the mutant flies and the expression pattern of the gene, it seems that this new gene may have an important role in oogenesis or embryogenesis. Therefore, a number of experiments were undertaken to study the function of this gene.

6.2 ESTs and Molecular Structure of *ntf-2* Gene

6.2.1 Identification of *ntf-2* gene

The *ntf-2* gene was identified as part of a P-element mobilisation experiment. The genomic DNA close to the P-element was obtained by using plasmid rescue technology. The flanking genomic DNA was sequenced. Using the BLAST search of the NCBI database, this DNA was found to be part of the sequence of a new gene called *ntf-2.*

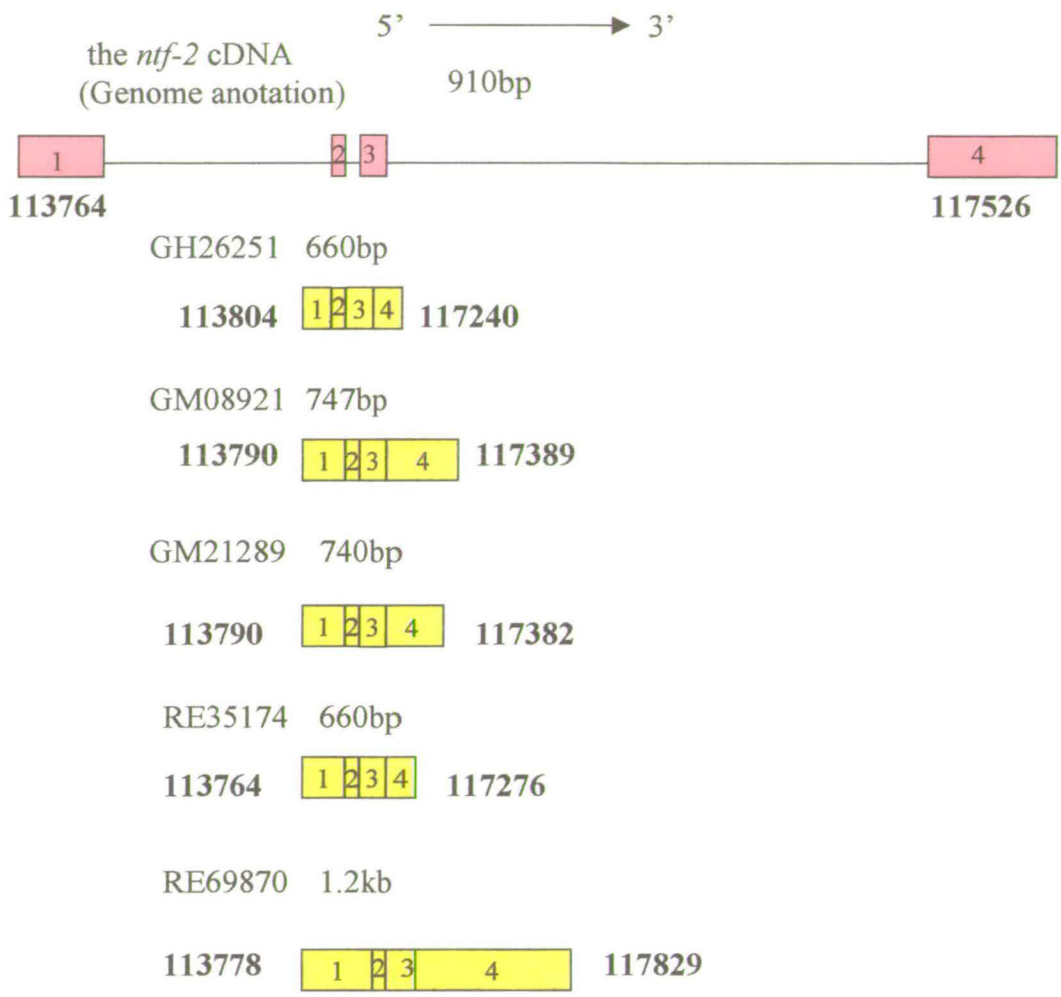
6.2.2 Analysis of ESTs

To obtain a full length cDNA for *ntf-2*, EST clones similar to *ntf-2* were searched for. Five EST clones were analysed. EST GH26251 was isolated from a adult head library and EST GM08921 and GM21289 were from an ovarian library. EST RE35174 and RE69870 were from an embryonic library. These five EST clones were sequenced. According to *Drosophila* genome anotaiton (AE003569.3), the *ntf-2* gene runs from 113764bp to 117526bp. EST GH26251 runs from 113804bp to117240bp; EST GM08921 runs from 113790bp to 117389bp; EST GM21289 runs from 113790bp to 117382bp; EST RE35174 runs from 113764bp to 117276bp; EST RE69870 runs from 113778bp to 117829bp. These ESTs are all close to the start point of the predicted *ntf-2* by comparison of their sequence at the 5' end (Fig.6.3). This suggests they might be the same transcript. The full length cDNA is 1.2kb (Fig.6.4).

6.2.3 *ntf-2* transcipts

To find how many transcripts *ntf-2* gene has, northern hybridisation was carried out. Since the ESTs were from ovarian and embryonic libraries, total RNA of ovary, embryo and first instar larva was extracted. The EST GH26251 cDNA was used as probe. The result of northern hybridisation revealed that two transcripts of 1.5kb and 2kb were present in ovaries and embryos(Fig.6.5). The 1.5kb transcript is in good agreement with that RE69870 EST which is 1.2kb long, assuming the presence of a 200-300 nucleotide polyA tail. But cDNA corresponding to the 2kb transcript has not been found.

Figure 6.3 EST clones similar to the *ntf-2* cDNA



Legend
There are five EST clones similar to the *ntf-2* cDNA. They are all close to the start point of the predicted *ntf-2* by comparison of their sequence at the 5' end. The bold numbers in the figure correspond to number of base pairs in the genome (AE003569.3).

Fig 6.4 Sequence of *ntf-2* cDNA

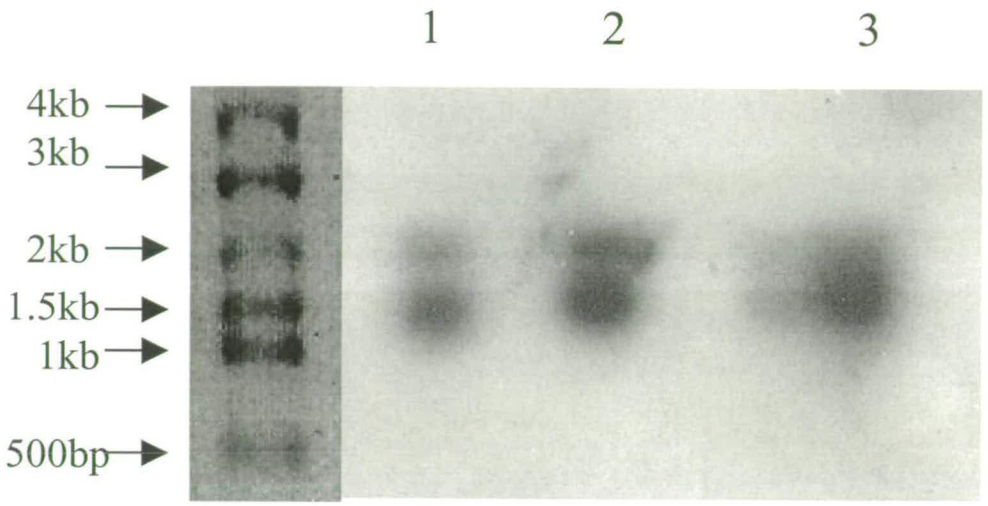
```
    ---------|---------|---------|---------|---------|
  1 GTTGTTCCACCCCTAACAAAGAGTGGTGATCTGAGCGCAGTCGGTTGATT 50

    ---------|---------|---------|---------|---------|
 51 TCATTTGGTTTTTTTTTTAATTATTCGTGTCGCCGCGATCGGATCGGATT 100

    ---------|---------|---------|---------|---------|
101 TCCCATAATCTCTGAGCGTTCCGCCTATCCTTCAAGTGAAatgtcgctga 150
                                          M  S  L  N

    ---------|---------|---------|---------|---------|
151 atccgcagtacgaggacattggcaagggatttgtgcagcagtactatgcg 200
      P  Q  Y  E  D  I  G  K  G  F  V  Q  Q  Y  Y  A

    ---------|---------|---------|---------|---------|
201 atattcgatgacccggcgaatcgggcgaacgtggttaatttctatagcgc 250
    I  F  D  D  P  A  N  R  A  N  V  V  N  F  Y  S  A

    ---------|---------|---------|---------|---------|
251 taccgactcattcatgacctttgaaggccaccaaatacaggggcaccca 300
     T  D  S  F  M  T  F  E  G  H  Q  I  Q  G  A  P  K

    ---------|---------|---------|---------|---------|
301 agattctggaaaaagttcagagtctgagctttcagaagattaccagagtg 350
      I  L  E  K  V  Q  S  L  S  F  Q  K  I  T  R  V

    ---------|---------|---------|---------|---------|
351 ataaccacagtggactcgcagccaactttcgatggcggagttctgatcaa 400
    I  T  T  V  D  S  Q  P  T  F  D  G  G  V  L  I  N

    ---------|---------|---------|---------|---------|
401 cgtccttggaagactacagtgcgatgacgatcccccacatgccttctcgc 450
     V  L  G  R  L  Q  C  D  D  D  P  P  H  A  F  S  Q

    ---------|---------|---------|---------|---------|
451 aggtctttttcctgaaggccaacgcaggcaccttctttgtggcccacgac 500
      V  F  F  L  K  A  N  A  G  T  F  F  V  A  H  D

    ---------|---------|---------|---------|---------|
501 atcttccgtctcaacatccacaactctgcctagGAGCACTCCACTTACCT 550
    I  F  R  L  N  I  H  N  S  A  *

    ---------|---------|---------|---------|---------|
551 ACGTATGCACACCACTCAGCACCACACATAATCGACATCCAAAGATGCCC 600

    ---------|---------|---------|---------|---------|
601 AGCGCCAGATGATAACAACAAGCTCGGCAGTGGAACTCAGAAAAAAAAAA 650

    ---------|---------|---------|---------|---------|
651 TATAACAAAGCCAGCCAGCGGTCTCACGATTATCAGCAAATACAAAAGTT 700
```

```
         ---------|---------|---------|---------|---------|
701 AGAAAAAGAAAAAAAAAAAAAAACAATAAAAAAATAAGAAAATAACGCT 750

         ---------|---------|---------|---------|---------|
751 AACTACTATTACGATGTCAACAAAATGGGCCGCCAGCTCACCTTCATTTG 800

         ---------|---------|---------|---------|---------|
801 TGTCCCATTGTTGCCGGTACTCTCGCTTTACGATCCACGGAACCCTAGAA 850

         ---------|---------|---------|---------|---------|
851 TTATATGTTCTGCGAACACCTGGATCTCGCGATCTAATTTAGCTAGGACT 900

         ---------|---------|---------|---------|---------|
901 ATCGAATTTGGTGGTATGTGCCGTGCTACAAGCTCGTATTTCGAATTCCC 950

         ---------|---------|---------|---------|---------|
951 GTGAAATGAGCAGGAACAGCAGGCCGGGATCATCGCCTACATAAGTTTAG 1000

         ---------|---------|---------|---------|---------|
1001 AGTGATTTTCTAGACCGTTTATATTTCCAATTGAATTATTTGTACTTTAA 1050

         ---------|---------|---------|---------|---------|
1051 TTATGAGGATTATTTTAGATAGCCGTATTGCAAATGTATTGTGTCGAGTG 1100

         ---------|---------|---------|---------|---------|
1101 TCTCGCTCTGTAAACAATTACTTGAGTACCGGGACTGCACCACACACAGT 1150

         ---------|---------|---------|---------|---------|
1151 CGCAAGTATCGACTCTTGCTTTCTCATATGACTAATTGAGCGATGGCGAA 1200

         ---------|--
1201 TTACTTTTTATT 1212
```

Legend

The *ntf-2* cDNA is 1212bp long. It is predicted to be translated from 141bp to 533bp, producing a peptide of 130 amino acids

Figure 6.5 Detection of *ntf-2* transcripts



Legend

Detection of *ntf-2* transcripts by Northern hybridisation. Lane1, RNA from first instar larvae; Lane 2, RNA from embryos; Lane 3, RNA form ovaries. GH 26251 DNA used as the probe. 18μg total RNA of each sample was loaded on the gel.

6.2.4 Structure of *ntf-2* gene

Comparing the *ntf-2* cDNA with the *Drosophila* genomic sequence established that the *ntf-2* gene is located on the X chromosome at position 19E7 region. It has 4 exons. The P-element is inserted in exon 1 (Fig.6.6). The *ntf-2* gene putatively encodes a single ORF, it is translated from 141bp to 533bp, producing a peptide of 130 amino acids.

