

**CHARACTERISATION OF NEWLY ISOLATED GENES
EXPRESSED IN THE OVARY OF
*DROSOPHILA MELANOGASTER***

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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.**

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ABSTRACT

Two genomic clones called λ ov5 and λ ov2 were isolated from a differential screen and found to encode ovary enriched transcripts. The genes within λ ov2 and λ ov5 have been initially characterised with the view to ultimately determining their function(s) during oogenesis.

λ ov5 consists of two EcoRI sub-fragments denoted ov5A and ov5B. Each of these fragments detect ovary enriched transcripts on Northern blots. The ov5A probe was used to screen a λ gt11 ovary cDNA library and three size classes of cDNA called λ ov5A1, λ ov5A2 and λ ov5A5 were identified. Each cDNA detects at least two ovary enriched transcripts, approximately 1.7 kb and 1.4 kb in length. The ov5A5 cDNA also hybridises with two additional ovary specific transcripts approximately 0.8 kb and 0.2 kb in length. The organisation of these ovary transcripts within the ov5A genomic region was determined by cross-hybridisation of cDNAs to Southern blots of restriction enzyme digests of ov5A sequences. The ov5B sub-fragment detects an ovary specific transcript approximately 1.4 kb in length. Full length cDNA copies of this transcript were isolated.

In situ hybridisation to whole mounts of *Drosophila* ovaries were carried out using digoxigenin-labelled ov5A2 and ov5B1 cDNA probes to determine the spatial and temporal distribution of the ovary enriched transcripts during oogenesis. ov5A2 detects transcripts in the nurse cell cytoplasm and in oocytes from stage 7 onwards. ov5B1 strongly detects transcripts in stage 10B nurse cell cytoplasm and in the degenerating nurse cells.

Two EcoRI sub-fragments of λ ov2, ov2A and ov2B, hybridise with female cDNA but not with male cDNA. ov2A detects two ovary and embryo specific transcripts approximately 4.0 kb and 3.0 kb in size and also a 1.6 kb sized male-specific transcript. ov2B detects two transcripts estimated to be 3.5 kb and 2.5 kb in length which are expressed exclusively in the ovary.

λ ov5 and λ ov2 were mapped on chromosome 3R at positions 88B/C and 89B respectively.

Within the cytological region 88-89, two female sterile mutations called *Spindle-B* and *fs293^{Y5}* have been genetically mapped. Experiments with a set of deficiency chromosomes were carried out to determine whether or not the ov5 or ov2 genomic sequences mapped close to these mutations.

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ABBREVIATIONS

amp	Ampicillin
ATP	Adenosine-5'-triphosphate
bp	Base pair
BSA	Bovine serum albumin
°C	Degrees centigrade
cDNA	Complementary deoxyribonucleic acid
Ci	Curies
cm	Centimetre(s)
cpm	Counts per minute
dATP	Deoxyadenosine-5'-triphosphate
dCTP	Deoxycytosine-5'-triphosphate
dGTP	Deoxyguanosine-5'-triphosphate
dNTP	Deoxynucleotide-5'-triphosphate
dTTP	Deoxythymidine-5'-triphosphate
dH ₂ O	Distilled water
DEAE	Diethylaminoethyl
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Diaminoethanetetra-acetic acid
EMS	Ethyl methyl sulphonate
fs	Female sterile
FSB	Formaldehyde sample buffer
g	Gram(s)
[³ H]	β-emitting isotope of hydrogen

HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
kb	Kilobase
Klenow	Large fragment of DNA polymerase I
Krpm	Kilorevolutions per minute
λ	Lambda bacteriophage
λ ov5	λ Charon 4A vector containing the 10.7 kb ov5 fragment
λ ov2	λ Charon 4A vector containing the 13.7 kb ov2 fragment
L	Litre
M	Molar
mA	Milliampere(s)
mAP	Messenger Affinity Paper
mCi	Millicurie(s)
mg	Milligram(s)
ml	Millilitre(s)
mm	Millimetre(s)
mm/L	Millimoles per litre
mM	Millimolar
mmol	Millimole(s)
min	minute(s)
MOPS	Morpholinopropanesulphonic acid
mRNA	Messenger ribonucleic acid
m wt/ml wt	Molecular weight
ng	Nanogram(s))
nmol	Nanomole(s)
OLB	Oligo labelling buffer
OD	Optical density
ov2A	3.25 kb EcoRI sub-fragment contained within λ ov2
ov2B	4.2 kb EcoRI sub-fragment contained within λ ov2

ov5A	5.0 kb EcoRI sub-fragment contained within λ ov5
ov5A1	1.6 kb cDNA which hybridises with the ov5A genomic fragment
ov5A2	1.8 kb cDNA which hybridises with the ov5A genomic fragment
ov5A5	0.9 kb cDNA which hybridises with the ov5A genomic fragment
ov5B	5.7 kb EcoRI sub-fragment contained within λ ov5
ov5B1	1.4 kb cDNA which hybridises with the ov5B genomic fragment
[³² P]	β -emitting isotope of phosphorous
pers.comm.	Personal communication
PEG	Polyethylene glycol
%	Percentage
pg	Picogram(s)
pH	$-\log_{10}$ (hydrogen ion concentration)
Poly (A) ⁺ RNA	Polyadenylated ribonucleic acid
psi	Pounds per square inch
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal ribonucleic acid
rpm	Revolutions per minute
[³⁵ S]	β -emitting isotope of sulphur
SDS	Sodium dodecyl sulphate
Tris	Tris(hydroxymethyl)-amino-methane
Triton-X-100	Octylphenoxypolyethoxyethanol
TCA	Trichloroacetic acid
U	Unit(s)
UTP	Uridine-5'-triphosphate
UV	Ultraviolet
μ Ci	Microcurie(s)
μ g	Microgram(s)

μl	Microlitres(s)
μM	Micromolar
μmol	Micromole(s)
V	Volts
v/v	Volume per volume
w/v	Weight per volume

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CHAPTER ONE
INTRODUCTION

1.1 Female Reproductive System in *Drosophila melanogaster*

The female reproductive system of *Drosophila melanogaster* has been studied at the morphological, genetic and molecular levels (reviewed King, 1970; Mahowald and Kambyzellis, 1980; Bownes and Dale, 1982; Mahowald and Hardy, 1985). It has been shown that the female reproductive system is derived from the genital disc in the embryo. At the posterior of the early embryo pole cells are formed which will give rise to germ cells in the female gonad. Very little is known about the growth of the gonad in the embryo but it can be observed and cell divisions have already occurred as early as 16 hours after egg deposition at 25°C. During the pupal stages complex cellular interactions are involved in the differentiation of the adult ovary. At eclosion only previtellogenic (i.e., there has been no yolk deposition) stages of oocyte development are present. In the adult female there are a pair of ovaries connected by a common oviduct (Figure 1.1A). Within each ovary there are 15-20 parallel ovarioles held together at their anterior ends by terminal filaments. The ovarioles are covered by a network of muscle fibres (peritoneal sheath).

The oocyte is derived from a stem cell at the tip of the germarium. The germline-derived stem cell divides to produce a daughter stem cell and an oogonium. The oogonium proceeds through eight incomplete cell divisions to produce a complex of 15 nurse cells and the oocyte, connected by intercellular bridges. The nurse cell-oocyte complex (cystoblast) is covered initially by a monolayer of somatically derived follicle cells. Together, the oocyte, nurse cells and follicle cells make up the egg chamber, and in each ovariole a series of different stages of egg chamber development can be observed. King (1970) divided oogenesis into 14 stages based on the size, shape and location of the egg chamber in each ovariole (see Figure 1.1B). In the germarium, stage 1 egg chambers can be observed using electron microscopy. During the previtellogenic stages of oocyte development, from stage 2 to stage 7 there are marked morphological events in the egg chamber which changes from being spherical to oval. During the vitellogenic stages (stage 8 onwards) there is a 10,000 fold increase in size of the oocyte due mainly to the uptake of yolk proteins from the follicle cells and the haemolymph. The follicle cells gradually surround the oocyte between stages 7-13 of oogenesis and exclude the nurse cells which are gradually

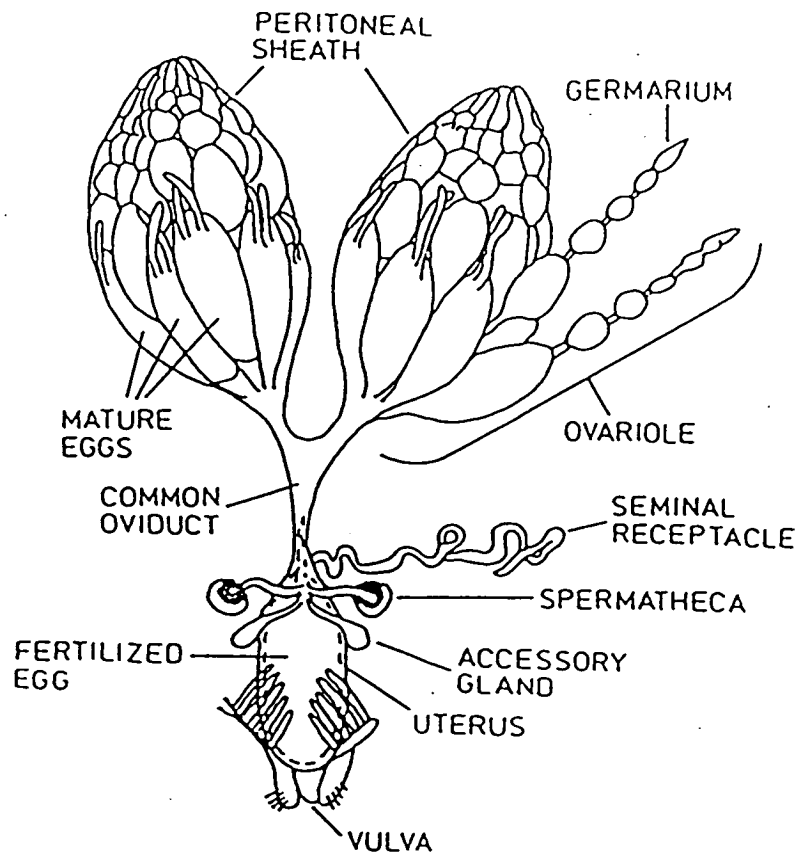
Figure 1.1

A. Structure of the Adult Female Reproductive System in *Drosophila melanogaster* (adapted from Bownes, 1982b).

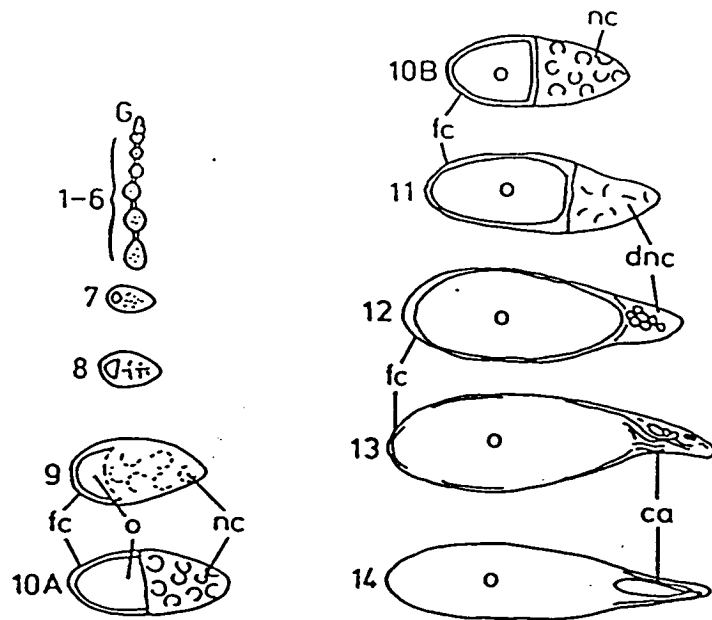
B. Stages of Oogenesis in *Drosophila melanogaster* (based on King, 1970)

- 1-14 = stages of oogenesis
- nc = nurse cells
- dnc = degenerating nurse cells
- ca = chorionic appendages
- fc = follicle cells
- o = oocyte

A



B



degenerating. During stages 9 and 10 the follicle cells secrete the protective vitelline membrane around the oocyte and in the final stages (11-14) the hard outer egg shell layers are formed by the ordered secretion of chorion proteins.

As the mature oocyte passes into the uterus, it is fertilised by sperm stored in the ventral receptacle and spermathecae.

1.2 Oogenesis

In the cystoblast there is an ordered network of intercellular bridges connecting the nurse cells and oocyte. There are eight cells that are connected to only one other cell, four cells connected to two cells, two cells connected with three other cells and finally two cells have four intercellular bridges and are connected to each other and three other cells (Mahowald and Strassheim, 1970).

It is usually one of the two cells with four intercellular bridges that becomes the pro-oocyte. By the time the 16 cell cyst has passed the middle of the germarium, one of the two cells will lose its synaptonemal complex and switch to nurse cell development; the other will have gone through meiosis forming a single pro-oocyte (Carpenter, 1975). The posterior position of the oocyte within the egg chamber determines the anterior-posterior polarity of the egg and this is important later on for the establishment of the polarity of the embryo. The nurse cells synthesise most of the RNA (mainly rRNA) required by the oocyte (King and Burnett, 1959; Brown and King, 1964). The nurse cells also contribute mRNAs to the oocyte, and some of these have been shown to encode positional information required for early embryogenesis. For example *bicoid* mRNA is synthesised in the nurse cells and is localised along the anterior periphery of the oocyte by stages 5-7 of oogenesis (Berleth *et al.*, 1988) and is thought to be the primary determinant of anterior-posterior polarity in the embryo (discussed in Section 1.3). Most of the maternal components contributed to the oocyte are transported late in oogenesis (stages 7-13) coinciding with degeneration of the nurse cells and a large increase in oocyte volume. The transport of cytoplasm from the nurse cells to the oocyte is through the intercellular bridges, possibly mediated by an osmotic pressure flow requiring the action of actin filaments (reviewed by Gutzzeit, 1986;

Cooley, 1990).

The nurse cells become polyploid, reaching 512 and 1024 times the haploid DNA content depending on their position in the egg chamber and this large increase in genome size presumably reflects the demands on the nurse cells to synthesise the large amount of products required by the oocyte (Jacob and Sirlin, 1959). The oocyte nucleus shows a brief period of transcriptional activity between stages 9 and 10 (Mahowald and Tiefert, 1970) but the majority of stored gene products are derived from the nurse cells. There has only been one gene cloned which is expressed specifically in the oocyte nucleus, called *fs(1) K10* (Wieschaus *et al.*, 1978; Haenlin *et al.*, 1987) and it is involved in dorso-ventral pattern formation in the egg and embryo.

There is still very little understood about how the germline derived cells (nurse cell-oocyte complex) interact with the somatically derived follicle cells. In the germarium of the adult fly there are approximately 1000 pre-follicle cells from which the follicle cell sub-types are derived (reviewed by Margaritis, 1985). There is a complex series of ovarian follicle cell movements, during which the pre-follicle cells (also called follicular epithelial cells) differentiate into different sub-types (Figure 1.2A). There are 6-10 follicle cells (called border cells) which migrate between the nurse cells and eventually align themselves along the anterior tip of the oocyte (Figure 1.2B). The nurse cells are covered in a layer of squamous follicle cells and so far their function is unknown. Surrounding the egg chamber are the columnar follicle cells and by stage 11 of oogenesis they will have differentiated into three different types; the anterior pole follicle cells, the posterior pole follicle cells and the columnar main body cells. The columnar follicle cells secrete the egg shell (discussed later in detail).

During the vitellogenic stages, three major yolk proteins are synthesised and are called YP1, YP2 and YP3 (Bownes and Hames, 1977; Warren and Mahowald, 1979). The yolk proteins aggregate in the oocyte to form α -spheres (stages 8-10) and β -spheres (stages 13-14) which also contain glycogen (Bownes, 1982). The sites of synthesis of the major yolk proteins are in the adult female fat body and the ovarian follicle cells that surround the developing oocyte (Bownes and Hames, 1978; Brennan *et al.*, 1982). The proteins are secreted from the fat body into the haemolymph, and from the follicle cells, probably into the interfollicular spaces (Brennan *et al.*,

Figure 1.2

A: Differentiation Of Ovarian Follicle Cells

(based on Margaritis *et al.*, 1980 and adapted from Logan and Wensink, 1990)

Each differentiation lineage is indicated by arrows. Cell type abbreviations are shown in parentheses. Under each cell type name the approximate number of cells (n) and the approximate developmental stage of appearance(s) are indicated. (?) denotes unknown information.

B: Drawings Of Sectioned Egg Chambers

(based on King, 1970 and adapted from Logan and Wensink, 1990)

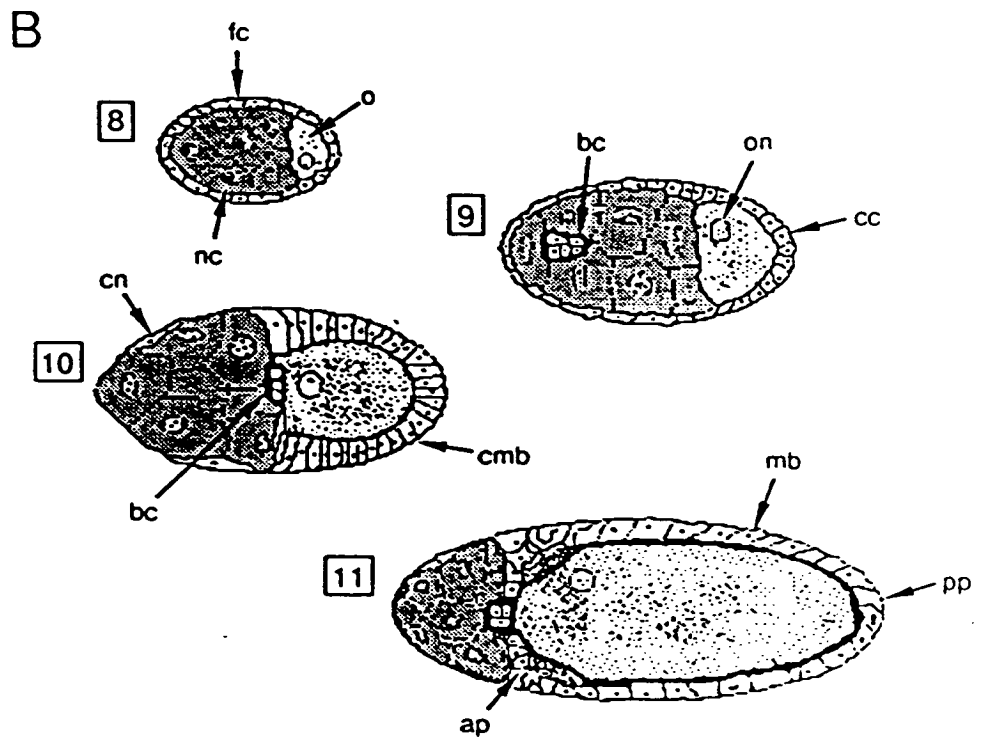
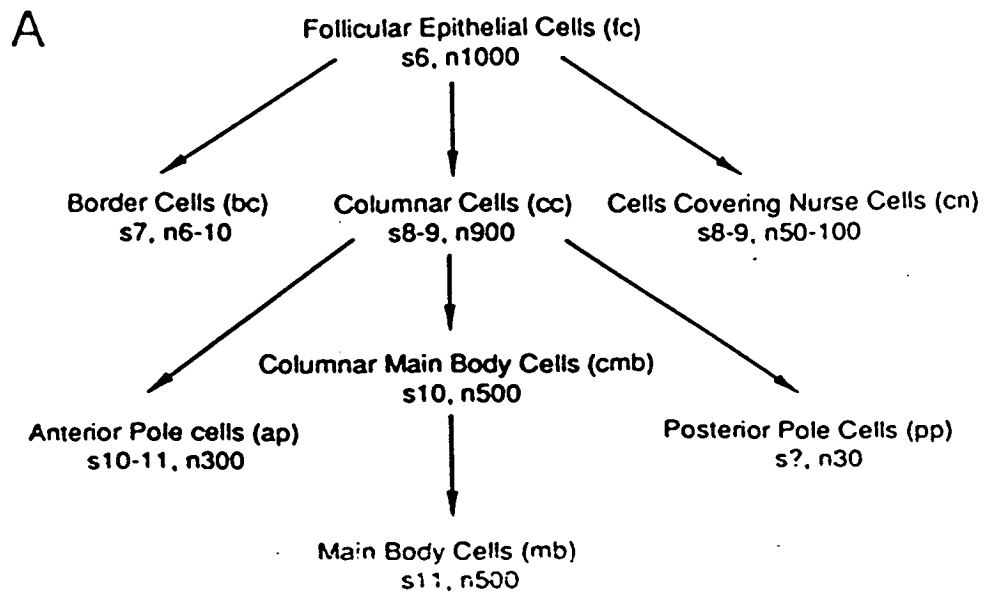
The boxed numbers indicate developmental stages.

Cell type abbreviations are detailed in Figure 1.2A.

o = oocyte

nc = nurse cell

on = oocyte nucleus



1982) and are selectively uptaken by the oocyte by receptor mediated endocytosis (Mahowald, 1972; Giorgi and Jacob, 1977). The yolk protein gene family is located on the X chromosome (Barnett *et al.*, 1980). The genes for *yp1* and *yp2* are closely linked and their expression in the female fat body and ovarian follicle cells is controlled by at least three regulatory elements located between and within these divergently transcribed genes (Garabedian *et al.*, 1986; Logan *et al.*, 1989; Logan and Wensink, 1990). The *yp3* gene is located at a distance of more than 1000 kb from the *yp1* and *yp2* genes. The *cis* acting sequences controlling the expression of *yp3* in the fat body and ovarian follicle cells have been localised to within a 3.1 kb DNA fragment including the *yp3* gene and 5' flanking sequences (Liddell, 1989). Logan and Wensink (1990) have shown that in the ovary the genes for *yp1* and *yp2* are expressed only in columnar follicle cells and their derivatives. There are two enhancers regulating this expression pattern called ovarian enhancer 1 which is located in the intergenic region between *yp1* and *yp2* and ovarian enhancer 2 which is located within the first exon of *yp2*. Transcripts from the *yp* genes are first seen after eclosion and reach a plateau after 24 hours (Barnett and Wensink, 1981), and the appearance of yolk protein transcripts coincides with the appearance of yolky oocytes in the adult ovary (Bownes, 1982).

By stage 11 the nurse cells are degenerating, and the oocyte has greatly expanded in size, occupying most of the egg chamber (Figure 1.1B). The follicle cells have differentiated into their sub-types and the columnar cells surround the oocyte, excluding the degenerating nurse cells. The chorion layers are secreted by the follicle cells during the later stages of oogenesis (stages 8-14). The fine structure of the *Drosophila* chorion is illustrated in Figure 1.3A. The first protective layer to be formed around the oocyte is the vitelline membrane. There are at least ten classes of vitelline membrane proteins (VMP) (Petri *et al.*, 1976; Fagnoli and Waring, 1982) but only a few VMP genes have been characterised. Burke *et al.*, (1987) have isolated two cDNA clones that encode VMPs synthesised by the follicle cells during stages 8-10. One clone is thought to encode a major protein component of the vitelline membrane and the other clone encodes a protein of unknown function which is likely to be an intracellular protein as it lacks a signal peptide sequence. Gigliotti *et al.* (1989) have isolated another VMP gene by carrying out a chromosomal

Figure 1.3

The Structure Of The *Drosophila* Chorion

A: Fine Structure Of The *Drosophila* Chorion

The *Drosophila* chorion consists of five layers which are sequentially deposited during egg shell formation.