6.2.5 Homologues of *ntf-2* gene in other species

The amino acid sequence of *ntf-2* was used to search the NCBI database. It was found that the gene has a nuclear transport factor 2 domain. Hence, this gene is called the *ntf-2* gene. Putative homologues were found in *C. elegans*, *X.laevis*, *A.gambiae*, *M.musculus*, and *H.sapiens*. Protein sequences were aligned with these homologues (Fig.6.7) and there are high percentages of identities and similarities in these sequences (Table6.1). This suggests that the *ntf-2* domain is highly conserved in these animals.

Table 6.1

Homologues	Sequence Identities	Sequence Similarities
Anopheles gambiae	75%	84%
Caenorhabditis elegans	47%	68%
Xenopus laevis	43%	58%
Mus musculus	41%	57%
Homo sapiens	39%	52%

Figure 6.6 Structure of the *ntf-2* gene

Legend
The intron/exon structure of the *ntf-2* gene. The boxes are exons. Green represents coding regions. The blue arrow indicates that the P-element insert in exon 1. The numbers in the figure correspond to position of the base pairs in the genome. Each splice junction correspond to number of base pairs inthe genome is shown in table II.

Table II

	Exon 1	Exon 2	Exon 3	Exon 4
Start	113764	114689	114829	117036
End	114011	114760	114927	117526

Figure 6.7 Homologues of the *ntf-2* gene

Legend

Alignment of protein sequence encoded by the *ntf-2* gene and its homologues. Amino acids shaded in black are identical and those shaded in grey are similar in these six sequences.

In the mammal, Ntf-2 is a homodimer of 14kDa subunits which stimulate efficient nuclear import of a cargo protein. Ntf-2 binds to RanGDP sufficiently strongly for the complex to remain intact during transport through nuclear pore complexes (NPCs) (Stewart, 2000; Bayliss et al., 2000). The small GTPase Ran (RanGDP) is required for the trafficking of macromolecules into and out of the nucleus. Ran is an evolutionary conserved member of the Ras superfamily of small GTPases that regulate all receptor-mediated transport between the nucleus and the cytoplasm. Import receptors bind their cargos in the cytoplasm where the concentration of RanGTP is low and release their cargos in the nucleus where the concentration of RanGTP is high (Steggerda and Paschal, 2002). When the *Drosophila ntf-2* mutant was obtained by P-element mobilisation, there were no papers published about this gene. The *ntf-2* mutant we obtained can help us to study the function of this gene in development.

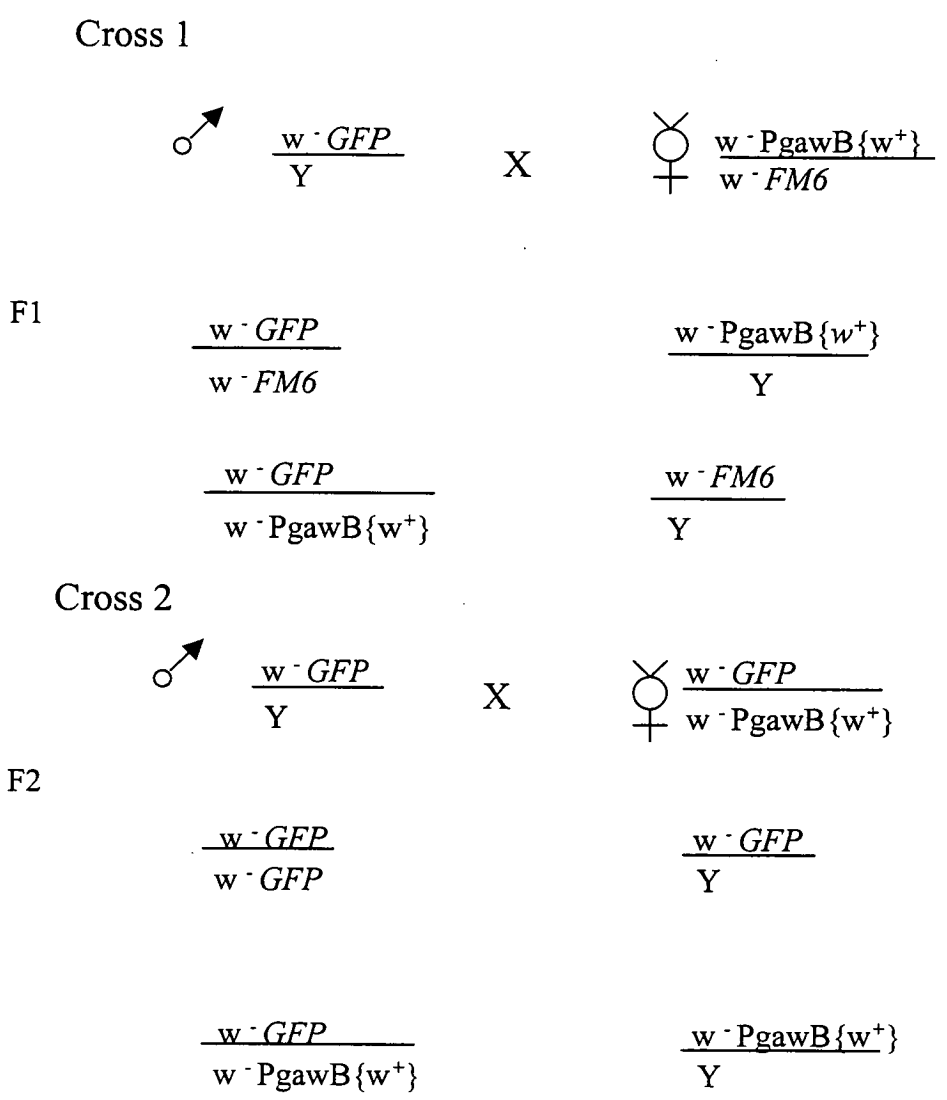
6.3 Analysis of the *ntf-2* mutant

To investigate when and why *ntf-2* mutants die, *ntf-2* mutant flies were crossed with flies expressed GFP (Fig6.8). The heterozygous flies with *ntf-2* and GFP were generated in the F1 generation. Using these flies crossed with GFP flies again, only the mutant embryos and larvae are non fluorescent. All the other embryos and larvae are fluorescent. The result of the cross is detailed in Table 6.2.

Table 6.2

Experiment number	Embryos (total)	Embryos (dead)	Larvae (hatched)	Larvae (dead)	Larvae (survival)
1	239	17	222	43	179
2	303	16	287	49	238
3	208	13	195	42	153

Figure 6.8 Cross scheme of *ntf-2* mutant flies with GFP flies



Legend

ntf-2 mutant female flies were crossed with GFP male flies. The GFP/*ntf-2* heterozygous flies were generated in the F1 generation. The heterozygous female flies were crossed with GFP male flies again. In the F2 generation, only the *ntf-2* male mutant flies are non fluorescent.

It was found that almost 25% mutants were dead as embryos and 75% died during larval stages. One possible reason for the death of the larvae is that their tracheal system is broken (Fig.6.9). They presumably survive to larvae stages because they have obtained maternal RNA from their heterozygous mother.

6.4 Expression Analysis of the *ntf-2* Gene

6.4.1 *ntf-2* expressed during oogenesis

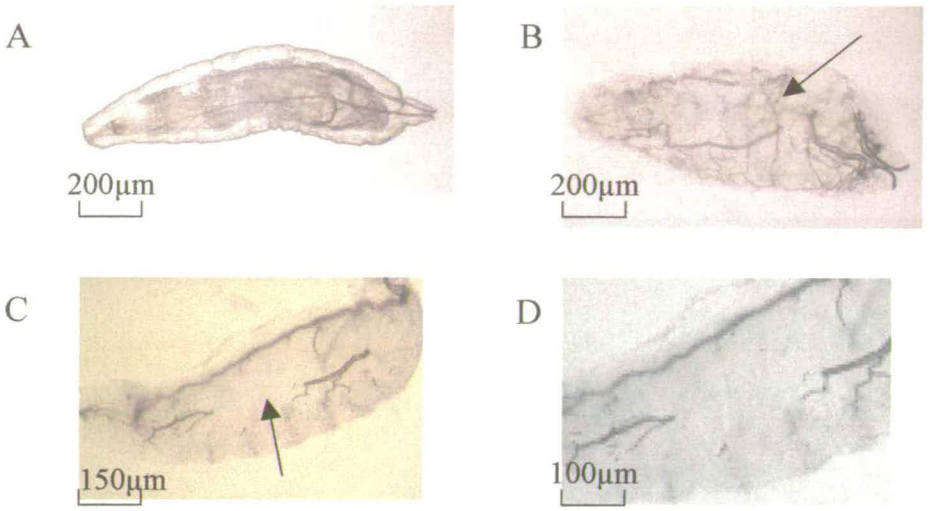
The *ntf-2* mutants die as late embryos and during larval stages. This suggests that they are provided maternal RNAs and proteins to the embryo. The maternal materials are synthesised during oogenesis. To investigate if the *ntf-2* gene is expressed, as expected during oogenesis, *in situ* hybridisation was carried out using an *ntf-2* cDNA as a probe. It was found that the *ntf-2* gene is expressed in the nurse cells at stage 10 of oogenesis (Fig.6.10).

6.4.2 *ntf-2* expression during embryogenesis

The *ntf-2* gene was found to be expressed in the nurse cell in the late stages of oogenesis. Since nurse cells transfer their cytoplasm to the oocyte, it is possible that *ntf-2* transcripts are transferred to the embryo. To test this hypothesis, RT-PCR was carried out to detect any *ntf-2* mRNA in the early stages of embryogenesis. The results revealed that *ntf-2* mRNA was found in 0-4 hour embryos (Fig.6.11). This suggests that the *ntf-2* mRNA is maternally provided. *ntf-2* mRNA was also found in 6-14 hour embryos, which could be maternal or newly expressed transcripts.

In situ hybridisation to embryos of wild type flies was carried out. The result revealed that *ntf-2* transcripts exist in the early cleavage stage of embryogenesis or

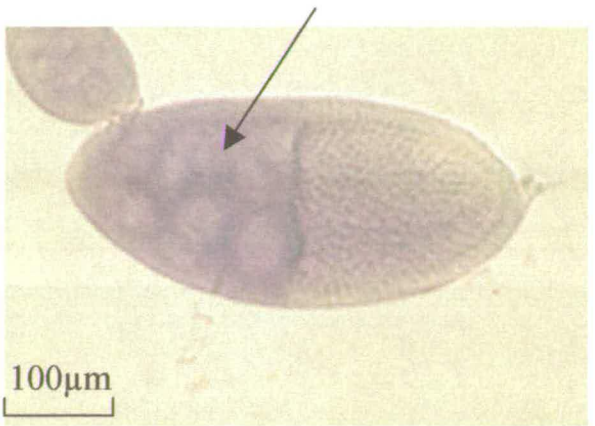
Figure 6.9 Analysis of *ntf-2* mutant



Legend
A. Wild type larva
B. Tracheal system is broken in the *ntf-2* mutant as 1st instar larva.
C.. Tracheal system is broken in the *ntf-2* mutant as 2nd instar larva.
D. Enlargement of C.
Anterior is to the left. Arrow indicated the breakpoint.

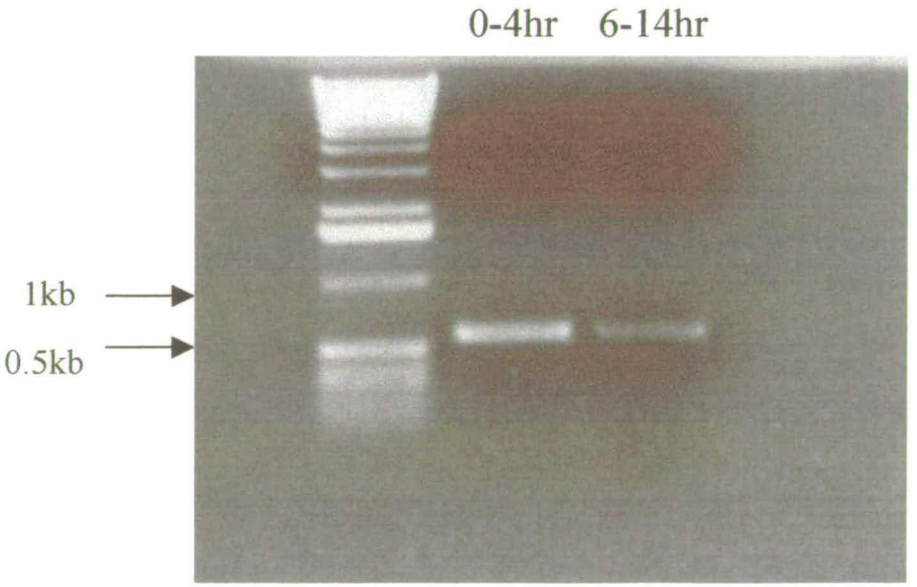
Figure 6.10 *ntf-2* gene expressed in egg chamber

A



Legend
A.The *ntf-2* gene is expressed in the nurse cells at stage10.

Figure 6.11 *ntf-2* mRNA in embryo detected by RT-PCR



Legend
ntf-2 mRNA was found in 0-4 hour embryos and 6-14 hour embryos.

even before this (Fig.6.12), then in the syncytial blastoderm and strongly in the cellular blastoderm. When gastrulation has started, *ntf-2* transcripts can still be found in the embryo. At very late stages of embryogenesis, the *ntf-2* gene is expressed in the cells of the tracheal system.

6.5 Investigation of the Role of *ntf-2* Gene in Embryogenesis

6.5.1 RNA interference

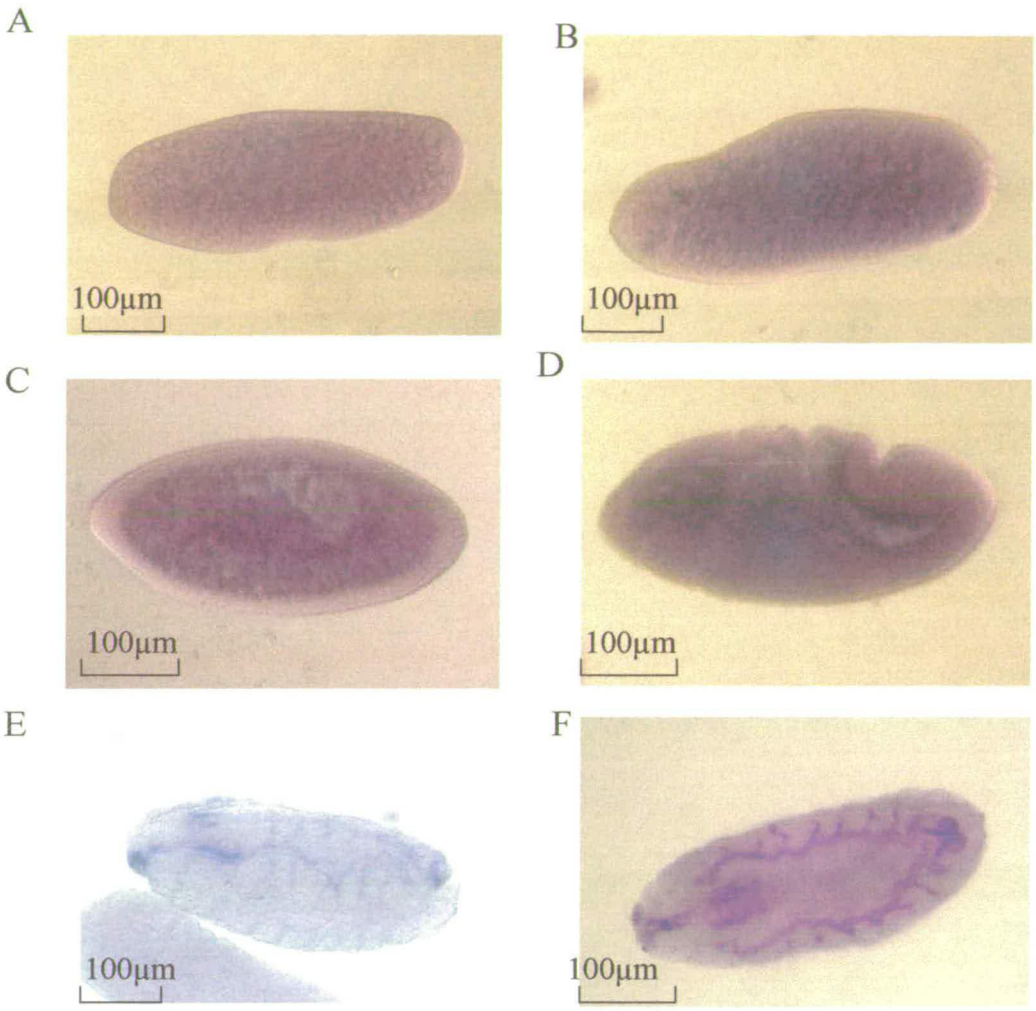
The *ntf-2* gene has a maternal effect. So to investigate how *ntf-2* affects the development of the embryo RNA interference (RNAi) was used to attempt to knock out the *ntf-2* maternal mRNA (Hannon, 2002). Four primers were designed to make double stranded RNA (Section 2.1.8, Kennerdell and Carthew, 1998). The double stranded RNA (ds RNA) was then injected into 0-30 minute OrR embryos. The injection buffer was also injected into 0-30 minute OrR embryos as control. The results of the microinjection are detailed in Table 6.3.

Table 6.3

	embryos (total)	embryos (dead)	larvae (hatched)	survival rate
OrR embryos (Injection buffer)	970	630	340	35%
OrR embryos (ds RNA)	770	480	290	37.6%

The results of microinjection revealed that embryos which were injected with ds RNA survive at the same level as controls. Thus no effect was observed, however, there is no evidence that the RNAi really removed all *ntf-2* transcripts. The reason maybe the low survival rate after microinjection, 35% injected embryos hatched to

Figure 6.12 *ntf-2* gene expression in the embryo



Legend

In situ hybridisation to the wild type embryos, *ntf-2* DNA used as the probe.

A.*ntf-2* transcripts exist in the early cleavage stage.

B. *ntf-2* transcripts can be found in the syncytial blastoderm.

C. *ntf-2* transcripts can be seen in the cellular blastoderm.

D. *ntf-2* transcripts can still be found in the embryo when gastrulation has started.

E. No staining in the control late stages of embryos (*Z14/Dmbve* DNA used as the probe.

F.The *ntf-2* gene is expressed in the tracheal system in very late stages.

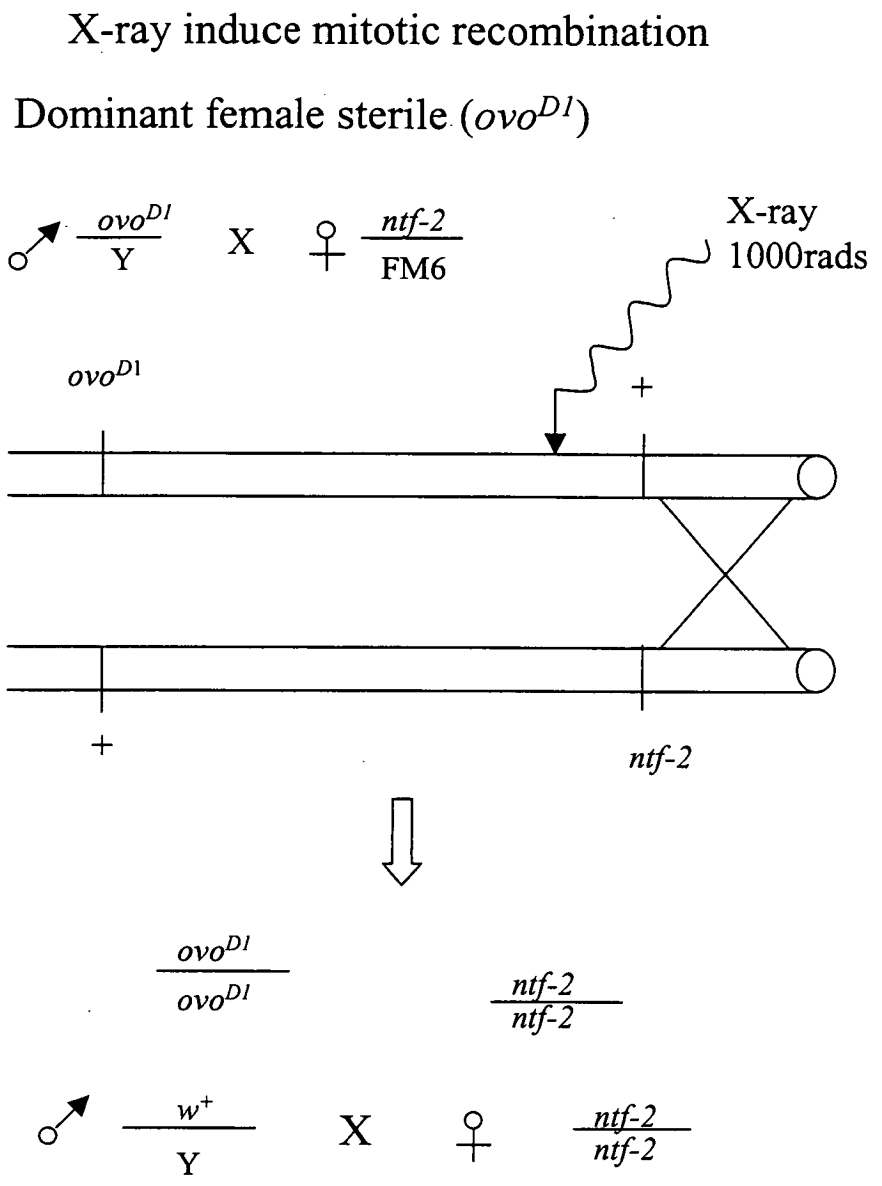
larvae. Most embryos were probably killed by the injection rather than by the ds RNA. Another reason could be *ntf-2* mRNA translates to protein very early, maybe just after fertilisation. So the ds RNA could knock out the *ntf-2* mRNA, but can not knock out the *ntf-2* protein.

6.5.2 Germline clone

6.5.2.1 X-ray induced mitotic recombination in the germline

Another effective approach to knock out n*tf-2* mRNA is the production of germline clone and thus produce *ntf-2* null embryos. An efficient method is to generate female germline mosaics by inducing site-specific homologous mitotic recombination with a yeast recombinase (*FLP*) which is driven by a heat shock promoter (Chou and Perrimon, 1992; Xu and Rubin, 1993). The *ntf-2* gene is located on X chromosome at position 19E7 region, very close to the centromere. Currently there are no *FRT/FLP* flies available to enable us to use the preferred technique of inducing germline clones, so X-rays were used to induce mitotic recombination (Perrimon et al., 1984; MacDougall et al., 2001). To take advantage of the dominant sterile system, the ovo^{D1} male mutant flies were crossed with *ntf-2* female mutant flies. The progeny were exposed to X-rays as first instar larva (Fig.6.13). The survival F1 female flies were crossed to wild type male flies. In the F1 generation only the female flies which have *ntf-2* germline clones can lay eggs. All the F2 flies are expected to have red eyes. The results of the mitotic recombination experiments are shown in Table 6.4

Figure 6.13 Germline clone methond



Legend
The ovo^{D1} male mutant flies were crossed with *ntf-2* female mutant flies. The progeny were exposed to X-rays when they were at first instar larva stage. In the F1 generation only the female flies which have the *ntf-2* germline clones can lay eggs.

Table 6.4

Female flies (total)	Egg-laying flies	Frequency of mitotic recombination
1850	42	2.27%

6.5.2.2 Analysis of *ntf-2* germline clones

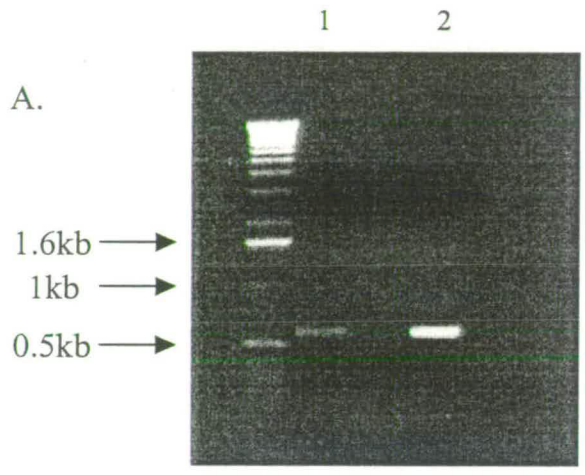
The eggs of female flies which have the germline clone were collected. It was found that almost one third of the progeny were dead in the early stages of embryogenesis, one third were dead in the larval stages and the rest survive to adults. The analysis of the progeny are shown in Table 6.5.