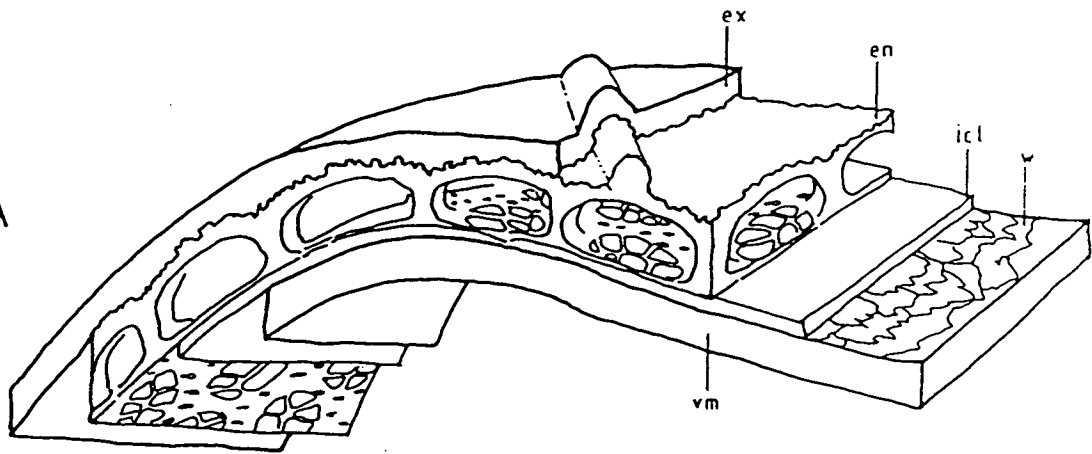
vm	=	vitelline membrane
w	=	waxy layer
icl	=	inner chorionic layer
en	=	endochorion
ex	=	exochorion

B: Specialised Features Of The Chorion Surface

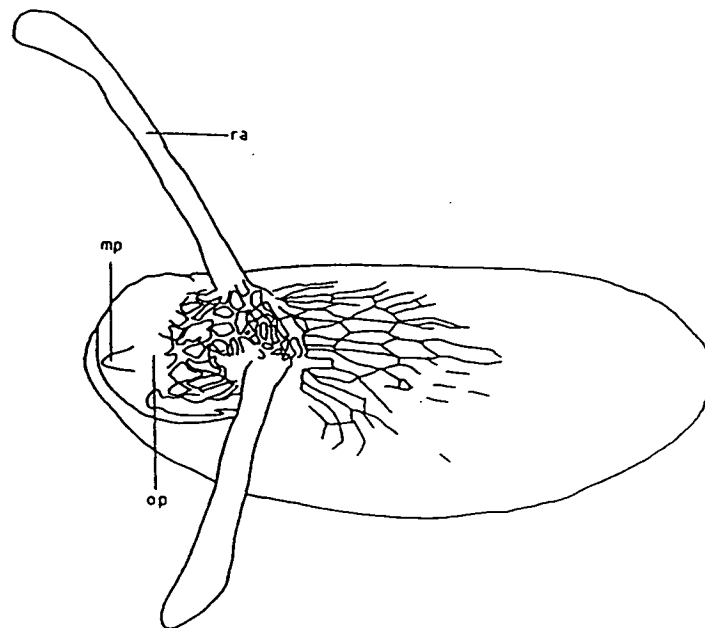
ra	=	respiratory appendages which facilitate embryonic respiration
mp	=	micropyle, which is the site of sperm entry
op	=	operculum, through which the larva hatches

Adapted from Margaritis *et al.*, 1985.

A



B



walk into the 32E region, within which four female sterile mutations affecting egg shell formation have been mapped.

Vitelline membrane formation begins at the anterior end of the oocyte and slightly later the posterior vitelline membrane is formed. Oil droplets are secreted during stage 10B to form a waxy surface on top of the vitelline membrane. The protective shell of the egg is constructed from the temporally regulated secretion of chorion proteins (Waring and Mahowald, 1979) which are synthesised by the columnar follicle cells and border cells. The inner chorionic layer has columnar structures which support the thick outer layer of endochorion and create air spaces which are thought to be involved in embryonic respiration. The final layer to be deposited is the exochorion. The exochorion has specialised structures (Figure 1.3B). The anterior pole follicle cells appear to form the respiratory appendages and operculum (through which the larva hatches) of the mature oocyte.

There is a great deal known about the chorion proteins. There are two chorion gene clusters, one on the third chromosome and one on the X chromosome (Spradling and Mahowald, 1980; Spradling *et al.*, 1980). To meet the demand for vast amounts of chorion protein during the late stages of oogenesis, a developmentally regulated system of sequential gene amplification has evolved to enable the follicle cells to synthesise sufficient amounts of chorion proteins in the appropriate order (Spradling and Mahowald, 1980; Spradling, 1981; Spradling *et al.*, 1987). There are at least 20 structural genes which are members of the chorion gene family, that are required for proper egg shell formation, and choriogenic activity lasts for 5-6 hours with some genes being expressed early and others expressed only in the later stages of chorion formation. The X-linked *s36* and *s38* chorion protein gene transcription begins at stage 11 of oogenesis, peaking at stage 12, whereas the third chromosome cluster of *s13*, *15*, *16*, *18* and *19* genes is transcribed at stages 13 and 14. Individual genes within each cluster have unique mRNA accumulation profiles rather than being under coordinate control. Mariani *et al.* (1988) have shown that at least one chorion gene (*s15-1*) is expressed only over a 2-hour period and is controlled by *cis*-acting, positive and negative regulatory sequences.

During the final stages of oogenesis there is visible polarity in the egg. There are

cytological markers at the posterior end of the ooplasm which are termed polar granules. Polar granules first appear at stage 9 (less than $0.2\ \mu\text{m}$ in diameter) and gradually increase in size (up to $0.5\ \mu\text{m}$ in diameter) in the later stages of oogenesis (Mahowald, 1962). The polar granules consist of RNA and protein and are the possible determinants of the germline, because these granules will induce nuclei in that region to become germ cells (Illmensee *et al.*, 1976). The polar granules become incorporated into cells in the posterior pole of the embryo (pole cells) at cellular blastoderm formation. The developmental potential of pole cells has been illustrated by transplantation experiments. For example, when pole plasm containing polar granules was transplanted into the anterior region of a 2 to 4 nuclei stage embryo, this embryo later developed pole cells in the anterior end (Illmensee and Mahowald, 1976). If the posterior end of the egg is treated with UV light, pole cell formation is disrupted and the resulting adult will lack a functional germline. The disruption of pole cells caused by the UV treatment directed at the posterior egg cytoplasm can be rescued by the injection of wild type cytoplasm extracted from the posterior end of normal eggs (Okada and Kobayashi, 1987). Mutations affecting pole cell formation will be discussed in the next section.

By stage 14 of oogenesis, the oocyte is fully mature and ready to be fertilised as it passes into the uterus. Meiosis is completed at fertilisation, prior to the fusion of the oocyte nucleus and sperm. After fertilisation, 13 mitotic divisions occur before cell membranes and nucleoli are formed. The egg will provide most of the RNA, protein and cellular apparatus required until cellular blastoderm formation, when the majority of zygotic RNA synthesis begins. The maternal genome contributes positional information to the egg and this information is required for the localised activation of zygotic genes necessary for the formation of the early embryo. This will be discussed in detail in the next section.

The processes involved in constructing a fully mature egg in *Drosophila* are complex and it is important to remember that these processes form part of a concerted program of a whole organism, involving a large number of activities within and outwith the ovary and environmental conditions. For example, there are several activities in the adult female responsible for regulating yolk protein synthesis. It is known that the hormones 20-hydroxyecdysone and juvenile hormone

both affect the levels of yolk protein gene transcription (Bownes and Blair, 1986), as does the availability of a food source (Bownes *et al.*, 1988). The regulatory genes of the somatic sex determination pathway must be expressed in the correct mode to ensure the correct sex-specific expression of yolk protein genes in the adult female fat body (Bownes and Nöthiger, 1981).

1.3 Genetic Analysis of Oogenesis

A lot of information about the processes involved in oogenesis has been obtained using genetic approaches. Genetic screens involving X-ray and EMS induced mutations on the 1st, 2nd and 3rd chromosomes of *Drosophila melanogaster*, have generated a large number of female sterile mutants (e.g. Bakken, 1973; Gans *et al.*, 1975; Mohler, 1977; Komitopoulou *et al.*, 1983; Schüpbach and Wieschaus, 1989). These female sterile mutations can be classed into two categories of defect: (I) those which affect egg development resulting in abortive egg production or obviously abnormal eggs and (II) the maternal effect mutations which allow mature eggs to be fertilised but disrupt early embryogenesis. King and Mohler (1975) classified female sterile mutants according to which stage of oogenesis had been disrupted (mainly class I mutations) (Table 1.1) and Schüpbach and Wieschaus (1989) classified category (II) mutants according to the stage of embryonic development affected by the various maternal effect mutations (Table 1.2). Within the first category of female sterile mutations it is noticeable that many of the mutations are pleiotropic, i.e., they affect other processes as well as disrupting oogenesis. For example, the partial deletion of the gene cluster coding for ribosomal RNA (rRNA) severely affects nurse cell function, as these cells are unable to meet the demands for the vast amounts of rRNA required by the developing oocyte and as a result females homozygous for the *bobbed* mutation lay fewer eggs (Mohan and Ritossa, 1970).

Mutants affecting the development of the ovary are classified according to whether the defect involves the ovary itself (ovary autonomous mutants) or is caused by a defect outwith the ovary (ovary non-autonomous mutants). As a consequence of the complexity of oogenesis (of the interactions within the ovary and of the ovary with the rest of the organism) it is often a difficult process to characterise newly isolated female sterile mutants. After determining what visible

Table 1.1

Classification Of Female Sterile Mutants
Based on King and Mohler (1975)

Category I Mutants: Female Sterile Mutants With Ovarian Defects

<u>Class</u>	<u>Mutant</u>	<u>Symbol</u>	<u>Defect</u>	<u>Reference</u>
1. Germ cell determination	<i>grandchildless</i>	<i>gs</i> ⁸⁷	Mutants lay eggs that develop into adults generally lacking gonads	Thierry-Mieg <i>et al.</i> , (1972)
	<i>Bicaudal-D</i>	<i>Bic-D</i>	Role in differentiation of oocyte from nurse cells. Involved in <i>nanos</i> localisation	Mohler and Wieschaus (1986) Wharton and Struhl (1990)
2. Differentiation of ovarioles	<i>female sterile(2)A10</i>	<i>fs(2)A10</i>	disruption of ovariole differentiation	Bakken (1973)
3. Cystocyte divisions	<i>female sterile(2)B</i>	<i>fs(2)B</i>	cell division is complete therefore cystocytes have no inter-cellular bridges; tumorous phenotype	Yarger and King (1971)
4. Meiosis	<i>c(3)G</i> ⁶⁸	<i>c(3)G</i> ⁶⁸	defective in chromatid crossing over	Hall (1972)

<u>Class</u>	<u>Mutant</u>	<u>Symbol</u>	<u>Defect</u>	<u>Reference</u>
5. Nurse cell function	<i>chickadee</i>		mutant females have undersized oocytes that remain attached to persistent nurse cells	Cooley <i>et al.</i> , (1990)
	<i>kelch</i>			
	<i>bobbed</i>	<i>bb</i>	partial deletion in genes coding for rRNA. Eggs are produced at a reduced rate by females homozygous for <i>bb</i> . Pleiotropic mutation affecting all cells.	Mohan and Ritossa (1970)
6. Follicle cell development	<i>tiny</i>	<i>ty</i>	Follicle cell migration affected and consequently their synthetic activity. Homozygous <i>ty</i> flies are late to eclose and have a small body covered in thin, short bristles	King (1970)
7. Vitellogenesis	<i>fs(1)1163</i>	<i>fs(1)1163</i>	homozygous females are defective in Yolk Protein-1 production, though this might not be the primary defect.	Gans <i>et al.</i> , (1975) Saunders and Bownes (1986)

<u>Class</u>	<u>Mutant</u>	<u>Symbol</u>	<u>Defect</u>	<u>Reference</u>
8. Ovulation	<i>hairy wing</i>	<i>hw</i> ^{49a}	failure of mature oocytes to pass from the oviduct to the uterus. Has other pleiotropic effects.	Holzworth <i>et al.</i> (1974)
9. Chorion synthesis	<i>ocelliless</i>	<i>oc</i>	mature oocytes produced which contain greatly reduced amounts of several major chorion proteins including c36 and c38. Fewer eggs are laid. Pleiotropic mutation.	Spradling <i>et al.</i> (1979)
10. Egg shape	<i>fs(1)K10</i>	<i>fs(1)K10</i>	dorsalisation of egg (and embryo). Ventrally fused eggs with enlarged chorionic appendages.	Wieschaus <i>et al.</i> (1978)
	<i>Spindle-B</i>	<i>Spn-B</i>		Haenlin <i>et al.</i> (1987)
				Tearle and Nüsslein-Volhard (1987)
				Nüsslein-Volhard (1990)
	<i>gurken</i>	<i>grk</i>	ventralisation of egg and embryo	Schüpbach and Wieschaus (1989)
	<i>torpedo</i>	<i>top</i>		Price <i>et al.</i> (1989)

Table 1.2

Classification Of Maternal Effect Mutations Which Disrupt Embryonic Development (Based On Schüpbach and Wieschaus, 1989)

Category II Mutants: Eggs Are Laid Which Fail To Develop Properly

<u>Class</u>	<u>Mutant</u>	<u>Symbol</u>	<u>Defect</u>	<u>Reference</u>
1. Defects occurring during pre-syncitial stages	<i>fruh</i>	<i>frh</i>	eggs show no visible signs of development; irregular cellular blastoderm	Schüpbach and Wieschaus (1989)
2. Syncitial blastoderm arrest	<i>cortex</i>	<i>cort</i>	narrow halo of clear cytoplasm appears at egg periphery	Schüpbach and Wieschaus (1989)
3. Irregular cellularisation at blastoderm stage	<i>valois</i>	<i>val</i>	some embryos form pieces of cuticle 80-90% of embryos fail to cellularise	Schüpbach and Wieschaus (1989)
4. Abnormal cell behaviour at onset of gastrulation - <u>Maternal Effect Loci Affecting Embryonic Pattern</u>				
A. Mutants Affecting the Formation of the Anterior-Posterior Pattern				
a) anterior group	<i>bicoid</i>	<i>bic</i>	embryos lack anterior structures (Primary anterior determinant)	Frohnhofer and Nüsslein-Volhard (1986) Driever and Nüsslein-Volhard (1988a, 1988b, 1989) Berleth <i>et al.</i> , (1988)
	<i>exuperentia</i>	<i>exu</i>	required for the	
	<i>swallow</i>	<i>swa</i>	correct localisation of <i>bicoid</i>	Frohnhofer and Nüsslein-Volhard (1987) Berleth <i>et al.</i> , (1988)

<u>Class</u>	<u>Mutant</u>	<u>Symbol</u>	<u>Defect</u>	<u>Reference</u>
	<i>hunchback</i>	<i>hb</i>	maternal gap gene regulated by <i>bic</i> . Also expressed zygotically	Lehmann and Nüsslein-Volhard (1984); Tautz (1988); Tautz and Pfeifle (1989)
b) posterior group	<i>oskar</i>	<i>osk</i>	deletion of abdomen	in: Nüsslein-Volhard <i>et al.</i> , 1987
	<i>pumilio</i>	<i>pum</i>	(excluding telson)	
	<i>nanos</i>	<i>nos</i>	All except <i>nanos</i> and <i>pumilio</i> lack polar granules.	
	<i>valois</i>	<i>val</i>		(<i>vasa</i> : Lasko and Ashburner, 1990)
	<i>staufer</i> <i>stau</i>			
	<i>vasa</i>	<i>vas</i>	(<i>nanos</i> may be the primary posterior determinant)	
	<i>bicaudal</i> group	<i>bicaudal</i> <i>Bicaudal-C</i> <i>bicaudal-F</i> <i>Bicaudal-D</i>	Females carrying homozygous mutations develop double abdomen embryonic phenotype. <i>Bicaudal-D</i> mutants cause mis-location of <i>nanos</i> . This leads to repression of <i>bicoid</i> and zygotic <i>hb</i> expression. Subsequently cues to orchestrate the development of the abdomen are initiated. This explains the double abdomen phenotype of the <i>Bic-D</i> mutants.	in: Lasko and Ashburner (1990) Wharton and Struhl (1989, 1990)

<u>Class</u>	<u>Mutant</u>	<u>Symbol</u>	<u>Defect</u>	<u>Reference</u>
c) terminal group	<i>torso</i>	<i>tor</i>	Mutants lack terminal anterior (acron) and posterior (telson) structures	in: Lehmann (1988)
	<i>trunk</i>	<i>trk</i>		
	<i>torsolike</i>	<i>tsl</i>		Sprenger <i>et al.</i> (1989)
	<i>fs(1)polehole</i>	<i>fs(1)ph</i>		
	<i>fs(1)Nasrat</i>	<i>fs(1)N</i>		
	<i>l(1)polehole</i>	<i>l(1)ph</i>		
B. Mutants Affecting The Formation of Dorsal-Ventral Pattern				
a) dorsalising group	<i>dorsal</i>	<i>dl</i>	all cells at blastoderm differentiate as if they were located in the dorsal position of the blastoderm giving rise to a dorsalisated phenotype	Reviewed in: Anderson (1984a, 1984b, 1987); Ingham (1988); Levine (1988)
	<i>easter</i>	<i>ea</i>		
	<i>gastrulation-defective</i>	<i>gd</i>		
	<i>Mat(3)2</i>	<i>Mat(3)2</i>		
	<i>Mat(3)4</i>	<i>Mat(3)4</i>		
	<i>snake</i>	<i>snk</i>		
	<i>nudel</i>	<i>ndl</i>		
	<i>tube</i>	<i>tub</i>		
	<i>pipe</i>	<i>pip</i>		
	<i>spatzle spz</i>			
	<i>pelle</i>	<i>pll</i>		
	<i>fs(1)K10</i>	<i>fs(1)K10</i>	dorsalisation of egg and embryo	Wieschaus <i>et al.</i> , (1978) Haenlin <i>et al.</i> , (1987)
b) ventralising group	<i>gurken</i>	<i>grk</i>	ventralisation of egg shell and embryo	Schüpbach (1987) Price <i>et al</i> (1989)
	<i>torpedo</i>	<i>top</i>		

<u>Class</u>	<u>Mutant</u>	<u>Symbol</u>	<u>Defect</u>	<u>Reference</u>
c) lateralising group	<i>Toll</i>	<i>Tl</i>	lateralisation of embryonic pattern May be responsible for setting up the gradient of dorsal-ventral information specifying the dorsal-ventral pattern.	Anderson (1987) Hashimoto (1988)
5. Abnormalities visible in the cuticle of embryos	<i>rubbish</i>	<i>rub</i>	variable head defects and holes	Schüpbach and Wieschaus (1989)
6. Apparently normal larvae that do not hatch	<i>reticent</i>	<i>ret</i>	larvae fail to hatch	Schüpbach and Wieschaus (1989)

abnormalities are present, experiments to find out which tissues are involved in the sterility can be performed.

Ovary transplant experiments show whether or not the mutation is ovary autonomous and involve transplanting mutant ovaries into wild type hosts and *vice versa*. If a mutant ovary transplanted into a wild type host develops mutant oocytes, the mutation can then be classified as being ovary autonomous. Conversely, if a mutant ovary is transplanted into a wild type host and normal oocytes develop the mutation can be classified non-autonomous. Further experiments can be carried out to determine whether the mutation affects the germ line directly or through interactions with somatic tissues.

Pole cell transplantation experiments have been used to find out if a female sterile mutation affects the germline directly. Pole cells are transplanted from a mutant embryo into a wild type host embryo and *vice versa* (Wieschaus and Szabad, 1979). Pole cells are the first cells to form from the syncytial blastoderm at the posterior end of the embryo, in the region where polar granules are present in the mature oocyte (Section 1.2). If wild type pole cells are transplanted into a female sterile mutant embryo and the resulting adult female ovaries form some normal oocytes and *vice versa* (i.e. mutant pole cells are transplanted into a wild type female embryo and the resulting adult ovaries form some mutant oocytes), then the mutation can be classified as germline dependent.

Another technique that has been used to define whether or not a female sterile mutation is germline dependent is clonal analysis following mitotic recombination (e.g. Wieschaus *et al.*, 1981; Gans and Perrimon, 1983). X-ray mutagenesis was used to induce mitotic recombination in 1st instar larvae. By using a marker closely linked to the female sterile (*fs*) mutation to follow mitotic recombination in the germline, e.g. the egg marker *fs(1)K10* which results in elongated eggs (Wieschaus *et al.*, 1978), it was possible to define clones which had been induced in the germline (Wieschaus *et al.*, 1981). In this way clones of germline cells homozygous for a particular *fs* mutation could be generated and assessed to find out whether or not the marked elongated eggs have been affected by the *fs* mutation. If the mutation affects a function normally required in the germline, the defects associated with the mutation should also be apparent in the

clonally derived *K10* eggs. If the fs mutation is affecting a somatic cell function, the clonally derived *K10* eggs should not be affected because the homozygosity for *K10* and the effect of the female sterile mutation are restricted to the germline. The success of this technique depends on suitable markers being available to determine the genotype of the germline clones. It also relies on the assumption that the mutation being tested will not affect both the somatic and the germline derived cells in the ovary. There is evidence to suggest that at least some functions of the germline and somatic ovarian cells are interdependent. If a mutation affecting both tissues was studied by clonal analysis the results would be difficult to interpret but still informative.

Once an fs mutant has been isolated, the affected chromosomal region involved can be localised genetically using extensive crossing experiments. Alternatively this may already be known if a linked marker has been used. It can then be assigned to a cytological region on the chromosome map using chromosomal deficiency strains with established breakpoints to find out which deficiencies uncover the mutant locus.

During oogenesis most of the information required for the first few hours of embryogenesis (up to cellular blastoderm formation) is provided within the egg. The maternal genome is required to provide the egg with positional information as well as factors required for general cellular differentiation and mitosis. Schüpbach and Wieschaus (1989) carried out a genetic screen to isolate maternal mutations which disrupted early development, and defined six classes of defect described in Table 1.2. In this classification system I have included maternal effect mutations isolated both fortuitously and in other genetic screens and although this list is not exhaustive, representatives of most groups of maternal effect mutations are present. The most characterised maternal effect mutations at both the genetic and molecular levels, are those which disrupt the formation of the major axes of the early embryo (reviewed by Nüsslein-Volhard *et al.*, 1987). These genes (Class 4 in Table 1.2) can be divided into three groups - those responsible for the organisation of the anterior-posterior axis, the dorsal-ventral axis and the termini of the embryo (the acron at the anterior end and the telson at the posterior end). The maternal organising genes form gradients of activity along the major axes which activate a number of zygotic loci which act in a cascade to control subsequent development (reviewed by Akam (1987, 1989),

and Ingham, (1988)).