Table 6.5

		embryos	larvae	adult flies	total
1	number	42	12	30	84
	percent	50%	14.3%	35.7%	100%
2	number	30	63	48	141
	percent	21.3%	44.7%	34%	100%
3	number	48	48	42	138
	percent	34.8%	34.8%	30.4%	100%

Theoretically there should be no maternal *ntf-2* mRNAs in the germline clone embryos. To test this, RT-PCR was carried out with RNAs prepared from 0-4 hour germline clone embryos. To our surprise, *ntf-2* mRNAs can still be found in the 0-4 hour germline clone embryos though in a much low level by comparison with that in the wild type embryos (Fig.6.14A). This suggests that *ntf-2* mRNAs still existed in some embryos. Later on, we found some embryos could survive to adult flies. The surviving adult flies were checked and we found females with red eye and male with white eye in the mixed population of flies. As explained earlier (Section 6.5.2.1), when *ntf-2*$^{-}$/*ntf-2*$^{-}$ female flies were crossed with OrR male flies, all the progeny should have red eyes. When male flies with white eyes appeared, it is possible that

Figure 6.14 Analysis of germline clone



Legend

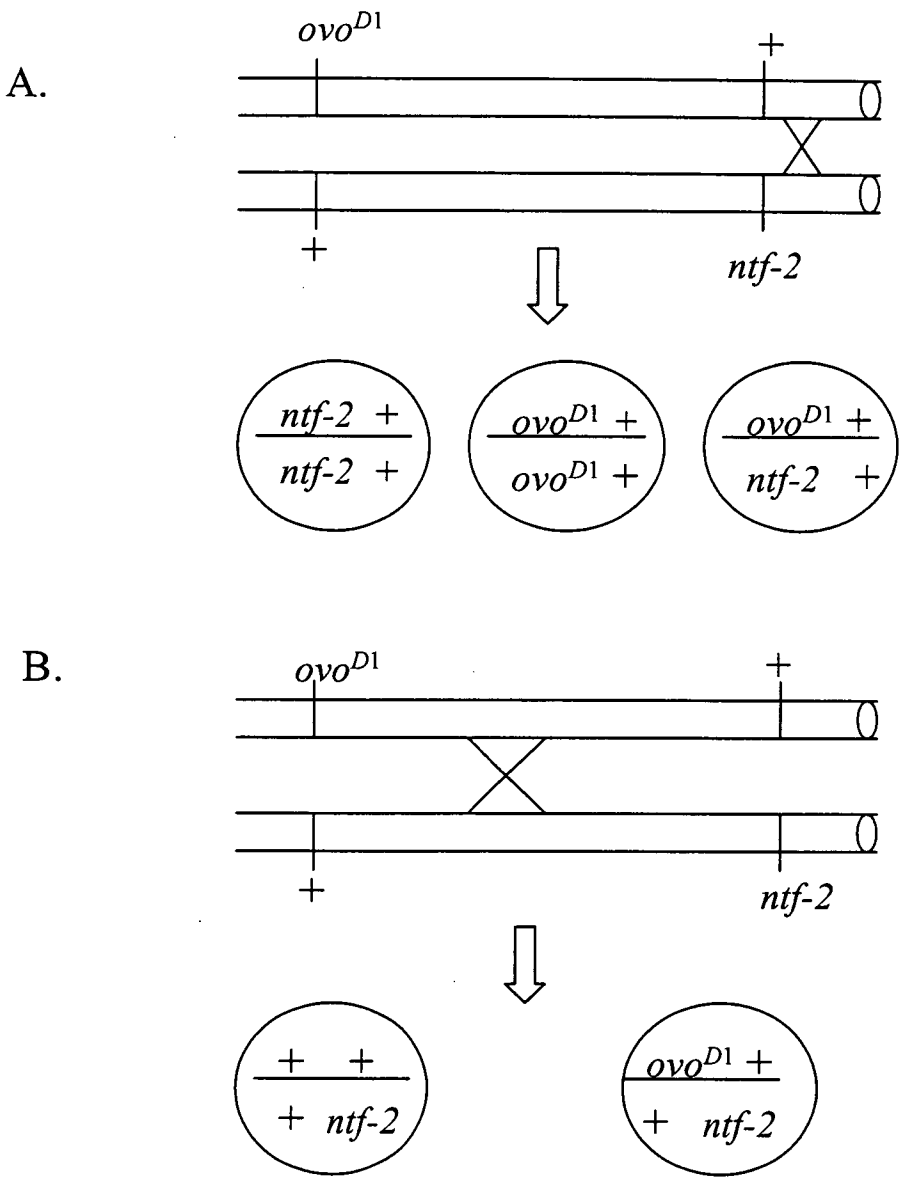
A.The result of RT-PCR revealed that some *ntf-2* mRNA can be found in the 0-4 hour germline clone embryos.

B.The result of Southern hybridisation revealed that there is no P-element in white eyed flies.

there is no P-element in these flies. To test this hypothesis, southern hybridisation was carried out and no P-elements were found (Fig.6.14B). Thus, the P-element is no longer inserted in the *ntf-2* gene. The embryos of these flies have maternal *ntf-2* mRNA. This result is consistent with the result of the RT-PCR. We need to understand why the P-element was excised in some flies to generate white eyed flies? One explanation is that X-ray treatment may trigger the P-element mobilisation. The other explanation is the P-element was removed by mitotic recombination. 70-75% of the mitotic recombination events occur in the heterochromatin (Perrimon and Gans, 1983). In the resulting proximal clone, ovo^{D1} was removed and *ntf-2* was thus homozygous. But there are also 25-30% of mitotic recombination events which take place in euchromatin at random sites (Wieschaus et al., 1981). This recombination will result a distal clone in which the ovo^{D1} was eliminated but the *ntf-2* remains heterozygous (Fig.6.15). Fertile female flies with clones of this type can generate white eyed male flies.

The germline clone mutant embryos died in the early stages of embryogenesis. These embryos were found to be dorsalised (Fig.6.16). It seemed that the *ntf-2* gene is involved in the establishment of dorsal-ventral polarity. Since the Ntf-2 protein has a nuclear transport domain, the function of *ntf-2* probably is to transport Dorsal protein into the nucleus. To test this hypothesis antibody staining of the wild type embryos and germline clone mutant embryos were carried out. The Dorsal antibody was kindly provided by Dr. Wasserman and checked using a western blot (Gillespie and Wasserman, 1994). Proteins from wild type ovaries and embryos were extracted and run on SDS-polyacrylamide gel. Western blots were treated with anti-Dorsal antibody. Strong bands were found around 86Kda which matches the molecular

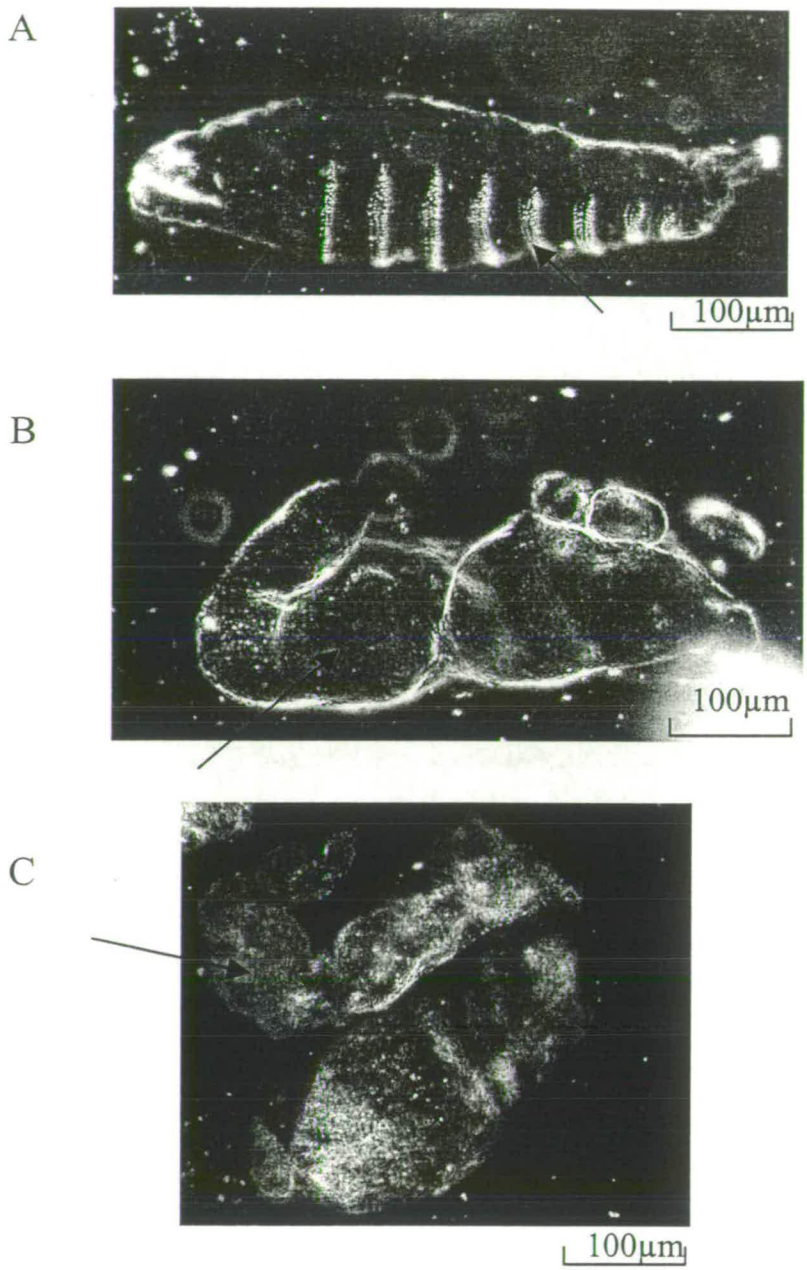
Figure 6.15 Analysis of mitotic recombination



Legend

A.Mitotic recombination in heterchromatin, *ntf-2* homozygous was generated in the proximal clone.
B. Mitotic recombination in euchromatin, *ntf-2* remains heterozygous in a distal clone.

Figure 6.16 Phenotype of germline clone embryo



Legend

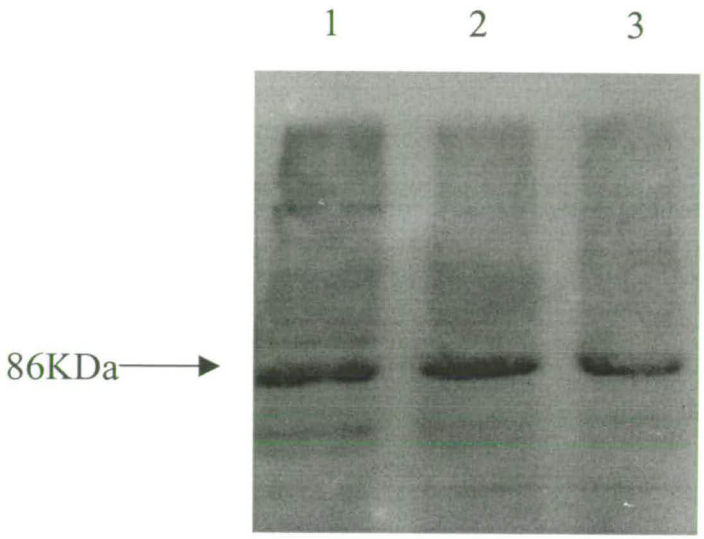
The embryos were dissected from the vitelline membrane and mounted in a mixture of Hoyer's medium and lactic acid (1:1). Cuticles of embryos were photographed using dark-field optics. A.wild type embryo. Arrow indicated the denticle of the embryo. B and C. Germline clone embryos were found, they are dorsalised. Arrow indicates that denticle disappeared.

weight of Dorsal protein (Fig.6.17). The antibody was considered as anti-Dorsal antibody and used for antibody staining. It was found that Dorsal protein entered the nuclei along the ventral side of the wild type embryos. The Dorsal protein has remained in the cytoplasm of germline clone embryos (Fig.6.18). This means the Dorsal protein can not be transported into the nucleus without *ntf-2*. Dorsal is known as a transcription factor. When it enters the nucleus, it can activate expression of some specific genes. One of these genes is *twist*. It is transcribed only in the ventral cell nuclei that have received high concentrations of the Dorsal protein, since its enhancer does not bind Dorsal protein with a very high affinity (Thisse et al., 1988,1991). To see if the Dorsal protein can enter the nucleus and affect gene expression in the germline clone embryos, *in situ* hybridisation was carried out using *twist* DNA as a probe. It was found that *twist* was expressed in the wild type embryo but not in the germline clone embryos (Fig.6.18). This certifies that the Dorsal protein cannot be transported into the nucleus without *ntf-2*. The result is consistent with the antibody staining.