According to the mutant phenotype, four main groups of genes regulating *Drosophila* development have been identified. The gap genes, if mutated, cause the embryo to develop with large gaps in the array of segments, and both the maternal organising genes (e.g. *bicoid* and maternal *hunchback*) and the zygotic regulatory genes activated by them (e.g. *krüppel*) fall into this class (this will be discussed further). The gap genes regulate the activity of the zygotically expressed pair-rule genes (e.g., *runt* and *hairy*). Mutations in pair rule genes cause embryos to develop with only half the normal number of segments, where elements of each alternate segment have been deleted. The segment polarity genes (e.g., *engrailed*) which are regulated by the gap genes and pair rule genes affect patterns within segments and mutations can lead to extensive pattern duplication or reversal of segment polarity. The segment polarity, pair rule and gap genes regulate the spatial transcription of the homeotic genes (e.g. the *ultrabithorax* complex and the *antennapedia* complex) which are responsible for specifying the identity of each segment. Thus, events which regulate the development of the fertilised egg through to the formation of the adult fruit fly form a hierarchy of regulatory gene activity and these processes are initiated during oogenesis.

Interactions of the maternal organising genes with zygotic genes which are responsible for the formation of the anterior-posterior axis of the embryo have been extensively studied at the genetic and molecular levels (reviewed by French, 1988; Manseau and Schüpbach, 1989; Nüsslein-Volhard *et al.*, 1987; Hartley and White, 1990). The maternal genes *bicoid*, *swallow* and *exuperantia* are required for the formation of the anterior body pattern (Frohnhofer and Nüsslein-Volhard, 1986, 1987). The effects of strong mutant alleles at the *bicoid* locus produce embryos lacking a head and thorax (Frohnhofer and Nüsslein-Volhard, 1986). Cytoplasm taken from the anterior tip of a wild type egg can rescue *bicoid* eggs to form a complete pattern if injected anteriorly. The *bicoid* gene has been cloned (Berleth *et al.*, 1988) and has been shown to be transcribed in the nurse cells and the RNA is localised along the anterior periphery of the oocyte by stages 5-7 of oogenesis. It remains strictly localised within the anterior 20% of the egg throughout the cleavage stages and disappears before cellularisation of the blastoderm. The

genes *exuperantia* and *swallow* are required for the correct localisation of *bicoid* RNA (Frohnhofer and Nüsslein-Volhard, 1987). The *bicoid* protein is not detectable during oogenesis but appears shortly after the egg is laid and forms a concentration gradient over the anterior 70% of the egg (Driever and Nüsslein-Volhard, 1988a). This broad concentration gradient of *bicoid* protein is thought to represent the primary determinant of anterior pattern and regulates the activity of zygotic gap genes (e.g. *hunchback* and *krüppel*). In *bicoid* mutants, zygotic *hunchback* activity is abolished (Driever and Nüsslein-Volhard, 1989) and the domain of *krüppel* expression is moved more anteriorly. From these observations it has been suggested that high levels of *bicoid* protein are responsible for activating zygotic *hunchback* which then represses *krüppel*. The sequence of the *bicoid* gene has revealed the presence of a homeobox binding domain in the *bicoid* protein which binds to high affinity and low affinity sites in the *hunchback* promoter (Driever and Nüsslein-Volhard, 1989).

The organisation of the posterior body pattern is controlled by a small group of maternally expressed genes called *tudor*, *staufer*, *pumilio*, *nanos*, *valois*, *vasa* and *oskar* (References in Table 1.2). All of these genes act together to form the abdomen and with the exception of *nanos* and *pumilio* are also required for pole cell formation. All of the posterior group mutants (with the exception of *pumilio* mutants) can be rescued by transplanting pole plasm from wild type mature oocytes or newly laid eggs, into the presumptive abdominal region. All mutant nurse cells extracts except *nanos* have rescuing activity (reviewed in Lehmann, 1988). The primary posterior determinant probably consists of *nanos* RNA which is synthesised during stages 9 and 10 of oogenesis. It has been suggested that in the case of *nanos* RNA, localisation occurs by degradation throughout most of the oocyte, except within the pole plasm (Ephrussi *et al.*, 1990).

It is thought that after fertilisation, the *nanos* protein product is translocated from the posterior pole in a process that requires the wild type *pumilio* gene product (Ephrussi *et al.*, 1990). The *nanos* gene product represses the activity of the maternal *hunchback* gene product in the abdominal region possibly by translational repression (Wharton and Struhl, 1990), which allows the transcription of the gap gene *knirps*. The *oskar* mutation seems to more specifically affect pole plasm formation (Lehmann and Nüsslein-Volhard, 1986). The *oskar* gene has been cloned

and encodes a maternal transcript which is localised within the posterior pole of early embryos. Examination of ovaries has revealed that *oskar* RNA is expressed as early as stage 3 in oogenesis (Ephrussi *et al.*, 1990). The *vasa* protein product is localised to the posterior pole of the oocyte and is sequestered by pole cells as they form (Lasko and Ashburner, 1990). The wild type activities of at least four genes *cappucino*, *spire* (Manseau and Schüpbach, 1989), *oskar* and *staußen* are required for the correct localisation of *vasa*. The *vasa* product is required for oocyte differentiation, pole cell formation and abdominal segmentation. It is also functional in pole cell derivatives throughout development in both sexes. *vasa* is active in the female germline although it is also expressed in the male germline (Hay *et al.*, 1988; Lasko and Ashburner, 1990). Since males carrying deletions at *vasa* are fertile and viable, its functional role if any remains unknown.

The *bicaudal* group is composed of four known loci: *bicaudal*, *Bicaudal-C*, *Bicaudal-D*, *bicaudal-F* (references in Lasko and Ashburner, 1990), and females with homozygous mutations at any of these loci produce double abdomen embryos. The most severe *Bicaudal-D* mutants completely fail to differentiate oocytes and therefore lay no eggs. The *Bicaudal-D* (*Bic-D*) gene has been cloned (Wharton and Struhl, 1989, 1990). In *Bic-D* mutants the posterior determinant *nanos* is mis-localised and is active in the anterior and posterior poles of the embryo. The presence of the *nanos* product in the anterior region of the embryo represses two anterior factors *bicoid* and *hunchback* which normally direct head and thoracic segmentation and repress abdominal segmentation. Therefore in *Bic-D* mutants the ectopic expression of *nanos* represses the anterior pattern and activates the posterior pattern forming processes resulting in the formation of abdominal structures in the anterior region of the embryo. This explains the double-abdomen phenotype observed in *Bic-D* mutants.

The establishment of the terminal ends of the *Drosophila* embryo is under the control of six known maternally expressed loci called *torso*, *l(1)polehole*, *torsolike*, *trunk*, *fs(1)Nasrat* and *fs(1)polehole* (reviewed by Lehmann, 1988). Females homozygous for mutations in any of the genes in the terminal class produce embryos with an identical phenotype which lack terminal structures. This suggests that these genes are part of a single pattern forming process that involves the localised activation of terminal zygotic genes such as *tailless*.

The maternal gene *torso* has been cloned and the *torso* product has been shown to be evenly distributed in the egg which was unexpected from genetic evidence (Sprenger *et al.*, 1989). However, structural similarities of the predicted *torso* protein with growth factor receptor tyrosine kinases suggest that the spatial restriction of *torso* activity results from a localised activation of the *torso* protein at the anterior and posterior egg poles. The activation of *torso* requires the expression of another terminal gene called *torso-like* which may encode the ligand which activates *torso* in a small group of somatic follicle cells at the poles (Stevens, 1990, discussed in Hartley and White, 1990).

Some of the maternally expressed genes which determine the dorsal-ventral axis of the embryo have been extensively studied (reviewed by Anderson and Nüsslein-Volhard, 1984a, 1984b; Anderson, 1987; Ingham, 1988). The separation of the major germ layers (mesoderm from ectoderm, neural ectoderm from epidermal ectoderm, ventral from dorsal ectoderm) occurs in the dorsal-ventral axis. Mutational analysis has identified at least 12 loci in *Drosophila* which produce totally dorsalised embryos - i.e., all cells at blastoderm differentiate as if they were located in the dorsal position of the blastoderm. Each locus must contribute to the dorsal-ventral pattern forming process. Rescue of mutant embryos with wild-type cytoplasm or RNA injections has provided most of the information about the function of the dorsalising loci. In *snake* embryos rescue can be obtained by wild type cytoplasm injections irrespective of the site of injection. It was concluded that *snake* function alone was not sufficient for generating assymetry. The *snake* gene has been cloned (De Lotto and Spierer, 1986) and shows significant similarity with human serine protease suggesting that the *snake* product may be regulating the activity of other spatially localised proteins by proteolytic cleavage. In contrast to *snake*, rescue of *dorsal* embryos only happens if the wild type cytoplasm is injected ventrally. This suggested that the *dorsal* product may only function in response to graded information in the dorso-ventral axis. It is thought that the *Toll* gene product may act as the primary morphogen along the dorso-ventral axis to which genes like *dorsal* respond. Rescue of embryos lacking *Toll* activity with wild type cytoplasm is position dependent. The site of injection defines the position at which the ventral-most structures will form. The *Toll* gene product has been cloned and although it is present throughout the

embryo, it acts locally to initiate polarity and may be transmitting signals to nuclei as its sequence predicts a strong similarity to transmembrane proteins (Hashimoto *et al.*, 1988; Levine, 1988).

Saturation mutagenesis screens have been carried out on the 1st, 2nd and 3rd chromosomes of *Drosophila melanogaster* to find maternally acting genes which affect early embryogenesis. Saturation mutagenesis involves mutagenising a large number of chromosomes until every gene of interest is represented by 3-4 mutant alleles. Thus, it is likely that most of the maternal genes involved directly in embryonic pattern formation have been identified although it is possible that genes involved in pattern formation and other processes have not been detected if they mutate to lethality.

1.4.1 P-Element Mediated Enhancer Detection Applied to the Study of Oogenesis

The P-element mediated gene transfer technique (Rubin and Spradling, 1982; Spradling and Rubin, 1982) has made a large contribution to our understanding of gene function and regulation. This technique has now been used to isolate and characterise developmentally regulated genes in *Drosophila* (O'Kane and Gehring, 1987; Bellen *et al.*, 1989; Wilson *et al.*, 1989). This gene detection system makes use of a P-element transposon which carries a weak promoter (the promoter of the P-element transposase gene) fused with a reporter gene, the β -galactosidase (*lacZ*) gene from *E.coli* and the *rosy* gene as a genetic marker. This P-element construct can be integrated at random into the genome of *Drosophila*. Once integrated, the P-element can then be mobilised genetically by using simple genetic crosses and in this way many strains carrying single insertions at new locations have been generated (Cooley *et al.*, 1988). The P-element transposase promoter may be influenced by nearby genomic regulatory elements (possible enhancer elements) and this can be visualised by staining whole mounts of embryos or dissected tissues with a chromogenic substrate. In one screen, in approximately 65% of the transposants tested (Bellen *et al.*, 1989), the β -galactosidase protein was detected in a spatially restricted pattern, presumably reflecting the influence of nearby regulatory elements on the P-element promoter. It is expected that such regulatory sequences will prove to be enhancer

elements.

Wilson *et al.* (1989) have shown by mapping the insertions of some transposants by *in situ* hybridisation to chromosomes, that at least some inserted P-elements are under the control of adjacent *Drosophila* genes. In some cases, genomic libraries from insertion strains were constructed and P-element sequences used as a probe to detect the P-element and flanking regulatory sequences. In other cases a specially designed P-element could be cloned from the genome of an insertion strain using the plasmid rescue technique. The cloning of developmentally regulated genes in this way has been called the "enhancer trap" technique, and has been applied to the study of oogenesis.

Fasano and Kerridge (1988) isolated about 100 transformed lines with specific staining patterns in the ovary. Some staining patterns were restricted to sub-populations of follicle cells not previously recognised by their morphology. A more extensive survey of ovary specific staining patterns was carried out by Grossniklaus *et al.* (1989), where almost 600 insertion strains were analysed. Their results confirmed and extended the types of staining patterns obtained by Fasano and Kerridge. A variety of staining patterns was observed in follicle cells. For example, within morphologically indistinguishable follicle cells, grades of staining were recognised with staining being most intense in anterior follicle cells and reducing gradually towards more posterior follicle cells. Double gradient patterns of staining in the follicle cells, e.g. along the anterior-dorsal axis were observed. Some staining patterns changed at different stages of oogenesis presumably reflecting temporal as well as spatial regulation of certain genes during oogenesis. Staining patterns in the nurse cells suggest that about 15% of genes involved in nurse cell activity are active early in oogenesis, and that about 85% of nurse cell gene activity occurs during stages 9 and 10 of oogenesis which is an expected period of busy gene activity. Only one transposant specifically labelled the oocyte which may suggest that few genes are exclusively expressed in the oocyte but that many more genes may be expressed in both the oocyte and other ovarian cells. In our laboratory, Roger Slee, Nian Zang and Mary Bownes have analysed many enhancer trap transposants exhibiting ovary specific and testes specific β -galactosidase staining patterns. Very localised areas of staining in subsets of ovarian follicle cells have been observed, such as in the

follicle cells bordering the nurse cells and oocyte. In a few cases, genomic sequences have been isolated that detect ovary specific and testes specific transcripts which probably correspond to the expression patterns similar to the reporter gene (Zang and Slee, pers. comm.).

This new method has the potential for the isolation of a more extensive range of genes involved in oogenesis than has previously been identified in classical genetic screens. The main advantage of this method over the classical genetic approach is that temporal and spatial gene expression patterns can be recognised directly in ovarian tissue, without relying on an observable mutant phenotype. This technique also overcomes the problem of pleiotropic mutations with a lethal mutant phenotype which would be undetected in conventional genetic screens. Furthermore, cloning and characterisation of important ovarian genes isolated by enhancer-mediated detection is a faster process than the cloning of ovarian genes identified genetically, where several alleles and a great deal of genetic information are required to clone the gene of interest. It is also very easy to cytologically map enhancer-trapped genes by *in situ* hybridisation to chromosomes using P-element sequences as a probe, whereas genetic mapping of genes causing female sterility takes generations of genetic crosses and is very time consuming. Genetic studies of the region of P-element insertion can be undertaken by inducing the P-element to excise and mutant alleles may be obtained by imprecise excision.

1.4.2 P-Elements And P-Element Enhancer Detectors As Insertional Mutagens Applied to the Study of Oogenesis

When P-elements are mobilised, occasionally they insert into a gene and give rise to a mutant phenotype. This is the basis of P-element insertional mutagenesis. Various genes involved in oogenesis have been cloned by this method. Montell *et al.* (1990) have identified a P-element induced mutation called *slow border cells (slbo)* that interferes with border cell migration and results in maternal effect lethality. Since the eggs laid by females homozygous for *slbo* are morphologically normal, they concluded that the border cells do not participate in egg shell formation, but are in some way required for fertility since *slbo* eggs are not fertilised. P-element induced female sterile alleles have now been obtained for two genes called *chickadee* and *kelch*

which were originally isolated in a conventional genetic screen by T. Schüpbach. Both mutations disrupt the transport of the nurse cell cytoplasm to the oocyte. The molecular characterisation of the genes disrupted by these mutations is underway (Cooley *et al.*, 1990).

Galanopoulos *et al.* (1989) carried out a screen of the X-chromosome, using P-element insertional mutagenesis to isolate mutants affecting chorion formation. They identified one soma-specific and seven germline specific mutations which disrupted the formation of the egg shell. This provides evidence to support the idea that activities of the germline and somatic cells are interdependent (other examples of interactions between somatic and germline cells are quoted in Schüpbach, 1987; Gutzeit, 1986). It will be interesting to find out how the germline specific genes and follicle cell specific genes cooperate during chorion formation. Perhaps there are genes active in different regions of the egg chamber which coordinate the localised activation of specific patterns of chorion gene expression. Alternatively (or additionally) they may be involved in regulating follicle cell movement and localised positional signals control specific patterns of chorion gene expression.

Some mutants disrupting oogenesis have also been identified using the P-element enhancer trap technique (Grossniklaus *et al.*, 1989; Couderac *et al.*, 1990). Grossniklaus *et al.* (1989) tested transposant lines with ovary staining patterns carrying insertions on the X chromosome and third chromosome to find out whether or not the insertion was associated with a mutant phenotype. Five insertion strains were found to have reduced fertility and another three exhibited a mutant ovarian phenotype. They were able to obtain wild type revertants for seven out of the eight insertion mutated lines. The mutations affected the organisation of the egg chamber and in one case nurse cell nuclei were present on both sides of the oocyte. Couderac *et al.* (1990) carried out a P-element enhancer trap screen to establish mutant lines of flies with staining patterns restricted to the germline that were also sterile. They established 2400 P-element enhancer trap lines, 500 of which were found to be lethal, 130 were female sterile, and among these 19 were also male sterile. One line which is male and female sterile shows β -galactosidase staining in the germ cells throughout oogenesis and spermatogenesis. Homozygous females fail to form early egg chambers and in males spermatocyte development and differentiation is also

defective. This mutation therefore is likely to affect functions common to both oogenesis and spermatogenesis in the early stages of germ cell differentiation.

One advantage of studying transposant lines which cause the ovary to fail to develop properly in homozygotes is that the staining pattern can be observed in heterozygotes. Presuming that heterozygotes will exhibit the wild type staining pattern it can be compared directly with the mutant staining pattern.

1.5 Using Molecular Methods to Isolate Genes Expressed in the Ovary of *Drosophila melanogaster*

Differential screening methods have been used to isolate cDNA representing highly expressed transcripts involved in oogenesis, including the yolk proteins (Barnett *et al.*, 1980), the chorion proteins (Spradling *et al.*, 1980) and vitelline membrane proteins (Burke *et al.*, 1987).

In the case of the chorion proteins a plasmid cDNA library was constructed from poly (A)⁺ RNA from stage 11-14 egg chambers, and blotted onto filters. Each filter was probed with radiolabelled cDNA made from poly (A)⁺ RNA extracted from pre-blastoderm embryos (selects oocyte-specific messages) or follicle cells. A set of twenty two clones were selected that hybridised only with the follicle cell probe. The next step involved hybridising [³H] labelled poly (A)⁺ RNA made from follicle cells to each putative follicle cell specific cDNA clone. The "hybrid-arrested" RNA was eluted, translated *in vitro* and the protein products analysed by gel electrophoresis. Three major protein bands were observed which were identical in size to previously observed major protein bands produced when follicle cell RNA itself was translated *in vitro*. There were found to be two groups of clones isolated in the screen hybridising to two chromosomal locations, on the third (66D 15) and X (7E11 - 7F12) chromosomes which corresponded with the map positions of two gene clusters encoding the major egg shell proteins (Spradling and Mahowald, 1979). Furthermore, an egg shell mutation called *ocelliless* (Spradling *et al.*, 1979) had been genetically mapped to the 7E11 region, which results in a marked reduction in two major chorion proteins (s36 and s38), which helped to confirm that the major egg shell proteins had been cloned.

In order to clone the yolk protein genes, a differential screening system was devised by

Barnett *et al.*, 1980. They used a size fraction of female poly (A)⁺ RNA which had been shown by *in vitro* translation to encode abundantly expressed protein in the oocyte. They used radiolabelled cDNA probes made from this yolk protein message enriched poly (A)⁺ RNA and male poly (A)⁺ RNA to differentially screen a *Drosophila* genomic library. Genomic clones hybridising only with female cDNA were selected, these fell into two distinct groups on the basis of their restriction maps and cross-hybridisation patterns. Within the first group there were two closely related sequences which hybridised to the same genomic location on the X chromosome (8F-9A). The second group contained genomic sequences which cross-hybridised with the first group but were located on the X chromosome at 12B-C. Female poly (A)⁺ RNA was hybridised with a member of each of the three classes of isolated DNA clones. The hybrid-arrested RNA was eluted and the *in vitro* translation products compared to the native female poly (A)⁺ RNA *in vitro* translation products. Each DNA clone selected an RNA encoding a product in the same size class as one of the major yolk proteins and with a similar α -chymotrypsin digestion pattern. From this result, they concluded that they had indeed isolated a family of three genes encoding the major yolk polypeptides in *Drosophila melanogaster*.

During the formation of the egg shell, the first layer to be secreted around the oocyte is the vitelline membrane (section 1.1). Burke *et al.* (1987) applied differential screening techniques to isolate proteins expressed during vitelline membrane formation. Firstly, they screened an ovarian cDNA library with end labelled RNA prepared from pre-blastoderm embryos to identify and eliminate clones representative of mitochondrial, housekeeping and oocyte-specific transcripts. The remaining clones were then hybridised with *in vivo* labelled RNA from previtellogenic ovaries (stages 0-7) or *in vivo* labelled RNA from stage 10 egg chambers. Forty clones were found which preferentially hybridised with the stage 10 probe. These clones were subsequently re-screened with *in vivo* labelled RNA from chorionic egg chambers and three clones were found to recognise transcripts that accumulate during the stages of vitelline membrane synthesis. On the basis of cross-hybridisation experiments it was found that two sequence classes had been isolated. Subsequent DNA sequencing analysis predicted that the size of the primary translation product of one cDNA is compatible with a major vitelline membrane protein previously reported (Fargnoli

and Waring, 1982). The other cDNA encodes a protein of unknown function. *In situ* hybridisation to ovarian tissue sections located the putative vitelline membrane message in the follicle cells of stage 10 egg chambers, which coincides with the time when vitelline membrane proteins are being produced in these cells.

The differential screens described were used to identify and isolate targetted genes whose products are highly expressed during specific stages of oogenesis. The functions of the gene products were previously determined on the basis of protein profiles during oogenesis and in some cases the occurrence of mutants whose altered function resulted in the loss or reduction of certain proteins (eg, *ocelliless* mutants which have reduced levels of major chorion proteins (Spradling *et al.*, 1979)).

In the past decade the use of differential screening has resulted in the isolation of previously unspecified genes involved in oogenesis. DiBeneditto *et al.* (1987) labelled populations of RNA isolated from male and female adult flies and used them to differentially screen replica lifts of a *Drosophila* genomic library. They isolated ten genomic clones encoding sex-specifically expressed transcripts. Three of these clones corresponded to yolk protein gene or chorion gene isolates, and one clone was shown by *in situ* hybridisation to ovarian tissue sections to encode a female specific transcript which is expressed in the nurse-cells and the oocyte. The other clones isolated encoded male-specifically expressed transcripts. Ait-Ahmed *et al.*, (1987) carried out a differential screen of a *Drosophila* genomic library with the aim of isolating genes expressed during oogenesis that are present in pre-blastoderm embryos. They selected clones which preferentially hybridised with poly (A)⁺ RNA isolated from pre-blastoderm embryos versus poly (A)⁺ RNA isolated from gastrula embryos. One clone hybridised with four transcripts which are abundant in pre-blastoderm embryos. They used this clone to carry out a bidirectional chromosomal walk which resulted in the isolation of a region containing additional genes expressed maternally during oogenesis. They called this region which is located at 98E/F on the cytological map, the *yema* region. All of the transcripts had a uniform distribution in the nurse cells and oocyte except for a 4.5 kb transcript which was visible in stage 7 oocytes and localised in the anterior region of stage 9 oocytes. The localisation is maintained until stage 10A then is uniform by late stage 10B

and remains evenly distributed in the pre-blastoderm embryo. This cluster of maternally expressed genes expressed in the mid- to late stages of oogenesis had not been previously identified using classical genetic methods.

In our laboratory Bownes and Smith (unpublished, 1984) carried out a differential screen of a standard *Drosophila* genomic library using radiolabelled cDNA isolated from adult male and female carcasses with the view of cloning genes expressed sex-differentially (discussed in detail in Introduction to Chapter 3). A set of genomic clones putatively encoding male and female specifically expressed transcripts were available for analysis at the start of this project.

After the preliminary analysis of several clones I became interested in two clones called λ ov5 and λ ov2 which were found to encode transcripts enriched in the ovary. The work in this thesis describes an initial molecular characterisation of these transcripts, their developmental regulation and their organisation within the corresponding genomic sequences. The possible functions of the ovary enriched transcripts during oogenesis are considered in the final chapter.

1.6 Discussion

Conventional genetic screens have provided many mutants which disrupt the normal processes involved in oogenesis. A lot of such mutants have increased our understanding of the complex processes involved in egg development. The discovery of the maternal effect mutations which disrupt embryonic pattern formation have made a great advance in our understanding of the molecular nature of the positional information provided within the egg during oogenesis. However, despite the large number of female sterile mutants that have been isolated there are still aspects concerning early events in oogenesis that remain to be understood at the molecular level. For example, there is little known about the processes which control stem cell differentiation in the germarium or what factors determine which cells in the cystoblast will form the nurse cells or which one will eventually form the oocyte. We need to understand more about how the germline derived nurse cells interact with the somatically derived follicle cells to co-ordinate the

development of the oocyte as it travels from the tip of the germarium along the length of the ovariole to the uterus. There are limitations involved in searching for genes involved in these processes using genetic methods. The techniques used in the characterisation of female sterile mutations are often difficult and time consuming and the female sterile mutant phenotype must be associated with an observable defect. Genes whose functions can be complemented by other genes or those which mutate to lethality due to pleiotropic effects would not be detected in conventional screens.

The P-element enhancer trap technique offers the potential to isolate genes involved in oogenesis (and other developmentally regulated genes). This relies on the assumption that the observed reporter gene staining patterns genuinely reflect the developmentally regulated expression of nearby genes. There is also the potential to obtain mutations in important ovarian genes using P-element mutagenesis.

The use of differential screening techniques to isolate ovary-specific genes has led to the identification of genes encoding the major chorion proteins, the yolk proteins and vitelline membrane proteins. The genes encoding each of these abundant ovary proteins are members of gene families which are developmentally regulated during oogenesis. Each gene encodes a single transcript expressed at a high level.

The study of genes with ovary enriched patterns of expression which have been isolated by molecular means (either in a molecular screen or because of their proximity to other cloned sequences) have revealed that these genes often exist in clusters, have complex regulation and frequently encode overlapping transcripts. Furthermore some genes have been shown to overlap. Although the functions of these genes are generally unknown, their molecular characterisation has provided a valuable insight into the complexity of gene organisation and regulation. The molecular investigations of gene clusters which encode ovary enriched transcripts will be discussed in detail in the final discussion of this thesis in relation to the results presented in the following results chapters.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Solutions

Chemicals were obtained from Sigma, BDH and Aldrich.

Enzymes (Restriction Endonucleases, T4 DNA Ligase, Klenow fragment of DNA Polymerase, DNase I, RNase A, Proteinase K) were obtained from BRL, Pharmacia and NBL.

Radioisotopes were obtained from Amersham.

Hybond-N nylon membranes were obtained from Amersham.

Standard solutions were made using sterile distilled water in sterile, baked glassware and were generally made sterile by autoclaving (15psi/15 min). Solutions not detailed in the text are described below.

TE 10mM Tris-Cl pH 7.4-8.0, 1mM EDTA

0.5M EDTA 0.5M Diaminoethanetetra - acetic acid, pH 8.0

Boehringer Mannheim Incubation Buffers for Restriction Enzymes :

Final concentrations in mm/L (i.e. 1:10 diluted buffer).

A : 33mM Tris HCl, 10mM Mg-Acetate, 66mM K-Acetate, 0.5mM Dithiothreitol (DTT), pH 7.5

B : 10mM Tris HCl, 5mm MgCl₂, 100mM NaCl, 1mM 2-Mercaptoethanol, pH 7.5

L : 10mM Tris HCl, 5mM MgCl₂, 1mM Dithioerythritol (DTE), pH 7.5

M : 10mM Tris HCl, 10mM MgCl₂, 50mM NaCl, 1mM DTE, pH 7.5

H : 50mM Tris HCl, 10mM MgCl₂, 100mM NaCl, pH 7.5

10X TBE gel buffer : 0.89M Tris-Borate, 0.89M Boric Acid, 10mM EDTA

10X DNA and RNA	:	
gel loading buffer		100mM EDTA pH 8.0, 0.1% (w/v) Bromophenol Blue, 20% (w/v) Ficoll (Type 400)
10X MOPS gel buffer	:	0.2M Na-MOPS pH 7.0, 50mM Na-Acetate, 10mM EDTA
RNA Formaldehyde	:	
Sample Buffer (FSB)		50% (v/v) formamide, 25% (v/v) formaldehyde (at 4% w/v), 25% (v/v) 10X MOPS buffer
RNA extraction buffer	:	100mM Tris HCl pH 7.5, 10mM EDTA, 150mM Lithium Chloride, 1% (w/v) SDS
OLB (oligo labelling	:	
buffer)		Solution 0 : 0.125M MgCl ₂ , 1.25M Tris HCl pH 8.0 Solution A : 0.95ml solution 0, 18ml 2-Mercaptoethanol, 25μl 20mM dATP, 25μl 20mM dTTP, 25μl 20mM dGTP Solution B : 2M Hepes pH 6.6 Solution C : Hexadeoxyribonucleotides (Pharmacia) suspended in TE buffer at 90 OD units/ml OLB is made by mixing Solution A, B and C in the ratio 2:5:3 (and is stored at -20°C)
20X SSC	:	3M NaCl, 0.3M Na- Citrate
20X SSPE	:	3.6M NaCl, 20mM NaHO ₄ pH 7.4, 20mM EDTA pH 7.4
Salmon Sperm DNA	:	10mg/ml stock which has been sonicated, phenol extracted and ethanol precipitated. Purchased from SIGMA.
50X Denhardt's Solution	:	1% (w/v) Ficoll, 1% (w/v) polyvinyl pyrrolidine, 0.2% (w/v) BSA
Ringer's Solution	:	3.2g NaCl, 3.0g KCl, 1.8g MgSO ₄ , 0.69g CaCl ₂ ·2H ₂ O, 1.79g Tricine, 3.6g glucose, 17.1g sucrose. Made up to 1 litre with dH ₂ O, pH adjusted to 6.95, filter sterilised and stored at 4°C
10X PBS	:	0.1M Sodium Phosphate pH 7.5, 1.3M NaCl

TM buffer	:	10mM Tris HCl pH 8.0, 10mM MgCl ₂
TE buffer	:	10mM Tris HCl pH 7.4-8.0, 1mM EDTA
NET	:	150mM NaCl, 0.1mM EDTA, 20mM Tris HCl pH 8.0
HNET	:	1.0M NaCl, 0.1mM EDTA, 20mM Tris HCl pH8.0

Table 2.1: List of *Drosophila melanogaster* Strains used and Their Relevant Features

<u>Stock</u>	<u>Relevant Features</u>	<u>Reference</u>
Oregon R (OrR)	A wild type strain	Lindsley and Grell (1968)
Deficiency Stocks (3R):		
<i>Df(3R)red^{P93}Sb/P18e, Me⁻</i>	Deficiency strains	Listed in
<i>Df(3R)red^{P52} /TM1, Me</i>	carrying deletions around	Lindsley and Zimm
<i>Df(3R)red¹ /MRS, Me</i>	the 88 region on 3R of	(1987)
<i>Df(3R)red^{P1} Xa/TM1, Me</i>	<i>Drosophila melanogaster</i> .	Strain <i>Df(3R)293^{Y5}</i>
<i>Df(3R)red^{P6} Xa/TM1, Me</i>	A map of these	was gifted by
<i>Df(3R)red³¹ /MKRS, Me</i>	deficiencies is	S. Parkhurst
<i>Df(3R)243⁸⁵ /TM6B, Tb</i>	provided in Figure 6.1	(University of Oxford)

Balancer Chromosomes

		<u>References</u>
<i>MRS, Me, ri, sbd¹</i>	Balancer	Lindsley and
<i>MKRS, Me, Kar, ry², Sb</i>	chromosomes used	Zimm (1990)
<i>P18e, Me⁻</i>	to balance the <i>Df(3R)</i>	Ashburner, M.
<i>TM1, Me, ri, sbd¹</i>	deficiencies.	(1989)
<i>TM6B, Tb, e</i>		

2.2 *Drosophila* Stocks and Strains

Drosophila strains are listed in Lindsley and Grell (1968) and Lindsley and Zimm (1987 and 1990). Strain *Df(3R) 293^{Y5}* was gifted from S. Parkhurst (University of Oxford, U.K.). Table 2.1 presents a list of stocks used, and provides a map of the deficiencies carried in the stocks used which span the 88 (3R) region is illustrated in Figure 6.1.

Drosophila stocks were maintained in vials or bottles at 18°C or 25°C on cornmeal food which consisted of cornflour 250g, sugar 500g, yeast pellets 175g and agar 100g made up to 10 litres with dH₂O. The food was then boiled, cooled to approximately 40°C and poured into bottles or vials and allowed to set. A fungicide, Nipagen was added to a final concentration of 4.5µg/L and on occasion antibiotics such as Gentamycin (to 40µg/ml) were added. Strips of filter paper that had been soaked previously in 3% (v/v) Benzyl Benzoate in ethanol and allowed to air dry, were placed on top of the food if mite infections occurred.

Table 2.2: Stages of *Drosophila melanogaster* development at 25°C (Reference: Bownes and Dale, 1982)

<u>Hours</u>	<u>Days</u>	<u>Developmental Stage</u>
0	0	Fertilization and fusion of pronuclei
1.5		Preblastoderm stage. Migration of cleavage nuclei and pole cell formation
3		Blastoderm stage. Migrated nuclei form cells in the previously syncytial blastoderm
3.5		Gastrulation begins
6-8		Segmentation visible
18		Larval differentiation nearly complete
24	1	Hatching from egg. Onset of first larval instar
48	2	First moult. Second larval instar begins
72	3	Second moult. Third larval instar begins
120	5	Puparium formation with white puparium
122		Puparium darkens
124		Prepupal moult
132		Pupation. Eversion of imaginal discs
216-240	9-10	Emergence of adult from pupal case

2.3 *Drosophila melanogaster* Life Cycle - Collection of Developmental Stages

2.3.1 Stages of *Drosophila melanogaster* Development at 25°C

Table 2.2 summarises the stages of *Drosophila melanogaster* development at 25°C for the strain used, Oregon R.

2.3.2 Setting Up A Population Cage

A population cage (40cm X 40cm X 40cm) was made from perspex. One side had a circular opening (10cm diameter) with a length of muslin attached, through which flies were entered. Approximately 500-1000 flies were reared in bottles before being delivered into the population cage. Once the flies were inside the population cage, they were fed on cornmeal food (2.2) which had been poured into petri dishes (9cm diameter) and kept in the dark at room temperature.

2.3.3 Collection of Eggs (0-24 hour embryos)

Eggs were collected on 3% (w/v) agar plates with a smear of yeast paste (dried yeast dissolved in 20% (w/v) glucose) on the surface. The eggs were harvested by washing the collection plate with water and using a paintbrush to transfer the eggs to a sieve where any traces of food were removed by rinsing with water. The eggs (which represent a collection of 0-24 hour embryos) were frozen in liquid nitrogen and stored at -70°C.

2.3.4 Collection of 1st and 2nd Instar Larvae

Eggs were collected on agar plates as described in 2.3.3 over periods of 2-3 hours. The eggs were placed in an incubator (25°C) for 24 hours to allow the development of 1st instar larvae and for 48 hours to allow the development of 2nd instar larvae. Extra yeast paste was added as a food source for the developing larvae. The addition of a saturated sucrose solution caused the larvae to float to the surface of the liquid. The larvae were rinsed with dH₂O using a sieve,

transferred to Eppendorf tubes, frozen in liquid nitrogen and stored at -70°C.

2.3.5 Collection of Early and Late 3rd Instar Larvae and Pupae

Egg laying flies were placed in fresh food bottles for 4 hours then removed. The bottle was then incubated for 72 hours, 96 hours and 120-240 hours. Early 3rd instar larvae were collected after 72 hours by floating them out of the food using a saturated sucrose solution, rinsed with dH₂O and frozen in liquid nitrogen. Late 3rd instar larvae were picked with a paintbrush as they crawled up the sides of the bottles after an incubation period of 96 hours, placed in Eppendorf tubes and frozen with liquid nitrogen, and stored at -70°C.

Pupae were collected by picking them off the inside of the food bottles after 120-140 hours incubation, placed in Eppendorf tubes and frozen in liquid nitrogen, and stored at -70°C.

2.3.6 Collection of Sexed Adults

Adult flies were collected ranging in age from newly eclosed to 10 day old flies. The flies were anaesthetised with di-ethyl ether, sexed, placed in Eppendorf tubes, frozen in liquid nitrogen and stored at -70°C.

Table 2.3: List of Microbial Strains, Vectors, Libraries, Plasmids and Recombinant bacteriophage Used.

<u>Hosts</u>	<u>Relevant Genotype</u>	<u>Comments</u>	<u>Reference</u>
HB101	F ⁻ , hsd520, recA13, ara14, proA2, lacY1, galK2, rpsL20 (Sm ^r), xy15, mtl1, supE44, λ ⁻	Used as general plasmid host	Boyer (1969)
DL307 (FS1585)	recD SuII ⁺ SuIII ⁺	Used for propagating λ Charon 4A bacteriophage	Frank Stahl (gift) Eugene Oregon University Also: D Leach Edinburgh University
Y1090 <i>hsdR</i>	hsdR supF Δ lac Δ lon pMC9	Contains a plasmid which is amp ^r and produces high levels of <i>lac</i> repressor and deficient in <i>lon</i> protease	Young and Davis (1983)
<u>Plasmids</u>	<u>Relevant Genotype</u>	<u>Comments</u>	<u>Reference</u>
pGem-1/2	Amp ^r	pGemini vector used for sub-cloning <i>Drosophila</i> DNA sequences	Promega Biotech. Melton (1984)
pGem YP1A	Amp ^r	pGem containing a 2.9 kb DNA fragment of the <i>yolk protein-1</i> gene from <i>Drosophila melanogaster</i> . This fragment was subcloned from another YP-1 containing plasmid (Barnett <i>et al.</i> , 1980)	Blair, M. This laboratory (pers.comm) Barnett <i>et al.</i> , (1980)
Gem α -tubulin	amp ^r	pGemini-2 plasmid containing a 1.7 kb α -tubulin-1 DNA fragment. This plasmid was constructed in this laboratory by M.Blair from a plasmid gifted by Kalfayan and Wensink	Kalfayan, L. and Wensink, P. (1982)

pGem Adh

amp^r

pGemini 1 plasmid
containing a 1.7 kb
pGemini XbaI fragment
the *Drosophila*
alcohol dehydro-
genase gene

Source:
Alan Shirras
University
of Edinburgh
(see Shirras
& Bownes,
1987)

Bacteriophage

Comments

Reference/Source

λMaIDI
λA05

λ vectors, containing
chorion DNA
from the chorion gene
clusters from the 3rd and
X chromosomes of *Drosophila*

Spradling (1980)
Gifted from
Alan Spradling's
laboratory in
1987.

λ8
λ9
λ6-6
λ9-24
λ8-15

λCharon 13 vectors
containing DNA sequences
spanning the *doublesex*
locus of *Drosophila*

Baker and
Wolfner (1988)
Gifted from
B.Baker's
laboratory

Libraries

Comments

References/Source

λCharon4A
Drosophila
Genomic
Library

Randomly sheared *Drosophila*
DNA had EcoRI linkers added
and was subsequently subcloned
into the λCharon4A vector

Maniatis (1978)
Gifted from the
Maniatis Laboratory

λgt11
Ovarian cDNA
Library

Whole ovaries were hand
dissected from the Canton S
strain of *Drosophila melanogaster*
and poly (A)⁺ RNA prepared. The
cDNA was made by oligo(dT)
priming and packaged into the
λgt11 vector

Huynh, T. *et al.*,
(1985); Jendrisak, J
(1987). Source:
The library was
constructed and
donated by P.Sullivan
and L.Kalfayan,
University
of North Carolina

2.4 Microbial Strains and Media

2.4.1 Microbial Strains

All bacterial strains, plasmid vectors, bacteriophage, plasmids and libraries used are listed in table 2.3.

Bacterial stocks were maintained on appropriate plates at 4°C and also as stabs at room temperature in the dark.

Lambda bacteriophage were stored at 4°C in phage buffer with a few drops of chloroform added.

Plasmid and Lambda DNA samples were stored suspended in TE buffer and kept at -20°C.

2.4.2 Media

All media used (listed in table 2.4) were sterilised by autoclaving. Antibiotics, vitamins and sugars were made up in dH₂O and filter sterilised. Where appropriate, antibiotics were added to plates and media, e.g. ampicillin to a final concentration of 50µg/ml.

Table 2.4: Media

L-broth	10g Difco Bacto Tryptone, 5g Difco Bacto Yeast Extract, 5g NaCl per litre, pH 7.2
L agar	16g Difco Bacto Tryptone, 10g Difco Bacto Yeast Extract, 5g NaCl per litre
BBL agar	10g Baltimore Biological Laboratories trypticase, 5g NaCl, 10g Difco agar per litre
BBL top agar	As BBL agar except, only 6.5g Difco agar per litre
Top agarose	0.7% (v/v) Pharmacia agarose in L-broth
Phage buffer	3g KH ₂ PO ₄ , 7g Na ₂ H PO ₄ , 5g NaCl, 10ml 0.1M MgSO ₄ , 10ml 0.1M CaCl ₂ , 1ml 1%(w/v) gelatin per litre

2.5 Methods

2.5.1 General Methods

2.5.1.1 Phenol Extraction

Phenol was redistilled and saturated with water (purchased from Raithburn Chemicals). It was then equilibrated with TE buffer and 8-hydroxyquinoline was added to 0.1% (w/v). Solutions of nucleic acid were deproteinised by shaking, or vortexing, with an equal volume of phenol or phenol/chloroform(1:1). The phases were then separated under centrifugation in a Sorval RC-5B centrifuge (5 Krpm, 10 minutes) or a microfuge (12 Krpm, 5 minutes) and the aqueous layer carefully removed to a fresh tube.

2.5.1.2 Ether Extraction

Diethyl ether was equilibrated with TE buffer. Solutions were shaken, or vortexed, with an equal volume of di-ethyl ether to remove any remaining phenol after de-proteinisation. The phases were allowed to settle before the ether phase was removed. Any remaining ether was removed by heating the sample to 65°C for 2 minutes.

2.5.1.3 Precipitation of Nucleic Acids

The solution was adjusted to 0.3M Sodium Acetate pH 5.5 then 2.5 volumes of ethanol or 0.6 volumes of propan-2-ol were added.

The samples were incubated at -20°C for a minimum of 60 minutes. Small quantities of nucleic acids (if less than 5 μ g) were precipitated at -20°C overnight. The precipitate was recovered by centrifugation in a Sorval centrifuge (10 Krpm, 20 minutes, 4°C) or a microfuge (12 Krpm, 10 minutes). The pellet was washed in 70% (w/v) ethanol, dried under vacuum and re-dissolved in TE buffer or sterile, distilled water.

2.5.1.4 Estimation of DNA and RNA Yields

The optical density (OD) at 260nm was used to quantify nucleic acids. An OD₂₆₀ of 1.0 is 50µg/ml for DNA and 40µg/ml for RNA. Nucleic acid was free of contaminating protein if the OD₂₆₀/OD₂₈₀ was 1.8-2.0. Estimates of nucleic acid concentrations were also obtained by ethidium bromide staining of agarose gels with known quantities of material.

2.5.1.5 Restriction Endonuclease Digestion of DNA

In general, DNA was digested with a 2-4 fold excess of the enzyme (2 - 4 units of enzyme/µg of DNA) for a minimum of 60 minutes in reaction buffers purchased from Boehringer Mannheim (Section 2.1). Reaction conditions were recommended by the manufacturers were used.

2.5.1.6 Extraction of DNA from Agarose Gels

- a) Low melting point agarose gels: DNA fragments were fractionated on low melting point agarose gels and the desired fragment was cut from the gel. An equal volume of TE buffer was added to the sample and heated to 65°C to melt the agarose. The sample was then phenol extracted (2.5.1.1). The aqueous layer containing the DNA fragment was then ethanol precipitated (2.5.1.3).
- b) DEAE Paper: DNA fragments were separated on agarose gels (2.5.6.1). The DEAE membrane (Schleicher and Schnell) was cut to the same width as the gel slot, soaked in NET buffer (150mM NaCl, 0.1mM EDTA, 20mM Tris pH 8.0) and placed into a cut in the gel, just ahead of the DNA fragment of interest. The fragment was electrophoresed onto the DEAE membrane at 100 volts for 10 minutes. The DEAE membrane was then removed from the gel, and placed in an Eppendorf tube. The DNA was eluted from the DEAE membrane by heating at 65°C for 10 minutes in 300µl of HNET buffer (1M NaCl, 0.1mM EDTA, 20mM Tris pH 8.0). The eluate was removed to a fresh tube, phenol:chloroform extracted (2.5.1.1) followed by an ether extraction (2.5.1.2). The DNA fragment was then ethanol precipitated (2.5.1.3).

2.5.1.7 Ligation of DNA Molecules

The vector and DNA insert were cut to completion with the appropriate endonucleases. Vector molecules were prevented from self ligation by removing the terminal 5'-phosphate groups by the addition of 0.01 units of calf intestinal alkaline phosphatase 5-15 minutes before digestion time was complete. Prior to ligation, the vector and insert DNA solutions were usually deproteinised by phenol extraction (2.5.1.1) and ethanol precipitated (2.4.1.3). Typically, between 10-20ng insert DNA fragment was ligated in a reaction with vector to insert DNA concentration in a 3:1 molar ratio. Ligations were carried out in a 10 μ l reaction volume containing 20mM Tris HCl pH 7.6, 5mM MgCl₂ and 5mM DTT, supplemented with 1mM dATP, and incubated overnight at 4°C. Ligations required 0.01 unit of T4 DNA "sticky" ligase for the ligation of cohesive ends. The ligation products were then transformed into *E.coli* (2.5.1.8).