6.6 Summary and Discussion

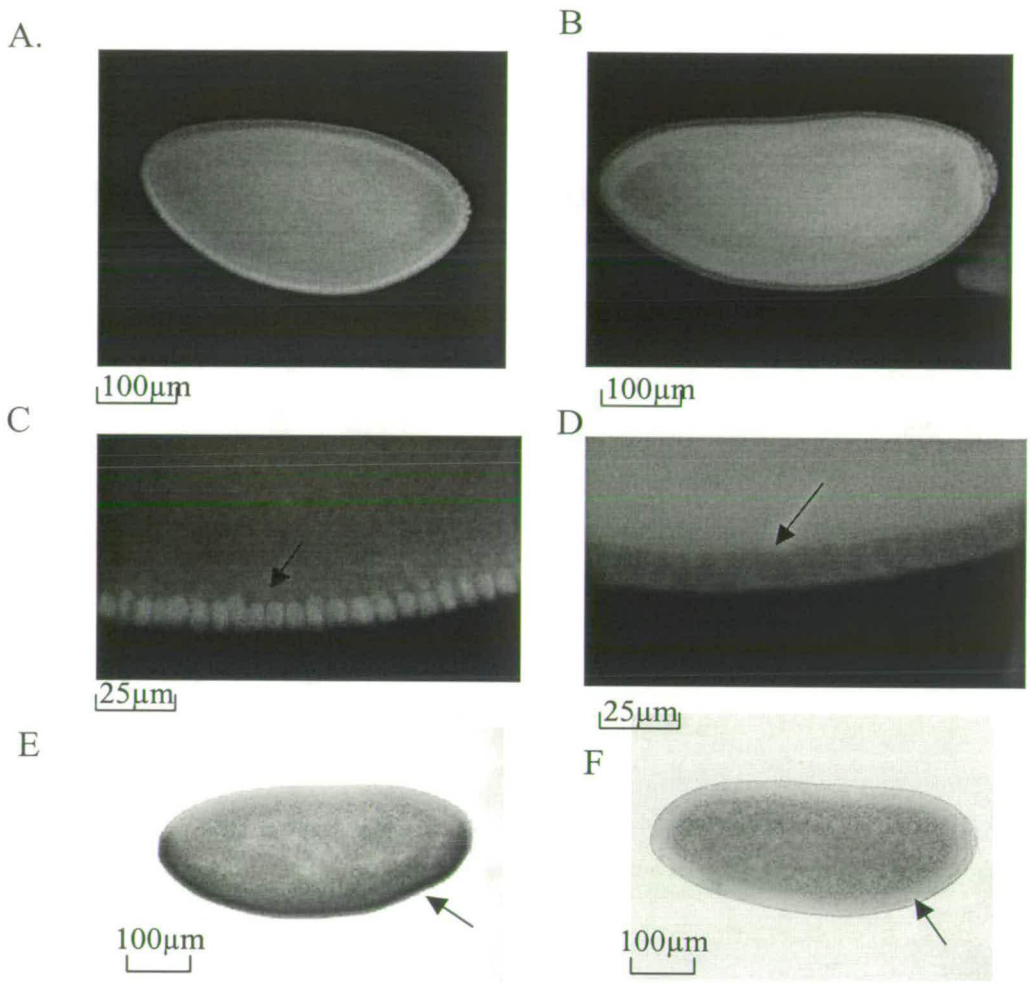
The *ntf-2* mutant was generated while looking for mutations in the *Z14/Dmbves* gene by mobilising the P-element. 4 homozygous lethal red-eyed fly lines were found. Why was there no mutants in the white eyed lines? We found that there is a large intron of 13kb between exon 2 and exon 3 in the *Z14/Dmbves* gene. The P-element is inserted in the middle of the intron. Perhaps the P-element removes some flanking genomic DNA when it excises imprecisely but it did not remove enough genomic DNA to cause a mutation. Thus, there are no white-eyed mutant flies

Figure 6.17 Specificity of the anti-dorsal antibody



Legend
Proteins from wild type ovaries and embryos were extracted and run on SDS-polyacrylamide gels. Western blots were treated with anti-dorsal antibody.Lane 1, ovaries; Lane 2, 0-4 hour embryos; Lane 3, 6-14 hour embryos.

Figure 6.18 Analysis of germline clone embryos



Legend

A–D. Antibody staining by using anti-dorsal protein antibody.
E and F. In situ hybridisation by using *twist* DNA as probe.
A.The Dorsal protein entered the nuclei at the ventral side of the wild type embryos.
B.The dorsal protein remained in the cytoplasm of germline clone embryos.
C. Enlargement of the cells along the ventral side in A.
D. Enlargement of the cells along the ventral side in B.
E. *Twist* is expressed in the wild type embryo on the ventral side.
F. *Twist* is not expressed in the germline clone embryos.
Anterior is to the left.

hybridisation and rescued plasmids of them were sequenced. They have inserted into genes either side of the *Z14/Dmbves* gene.

In one of the mutant fly lines, the P-element was found to be inserted in a new gene which is named *ntf-2*. The *ntf-2* gene has a nuclear transport factor 2 domain. Homologues were found in other species. The *ntf-2* gene is expressed during *Drosophila* oogenesis and embyrogenesis. To study the function of the *ntf-2* gene, RNAi was first used to attempt to knock out the maternal *ntf-2* mRNA. Double stranded *ntf-2* RNA was injected into very early stage embryos. There was no difference between ds RNA injected embryos and the control embryos which were injected with injection buffer. The reason maybe the low survival rate after microinjection, only 35% of injected embryos hatched to larvae. Most embryos were probably killed by the injection rather than by the ds RNA. Another reason could be *ntf-2* mRNA translates to protein very early, maybe just after fertilisation. So the ds RNA could knock out the *ntf-2* mRNA, but can not knock out the *ntf-2* protein. Another approach to knock out the maternal *ntf-2* mRNA was to generate germline clones. We used the dominant sterile system, the ovo^{D1} male mutant flies were crossed with *ntf-2* female mutant flies. X-rays were use to induce the mitotic recombination (Perrimon et al., 1984; MacDougall et al., 2001). 42 female flies were found to lay eggs. The eggs were analysed and it was found one third of them can survive to adult. Among the surviving adult flies, there are some white eyed male flies. We showed these flies have lost the P-element from their genome by Southern hybridisation. The conclusion is that X-rays may trigger P-element mobilisation or the P-element was removed by mitotic recombination. The P-element is no longer inserted in the *ntf-2* gene, therefore these white-eyed male flies can survive and will

not *ntf-2* nulls as embryos. For the same reason, the red eyed female flies are heterozygous. They have one copy of maternal *ntf-2* RNA which allows them to survive as embryos. Thus the embryos are a mixed population.

The germline clone embryos were found to be dead in the early stages of development. The phenotype of the mutant embryos was dorsalised. To find out if *ntf-2* is involved in the establishment of dorsal-ventral polarity, *in situ* hybridisation and antibody staining were carried out. The results revealed that the *ntf-2* gene is involved in the Toll signalling pathway. We propose that Ntf-2 translocates the dorsal protein into the nucleus. Then Dorsal protein activates the expression of *twist* gene, which establishes the dorsal-ventral polarity in the embryo. Further discussion of the function of *ntf-2* is presented in chapter seven.

Chapter Seven: Final Discussion

The data in this thesis revealed that the molecular structure of *Z14/Dmbves*. It has 8 exons with a single ORF encoding 414 amino acids. The *Z14/Dmbves* gene is the homologue to a gene encoding blood vessel/ epicardial substance (bves) in Chicken and humans. We found that *Z14/Dmbves* is expressed in the nurse cell and all the follicle cells surrounding the oocyte except those in the anterior-dorsal and posterior regions, which receive the *gurken* (Grk) signal from the oocyte. Further analysis indicated that the expression of *Z14/Dmbves* is repressed by the Grk signal. P-element mobilisation to generate mutants was undertaken but no mutants of *Z14/Dmbves* were obtained. Later on, investigation of transgenic flies revealed that disruption of the function of *Z14/Dmbves* caused lethality to both embryos and larvae. This is controversial to the result from P-element mobilisation. This problem is discussed further in this chapter.

The *ntf-2* gene was identified as part of a P-element mobilisation screen for generating mutants of *Z14/Dmbves*. It has a nuclear transport factor 2 domain. Homologues were found in other organisms. It was found that *ntf-2* transcripts are present during oogenesis and embryogenesis. *ntf-2* is provided maternally to the embryo since its mutants only die as late embryos and during larval stages. Germline clones were generated to knock out the maternal *ntf-2* mRNA. Homozygous *ntf-2* null embryos generated from germline clones are found to be dead in the early stages of embyrogenesis and show a dorsalised phenotype. The result of *in situ* hybridisation and antibody staining of *ntf-2* null embryos revealed that *ntf-2* is involved in the Toll signalling pathway. The mechanism of *ntf-2* involved in the translocation of Dorsal protein into nucleus is discussed in this chapter.

7.1 Analysis of *Z14/Dmbves*

Z14/Dmbves was identified as part of a GAL4/UAS enhancer trap scheme. It was found that *Z14/Dmbves* has a specific expression pattern during oogenesis. To generate a *Z14/Dmbves* mutant, P-element mobilisation was carried out. No mutant phenotypes, such as female sterility, aberrant egg shells or adult flis were observed. 4 homozygous lethal fly lines were obtained, but unfortunately these 4 mutant lines were found not to be mutants of *Z14/Dmbves* by sequencing the rescue plasmids. It was therefore possible that *Z14/Dmbves* was a redundant gene. To further analyse the function of *Z14/Dmbves*, transgenic flies were generated. pUAST antisense fly lines were crossed with different GAL4 lines, but again no abnormal phenotype was found possibly due to lower level of *Z14/Dmbves* antisense RNA expression . Things changed when the pCaS-*Z14/Dmbves* inducible heat shock antisense flies were used. It was found that almost 40-50% of the eggs laid by heat shock female flies died after heat shock in comparison to 5-10% of the eggs laid by wild type female flies. This suggests that the reason for the death of the embryos of heat shock lines is the expression of *Z14/Dmbves* antisense RNA. The native *Z14/Dmbves* mRNAs were knocked out which leads to a disruption of the function of *Z14/Dmbves*.

As it was mentioned early that *Z14/Dmbves* possibly was a redundant gene. The fact that heat shock *Z14/Dmbves* antisense flies caused the lethality of hs embryos excluded this possibility. Normally heat shock expression of antisense RNA caused 10-20% phenotypes (Zhao et al., 2000; Pathirana et al., 2001). But knocking out expression of *Z14/Dmbves* by heat shock of *Z14/Dmbves* antisense flies caused much high rate of death (40-50%). Since the heat shock is transient, only the egg chamber

at proper stage can express *Z14/Dmbves* antisense RNA, it is impossible that all the hs embryos will dead after heat shock. Thus, we proposed that *Z14/Dmbves* is essential for normal embryogenesis and it might still be a lethal gene. Why did we not get lethal lines from P-element mobilisation? One explanation is that *Z14/Dmbves* maybe a haplo-insufficient gene. But we found that some deficiency lines which deletes all the 19E7 region still be able to survive. Thus it is impossible that *Z14/Dmbves* is a haplo-insufficient gene. The another possibility is that we maybe have not got enough lines to generate a mutant. According to the experience of previous students in our lab, almost 5% mutants can be generated by P-element mobilisation. We have generated 316 balance lines and believe that we are able to obtain mutant within these balance lines. Probably the number of balance lines are not enough. One problem is still not solved, we obtain 3 homozygous lethal lines which the P-elements all inserted in the distant genes. Theoretically the P-element inserted in the local gene easier than in the distant gene. Thus, we only can explain that we are unlucky.

Z14/Dmbves encodes a novel protein. The function of *Z14/Dmbves* homologues is also not clear. This causes difficulty to know what the exact function of *Z14/Dmbves* is. Due to time constraints, we have not analysed the deficiency lines that may delete *Z14/Dmbves*. Future direction of this research could be on searching deficiency lines and P-element insertion lines related to *Z14/Dmbves*. The deficiency lines can be analysed by southern hybridisation and found the line which deletes the *Z14/Dmbves* gene. The P-insertion lines can be analysed by sequencing the rescued plasmid to identify the position of the P-element. If the P-element is inserted in the coding region of *Z14/Dmbves*, we obtain a mutant of *Z14/Dmbves*. Other work can be on the

epistasis of the Grk/EGFR signalling pathway. If a *mirror* P1 mutant could be obtain from other lab, the relation between *mirror* and *Z14/Dmbves* could be studied. Which gene functions before another? Which gene really determines the phenotype? The answer to these questions will give us more knowledge and understanding of the role of *Z14/Dmbves* during oogenesis and embryogenesis.

7.2 Analysis of *ntf-2*

7.2.1 Mechanism of nuclear import

The trafficking of macromolecules, ions and small molecules between the cytoplasm and nucleus is fundamental to eukaryotic cells (Nakielny and Dreyfuss, 1999; Gorlich and Kutay, 1999). Molecular trafficking between the cytoplasm and nucleus occurs through large, proteinaceous structures called nuclear pore complexs (NPCs) (Forbes, 1992; Fabre and Hurt, 1994). Nuclear proteins such as histones and transcription factors synthesised in the cytoplasm need to be imported into the nucleus. On the other hand, mRNA, tRNA and rRNA which are synthesised in the nucleus need to be exported to the cytoplasm. There are two types of transport: passive diffusion and active transport through NPCs. Ions and other small molecules passively diffuse through the aqueous channels in the NPC. The macromolecules cannot pass through these channels and need to actively transport through central channel in the NPC.