2.5.1.8 Transformation into *E.coli*

L-broth (100ml) was inoculated with 1ml of an overnight culture of the relevant bacterial strain and grown at 37°C with shaking until the OD₆₅₀ reached 0.45-0.55. The culture was chilled on ice for 10 minutes. Cells were made competent and transformed with plasmid DNA by using either of the following methods:

- a) CaCl₂ Method: The cells were pelleted by centrifugation (2 Krpm, 10 minutes, 4°C) and resuspended in 50ml of ice-cold, sterile 50mM CaCl₂. The suspension was left on ice for 15 minutes and the cells were re-pelleted and resuspended in 2.5ml of ice-cold 50mM CaCl₂. One 0.1ml aliquot of this suspension was removed into a 5ml glass tube for each transformation, mixed with the ligation mixture and incubated on ice for 30 minutes. The cells were then heat-shocked at 42°C for 2 minutes and returned to ice for 1 minute. 0.9ml of L-broth was added to each tube and the cells incubated at 37°C for 1 hour.
- b) Transformation in the presence of DMSO: The cells were pelleted by centrifugation (2 Krpm, 10 minutes at 4°C) and resuspended in 1/10th volume of Transformation Buffer which consists of L-broth containing 10% (v/v) PEG 3000, 5% (v/v) DMSO, 10mM MgCl₂

and 10mM MgSO₄. The cells were incubated on ice for 10 minutes. A 0.1ml aliquot of this suspension per transformation was added to each ligation mixture and incubated on ice for 30 minutes. 0.9ml of Transformation Buffer and 20μl of a 20% (w/v) glucose solution were added to each transformation, and the cells were allowed to grow at 37°C for 1 hour.

Samples of 100-200μl from each transformation were then plated out on to L-agar plates supplemented with ampicillin (to a final concentration of 50μg/ml). The plates were inverted and incubated at 37°C overnight.

2.5.1.9 Preparation of Dialysis Tubing

Dialysis tubing (32mm flat width) was purchased from SIGMA. The tubing was cut into pieces of convenient length (10-20 cm), and boiled for 10 minutes in 2 litres of 2% (w/v) sodium bicarbonate/1mM EDTA. The tubing was then thoroughly rinsed in dH₂O followed by boiling in dH₂O for 10 minutes, allowed to cool and stored submerged in 70% (w/v) ethanol at 4°C. Prior to use the tubing was rinsed in dH₂O.

2.5.1.10 Densitometry

The amount of material present in bands on autoradiographic film was estimated using computerised scanning densitometry.

2.5.2 Preparation of Genomic DNA from Adults of *Drosophila melanogaster*

A rapid method for DNA extraction from single flies was used, essentially this method is that of Marcus (1985). Care was taken to prevent shearing of genomic DNA.

One or two flies were etherised, frozen in liquid nitrogen and homogenised in 50μl Genomic DNA Extraction Buffer (0.15M NaCl, 50mM Tris HCl pH 8.0, 15mM EDTA). The flies were homogenised in a 1.5 ml Eppendorf tube with a pasteur pipette, the tip of which had been melted into a ball to fit snugly in the bottom of the tube. A further 50μl of the same buffer containing 0.04% (w/v) SDS was added. Proteinase K was added to a final concentration of

50 μ g/ml and the homogenate incubated at 65°C for 30-60 minutes. The mixture was then phenol extracted twice and ethanol precipitated for at least 3-4 hours. The nucleic acid pellet was left to resuspend in TE buffer at 4°C overnight.

Genomic DNA was digested for 2 hours with 10 units of chosen restriction enzyme, in the appropriate buffer conditions (2.1), and RNaseA (to a final concentration of 0.1 μ g/ μ l) was also added to the reaction mixture.

2.5.3 Preparation of RNA from Different Developmental Stages and Tissues of *Drosophila melanogaster*

2.5.3.1 Large Scale Total RNA Preparations from Eggs, Larvae, Pupae and Adults

Usually up to 3g of eggs (0-24 hours), 4g of larvae (1st, 2nd, early third and late third instar), 5g of pupae, 2g of female adults and 2g of male adults were collected as described in section 2.3. Each sample was frozen in liquid nitrogen and homogenised in 5mls of RNA Extraction Buffer (100mM Tris HCl pH 7.5, 10mM EDTA, 150mM LiCl, 1% (w/v) SDS), in a 10ml sterile glass homogeniser (which had been previously baked at 200°C for 2 hours rendering it RNase free). The homogenate was extracted 4 times with a 1:1 ratio of phenol:chloroform (2.5.1.1) and then ethanol precipitated overnight at -20°C (2.5.1.3). The precipitate was pelleted by centrifugation in a Sorval RC-5B centrifuge (7 Krpm, 10 minutes), washed in 70% (w/v) ethanol and dissolved in 1-2 mls of sterile distilled water, and 2 volumes of 3M Lithium Chloride added. The sample was incubated at 4°C overnight, centrifuged and the pellet washed in 70% (w/v) ethanol, vacuum dried and resuspended in sterile distilled water.

2.5.3.2 Small Scale Total RNA Preparations from Ovaries and Carcasses

Up to 200 female adults were hand dissected using watchmaker's forceps. The pairs of ovaries and the remainder of the female carcass were transferred to separate Eppendorf tubes which contained 100 μ l of RNA extraction buffer (2.5.3.1) and kept on ice. The samples were frozen in liquid nitrogen and homogenised in a 5ml sterile, RNase free glass homogeniser, in a

total volume of 1-2mls RNA extraction buffer. Total RNA was extracted as above (2.5.3.1).

2.5.3.3 Preparation of poly (A)⁺ RNA

a) Selection of poly (A)⁺ RNA using HybondTM-messenger Affinity Paper (mAP).

A piece of HybondTM-messenger affinity paper (mAP) was cut to 1cm² in size. This paper binds at least 25µg of poly (A)⁺ RNA/cm². Selection of poly (A)⁺ RNA from populations of total RNA was carried out according to the manufacturer's instructions (Amersham). The total RNA sample was heated to 65°C, cooled on ice and applied to the messenger affinity paper in high salt conditions (0.5M NaCl), which allows poly (A)⁺ RNA to bind to the mAP paper. Any unbound RNA is removed by washing with 0.5M NaCl followed by 70% (w/v) ethanol washes. The poly (A)⁺ RNA was then recovered by boiling the mAP paper in sterile dH₂O for 5 mins followed by ethanol precipitation (2.5.1.3).

b) Selection of poly (A)⁺ RNA using oligo (dT) Cellulose Powder - Spin Method.

Oligo(dT) cellulose powder was purchased from Boehringer Mannheim, and the manufacturer estimates a binding capacity of 40mg of poly (A)⁺ RNA/g oligo(dT) cellulose powder. Total RNA was made up to a volume of 500µl with sterile dH₂O, heated to 65°C and cooled on ice. An equal volume of 2X Loading Buffer (40mM Tris HCl pH 7.6, 1M NaCl, 2mM EDTA, 0.2% (w/v) SDS) was added, and the RNA sample applied to 0.01g of dry oligo(dT) cellulose powder in an Eppendorf tube. The RNA sample and oligo(dT) cellulose powder were mixed by shaking vigorously for 5 minutes. The oligo(dT) cellulose powder was pelleted by centrifugation in a microfuge, for 5 minutes. The supernatant was removed, heated at 65°C for 5 minutes, cooled on ice and reapplied to the oligo(dT) cellulose powder as before. This procedure was repeated once more. Any unbound RNA was removed by washing 5 times with 1X Loading Buffer (20mM Tris HCl pH 7.6, 0.5M NaCl, 1mM EDTA, 0.1% (w/v) SDS). The poly (A)⁺ RNA was recovered from the oligo(dT) cellulose powder by adding 500µl of Elution

Buffer (10mM Tris HCl pH 7.5, 1mM EDTA, 0.05% (w/v) SDS), followed by ethanol precipitation (2.5.1.3).

c) Estimation of poly (A)⁺ RNA Yields.

The poly (A)⁺ RNA yield was estimated to be 2% of the total RNA yield. Using both methods (see above) for poly (A)⁺ RNA extraction usually about 70% of the predicted poly (A)⁺ RNA yield was obtained. The OD₂₆₀ of a sample from a poly (A)⁺ RNA preparation was occasionally used as a guide for estimating the yield. The most reliable method of assessing the quantity and quality of a given poly (A)⁺ RNA preparation involved radioactively probing (2.5.8) Northern Blots (2.5.9) containing poly (A)⁺ RNA and measuring the strength and quality of the signal obtained compared to the signal obtained from a track loaded with a known quantity of poly (A)⁺ RNA.

2.5.4 Preparation of Plasmid DNA

2.5.4.1 Small Scale

Small scale plasmid DNA was prepared by modification of the rapid extraction method of Birnboim and Doly (1979).

A single bacterial colony was used to inoculate 5ml of L-broth and grown with shaking, at 37°C overnight. A 1.5 ml aliquot of this culture was pelleted in a microfuge (12 Krpm, 1 min) and the supernatant discarded. The cells were resuspended in 100μl of 50mM glucose, 10mM EDTA, 15mM Tris.HCl pH 8.0 and 10μl of lysosyme (10mg/ml) were added, vortexed, and the mixture was incubated on ice for a further 2 minutes before the addition of 150μl of 3M sodium acetate pH 5.5 and vortexing. The sample was then phenol extracted (2.5.1.1), the DNA precipitated with ethanol (2.4.1.3) and the DNA pellet resuspended in 20μl TE buffer. Aliquots of 2μl were used in restriction digests (2.4.1.5) with RNase-A added to a final concentration of 0.1μg/μl in the reaction mixture.

2.5.4.2 Large Scale

A fresh overnight culture (as small scale) of bacteria containing the plasmid required was used to inoculate 500ml L-broth supplemented with the appropriate antibiotic and grown overnight, with shaking, at 37°C. The cells were pelleted in a Sorval RC-5B centrifuge (5 Krpm, 5 minutes) and resuspended in 10ml of ice cold 50mM glucose, 10mM EDTA, 25mM Tris HCl pH 8.0 with the addition of 200 μ l lysosyme (5mg/ml). After the suspension was incubated on ice for 5 minutes, 20ml of 0.5M NaOH, 1% (w/v) SDS were added, with vigorous mixing, and incubated on ice for 5 minutes after which 15ml of 3M Sodium Acetate pH 5.5 were added and the mixture incubated on ice for 60 minutes. After centrifugation (12 Krpm, 10 minutes), the supernatant was phenol extracted (2.5.1.1) and the DNA precipitated by the addition of 0.6 volumes of propan-2-ol and incubation at -20°C for 60 minutes. The DNA was pelleted (12 Krpm, 20 minutes), resuspended in 10ml TE buffer and 10g Caesium Chloride and 1ml of Ethidium Bromide (10mg/ml) were added. The resulting solution was centrifuged in a Sorval OTD 50B ultracentrifuge at 38 Krpm at 18°C, for 48 hours. The DNA bands were visualised using UV illumination and the plasmid band collected. Ethidium Bromide was removed by extraction with Caesium Chloride saturated butan-2-ol, the DNA precipitated with ethanol (2.5.1.3) and resuspended in TE buffer.

2.5.5 Manipulations with Lambda

2.5.5.1 Plating of Phages

Plating cells were prepared by growing host cells overnight in L-broth supplemented with 0.04% (w/v) maltose, pelleting the cells by centrifugation and resuspending in ½ volume 10mM MgSO₄. Appropriate dilutions of phage were mixed with 0.1ml of plating cells and incubated at 37°C for 15 minutes. 3ml of molten BBL top agar was added and poured onto BBL agar plates (9cm diameter). The plates were allowed to set then inverted and incubated at 37°C for 6-16 hours.

2.5.5.2 Plate Lysates - Phage Storage

A well isolated phage plaque was picked into 1ml of phage buffer and left to elute from the agar plug at 4°C overnight. The next day, 50µl of phage suspension was incubated with 100µl of plating cells (prepared as in section 2.5.5.1) and poured onto L-agar plates (9cm diameter). Phage were grown non-inverted for 12-16 hours at 37°C, until the surface of the plate was confluent. The phage were harvested by adding 5ml of phage buffer onto the surface of the plate and incubating for 2-3 hours at 4°C. The liquid layer was removed, vortexed briefly and cells and agar removed under centrifugation (Sorvall RC5-B, 5 Krpm, 5 minutes). The supernatant was removed, titred and stored at 4°C with the addition of a few drops of chloroform.

2.5.5.3 Phage DNA Extraction

a) Miniscale

A single plaque was picked and transferred into 0.3ml of adsorption buffer (10mM MgCl₂, 10mM MgSO₄), and left at 4°C overnight. The next day, 1ml of host plating cells (see section 2.5.5.1) was added to the phage suspension and incubated at 37°C for 15 minutes. The cells and phage were added to 10mls of L-broth in a flask which was supplemented with 10mM MgCl₂ and 0.1% (w/v) glucose, and the phage allowed to lyse the host cells by shaking at 37°C for 3-8 hours. After lysis had taken place, the cell debris was removed by centrifugation (10 Krpm, 30 minutes). The supernatant was carefully removed to a fresh sterile flask and DNase I and RNase A were added, each to a final concentration of 1µg/ml, and incubated at 65°C for 30-60 minutes. The phage were pelleted by ultracentrifugation (Sorvall OTD-50B, 30 Krpm, 60 minutes) and resuspended in 200µl of TM buffer (50mM Tris HCl pH 7.5, 10mM MgSO₄). Proteinase K was added to the resuspended phage pellet to a final concentration of 1µg/µl and incubated at 65°C for 1 hour. Phage DNA was extracted by gentle shaking with an equal volume of phenol (section 2.5.1.1), followed by a phenol : chloroform (1 : 1) extraction and finally traces of phenol were removed by extraction with an equal volume of chloroform. The λ DNA was then ethanol precipitated (2.5.1.3), and resuspended in TE buffer for several

hours at 4°C and stored at -20°C.

b) Maxi Scale

Host cells were grown overnight in a 5ml culture of L-broth supplemented with 0.04% (w/v) maltose. A 0.5ml sample was removed and the cells pelleted by centrifugation in a microfuge and resuspended in 0.5mls of phage buffer. To this preparation of cells, 0.5ml 10mM MgCl₂ and a 0.5ml suspension of phage containing 2.5×10^8 plaque forming units (pfu) were added, and incubated at 37°C for 15 minutes. The cells and phage were then added to 200mls of L-broth supplemented with 10mM MgCl₂ in a 2 litre flask and grown for 6-7 hours, shaking at 37°C until lysis was apparent by the presence of cell debris. Chloroform (0.5mls) was then added to complete lysis. The cell debris was pelleted at 10 Krpm for 10 minutes, and the supernatant was removed and treated with RNaseA and DNase (both at a final concentration of 1μg/ml) at room temperature for 1-2 hours. The degraded cellular RNA and DNA was removed by centrifugation (10 Krpm, 10 minutes). The phage was precipitated by adding 8g of NaCl and 8g of polyethylene glycol 6000 and incubating at 4°C overnight. The phage were pelleted by centrifugation (7 Krpm, 15 minutes), and resuspended in 8mls of phage buffer and extracted once by gentle shaking with an equal volume of chloroform. The phage particles were further purified on a CsCl step gradient. Three 2ml CsCl steps (in phage buffer) were used with densities of 1.3, 1.5 and 1.7g/ml. The phage sample was carefully layered onto the step gradient in 14ml MSE centrifuge tubes and the phage banded at 35 Krpm at 20°C for 2 hours in a 6 x 14ml Ti swing out rotor (Sorvall Ultracentrifuge OTD 50B). The bluish phage band was collected through the side of the tube with a syringe. The phage suspension was then dialysed twice for 1 hour at room temperature against 1 litre of 10mM MgCl₂, 10mM NaCl, 50mM Tris HCl pH 8.0 to remove CsCl. Dialysis tubing was prepared in the standard way (2.5.1.10). The dialysed phage suspension was diluted to 2ml with TE and 100μl 0.1M EDTA, 100μl 10% (w/v) SDS and 10μl 20mg/ml proteinase K added and incubated at 65°C for 1 hour. The phage DNA was then deproteinised by phenol extraction (2.5.1.1) and ethanol precipitated (2.5.1.3). The DNA

pellet was left to dissolve in 50 μ l of TE buffer overnight at 4°C before storing at -20°C.

2.5.5.4 Plating an Ovarian (λ gt11) cDNA Library

A *Drosophila melanogaster* ovarian cDNA library constructed using the λ gt11 vector (see table 2.3) was plated out on L-amp bottom plates.

Approximately 5×10^5 phages were incubated with 1ml of Y1090 plating cells (Method, 2.5.5.1) at 37°C for 15 minutes. Once the freshly made 25mls of top L-agarose (10mM MgSO₄, 0.7% (v/v) agarose (Pharmacia NA) in L-broth) had been boiled and cooled to 49°C, it was added to the phage and cell suspension and quickly poured onto the surface of a large dry L-amp plate (23cm x 23cm). Once the top layer had set, the plate was inverted and the plaques left to grow until they were just touching each other (6-9 hours).

2.5.5.5 Plaque Lifts

DNA from phage plaques were transferred to Hybond-N nylon membrane filters (Amersham). Phage were added to top L-agarose (2.5.5.4) and plated onto BBL bottom (λ Charon4A, λ Charon13) or L-amp bottom (λ gt11) plates and allowed to grow for 6-12 hours at 39°C. After cooling at 4°C for 1 hour a single sheet of Hybond-N nylon membrane (circles 9cm in diameter or 20cm x 20cm sheets for library lifts) was laid on the surface for 1 minute and marked with needle holes for subsequent orientation. The membrane was removed and placed plaque side up on blotting paper soaked in 0.5M NaOH, 1.5M NaCl for 7 minutes then neutralised by placing on blotting paper soaked in 1M Tris HCl pH 8.0, 1.5M NaCl for 6 minutes, and finally placed on blotting paper soaked in 2 X SSC for 5 minutes. The filters were air dried for 30 minutes and the DNA fixed to the membrane using ultra violet light as described in section 2.5.7.1.

2.5.6 Gel Electrophoresis

2.5.6.1 Agarose Gel Electrophoresis of DNA

Agarose gels were made and run in 1 X TBE gel buffer. Ethidium Bromide was added at a concentration of 0.5mg/ml to both the gel and the running buffer. Gel loading sample buffer was added to the DNA samples to 10% (v/v).

Mini-gels were cast and run in a Cambridge Biotechnology model CB1000 mini-gel apparatus. These gels were run at 50-100V for 1-2 hours. Larger agarose gels (16 x 10 x 1cm) were run submerged in home-made gel kits at 100-150V for 1-4 hours.

After electrophoresis DNA bands were visualised on a chromatovue C-70G UV viewing system (254nm short, 265 long).

2.5.6.2 Agarose Gel Electrophoresis of RNA

RNA samples (typically 10 μ g total RNA in dH₂O or 2 μ g of poly (A)⁺ RNA in dH₂O) were added to an equal volume of formaldehyde sample buffer (FSB) and heated to 60°C for 5 minutes, then immediately cooled on ice before adding 0.25 volumes of Ficoll-dye-EDTA (FDE). The samples were immediately loaded on to 1.3% (w/v) agarose gels which were made and run in 1X MOPS buffer. The gels also had formaldehyde added to 17.3% (v/v). The gels (16 x 10 x 1cm) were run submerged in home-made gel kits at 100 volts for 3-4 hours.

2.5.6.3 Molecular Weight Markers

DNA restriction fragments were sized by comparing their distance migrated in the gel with the migration distances of DNA fragments of known molecular weight. λ DNA digested with HindIII DNA (purchased from NBL) and a set of DNA fragments differing in size by 1kb (NBL) were used as standard molecular weight markers.

2.5.7 Transfers to Membrane Filters

2.5.7.1 Southern Blotting (Southern, 1975)

Gels for Southern transfers were prepared with Pharmacia NA agarose. *Drosophila* genomic DNA samples were run in 1% (v/v) agarose gels. Plasmid and λ DNA restriction fragments were sized on 0.7 -1.2% (v/v) agarose gels. When electrophoresis was complete, and after photographing, gels were gently shaken for 30 minutes in 0.5M NaOH, 1.5 NaCl then soaked for 30 minutes in 1M Tris HCl pH 5.5, 3M NaCl and washed in 2X SSC.

A blotting paper wick was placed on a platform supported above a reservoir of 20X SSC with the ends of the wick immersed in the buffer. The wick was thoroughly wet with 20X SSC and the gel laid on top. A sheet of Hybond-N membrane filter (Amersham) was cut to the same dimensions as the gel and was wetted with 2X SSC before being laid on top of the membrane. Care was taken during these steps to avoid air bubbles being trapped. A few pieces of blotting paper, cut to the same dimensions as the gel were soaked in 2X SSC and placed on top of the membrane. Further pieces of blotting paper (again cut to the same dimensions as the gel) were laid, dry, on top of the wet blotting paper to a thickness of 3-5cm, followed by a stack of paper towels to a thickness of 5cm. A sheet of perspex was laid on top of the paper towels and a weight placed on top. Transfer was allowed to occur for 16-24 hours after which the membrane filter was removed, rinsed briefly in 2X SSC and wrapped in clingwrap (Vitafilm, Goodyear Tyre and Rubber Co. Ltd, Staffordshire, GB). The DNA was fixed to the filter by UV irradiation in a UV light box (ChromatoVue C-70G, UV viewing system), with the DNA side towards the source of UV light (254nm short and 365nm long), for 5 minutes. The membrane was sealed and stored in a plastic bag.

2.5.7.2 Bidirectional Southern Blotting

Some DNA gels for Southern transfers were blotted onto two sheets of Hybond-N nylon membrane filters. After gel electrophoresis, the gel was denatured for 30 minutes in 0.5M NaOH, 1.5 M NaCl then soaked for 30 minutes in 1M Ammonium Acetate/0.02M NaOH for 1 hour. Two

sheets of Hybond-N membrane were cut to the same size as the gel and soaked in 1M Ammonium Acetate/0.02M NaOH for 5 minutes. To set up the blot a stack of paper towels 3-5cm in height were cut to the same size as the gel and 3 sheets of blotting paper moistened with 1M Ammonium Acetate/0.02M NaOH were placed on top. Next, a sheet of moistened Hybond-N membrane (with 1M Ammonium Acetate/0.02M NaOH) was laid on top of the blotting paper and the gel placed on top. Another piece of Hybond-N membrane was placed on the upper side of the gel, so that the gel was now sandwiched between 2 sheets of transfer membranes. The blot was stacked up by adding moistened (as before) blotting paper followed by 3-5cm of paper towels. A weight was placed on top of the blot and DNA transferred equally onto each membrane after a few hours. The two membranes were removed and the DNA fixed using UV light (2.5.7.1).

2.5.7.3 Northern Blotting (Thomas, 1980)

Gels for Northern transfers were prepared (2.5.6.2) with Miles Seachem agarose. After electrophoresis, and without further treatment, the gels were transferred to a platform with a blotting paper wick in a reservoir of 20X SSC. The remainder of the transfer procedure was carried out as described for Southern blotting (2.5.7.1).

2.5.8 Radioactive Labelling of DNA and Autoradiography

2.5.8.1 Radioactive Labelling of DNA by Random Priming (Feinberg and Vogelstein, 1983, 1984)

The DNA fragment to be labelled was denatured by boiling for 2 minutes followed immediately by rapid cooling on ice. If the DNA fragment was isolated in low melting point agarose, the fragment was denatured by boiling for 5 minutes, allowed to cool for 1 minute at room temperature then added directly to the reaction mixture. Between 30 - 100ng of DNA was labelled in the reaction in a total volume of 50 μ l containing 10 μ l OLB (detailed in section 2.1), 1 μ l 20mg/ml BSA, 20-50 μ Ci [^{32} P]-dCTP, 1 μ l (5 units/ μ l) Klenow fragment of DNA polymerase I. The reaction was incubated for 4-16 hours at room temperature.