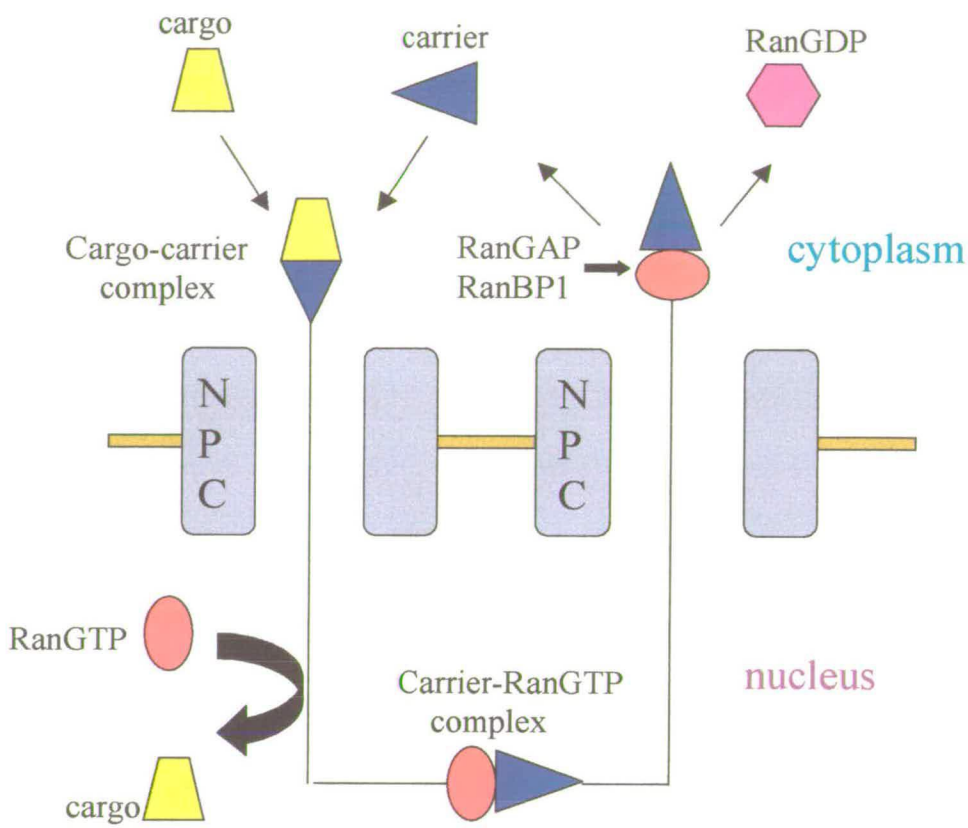
Although the mechanism for active transport of proteins and RNAs through the NPC is not clear, it basically involves a multistep process requires interactions between several cytoplasmic factors and the NPC (Forbes, 1992; Fabre and Hurt, 1994). Until now, five distinct cytosolic transport factors have been identified: NLS

receptor (Gorlich et al., 1994), Hsp70 (Shi and Thomas, 1992), Ran/TC4 (Moore and Blobel, 1993; Melchior et al., 1993), p97 (Adam and Adam, 1994) and NTF-2 (Paschal and Gerace, 1995). Proteins which contain a nuclear localization sequence (NLS) can be imported into the nucleus (Robbins et al., 1991). This event first occurs in the cytoplasm where an NLS-containing protein (cargo) binds to the NLS receptor (carrier) (Adam and Gerace, 1991). The cargo-carrier complex then docks at the cytoplasmic face of the NPC (Jarnik and Aebi, 1991). Subsequently, the cargo-carrier complex is delivered to the central channel of the NPC and translocated into the nucleus. Translocation is followed by displacement of the cargo from the carrier by nuclear RanGTP. Afterwards, the carrier-RanGTP complex is recycled back through the NPC to the cytoplasm where RanBP1 and RanGAP act to dissociate the complex and release Ran from the carrier in preparation for another round of nuclear importation (Fig.7.1).

7.2.2 The function of NTF-2

Efficient protein importation into the nucleus requires both the GTPase Ran and the homodimeric NTF-2. The interaction of the carrier with its cargo is orchestrated by the nucleotide state of GTPase Ran (Gorlich, 1998; Melchior and Gerace, 1998), however, the nuclear importation of Ran itself is mediated by NTF-2 (Ribbeck et al., 1998; Smith et al., 1998). NTF-2 binds the RanGDP in the cytoplasm. The NTF-2-RanGDP complex is then translocated through the NPC into the nucleus. It has been established that NTF-2 interacts with FxFG nucleoporins when NTF-2-RanGDP passes through the NPC (Paschal and Gerace 1995; Chaillan-Huntington et al, 2000).

Figure 7.1 Translocation of cargo-carrier through the NPC



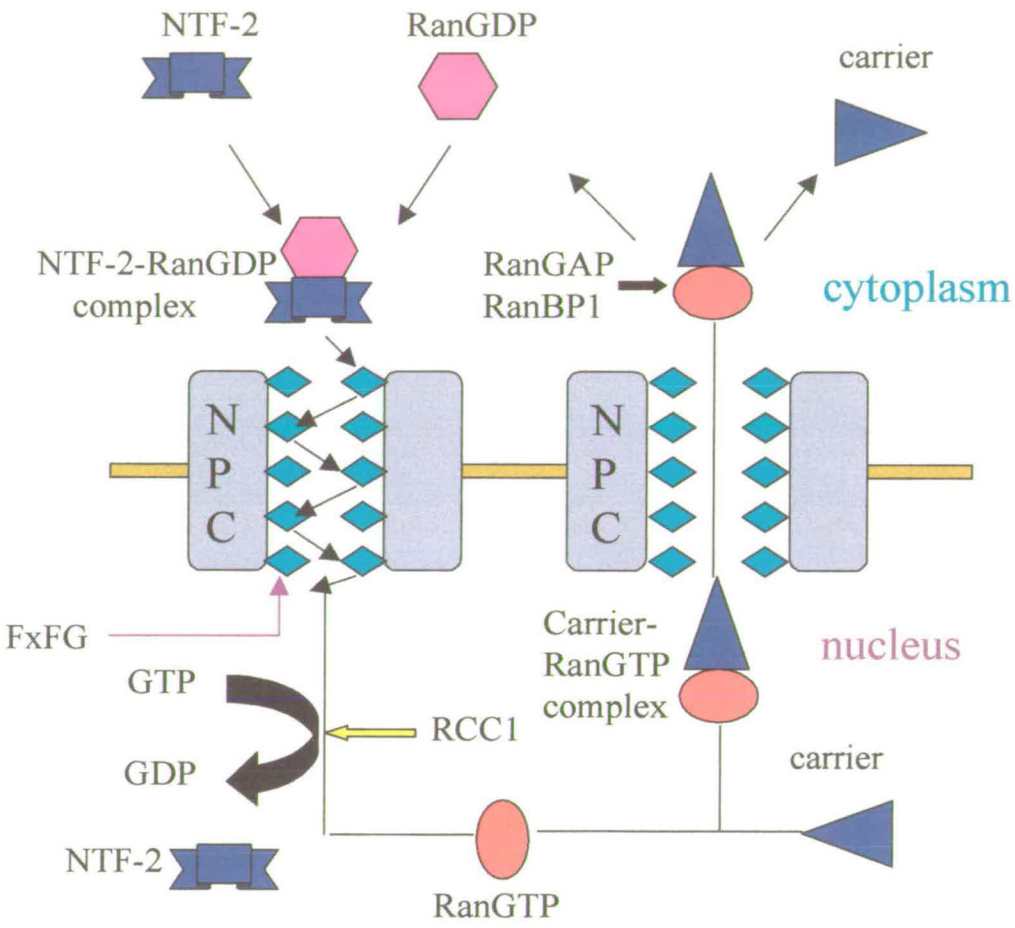
Modified from Stewart,2000

Legend
Cargo and carrier bind in the cytoplasm and be translocated through the NPC. The complex is dissociated in the nuceus by RanGTP. The carrier-RanGTP complex is then recycled to the cytoplasm to participate in further rounds of transport.

Binding of NTF-2 to FxFG repeats are much weaker and would be transient. The transient interaction between NTF-2 and the nucleporins allows the NTF-2-RanGDP complex to move through the NPC by jumping from one FxFG repeat to the next (Bayliss et al., 1999), this facilitates rapid translocation through NPCs during nuclear importation. When the NTF-2-RanGDP complex enters the nucleus, RCC1 catalyzes nucleotide exchange and recharges Ran with GTP. The RanGTP then binds a carrier molecule to export to the cytoplasm for another round of transport (Fig.7.2).

It seems that there are two parallel systems of nuclear importation. One includes the cargo proteins binding their receptors or carriers through the NPCs. The other includes NTF-2 binding RanGDP and passes through the NPCs. Is there a link between these two systems? We propose below a model base upon our new data and earlier studies. Our study reveals that NTF-2 plays a key role in mediating nuclear importation of the Dorsal protein. When *ntf-2* germline clones are induced embryos lacking Ntf-2 protein are produced and we observed that Dorsal cannot enter the nucleus in the germline clone embryo. The Dorsal protein containing an NLS is considered to be a cargo protein. The receptor of Dorsal is not known but is probably from the importin α and β superfamily (Chook and Blobel, 2001; Gorlich and Kutay, 1999). NTF-2 might be not bind Dorsal directly, but mediates the nuclear importation of the Dorsal-receptor complex. Evidence from a recent report demonstrates that *Drosophila* NTF-2 is required for nuclear translocation of the Rel proteins Dorsal, Dif and Relish to the nucleus of larval fat bodies (Bhattacharya and Steward, 2002).The signalling pathway which disrupt Dorsal and Cactus complex in the larvae fat body is the same as in the embryo. These three proteins all have an NLS, but their NLS are not identical. Therefore, they might be recognised by

Figure 7.2 The role of NTF-2 in the nuclear import

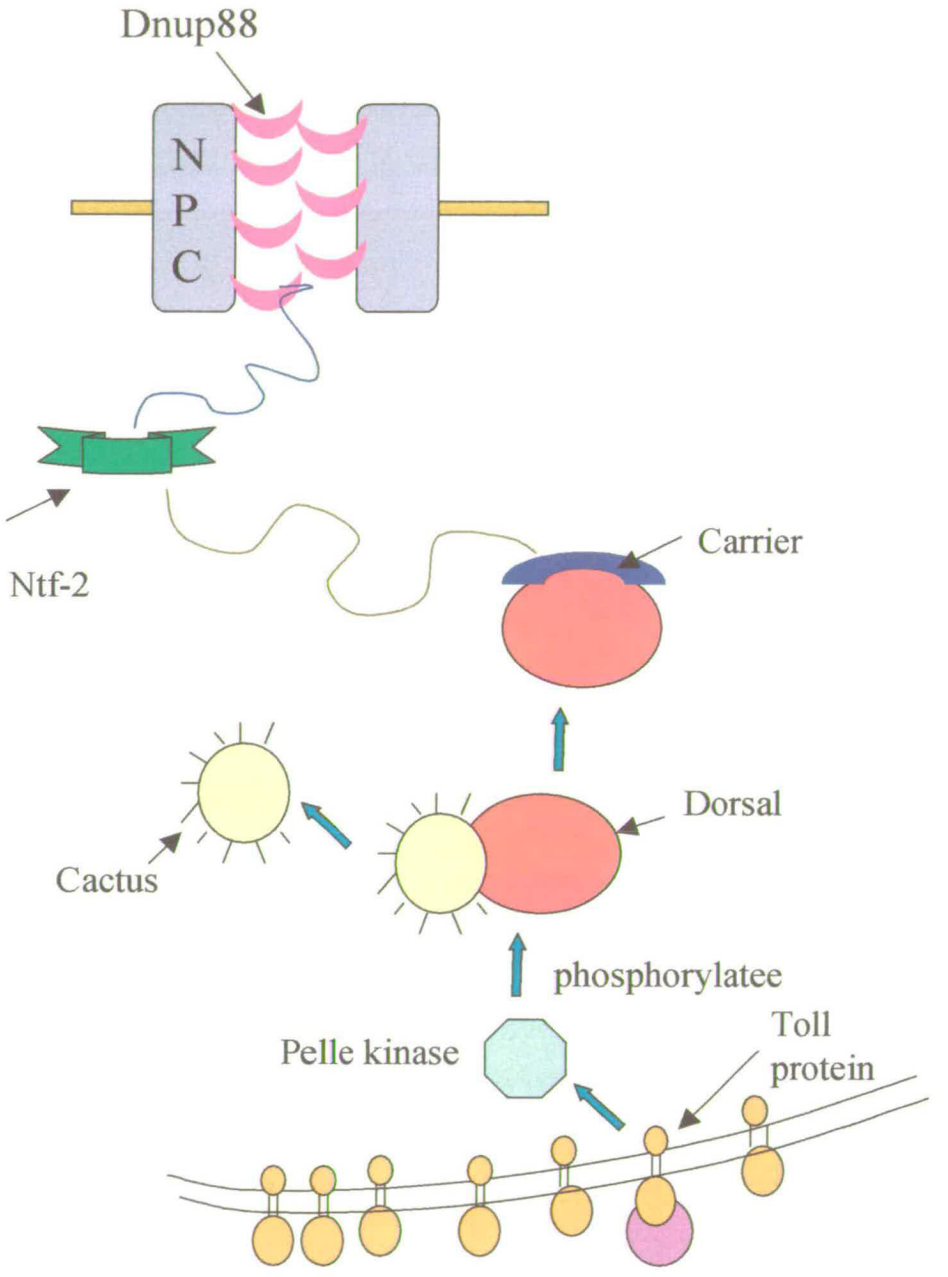


Modified from Stewart,2000

Legend
NTF-2 binds RanGDP in the cytoplasm and interacts with FxFG when the complex through the NPC. RCC1 recharges Ran with GTP. The RanGTP binds carrier and then is exported to the cytoplam.

different NLS receptors. All these Rel proteins failed to enter into the nucleus in the *Dntf-2* mutant. This suggests that DNTF-2 proteins mediate the cargo-carrier complexs rather than cargo proteins themselves. NTF-2 binds RanGDP and interacts with FxFG nucleoporins when NTF-2–RanGDP complex passes through the NPC. This has been established *in vitro*, but it is not so simple *in vivo*. NTF-2 probably has multiple roles for nuclear transport. NTF-2 proteins without the FxFG-binding domain can still bind to the cytoplamic and nucleoplamic sides of the nuclear pore (Bayliss et al., 1999). *Drosophila* NTF-2 is also found to interact directly with DNup88 (Bhattacharya and Steward, 2002). DNup88 is a *Drosophila* nucleoporin encoded by *members only* (*mbo*) which is required for the Rel proteins Dorsal and Dif importation (Uv, et al.,2000). In *mbo* mutants the signal transduction cascade leading to Cactus degradation is functional, but Dorsal and Dif remain in the cytoplasm. mRNA export and classic NLS-mediated protein import are unaffected in *mbo* mutants. This demonstrates that distinct nuclear import events require different nucleoporins *in vivo*. So from our data we suggest that there is a model for NTF-2 to translocate the Dorsal protein *in vivo* (Fig.7.3). DNTF-2 interacts with nucleoporin DNup88 which leads to a change in the structural components of the NPC. The DNTF-2-DNup88 multiprotein complex then selectively translocates Dorsal-receptor complex into the nucleus. Without DNTF-2, Dorsal cannot be imported into the nucleus or resulted in a very low level of transport. Thus, DNTF-2 is essential for the Dorsal protein importation.