2.5.8.2 Measurement of Radioactivity Incorporated into DNA

The efficiency of incorporated radiolabel was measured by precipitation with trichloroacetic acid (TCA). One aliquot of 1 μ l from the reaction mixture was added to 200 μ l ice cold dH₂O containing 10 μ g BSA and incubated on ice for 10 minutes. The sample was made 10% (w/v) TCA and the precipitate collected on 2.5cm Whatman GF/C glass fibre circles using a Buchner funnel equipped with a water pump. The discs were washed with 5% (w/v) TCA and dried. Another sample (1 μ l) was spotted directly on to a Whatman GF/C glass filter and dried. Each filter was inserted into a scintillation vial, immersed in scintillant (6g/L butyl-PBD in toluene) and the radioactivity measured by counting in a liquid scintillation spectrometer (Intertechnique SL 3000). The percentage incorporation of label into nucleic acid was estimated by comparing the two values and usually probes used achieved 50-90% incorporation and were 10⁷-10⁹cpm/ μ g DNA.

2.5.8.3 Precipitation of Radiolabelled DNA Molecules

Radiolabelled DNA fragments were precipitated from the labelling reaction by the addition of an equal volume of 4M Ammonium Acetate (pH 6.6) and 4 initial volumes of ethanol. After incubation in a dry ice/methanol bath for 15 minutes, the DNA fragments were pelleted by centrifugation in a microfuge for 5 minutes, washed in 70% (w/v) ethanol, air dried and resuspended in hybridisation buffer (see section 2.5.9). This precipitation is successful in removing >90% of unincorporated labelled triphosphates from labelling reactions (Okayama and Berg, 1982).

2.5.8.4 Authoradiography

Authoradiography of nylon membrane filters was performed using Cronex 4 (Dupont) X-ray film and cassettes. Films were developed in an Agfa 1 automatic film processor.

2.5.9 Hybridisation of Northern Blots, Southern Blots and Plaque Lifts

Hybond-N nylon membranes used for Northern blots, Southern blots and Plaque lifts were prehybridised in hybridisation solution (0.5M Sodium Phosphate pH 7.0, 7% (v/v) SDS, 50% (v/v) Formamide) in sealed plastic bags at 42°C for a minimum of 2 hours but usually filters were prehybridised overnight. A volume of 10mls of hybridisation solution was used for most blots (16cm x 10cm) and plaque lifts (9cm diameter). For library lifts (20cm x 20cm) 20mls of hybridisation solution was used.

Once the radiolabelled probe had been denatured (90°C, 5 minutes) it was added to the bag containing the membrane and hybridisation solution and the probe allowed to anneal at 42°C overnight.

2.5.9.2 Post-Hybridisation Washing

After the probe had been removed, membrane filters were washed at varying levels of stringency as follows.

a) Northern Blots

Northern blots were washed in two changes of 1X SSC 0.5% (v/v) SDS at 42°C for 1 hour each and exposed to X-ray film (2.5.8.4). If an overnight exposure detected non-specific background on the filter, a further wash in 1X SSC, 0.5% (v/v) SDS at 65°C for 1 hour was usually sufficient to remove most background.

b) Southern Blots and Plaque Lifts

Genomic blots were washed in two changes of 1X SSC, 0.5% (v/v) SDS at 42°C for 1 hour each followed by a further wash in the same solution at 65°C for 1 hour and exposed to X-ray film (2.5.8.4). Plasmid DNA and λ DNA Southern filters and Plaque lifts were washed briefly (15 minutes) at 42°C in 1X SSC, 0.5% (v/v) SDS followed by 2 washes in the same solution at 65°C for 1 hour each. Exposure to X-ray film allowed signal detection usually within 1-2 hours if probed with homologous DNA.



2.5.10 Screening an Ovarian (λ gt11) cDNA Library

Plaque lifts were performed as described in section 2.5.5.5 and filters hybridised as described in section 2.5.9.1 and washed as described in section 2.5.9.2. Positive plaques were picked with a sterile blue tip (cut off at the end) attached to a P1000 Gilson Pipetteman so that an area of approximately 3mm in diameter was picked and transferred to 1ml of phage buffer. This phage suspension was then rescreened at a density of approximately 100 plaques per 9cm plate until a positive signal corresponded to an individual well isolated plaque. Each positive plaque isolate was grown and stored as a plate lysate (2.5.5.2).

2.5.11 Radioactive Labelling of poly (A)⁺ RNA by Reverse Transcription

Transcription of poly (A)⁺ RNA into cDNA was performed according to the method of Gubler and Hoffmann (1983) using a cDNA synthesis kit purchased from Boehringer Mannheim. At least 1 μ g of poly (A)⁺ RNA was added to a 20 μ l labelling reaction containing first strand synthesis buffer, RNaseA inhibitor, dGTP, dTTP, primer oligo(dT)₁₅, redistilled sterile water and 50 μ Ci of [³²P]-dCTP, 50 μ Ci of [³²P]-dATP and AMV reverse transcriptase. The reaction was incubated at 42°C for 1 hour and the amount of synthesis of [³²P]-dCTP and [³²P]-dATP labelled cDNA was measured by precipitation with TCA (2.5.7.2). Usually ~3 μ g of labelled cDNA was synthesised from 1 μ g of poly (A)⁺ RNA. Removal of unincorporated nucleotides was carried out as described previously (2.5.8.3). The labelled cDNA was resuspended in 1ml of sterile dH₂O.

2.5.12 Probing Southern Blots with Radiolabelled cDNA

Radiolabelled cDNA made from poly (A)⁺ RNA prepared from male and female adults of *Drosophila melanogaster* (2.5.3) was used to probe a set of restriction endonuclease digested recombinant λ bacteriophage clones which had been bidirectionally Southern blotted (2.5.7.2). The membranes were prehybridised in a sealed plastic bag containing 50% (v/v) formamide, 5X SSPE, 1X Denhardt's solution, 0.1% (v/v) SDS and 0.1mg/ml sonicated salmon sperm DNA for 24 hours. The cDNA probe (2.5.11) was denatured at 90°C for 5 minutes, snap cooled on ice for 5 minutes and added to the hybridisation bag. The hybridisation was carried out at 42°C for 4

days. The probe was removed and the filter washed in 3 changes of 0.1X SSPE, 0.1% (v/v) SDS for 1 hour each wash. The excess moisture on the membrane surface was removed with blotting paper and the filter then exposed to pre-flashed Hyperfilm (Amersham).

2.5.13 In situ Hybridisation to Third Instar Polytene Chromosome Preparations

2.5.13.1 Preparation of Third Instar Polytene Chromosomes

Microscope slides were washed in 1M HCl and then subbed by dipping into a solution of 0.1% (w/v) gelatin, 0.025% (w/v) chromic Potassium Sulphate, and air dried. Cover slips were washed in 1M HCl, air dried and siliconised by dipping into a solution of Dimethyldichlorosilane (about 2% (v/v) in 1,1,1-trichloroethane) followed by air drying.

Salivary glands were dissected in Ringer's Solution (2.1) from late third instar larvae and placed in a small drop of aceto-orcein stain (BDH) for five minutes. A few drops of 45% (w/v) acetic acid was added to allow the salivary gland chromosomes to be easily visualised. The glands were then transferred to a drop of 45% acetic acid on a microscope slide. A coverslip (18mm²) was placed on top of the glands. The cells were broken by pressing the coverslip at one edge with a tissue followed by gentle tapping with a stiff, pointed probe starting over the glands and then gently working towards the edge in a spiral pattern. The slide was then turned over, onto a paper towel, and pressed at one edge so that the stain flowed to the opposite edge thus separating cell debris from the nuclei. The slide was inverted again, held at the edge with a paper towel and the chromosomes were spread by streaking across the coverslip in a zig-zag motion using a pointed probe. Finally the chromosomes were flattened by placing the slide between filter paper circles and pressing firmly. The slide was frozen in liquid nitrogen, the coverslip removed, and then the chromosomes were dehydrated in 95% (w/v) ethanol for 5 minutes, and air dried. The chromosomes were then examined under phase contrast microscopy and chosen for subsequent hybridisation experiments if the chromosome morphology was in good condition. The slides were stored at 4°C in an air tight dry plastic container.

2.5.13.2 Prehybridisation of Polytene Chromosome Preparations

Before hybridisation, the slides were incubated in 2X SSC at 65°C for 30 minutes, then dehydrated through 2 changes of 70% (w/v) ethanol, 10 minutes each and 1 change of 95% (w/v) ethanol for 5 minutes and air dried. 200µl of 100µg/ml RNaseA in 2X SSC was pipetted onto the chromosomes and a large coverslip (25mm²) was placed on top. The slides were incubated at 37°C in a moist chamber, then rinsed in 2X SSC (3 changes at 5 minutes each) followed by dehydration through an ethanol series as described above. The chromosomal DNA was then denatured by soaking the slides in 7mM NaOH for 3 minutes followed by dehydration as before.

2.5.13.3 Preparation of [³H]-dCTP Labelled DNA Probes and Hybridisation

Probe DNA was labelled using the random priming method. EcoRI restriction fragments of genomic DNA in the range of 3.0 kb to 5.0 kb in length were isolated from low melting point agarose gels (2.5.1.6). Approximately 50ng of DNA in low melting point agarose was labelled as described in section (2.5.8.1) using [³H]-dCTP instead of [³²P]-dCTP. The reaction components were added to 40µCi of [³H]-dCTP which has to be dried before use as it is supplied in 50% (v/v) ethanol. Labelled probe DNA was separated from unincorporated [³H]-dCTP by the addition of 50µl of 4M Ammonium Acetate pH 6.6 and 200µl of absolute ethanol and placed on a dry ice/methanol bath for 30 minutes. 10µg of sonicated salmon sperm DNA was also added to the ethanol precipitation. The labelled DNA was pelleted by centrifugation in a microfuge for 7 minutes, washed in 70% (w/v) ethanol, vacuum dried and resuspended in hybridisation buffer (50% (w/v) formamide, 4X SSC, 1X Denhardt's). A volume of 10µl to 20µl of probe with approximately 5X 10⁵ cpm was pipetted onto each squash, and covered with an acid washed coverslip. The slides were sealed with cow gum (diluted with diethyl ether to a consistency easily pipetted using a glass pasteur pipette) and placed in a moist chamber at 42°C overnight.

2.5.13.4 Washing Steps After Hybridisation

After hybridisation the cow gum seal was easily removed using tweezers and the microscope slide floated off in the first wash. Slides were washed twice in 2X SSC, 50% (v/v) formamide for 10 minutes, at room temperature, then dehydrated through ethanol as described above (2.5.13.2).

2.5.13.5 Autoradiography

Prior to dipping, 5ml of 2% (w/v) glycerol was measured out into a dipping chamber and clamped immersed in a water bath at 42°C, in the darkroom. In complete darkness, approximately 5ml of Ilford IL4 photographic emulsion was melted in a beaker pre-clamped in the water bath, and then added to the glycerol in the dipping chamber. The slides were dipped one at a time, and the excess emulsion was removed by vigorous shaking. The slides were stored in a lightproof box for approximately 10 days at 4°C. The slides were developed in complete darkness for 3 minutes, stopped with 3% (w/v) acetic acid, fixed in Hypon solution for 1½ minutes, and washed in running water for 10 minutes, followed by air drying. The developer was 0.22% (w/v) p-methylamino phenol sulphate, 7.2% (w/v) NaSO₃, 0.88% (w/v) hydroquinone, 4.8% (w/v) Na₂CO₃, 0.4% (w/v) KBr. This can be purchased from Kodak (D19 developer).

2.5.13.6 Microscopy and Chromosome Staining

After developing, the slides were examined using light microscopy to determine if the hybridisation signal visualised as black grains at the site of hybridisation on the chromosome arm were detectable. The slides were then stained as follows. The slides were soaked in 10mM Sodium Phosphate buffer, pH 7.0, for 3 minutes, then transferred to a 5% (v/v) dilution of Giemsa stain (supplied by SIGMA) in 10mM Sodium Phosphate buffer pH 7.0. Slides were stained for 10 minutes, then placed with 10mM Sodium Phosphate, pH 7.0 for 5 minutes and finally rinsed extensively in running water. The slides were air dried and viewed at X25, X40 and under immersion oil at X100 magnification to locate the precise band within which the signal was present. The *in situ* hybridisation signals were photographed using both black and white film

(Ilford Pan F) and also colour film (Kodak Ektachrome 160).

2.5.14 In situ Hybridisation To Whole Mounts of *Drosophila* Ovaries (Tautz and Pfeifle, 1989)

2.5.14.1 Preparation of Tissues - Paraformaldehyde Fixation

Ovaries and testes were hand dissected from adults of *Drosophila*. The method of fixing is essentially the same method described in Tautz and Pfeifle (1989). The flies were anaesthetised and dissected in fixing solution which consists of 0.1M Hepes pH 6.9, 2mM MgSO₄, 1mM EGTA (ethylen-glycol-bis(2-aminoethylether)-N,N-tetraacetic acid) and 5% (v/v) paraformaldehyde. The paraformaldehyde stock solution (20% w/v) was dissolved by heating to 65°C, neutralised with NaOH and stored at -20°C. Once dissected, the ovaries were transferred to sterile universal tubes containing 2 mls of fixing solution. An equal volume of methanol was added and the tissues were gently shaken for 5 minutes, and left to fix for 30 minutes - 2 hours. The fixing solution was removed and the ovaries were transferred to eppendorf tubes containing 1 ml of 90% (v/v) methanol and 10% (v/v) 0.5M EGTA (ME). The ovaries were then refixed and dehydrated by passage through a series of steps consisting of ME and PP (PP consists of 4% (v/v) paraformaldehyde in phosphate buffered saline (PBS, 130 mM NaCl, 10 mM sodium phosphate pH 7.2)). The first step is for 5 minutes in 7/3 ME/PP, the second is for 5 minutes in 1/1 ME/PP, the third for 5 minutes in 3/7 ME/PP and the last step for 20 minutes in PP alone. The ovaries were then washed in PBS for 10 minutes and directly subjected to the following steps. They can, if desired be stored at this point by dehydrating them in a 30%, 50% and 70%(w/v) ethanol series and leaving them at -20°C. Before proceeding with the next stage they must be rehydrated.

2.5.14.2 Probe Labelling Using Digoxigenin - Non Radioactive Method

Probes consisted of linearised plasmids containing cDNA inserts and were labelled according to the protocol supplied with the Boehringer Kit. The DNA to be labelled was denatured at 90°C for 10 minutes and rapidly cooled for 10 minutes on ice. Buffer containing unlabelled dATP, dGTP, dCTP, dGTP and digoxigenin labelled dUTP, the correct reaction salt

conditions and primers, and Klenow were added and the mixture incubated overnight at 37°C. The labelling was done by random priming (Feinberg and Vogelstein, 1983). The probes were precipitated with an equal volume of 4 M Ammonium Acetate pH 6.6 and four volumes of absolute ethanol at -20°C overnight. 10µg of sonicated salmon sperm DNA was also added to the precipitation. The labelled DNA fragments and salmon sperm DNA were recovered by centrifugation in a microfuge for 5 minutes, washed in 70% (w/v) ethanol, air dried and resuspended in hybridisation solution (2.5.14.4).

2.5.14.3 Pretreatment of Tissues

All pretreatment steps were performed in 1.5 ml eppendorf tubes in 1 ml solution at room temperature on a revolving wheel. The ovaries were first washed 3 times for 5 minutes each in PBT (PBS plus 0.1% (v/v) Tween 20). They were then incubated for 3-5 minutes in 50 µg/ml Proteinase K in PBS. The digestion was stopped by incubating for 2 minutes in 2 mg/ml glycine in PBT. The ovaries were then washed 2 times for 5 minutes each in PBT, refixed for 20 minutes with PP and finally washed 3 times for 10 minutes each in PBT.

2.5.14.4 Hybridisation and Washing

The hybridisation solution (HS) consists of 50% (v/v) formamide, 5X SSC, 50 µg/ml heparin, 0.1% (v/v) Tween20 and 100 µg/ml sonicated and denatured salmon sperm DNA. (HS maybe stored at -20°C.) The tissues were washed for 20 minutes in 1/1 HS/PBT then for 20-60 minutes in HS. They were then transferred to a 45°C water bath for 20-60 minutes. Most of the supernatant was then carefully removed and the heat denatured probe (90°C, 5 minutes) was added (refer to 2.5.14.2). The probe concentration was aimed to be at approximately 0.5 µg/ml. The tissues were hybridised overnight at 42°C. The tissues were washed at room temperature for 20 minutes at each step. The first wash was in HS, the second in 4/1 HS/PBT, the third in 3/2 HS/PBT, the fourth in 2/3 HS/PBT, the fifth in 1/4 HS/PBT and the last two 20 minutes each in PBT.

2.5.14.5 Signal Detection

The antibody-conjugate solution (supplied with the Boehringer Kit) was used to detect the digoxigenin signal. The antibody was freshly diluted 1/2000 to 1/5000 in PBT and was preabsorbed for 1 hour with fixed tissues as this was found to be a critical step in reducing the background signal. The antibody solution (500 μ l) was then transferred to the experimental tissues and incubated for 1 hour at room temperature on a revolving wheel. The ovaries were washed 4 times for 20 minutes each in PBT, then 3 times for 5 minutes each in 100 mM NaCl, 50 mM MgCl₂, 100 mM Tris pH 9.5, 1mM Levamisole (a potent inhibitor of lysosomal phosphatases), 0.1% (v/v) Tween 20. The ovaries were then transferred to a microtitre dish with 250 μ l of the above solution. The antibody signal was detected using a colour reaction by adding 1.25 μ l of NBT (nitroblue tetrazolium salt, 75 mg/ml in dimethyl formamide, 70% (v/v)) and 0.9 μ l of the X-phosphate solution (5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt, 50 mg/ml in dimethylformamide) both of which were supplied with the Boehringer kit. The colour developed after approximately 2 hours in the dark. The colour development was stopped by adding PBT before background colour appeared. The tissues were mounted in Gurr's Water Mounting Medium and flattened by placing a brass weight on top of the coverslip overnight. The tissues were photographed at X10 magnification using Kodak Vericolor III professional film.

CHAPTER THREE

IDENTIFICATION OF FEMALE SPECIFIC TRANSCRIPTS IN

DROSOPHILA MELANOGASTER

Introduction

Background Information About The Differential Screen Used To Isolate Sex-Specific Transcripts

In *Drosophila melanogaster*.

In order to isolate genes expressed sex-specifically in adults of *Drosophila melanogaster* a differential screen was carried out in this laboratory by Smith and Bownes (1984, unpublished). The view at that time was that genes involved in male and female somatic sexual differentiation would be switched on or off at the level of gene transcription. The chorion genes (Spradling *et al.*, 1980) and the yolk protein genes (Barnett *et al.*, 1980) were the only genes that had been cloned which were known to encode sex-specific transcripts. The chorion and yolk protein gene transcripts were shown to be absent in males.

A small number of regulatory loci controlling sexual differentiation in somatic tissues had been identified genetically. Extensive mutational analysis of these loci revealed a hierarchy of gene activity (reviewed in Baker and Belote, 1983) (refer to Figure 3.1). The primary sex determining signal consists of the ratio of the number of X chromosomes (X) to autosome sets (A); a ratio of 2X:2A triggers the female pathway of development and a ratio of 1X:2A triggers the male pathway of development. The reading of the primary signal depends on the dosage of a number of discrete X-linked loci which signal the number of X chromosomes and have been called numerator elements. The master regulatory locus, *Sxl* (*Sex-Lethal*) is activated only in females. For female development, the genes *intersex* (*ix*), *transformer* (*tra*) and *transformer-2* (*tra-2*) are switched on and this results in the female mode of expression of the bifunctional *doublesex* (*dsx*) locus. In males, *Sxl* is not activated and the *doublesex* locus is expressed in its basal mode resulting in the activation of the male pathway of development.

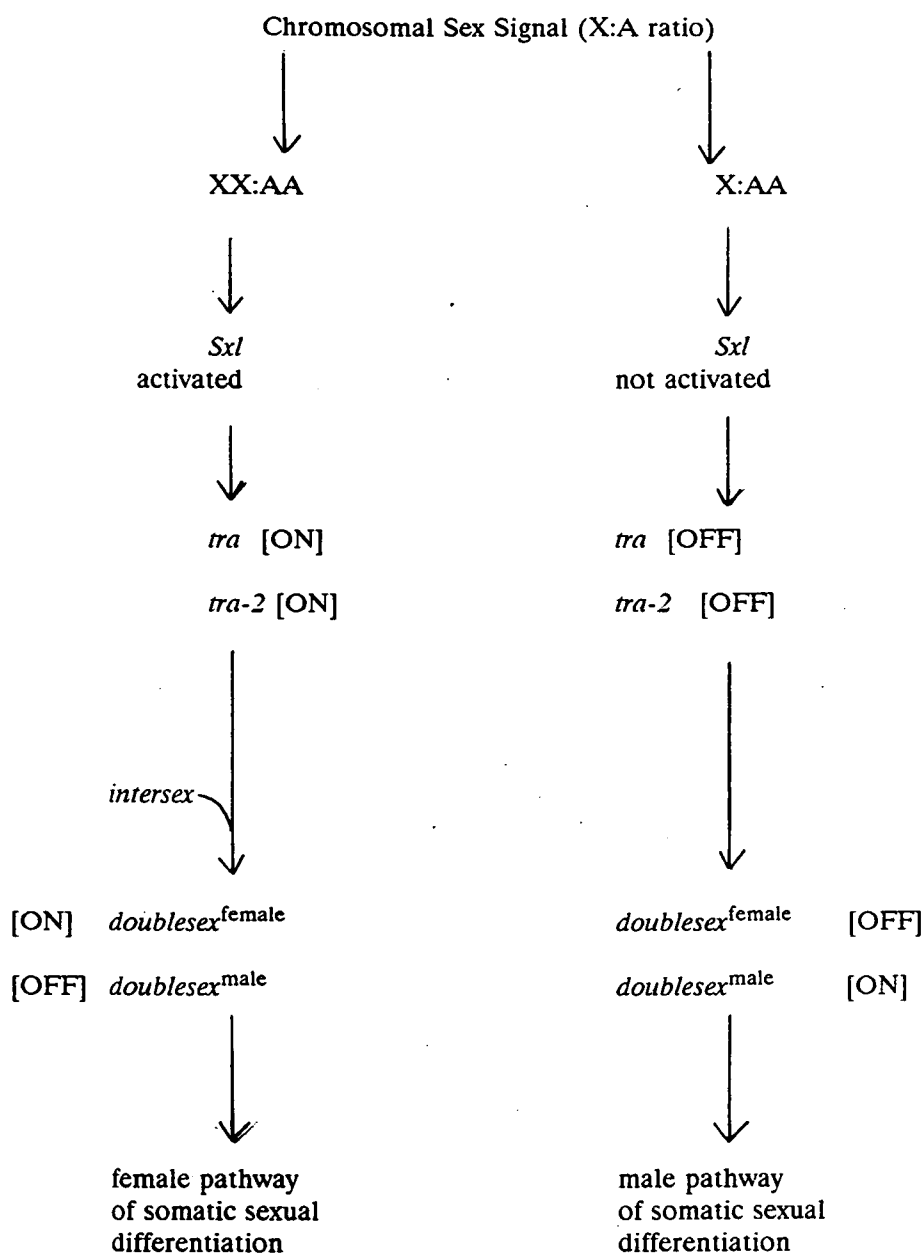
Therefore, on the basis of the results from gene expression studies on the yolk protein and the chorion protein genes, and the genetic evidence for on/off regulation of sex specific gene expression, the following differential screen was carried out in an attempt to identify male and female sex-specifically expressed transcripts which would be under the control of the somatic sex determination hierarchy. It is important to note that the determination of the sex of the germline

Figure 3.1

Control Of Somatic Sexual Differentiation In *Drosophila melanogaster*

This figure illustrates the interactions of the regulatory loci which control somatic sexual differentiation in *Drosophila*, based on the model proposed by Baker and Belote (1983)

<i>Sxl</i>	:	<i>Sex lethal</i>
<i>tra</i>	:	<i>transformer</i>
<i>tra-2</i>	:	<i>transformer-2</i>



cells (e.g. the oocyte in females and sperm in males) appears to be controlled, at least in part, by a separate regulatory mechanism (reviewed by Pauli and Mahowald, 1990). There is at least some overlap between the somatic and germline sex determination pathways, and *Sxl* has been shown to be required for correct determination in both. To date, the genes controlling the differentiation of germline cells have not been identified with certainty although the genes *ovo* and *onu* (ovarian tumour) seem likely to be involved (Pauli and Mahowald, 1990).