Figure 7.3 The role of *Drosophila* Ntf-2 in the nuclear import



Legend
Drosophila Ntf-2 interacts with nucleoporin DNup88 and mediates the Dorsal-receptor complex into the nucleus.

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Appendix

1. Sequence of original *Z14Dmbves* cDNA

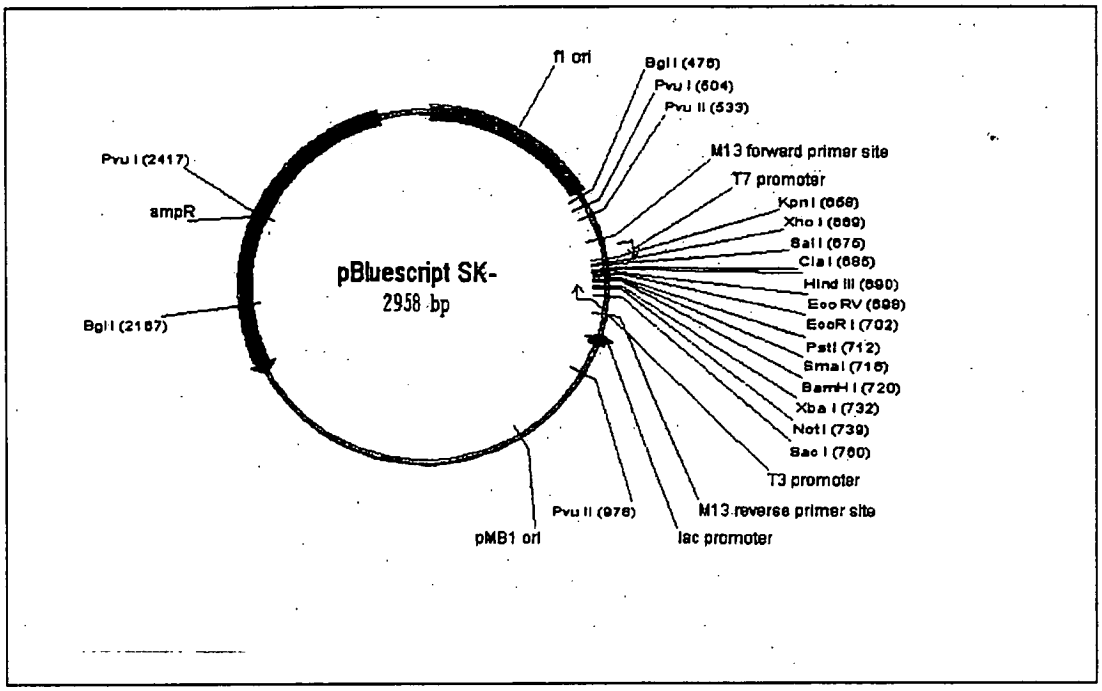
```
          10         20         30         40         50
----:----|----:----|----:----|----:----|----:----|
CGCAATAACAAAACAAACCACCACCAACAAAGGTTGAGGAAAACCGCGCG

          60         70         80         90         100
----:----|----:----|----:----|----:----|----:----|
TAAGCAACAACAAACGAATTAGGCGAAGGAGAGAAAGGTGGAAGATAGAA

          110        120        130        140        150
----:----|----:----|----:----|----:----|----:----|
ACAAAAGGCAGATTTTTGGCATTCGCCTAGCGTGGACAACAACCAACCAG

          160        170        180        190        200
----:----|----:----|----:----|----:----|----:----|
TACATCAACCAATCAATTTGGTAATTAGGCAAAATACGTGCTGCAAAAAA

          210        220        230        240        250
----:----|----:----|----:----|----:----|----:----|
TAGCTCAACGATCAGGCTGAGAGGAGAGTGACAGACTGAAACGAAGGCAG

          260        270        280        290        300
----:----|----:----|----:----|----:----|----:----|
TTCGCCTGGATCAACGGAATGCCCAGCACGGCGGGCAGTGCAGCTGGCGT
                    M  P  S  T  A  G  S  A  A  G  V

          310        320        330        340        350
----:----|----:----|----:----|----:----|----:----|
TGGTATGGGCGCCCTGATCAACAGTGCCGGCAGCAGCGCCAGTAGTGTCA
 G  M  G  A  L  I  N  S  A  G  S  S  A  S  S  V  M

          360        370        380        390        400
----:----|----:----|----:----|----:----|----:----|
TGGGCATTGGCATGGGCAGAGCAGCTGCTACGGGAGCAGGAGCACCAGGC
  G  I  G  M  G  R  A  A  A  T  G  A  G  A  P  G

          410        420        430        440        450
----:----|----:----|----:----|----:----|----:----|
AGTTCGGGATCTGGAGGAGATGCCTCCGCCGCCGGCACTTTAATCGCCCA
```

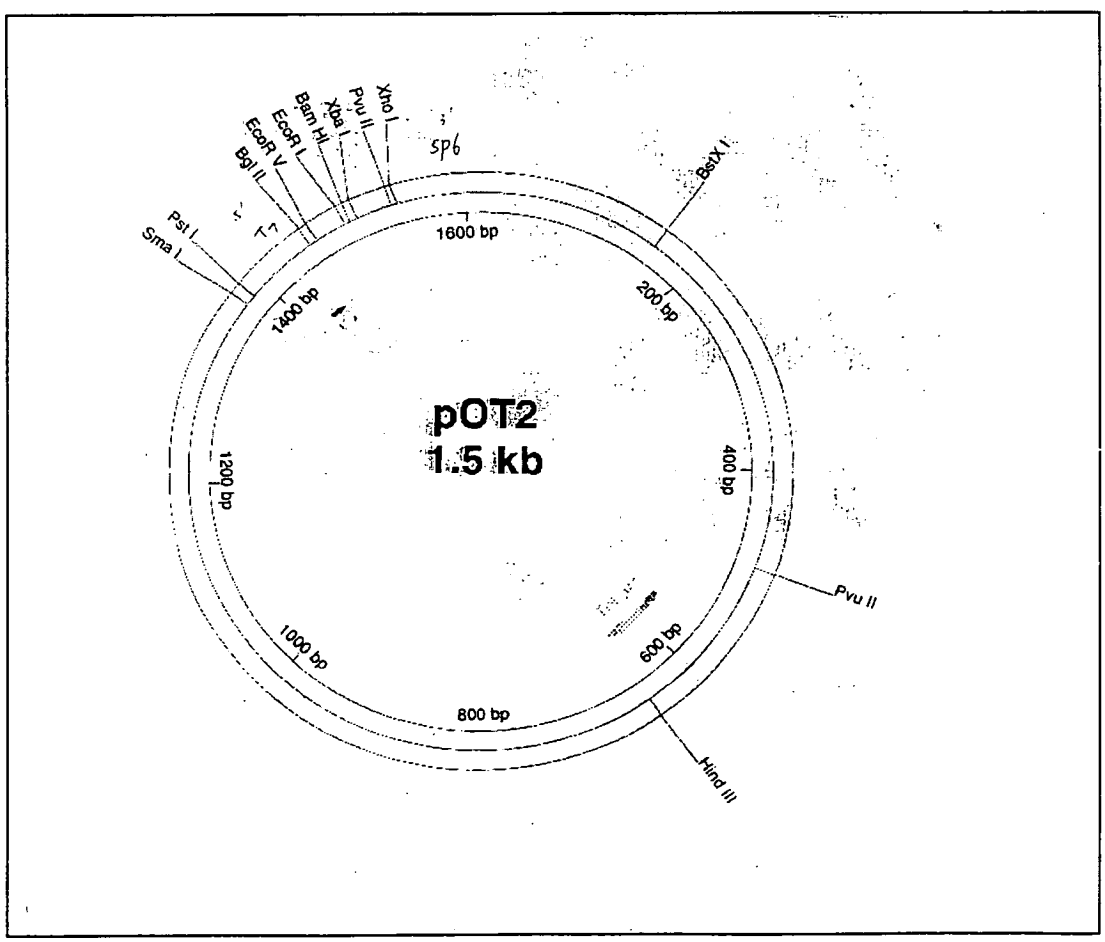
```
S  S  G  S  G  G  D  A  S  A  A  G  T  L  I  A  Q

         460        470        480        490        500
----:----|----:----|----:----|----:----|----:----|
GAGCACGGCGGGAACGAGCGCCGCCAGCAGTGGAACGATCACCTGGGACA

 S  T  A  G  T  S  A  A  S  S  G  T  I  T  W  D  N

         510        520        530        540        550
----:----|----:----|----:----|----:----|----:----|
ACAATGGAACCCTGCGATCGATCAATCCCGGCGATTGGTCCATCGAACAG

  N  G  T  L  R  S  I  N  P  G  D  W  S  I  E  Q

         560        570        580        590        600
----:----|----:----|----:----|----:----|----:----|
TGCCTCGGGCCACATCACCTGTACTTTCAGCTCGGCTGGGCCTTTCTCTT

C  L  G  P  H  H  L  Y  F  Q  L  G  W  A  F  L  F

         610        620        630        640        650
----:----|----:----|----:----|----:----|----:----|
CCTAGCCTTTTTGGCTCCACACGGTCCCTACGGCGCCCTGTGGATGCGTG

 L  A  F  L  A  P  H  G  P  Y  G  A  L  W  M  R  A

         660        670        680        690        700
----:----|----:----|----:----|----:----|----:----|
CCATGCTGCTCATTGGCTGCCTGATGATGGGCATGCATGGCTACTTGGTG

  M  L  L  I  G  C  L  M  M  G  M  H  G  Y  L  V

         710        720        730        740        750
----:----|----:----|----:----|----:----|----:----|
ACCTTCTCCTGGGCGTAGACCTCCTGGTACTTCAGGCACGTATCACCTTC

T  F  S  W  A  *

         760        770        780        790        800
----:----|----:----|----:----|----:----|----:----|
ATGCAGTTGAGCACCTTCTTGAACTGGTGGCGCGTCACGTGCAGCGGCTG


         810        820        830        840        850
----:----|----:----|----:----|----:----|----:----|
GAAAAGTGCCAGGTAGACC
```

Legend

Fig1 Original *Z14/Dmbves* cDNA is 819bp long. It is translated from 269bp to 716bp, producing a peptide of 149 amino acids.

2. Maps of pBluescript and pOT2 vector





3. Sequences and annotation of rescued plasmids

1) T1 (No.1, pst I digestion)

CCCACCGCGGTGGCGGCCCCTCGAGGTCGACCTGTCCNTACTNNNGGTGA
ANGCCCACAGCTTCCAGCGATGGCTTTCACGTTGCCGTGACATAGTCGAT
GCACATACACCCACCTCGGTGACCCTGCAGGAATAGGAAGGGTATGCAA
AATATTATACATATGCCCATGACTGTATATAATCAGGACTTACCTGNNTT
TTTTCCAGAANGNTGGGTGCCCCCTGTATTTGGTGGCCTTCAAAGGTCAT
GAATGAGTCGGTAGCCTGGAAGTATAAAATATATAATGATATAAGTGTTT
T
ACTTAGTAATGATATTTAGTATTATTAAGAACAAAATTGGTTATAAAACT
GGTAATATTTAAATTAAAAACATATCTAATAGATAGNTTTATTTTCAATA
CTTNATCATTATAGACTTTTTTATTCTTTCTTTTACTCTTTTATGTGACAAA
AAACTGAAATGATTGAAACTAATGAAACACAATTATGCCATATTTTTCAA
GATATTAATACAATTTTTTCACGAATTTATATCGTATATTGGGGCCCTATT
GTGCTAACCGCAGTNTGTATGTTNGGCCATATATTTNCGGATGGGGGGNA
TTTATTTNATGNNGAGCATGGGGCGTAATGGGGNNCCCCGAAACTTTNN
AGAATTGG

Note: Searched in gb|AE003569.3| *Drosophila melanogaster* chromosome X section 70 of 74 of the complete sequence. Sequence from 114832 to 114301. P-element inserted in the *ntf-2* gene which is from 113764 to 117526.