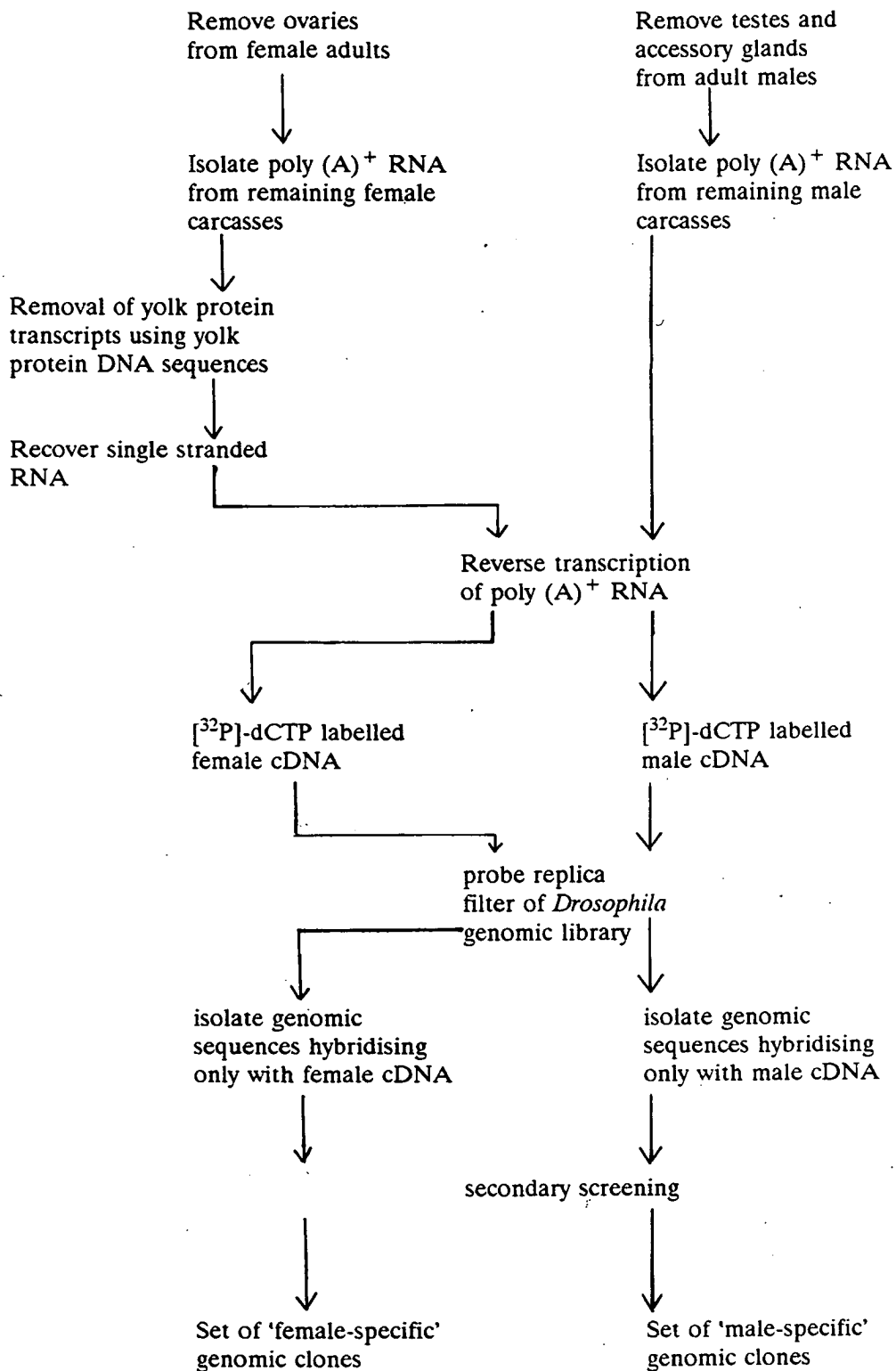
Differential Screening Of A *Drosophila* Genomic Library With Populations Of [³²P]-dCTP Labelled cDNA Made From Male And Female Adult Carcass RNA

This screen was designed with the aim of isolating sex-specifically expressed genes under the control of the somatic sex determination hierarchy. In order to avoid isolating genes expressed in the gonads, the ovaries and testes were removed from 200-300 female and male adults, respectively. Poly (A)⁺ RNA was extracted from the remaining male and female carcasses. In order to avoid isolating the *Drosophila* yolk protein genes, the female poly (A)⁺ RNA was hybridised with yolk protein DNA sequences. The fraction of the female carcass RNA sample which did not hybridise with the yolk protein sequences was recovered by passage through a hydroxyapatite column which separates single stranded nucleic acids from double stranded nucleic acids. The male and female carcass poly (A)⁺ RNA preparations were then reverse-transcribed into [³²P]-dCTP labelled cDNA. Each male and female cDNA probe was used to screen replica filters of a *Drosophila* genomic DNA library (Maniatis *et al.*, 1978). Genomic clones which hybridised only with the female cDNA probe formed a set of clones encoding putative female-specific transcripts. Clones which hybridised only with the male cDNA probe were selected and proposed to encode male-specific transcripts. Both sets of clones were re-screened with male and female radiolabelled cDNA probes until a set of eight putative 'female-specific' and eight 'male-specific' transcript encoding genomic sequences were identified. This method of differential screening is summarised in Figure 3.2.

Figure 3.2:

Differential Screening Technique Applied To The Isolation Of Sex-Specifically Expressed Genes
In Adults Of *Drosophila*

Illustration of differential screen carried out by Smith and Bownes (1984, unpublished) aimed at isolating genes expressed sex-specifically in adults of *Drosophila melanogaster*.



RESULTS

A. Preliminary Analysis Of Female Specific Clones : Cross Hybridisation Studies

At the beginning of this project the eight female-specific clones were digested with EcoRI, Southern blotted and tested for cross-hybridisation. This thesis is concerned with the study of two independent and unrelated isolates, clone 2 (λ ov2) and clone 5 (λ ov5). Clone numbers 3, 4, 6 and 7 which were shown to cross-hybridise were studied by Claire Gilman. The putative 'male-specific' clones have not been analysed.

B. Identification Of Two Genomic Clones Encoding Transcripts Expressed In Adult Females

Northern blot analysis was used initially to investigate the putative sex-specific transcripts encoded by several female-specific genomic clones. Typically, 10 μ g of total male RNA and 10 μ g of total female RNA were Northern blotted and probed with a number of female specific clones.

In most cases, no hybridisation signals were observed. In order to increase the sensitivity of the Northern analysis, the following approaches were taken.

1. Identification Of Genomic Sub-Fragments Hybridising Only With Radiolabelled Female cDNA

Each putative female-specific clone was digested with EcoRI and the DNA restriction fragments separated by agarose gel electrophoresis. The DNA was transferred onto two Hybond-N membranes, one being placed on each side of the gel allowing equal transfer of DNA to each filter (Bidirectional Southern Blotting). Each replica blot was probed with radiolabelled male or female cDNA probes. The results of this experiment are presented in Figure 3.3. λ ov5 consists of two EcoRI fragments, 5.0 kb (ov5A) and 5.7 kb (ov5B) in length. The 5.0 kb EcoRI DNA sub-fragment of λ ov5 (ov5A) hybridised only with the female cDNA probe (Figure 3.3, lane 5). λ ov2 consists of 7 EcoRI sub-fragments, 4.2 kb (ov2B), 3.25 kb (ov2A), 2.0, 1.9, 1.4, 0.75 and 0.2 kb in length. The 4.2 kb (ov2B) and 3.25 kb (ov2A) insert EcoRI DNA fragments from λ ov2 hybridised only with the female cDNA probe (Figure 3.3, lane 2). The DNA fragments which selectively hybridised with the female cDNA probe were used to probe Northern blots of RNA

Figure 3.3

Identification of Sub-fragments Within λ ov2 And λ ov5 Which Hybridised With Female cDNA But Not With Male cDNA Probes

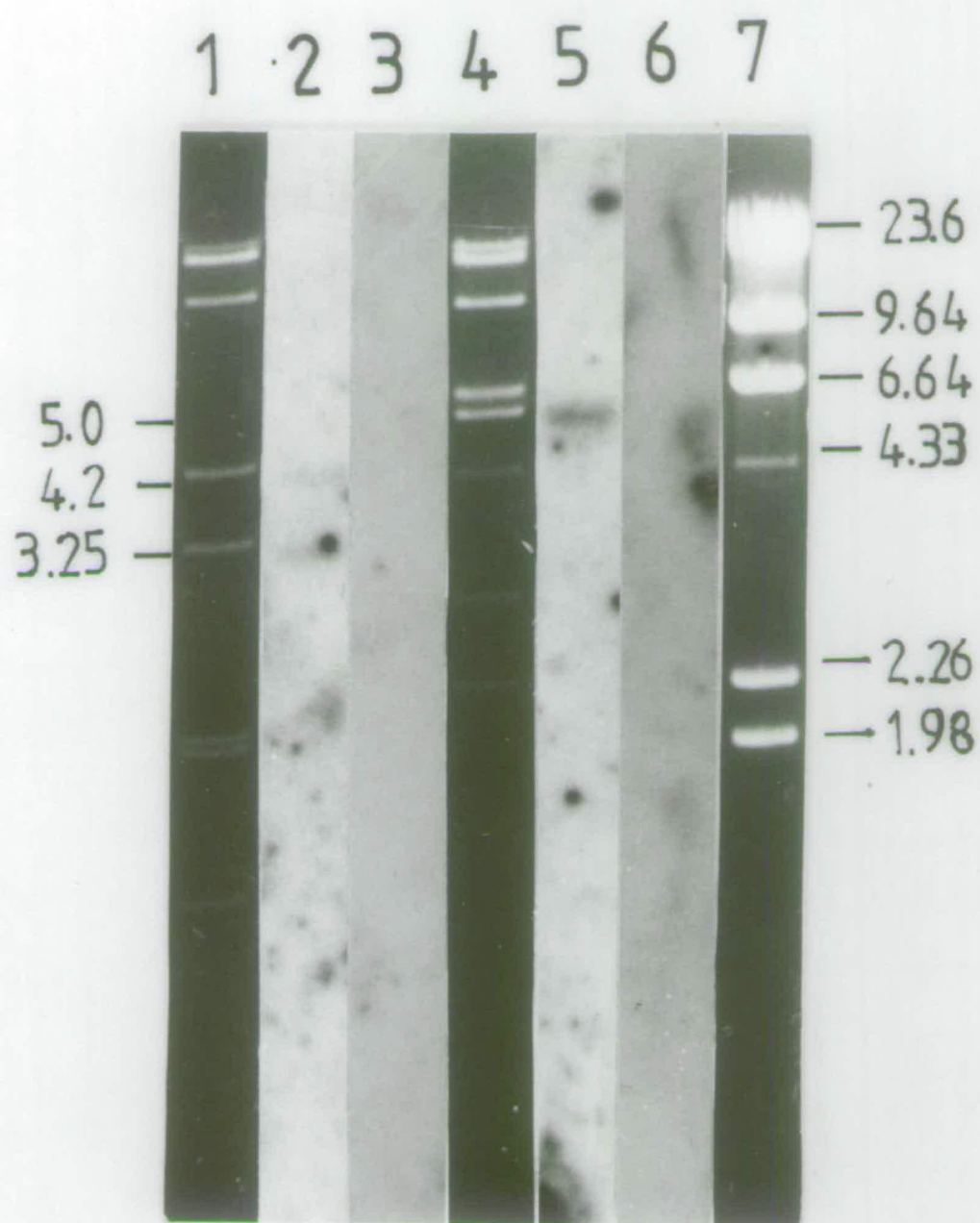
λ ov2 and λ ov5 DNA samples were digested with EcoRI and the fragments generated separated by electrophoresis on a 1% (w/v) agarose gel. The DNA was transferred equally onto two separate membranes (Bidirectional Southern Blotting Technique). Each membrane was then hybridised with [^{32} P]-dCTP and [^{32} P]-dATP labelled cDNA made from female or male poly (A)⁺ RNA. EcoRI sub-fragments from each clone which hybridised only with the female cDNA probe were identified and proposed to encode female-specific transcripts.

Lanes 1 and 4: Ethidium Bromide stained 1% (w/v) agarose gel containing EcoRI digestion products from λ ov2 (lane 1) and λ ov5 (lane 4) DNA samples.

Lanes 2 and 5: Autoradiograph of EcoRI digested λ ov2 (lane 2) and λ ov5 (lane 5) DNA samples after hybridisation with the radiolabelled female cDNA probe. The sizes of the sub-fragments (in kb) of each clone which hybridised with the female cDNA probe are indicated on the left hand side. λ ov2 (lane 2) contains two sub-fragments, 4.2 kb (ov2B) and 3.25 kb (ov2A) in length, which hybridised with the female cDNA probe. λ ov5 (lane 5) contains a 5.0 kb (ov5A) sub-fragment which hybridised with the female cDNA probe. In each case 4 days exposure was required to visualise the hybridisation signals.

Lanes 3 and 6: Autoradiograph of EcoRI digested λ ov2 (lane 3) and λ ov5 (lane 6) DNA samples after hybridisation with a radiolabelled male cDNA probe. No hybridisation signals were observed after a 10 day long exposure.

Lane 7: Molecular weight markers. Ethidium Bromide stained agarose gel containing λ DNA digested with HindIII. The size of each restriction fragment (in kb) is indicated on the right hand side of the figure.



extracted from sexed adults.

2. Enriching For Poly (A)⁺ RNA: Increasing The Sensitivity Of Northern Analysis.

In order to increase the sensitivity of transcript detection poly (A)⁺ RNA was extracted from sexed adults. Total RNA was also prepared from adult female carcasses and ovaries to enrich for transcripts present in each tissue.

C. Clone λ ov5: Identification Of Female Specific Transcripts

The 5.0 kb EcoRI fragment (ov5A) was isolated from λ ov5 and used to probe a Northern blot containing adult male and female poly (A)⁺ RNA, and ovary and female carcass total RNA.

Two female enriched transcripts were detected (Figure 3.4, panel A, lane 1). The 1.7 kb sized transcript was detected only in female poly (A)⁺ and ovary total RNA. The 1.4 kb sized transcript was detected at a higher level in female polyA⁺ RNA than in male poly (A)⁺ RNA. The 5.7 kb EcoRI fragment (ov5B) from λ ov5 hybridised with a 1.4 kb sized transcript only in adult female poly (A)⁺ RNA (Figure 3.4, panel B, lane 1).

D. Clone λ ov2: Identification of Female-Specific Transcripts

The 4.2 kb (ov2B) and 3.25 kb (ov2A) EcoRI DNA fragments were isolated from λ ov2 and used together to probe adult male and female poly (A)⁺ RNA, and female carcass and ovary total RNA. The results are illustrated in Figure 3.4, Panel D. In ovary total RNA a transcript approximately 4.0 kb in size was detected (Figure 3.4, panel D, lane 4). No transcripts were observed in the female carcass total RNA sample.

E. Estimation of Transcript Sizes

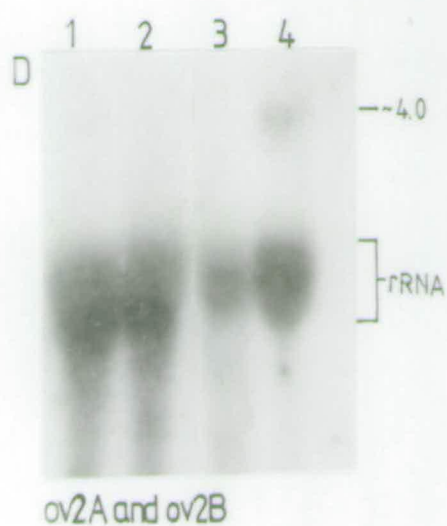
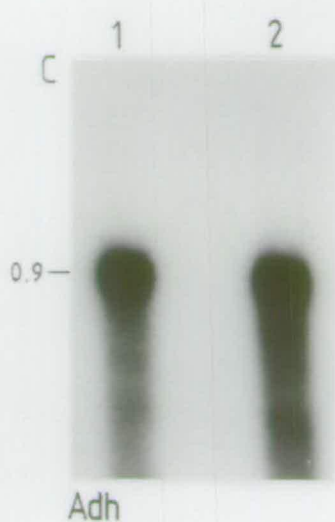
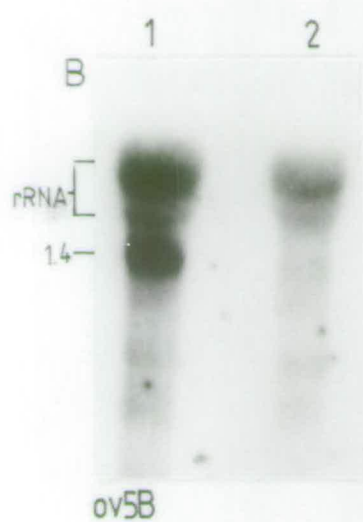
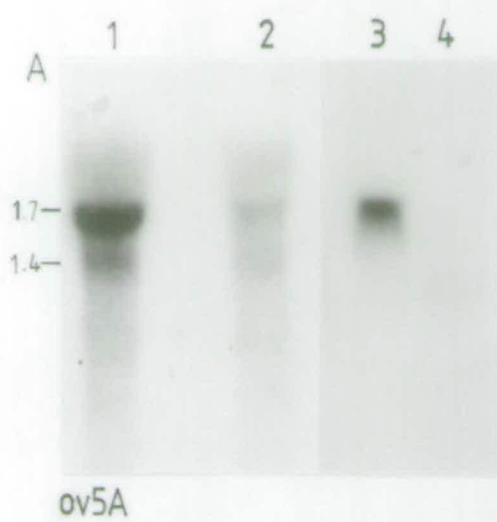
The sizes of the newly identified female-specific transcripts were estimated by comparing the location of the transcript hybridisation signals with the migration distance of transcripts of known length. In each of the blots shown in Figure 3.4 (panels A, B, and D) a non-specific hybridisation signal with rRNA (ribosomal RNA) was observed. This phenomenon has been

Figure 3.4

Preliminary Northern Analysis - Identification Of Female And Ovary Specific Transcripts

- A: Autoradiograph of a Northern blot containing 5 μ g of female poly (A)⁺ RNA (lane 1), 5 μ g of male poly (A)⁺ RNA (lane 2), 10 μ g of ovary total RNA (lane 3) and 10 μ g of female carcass total RNA (lane 4). This blot was probed with the ov5A sub-fragment. The autoradiograph was exposed for 10 days.
- B: The Northern filter containing lanes 1 and 2 from filter (A) was stripped and reprobed with the ov5B sub-fragment. A non-specific background hybridisation signal with rRNA sequences was observed after 10 days exposure.
- C: A duplicate Northern filter loaded with 5 μ g of female (lane 1) and male (lane 2) poly (A)⁺ RNA (as used in (A)) was probed with *Drosophila* alcohol dehydrogenase (*Adh*) sequences, to show that the samples were equally loaded. The autoradiograph was exposed for 5 hours. The 0.9 kb sized *Adh* transcript is indicated.
- D: Northern blot containing 2 μ g of female poly (A)⁺ RNA (lane 1), 2 μ g of male poly (A)⁺ RNA (lane 2), 10 μ g of female carcass total RNA (lane 3) and 10 μ g of ovary total RNA (lane 4). This blot was probed with the ov2A and ov2B fragments simultaneously.

The estimated sizes (in kb) of transcripts detected by each probe are indicated on each photograph.



observed with a variety of DNA probes when used to probe Northern blots to detect transcripts of relatively low abundance. For example, a non-specific hybridisation signal with rRNA is commonly observed when ovary poly (A)⁺ RNA was probed with DNA sequences from the *Drosophila* I-factor transposable element (J. Prosser, personal communication). The Northern blots shown in Figure 3.4 were probed with ribosomal DNA which hybridised with a much greater signal intensity to the same region as the non-specific rRNA signal obtained with the λ ov5 and λ ov2 sub-fragment probes (data not shown). This non-specific rRNA signal observed in Northern blots A, B and D (Figure 3.4) also served as a size marker for the newly identified transcripts. The upper ribosomal RNA signal is 1.9 kb in length and the lower ribosomal RNA signal is 1.7 kb in length. The *Drosophila* alcohol dehydrogenase gene (*Adh*) which hybridises with a 0.9 kb sized message on Northern blots was also used as a RNA size marker and for loading checks (Figure 3.4, panel C). The size of the ovary specific transcript detected by the ov2A and ov2B sub-fragments was estimated by eye.

F. λ ov2 And λ ov5: Was There Any Cross Hybridisation With *Drosophila* Chorion Protein Or Yolk Protein DNA Sequences?

Southern blots of the set of female-specific clones digested with EcoRI were Southern blotted and probed with chorion DNA sequences spanning the clusters on the third and X chromosomes (gifted by A. Spradling) and also with yolk protein DNA sequences. No hybridisation signal was observed with either of these probes which illustrates that the newly isolated female-specific clones are not re-isolates of this group of cloned female-specifically expressed genes (data not shown).

DISCUSSION

Discussion Of Results

In this chapter the preliminary analysis of transcripts encoded within two genomic clones called λ ov2 and λ ov5 which were isolated from a differential screen has been presented. Each clone was found to contain sub-fragments which detect female-specific or female enriched transcripts.

λ ov5 consists of two EcoRI sub-fragments, ov5A and ov5B, which detect two female enriched transcripts. The ov5A probe detects a 1.7 kb transcript that is female and ovary specific, and a 1.4 kb transcript which is enriched in female poly (A)⁺ RNA. The ov5B probe hybridises with a 1.4 kb sized female-specific transcript. λ ov2 includes two EcoRI sub-fragments, ov2A and ov2B, which were radio labelled together and found to hybridise with an ovary specific transcript approximately 4.0 kb in size.

The transcripts detected by each of the genomic clones λ ov5 and λ ov2 are expressed at a relatively low level. For example when the ov5A fragment (5.0 kb EcoRI fragment) was labelled to a specific activity of 5×10^8 cpm/ μ g DNA and used to probe a Northern blot containing 5 μ g of adult female poly (A)⁺ RNA, an exposure length of 10 days was required to visualise a clear hybridisation signal from the 1.7 kb female-specific transcript (Figure 3.4, panel A, lane 1). When a duplicate Northern blot containing an equivalent amount (5 μ g) of adult female poly (A)⁺ was probed with the *Adh* gene labelled to a similar specific activity, a very strong hybridisation signal was observed after 5 hours (Figure 3.4, panel C).