2) T1(No.4)

GGTCGACCTGCTTCTTGCGAAATCGCGGGCAGAATCGTAAGAGGTCGCTT
CTGGGTAGTAGGGAGGCCAGGTGAGATGACCTTTGGACTGGTTGTCATAT
CCGCCGACGGCGACGATTTCAAAACGGTTTCGGTAACGCCGCTATCCTCG
CTATCGCTGCCGGTGACCGAGGAGGACGAGGAGGAGGAGGAGGAGTCG
CTGGCGCTACTGCTTAGCTCGAGTTCCTCAAGGAACTGCTGTTCCCTGTG
CAGATCCTTTTCCAGATCTCTCCGCTGCTGCTCCTTCTTCTCGCGCTTCTTC
AGCGTGTACTTGACACTCTTCACCGCCTTCGATGGCATCCCACAGGCCAT
GTAGCGCTCGATCTGCTGCTCGTTGTTTTTCTCCACGATCCCTGAATTGTG
GCACATCTTTGCATTGGTTTCTGTGGCTCCTCTTTGAATTGATAATCCTTT
TTAGATGCTCTGTATTCTTTTTGGCACTCTTCTTTGTCTCGCTTCGCACAGC
AAAAAAATTTAATGGGGGTTACTTAACTTTCCGTTTGTATATTTTAAATAT
TGGATTTTGGGATTTTTCTACACCGCGCTTTCGGATCGGTTTGTGGGTTTT
GACTGCGATTCTAACGTGGGGTTGGGTTAATATTTGGGGGTGGTTATTTT
TTTT

Note: Searched in gb|AE003569.3| *Drosophila melanogaster* chromosome X section 70 of 74 of the complete sequence. Sequence from 145120 to 145761. P-element inserted in the *bves* gene which is from 139299 to 155397.

3) Th1

CCCTCGAGGTCGACGGCAACNNTTACTGAGAAACTGAGAAACTGAGGAG
CCGAGAAACTGAGAAAGTGCGAGACTGAGCAACTGAGAGACTGAGACTG
CGACGGCGATGCCTCAGAAGCACTCGAATTCATGGGTAACGCAGGAAAA
ATTAAATGAAGATGCTGTCACAGGCAACGTTGCGTATACGTAACGTGGG
CTGCGCGTGTTTCGCGATTATGCGTGTTTTTGTTGGCCGAAACAACAACA
AATCAGAACAACGACGACGGCGATGATGGTGCAGATAATGACGTGACTA
ACGATGATGCTTACGCTGATGTTCGTACGTCATCAGCATGATGCAGCGGC
CACATTGCGTATACGTAACGTGCGCCAAACGCGTTCGCGATTGTGTTAAT
AAATAAATGCGGCACTTGTAGAACAACAAACGAACGTATTAAAAATAAT
GACAACAAGCGGGTATTATTTCGATTGTTATTAAACAAATGCGCCGCGTG
CGTAGAAAAACAACACAAATGTGAAACGTATATTTTTTATTATTTTTTTTT
CTATAGTGTGTGTCAATTATGTGCAAGAATGCAATGCCAATTTCAACACT
AGTG
CGGCTACCACGCCACTGCGACTGCGAGCGGTNAATTGCCGCTGACACAA
TCTATAGCCTC

Note: Searched in gb|AE003513.3| *Drosophila melanogaster* chromosome X section 65 of 74 of the complete sequence. Sequence from 176266 to 176856. P-element inserted in the gene *CG32529* which is from 168912 to 207189.

4) F1

AACTTCCTTTTTTAAATTGAGCGCCATTCCAGTTAGTTGATGTCATAGCGG

AGCGCTAGTGGTCCTGTTCAAGAGGGGCTAGTGCTAAGTTTTTTCCCGAC

ACGGTTCAGTCGCCATTATGCTTGCAATAGTGAGGAGCTTGCCCTTGCCG

TTGAGGCCTACTTTTCAAGEGGATGTTCGGTTATTAAGACACAACTTGCA

TTTC

Note: Searched in gb|AE003569.3| *Drosophila melanogaster* chromosome X section 70 of 74 of the complete sequence. Sequence from 233023 to 232818. P-element inserted in the gene *CG32511* which is from 234651 to 235352.

4. Sequence of Z14/Dmbves from genome annotation

ACAAGAAAAC ATCGGATGAA TCGCCGGGCA GGAAAATCAC GGTTTCCACT
GAAAAACTAC TGATAGTTAT CACATATCCG AAATATATGT ATTTTCAATC
GAAACGAGAA CCAAATCCCC GAAAAAACGC GAATCGGAGT AAATACAAGT
TGACTTCTGC TGCAGCTGCC TAAGCGATGC AAAATGTGAC AGGCGGCAGC
AGCAGTAGCC TCATCAAAGG TTAGCTCACC AATAGCCCCA GCTAAATATT
AAACAAATAC GGAAATAAGA GTCTAAGGCC ATTAACTAAA CGGCTGACTA
AGCTGTAAAA TGCAAAGTAA TTCAAATTTC AACTAGCTAG GATATGAACC
GCAAGCAAAA AATACATTCA AGACACCAAC CGCAAAGCAC AAACATTTTG
AAAAGGGCAG CAGCAATAAC AAAACAAACC ACCACCAACA AAGGTTGAGG
AAAACCGCGC GTAAGCAACA ACAAACGAAT TAGGCGAAGG AGAGAAAGGT
GGAAGATAGA AACAAAAGGC AGATTTTTGG CATTCGCCTA GCGTGGACAA
CAACCAACCA GTACATCAAC CAATCAATTT GGTAATTAGG CAAAATACGT
GCTGCAAAAA ATAGCTCAAC GATCAGGCTG AGAGGAGAGT GACAGACTGA
AACGAAGGCA GTTCGCCTGG ATCAACGGAA TGCCCAGCAC GGCGGGCAGT
GCAGCTGGCG TTGGTATGGG CGCCCTGATC AACAGTGCCG GCAGCAGCGC
CAGTAGTGTC ATGGGCATTG GCATGGGCAG AGCAGCTGCT ACGGGAGCAG
GAGCACCAGG CAGTTCGGGA TCTGGAGCAG ATGCCTCCGC CGCCGGCACT
TTAATCGCCC AGAGCACGGC GGGAACGAGC GCCGCCAGCA GTGGAACGAT
CACCTGGGAC AACAATGGAA CCCTGCGATC GATCAATCCC GGCGATTGGT
CCATCGAACA GTGCCTCGGG CCACATCACC TGTACTTTCA GCTCGGCTGG
GCCTTTCTCT TCCTAGCCTT TTTGGCTCCA CACGGTCCCT ACGGCGCCCT
GTGGATGCGT GCCATGCTGC TCATTGGCTG CCTGATGATG GGCATGCATG
GCTACTTGGT GGCCTTTGCA CCGGACGTCA TCCTGTGGTC GGGCATGGGA
CTTTTTGTCA ACTTCATCTA TCTCGTCGTG GTGCTATGCC GGCTGAGGCC
TGTGCGATTC GAGCAGGAAA TCGAAGCGGT CTACCTGGCA CTTTTCCAGC
CGCTGCACGT GACGCGCCAC CAGTTCAAGA AGGTGCTCAA CTGCATGAAG
GTGATACGTG CCCTGAAGTA CCAGGAGGTC TACGCCCAGG AGAAGGTCAC
CAAGGTCGAC AGCCTGTCGC TGGTGCTGAG CGGCAAACTG GTGGTGTCGC
AGCATCAGCG CGCCCTGCAC ATTGTGTTTC CCCATCAGTT CCTAGACTCG
CCAGAATGGT TTGGCGTCTC GACCGATGAC TACTTTCAGG TCTCCATCAT
GGCCATGGAG GAGTCGCGGG TGCTGATCTG GCATCGCGAC AAGCTCAAAT
TGTCAATTAT GGCCGAGCCC TTCTTGCAGA CCGTCTTCGA TCACATTCTG
GGCCGGGATG TGGTCAAGAA GCTGATGCAG GTCACCCAGG TGAGCGAGTC
GATAGCCAGC AATGGCTTCC TGCCCTCTGG TGGATATGCT GAGGATGCGG
AGGACAAGCC CATGTTGATA CTAAAGAAGA GTGTGGATGT GGGACACGGA
CTGACGGCCC TGATCAACCG GCAGCTACAG GACGAGCATG TTCCTTTACT
CGGTCGCACG TACAAACAAC AACAACAACA ACAACAACTA CTGCTACTGC
AACAACAACT AGAACAACAA CGCGTACAAG AAGCAACAAC CAGCGCCAAC
AACATCGAGC AAAGTGCAAT CTGAATTTAT GCAACCGCCC GCTGCTGCTC
AACTTGCCAC GAACAGAAAT CAGAGCACCC ATCCGTCCCT TCACCTTTGA
CCTGCACAAG AACTACCAGA AAAACAACCC CACCCACACA TGCAAACAGC
CATCAGCCAT CAGTCATCAG TCAGTCAGTC ATCAATCATG CGCAGCCACA
CCAACAGCAA CATCGATCAC GATCATCAGA AAAGAGCGAA AAGAGCTGTC
GAAGATATCT GGAAGCTATC CATCTAAATC CGCATCCTAA GTTCCATATA
TAATAATCTC ATCTTTAGCA TAGCAACTAG CACTAGCTAA ACAAGCATCT
AACACATCTA ATCGTTCTAA TAGCAAACTT TGTATTGCCC CCACAACTGC
AAACAATTAG CCAGAGACTT GAGTTTTTGT GTAAAGTAAT TGACTTTGGG
AAACATAAAC TTTTCGTCTC AGCGGAAAAG TGCTAAAACA CCGTGTTACC
ATTTAGAAGA AACTTGGAGA AGTTACTAAA GAAGTTTACA GGCGTGAAAA
AAAAAATGAT TCGCCATAAA ACATAAGTAG TCTTTTATCA AACGTACACT
GACTTAAATC TAGGAAACTG GGAGAACTGC CTAAAATGAT TTGTAGTTTC
ACCTGACAAC TCTATTTAGC AGTGTAGAGA AAAACGTAAC ATCGTAGACC
GTTATTaaaa aaaaaaaaca agaaaaaaaa
ctattcaatt aaacccgtag taatatactt gtattccatg aagataaaga

aaccaattat ttcacaaaga aaacacaaaa acaagtagga aacttactta
cattgcaaca ctgttgaatt caatatgctc cctgaatcct tcgcaaaaac
tatcgttaac tgcagtcaaa ccagttgtaa aaattgtaat ttattttgac
ttatcatggt tgtatgtatg ttgagttgta gtcagacaag acacttgatt
gcacatgaat tagtttcagt ttgagtgggg ttttcaattg aacacgatca
gaaagccaac caaggtttaa atttgaaatt aattgatcgt ggttttgagt
ttgaattgga attagtaata tttgatatat cacgcagtat acaaacattt
tatattagtg gaagtttttg agtcgatctt ctacaatata aacgattatt
acactcgtgt aataaaggaa acctttgtag acgcagacta ttttccaaga
acctattata agtataatga ctagcattga aactggtttg attacagctc
ttcagcttca attaccgatt ctagatctca agatcgatca gtctagtgac
aggtgatagg taattaatga acttttccgc tgggccagga gttttcaacg
ctttatttga ttaaattctt atttcttacc gcttggcatt tactggaaat
ttactgtgct ggcacatcaa tcaagtctct aaatcttcca ttaacacatt
attattttat ttgtatttct atatgcatac gtattttgcg ttgattcgtt
gattgatttg catacaaatt gagcttgaag aaagcgtgaa aactaattag
aattaatatc gaataatatt tgataatgtt aagcaataca tagcctaagt
tgttgtacat acgctatata tatatatttg ctgtacataa tcccgagtgc
cctcaaatcc gagaaccaca cccccgtccc ctaatctcca ctaatactcc
cccaagaaag tgtaataaac gtagtggaat caatgtgaat gtaaacaagc
caaaaaccga aagctaataa aacaaaaaaa aaacacacat aatcaa

Legend

Letters in capital is the sequence we submitted to gene bank.