The results shown in Figure 3.3 illustrate how the sub-fragments encoded within λ ov2 and λ ov5 were identified as being likely to encode female-specific transcripts. Sub-fragments which hybridised with female cDNA but not with male cDNA probes were selected for use in subsequent Northern analysis. This was particularly useful in the case of λ ov2 as it encodes seven EcoRI sub-fragments. The 5.7 kb EcoRI fragment (ov5B) within λ ov5 did not hybridise with the female cDNA probe yet it detected a 1.4 kb female specific transcript in adult female poly (A)⁺ RNA (Figure 3.4, panel B, lane 1). This may indicate that in the population of female poly (A)⁺ RNA labelled the 1.4 kb transcript was represented at such a low level that it failed to be reverse

transcribed. It is also possible that not all mRNAs are efficiently reverse transcribed during cDNA labelling reactions perhaps due to secondary structural characteristics.

During the preliminary analysis of female-specific transcripts encoded within λ ov2 and λ ov5 it became apparent that at least two of the transcripts were expressed specifically in the adult female ovary. It was not expected that genomic sequences encoding ovary specific transcripts would have been detected in the differential screen used to isolate λ ov2 and λ ov5 (this will be discussed shortly). Nevertheless I became interested in the potential function of the ovary enriched transcripts during oogenesis. Moreover, at this stage of the project very few genes involved specifically in oogenesis had been cloned (the yolk protein genes and chorion genes). Some female sterile mutants disrupting oogenesis and early embryogenesis had been identified and cloning of the loci responsible for the female sterility was underway. The next two chapters in this thesis present a more detailed molecular analysis of the ovary transcripts detected by λ ov2 and λ ov5. In the final chapter experiments are described which were carried out in an attempt to evaluate the function(s) of the ovary enriched transcripts during oogenesis.

Examination Of The Differential Screening Technique - Did It Work?

It was not expected that genomic sequences encoding ovary specific transcripts would have been isolated in the differential screen described in this chapter. The screen was designed to isolate sequences encoding sex-specific transcripts in the somatic tissues of adults of *Drosophila melanogaster*. Of the set of female-specific isolates only one genomic clone was shown to hybridise with a female somatic specific transcript (C. Gilman, pers. comm.). Six out of the eight female-specific clones were found to encode ovary and/or embryo enriched transcripts. No transcripts have been detected with the eighth female-specific clone. So why then were sequences isolated in this screen which detect ovary enriched transcripts? It seems that (with one exception) there was no differential selection of genomic sequences on the basis that they encoded sex-specifically expressed somatic transcripts. However it does seem that some non-random selection of sequences has taken place as three female-specific isolates evidently contain related sequences since they cross hybridise with each other (C. Gilman, pers. comm.). Furthermore it is possible

that when the female carcasses were being collected that the egg was not removed from the uterus and if this was the case then the female carcass cDNA probe (refer to Figure 3.2) would be expected to contain reverse transcribed maternally expressed transcripts. It is also worth noting that the removal of yolk protein transcripts from the female poly (A)⁺ RNA using cloned yolk protein DNA sequences appears to have been successful since no yolk protein sequences were isolated.

Possible Reasons For The Failure Of The Differential Screen To Detect Sex-Specific Transcripts Expressed In Somatic Tissues

After this screen was performed and the initial characterisation of the isolated genomic sequences was underway, new information about how some sex-specifically expressed genes are regulated became available. Most of the regulatory genes of somatic sex determination hierarchy have now been cloned (reviewed in Slee and Bownes, 1990). For example, it was expected that the product of the *tra* locus would not be expressed in males, since genetic studies showed that it was required only for normal female somatic sexual development. When the *tra* locus was cloned and subsequently used to probe Northern blots of male and female RNA, a 1.2 kb non sex-specific transcript and a 1.0 kb female-specific transcript were detected (Boggs *et al*, 1987). Using the cloned *tra* gene as a probe, cDNA copies of both size classes of transcript were isolated. Comparisons of the genomic and cDNA sequences showed that the different *tra* mRNAs were generated by means of alternative splicing. The non sex-specific transcript results from the use of a 3' splice acceptor 125 bp upstream of the acceptor site used to generate the female-specific *tra* transcript. Sequence data also revealed that the *tra* mRNA present in both sexes does not contain a long open reading frame, while the female transcript has the potential to encode a polypeptide of 211 amino acids. Several other genes involved in regulating sexual differentiation have been cloned including *Sxl* and *dsx* (refer to Figure 3.1). These loci also appear to be under post-transcriptional regulation and are controlling sexual differentiation by means of alternative splicing of the pre-message to produce sex-specific gene products. It is therefore not surprising that genes whose transcripts are present in both sexes but functional only in one sex would not

be detected in a differential screen using male and female cDNA probes.

Moreover, it is possible that very few transcripts are expressed exclusively in males or females. Many genes involved in somatic sexual differentiation may be active in both sexes but have different spatial and/or temporal expression patterns in the two sexes. For example the genes involved in pigmentation patterns and bristle distribution in the adult abdomen could fall into this category.

Other differential screens carried out in search of sex-specifically expressed genes have only ever identified genes expressed in sex-specific tissues, namely the gonads (Schafer, 1986; DiBeneditto *et al*, 1987; Monsma and Wolfner, 1988). These genes do not seem to be under the direct control of the somatic sex determination hierarchy. It seems that the sex determining genes are responsible for the decision to develop either male or female gonads and from then on the genes are regulated by tissue-specific factors (Slee and Bownes, 1990). It is of interest to note that in *Drosophila* the yolk protein genes are the only known sex-specific genes to be expressed in a non-sex specific tissue (i.e. female fat body) that have been isolated.

CHAPTER FOUR

CHARACTERISATION OF λ ov5

IDENTIFICATION OF TRANSCRIPTS EXPRESSED IN THE OVARY

Introduction

As described in Chapter 3, λ ov5 was isolated in a differential screen and contains two EcoRI sub-fragments which detect female specific and female enriched transcripts. This chapter describes a more detailed analysis of these transcripts and their organisation within the corresponding ov5 genomic sequences.

Part One

Restriction Mapping Data : λ ov5

λ ov5 contains of two EcoRI fragments, 5.7 kb and 5.0 kb in length. Both fragments were sub-cloned into the p-Gemini vector and will be referred to as pGem-ov5A (5.0 kb EcoRI fragment) and pGem-ov5B (5.7 kb EcoRI fragment). Figure 4.1 provides a restriction map of λ ov5 and detailed restriction maps of each EcoRI sub-fragment within p-Gemini-1 vectors.

Part Two

Detection Of Transcripts In The Ovary Using The ov5A And ov5B Sub-Fragments

As shown in Figure 3.4, λ ov5 sequences detect a 1.7 kb female specific transcript and a 1.4 kb female enriched transcript. In order to determine the site of expression of the female enriched transcripts, poly (A)⁺ RNA was extracted from adult female carcasses and ovaries. When either ov5A or ov5B was used to probe Northern blots containing female carcass poly (A)⁺ RNA, no transcripts were detected (data not shown).

In poly (A)⁺ RNA extracted from ovaries, ov5A detected a 1.7 kb ovary specific transcript and a 1.4 kb ovary enriched transcript (Figure 4.2, Panels A and B). The 1.4 kb transcript detected with ov5A was present at a lower level in adult male poly (A)⁺ RNA compared with ovary poly (A)⁺ RNA. When ov5B was used to probe the same blot, a 1.4 kb ovary specific transcript was detected (Figure 4.2, Panel C). The simplest interpretation of this data was to invoke that the ov5A and ov5B fragments both detect the same 1.4 kb transcript. On this basis

Figure 4.1

λ ov5 Restriction Map

The ov5 sequence was isolated from a *Drosophila* genomic library which was constructed in the λ Charon 4A vector.

Restriction enzyme maps of the ov5 region and the two EcoRI sub-fragments are shown.

pGem-ov5B = 5.7 kb EcoRI fragment (ov5B) from λ ov5 was sub-cloned into the pGemini-1 vector. No sites for SmaI except in the polylinker.

pGem-ov5A = 5.0 kb EcoRI fragment from λ ov5 was sub-cloned into the pGemini-1 vector. No SmaI, SalI or HindIII sites except in the polylinker.

AvaI' = An AvaI site is located at one of these two positions

PstI' = There are a further four PstI sites in the ov5A sub-fragment whose positions have still to be determined.

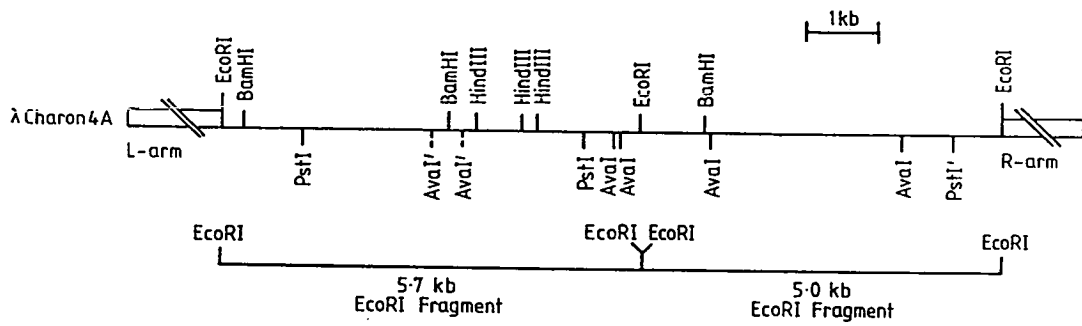
(P) = Polylinker multiple cloning site in the pGemini-1 vector.

 = pGemini vector sequences in pGem-ov5B and pGem-ov5A

 L-arm = Left arm of λ Charon 4A vector (19.8 kb)

 R-arm = Right arm of λ Charon 4A vector (10.8 kb)

λov5 Restriction Map



ov5B

ov5A

pGem-ov5B

pGem-ov5A

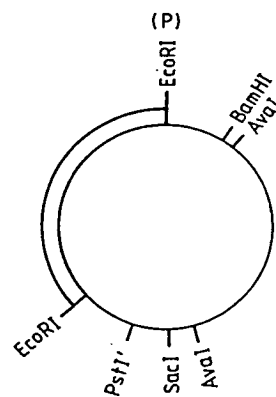
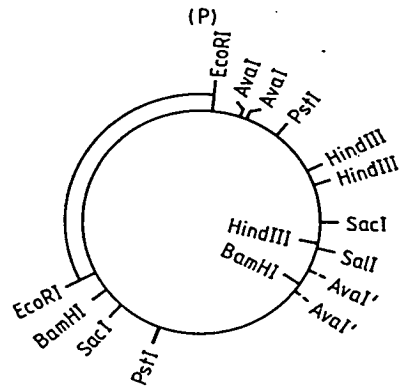


Figure 4.2

Detection Of Ovary-Specific Transcripts Using The ov5A And ov5B Genomic Sub-Fragment Probes

A Northern blot containing 2 μ g of ovary poly (A)⁺ RNA and 2 μ g of adult male poly (A)⁺ RNA was probed with ov5A (panels A and B), ov5B (panel C) and α -tubulin sequences (panel D).

In each case 1 = ovary poly (A)⁺ RNA

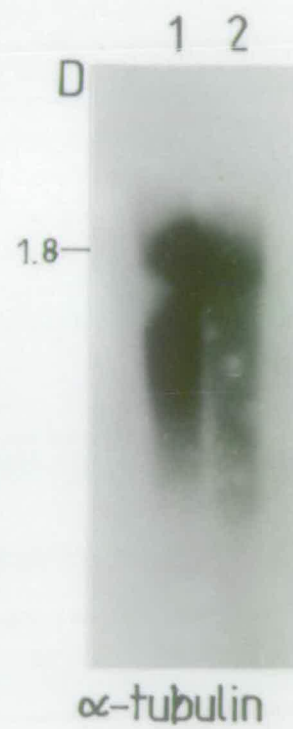
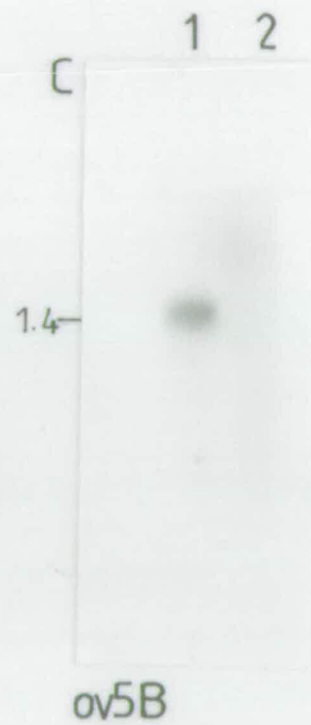
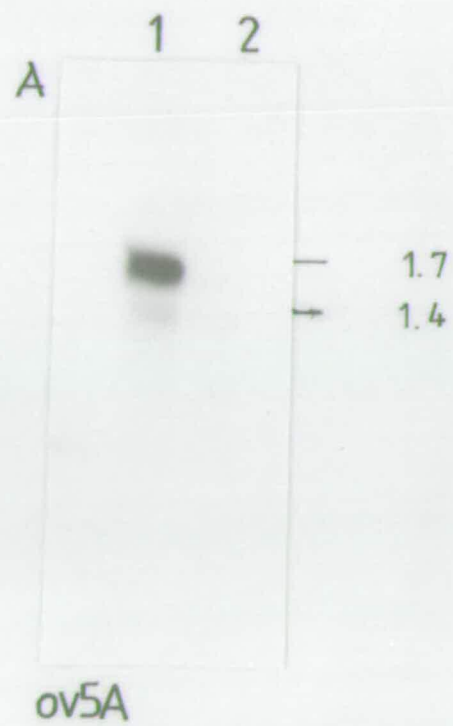
 2 = male poly (A)⁺ RNA

A and B: Autoradiographs of ovary and male poly (A)⁺ RNA probed with the ov5A genomic sub-fragment from λ ov5. A = 12 hours exposure; B = 24 hours exposure.

C: Autoradiograph of the blot used in A and B after stripping and subsequent reprobing with the ov5B genomic sub-fragment from λ ov5. 18 hours exposure.

D: Autoradiograph of the same blot used in A, B and C after stripping and reprobing with α -tubulin sequences. 24 hour exposure.

The estimated sizes of transcripts (in kb) detected by each probe are indicated.



an initial model of transcript organisation within λ ov5 was proposed (Figure 4.3). It seemed likely that at least some of the sequences coding for the 1.7 kb transcript were encoded within ov5A, and that sequences encoding the 1.4 kb transcript were present within ov5A and ov5B, spanning the internal EcoRI site within λ ov5.

Part Three: Analysis Of Ovary Enriched Transcripts Detected By ov5A

Isolation Of cDNAs Hybridising With ov5A From An Ovary cDNA Library

Initially, ov5A was used to screen a λ gt11 ovary cDNA library (gifted by P. Sullivan and L. Kalfayan, University of North Carolina) since this DNA fragment was predicted to encode sequences that would detect cDNA copies of the 1.7 kb and 1.4 kb ovary enriched transcripts. 50,000 plaques were screened and six positive signals were obtained. A small area containing 20-30 plaques corresponding to each primary signal was re-screened until well isolated plaques hybridising with the ov5A fragment were obtained. After the re-screening procedure three size classes of positive cDNA clones called λ ov5A1, λ ov5A2 and λ ov5A5 were identified. Each recombinant bacteriophage was digested with EcoRI and cDNA insert lengths of 1.6 kb (λ ov5A1), 1.8 kb (λ ov5A2) and 0.9 kb (λ ov5A5) were identified. Each of the ov5A1, ov5A2 and ov5A5 cDNA sequences cross hybridise with each other (data not shown). After EcoRI digestion, the cDNA isolates were Southern blotted and probed separately with the ov5A and ov5B genomic fragments. Each of the cDNA insert sequences ov5A1, ov5A2 and ov5A5 hybridised only with ov5A and not with ov5B (data not shown).

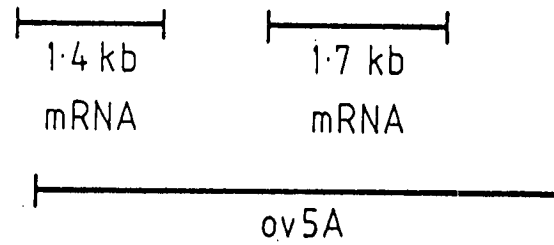
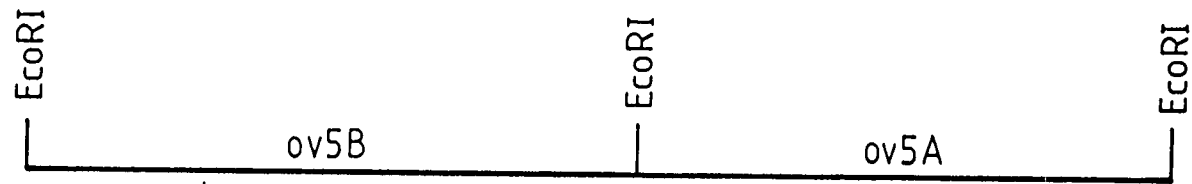
In accordance with the proposed model it seemed most likely that the ov5A1, ov5A2 and ov5A5 cDNAs represented cDNA copies of the 1.7 kb (estimated size) ovary specific transcript. If any of ov5A1, ov5A2 or ov5A5 represented cDNA copies of the 1.4 kb transcript, then they would have been expected to show homology with the ov5B fragment. In order to investigate the transcript(s) detected by ov5A1, ov5A2 and ov5A5, each cDNA was used to probe Northern blots containing ovary poly (A)⁺ RNA.

Figure 4.3

Initial Model Of Transcript Organisation Within λ ov5

Initial model of organisation of the 1.7 kb and 1.4 kb transcripts within λ ov5 based on Northern analysis using the ov5A and ov5B sub-fragment probes.

The ov5A fragment was used to screen an ovary cDNA library with the expectation that cDNA copies of both transcripts encoded within ov5A and ov5B would be isolated.



↓
screen ovary cDNA library

↓
expect to obtain copies of
the 1.7 kb and 1.4 kb transcripts

Table 4.1**Detection Of Multiple Transcripts In The Ovary With cDNAs ov5A1, ov5A2 and ov5A5**

cDNA	Length of cDNA Insert	Approximate Sizes of Ovary Enriched Transcripts
λ ov5A1	1.6 kb	1.7 kb, 1.4 kb
λ ov5A2	1.8 kb	1.7 kb, 1.4 kb
λ ov5A5	0.9 kb	1.7 kb, 1.4 kb, 0.8 kb, 0.2kb

Analysis Of Ovary Enriched Transcripts Detected By ov5A1, ov5A2 And ov5A5 cDNA Probes

A number of interesting results were obtained when each cDNA ov5A1, ov5A2 or ov5A5 was used to probe poly (A)⁺ RNA extracted from ovaries and adult males. When the longest cDNA (1.8 kb) ov5A2 was used to probe ovary poly (A)⁺ RNA, two transcripts 1.7 kb and 1.4 kb in length were detected (Figure 4.4, panel A, lane 8). The ov5A1 (1.6 kb in length) cDNA detected the same pattern of transcripts as ov5A2 (data not shown). This pattern of transcription in ovary RNA is similar to that obtained with the ov5A genomic fragment probe (Figure 4.2, panel A). These results clearly show that the 1.7 kb and 1.4 kb transcripts must overlap. It was unexpected to find that the ov5A2 cDNA probe detected the 1.7 kb transcript in male poly (A)⁺ RNA (Figure 4.4, Panel A, lane 6). The 1.7 kb transcript had never been detected in male poly (A)⁺ RNA using the ov5A genomic fragment probe (Figure 3.4, panel A; Figure 4.2 panels A and B). Presumably the abundance of the 1.7 kb transcript in males is below the detection threshold of the less specific and hence less sensitive genomic probe. Nevertheless, it does seem that the 1.7 kb and 1.4 kb transcripts are enriched in the ovary. Since the longest cDNA sequence is 1.8 kb in length, the original estimated transcript size of 1.7 kb for the ovary specific transcript is an underestimate, by 0.1 kb. For the purposes of this thesis this transcript will be referred to as 1.7 kb to make the overall description easier to follow.

When the ov5A5 cDNA (0.9 kb insert length) was used to probe ovary and male poly (A)⁺ RNA four transcripts were detected (Figure 4.4, panel B). The ov5A5 probe detected the 1.7 kb and 1.4 kb ovary enriched transcripts detected by ov5A1 and ov5A2, but it also detected 0.8 kb and 0.2 kb ovary specific transcripts (Figure 4.4, lanes 8 and 9).

The results of the Northern analysis with each of the ov5A1, ov5A2 and ov5A5 cDNAs clearly showed that the ov5A fragment must encode overlapping ovary enriched transcripts. The number and estimated sizes of transcripts detected by each of ov5A1, ov5A2 and ov5A5 are listed in Table 4.1.

Since it was discovered that the cDNAs ov5A1, ov5A2 and ov5A5 detected overlapping transcripts which were unrelated to the ov5B genomic sequences, the initial model of transcript organisation within λ ov5 was called into question (refer to Figure 4.3). It was still possible that

Figure 4.4

Detection Of Overlapping Transcripts In The Ovary Using The ov5A2 and ov5A5 cDNA Probes, And Their Developmental Regulation

In each of the Northern blots A, B and C, 2 μ g of poly (A)⁺ RNA from the following samples were loaded:

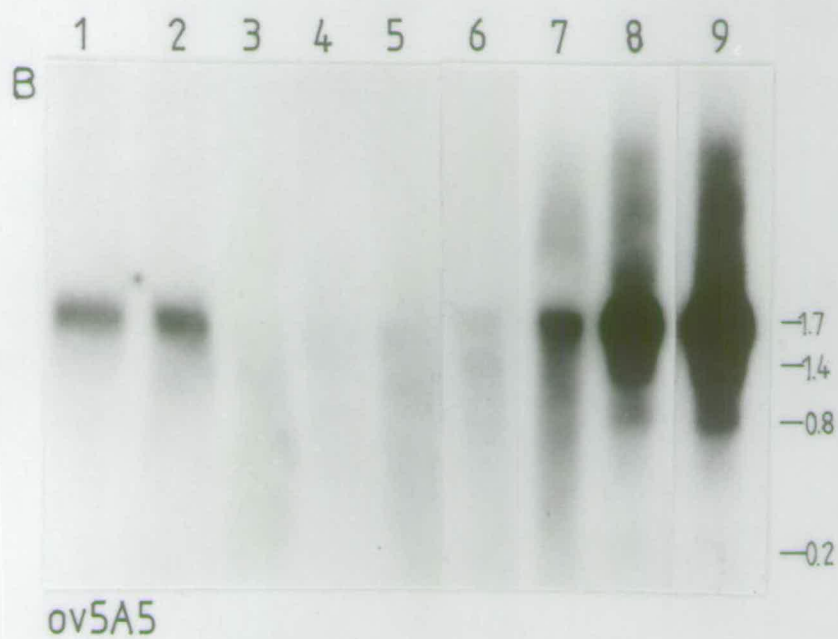
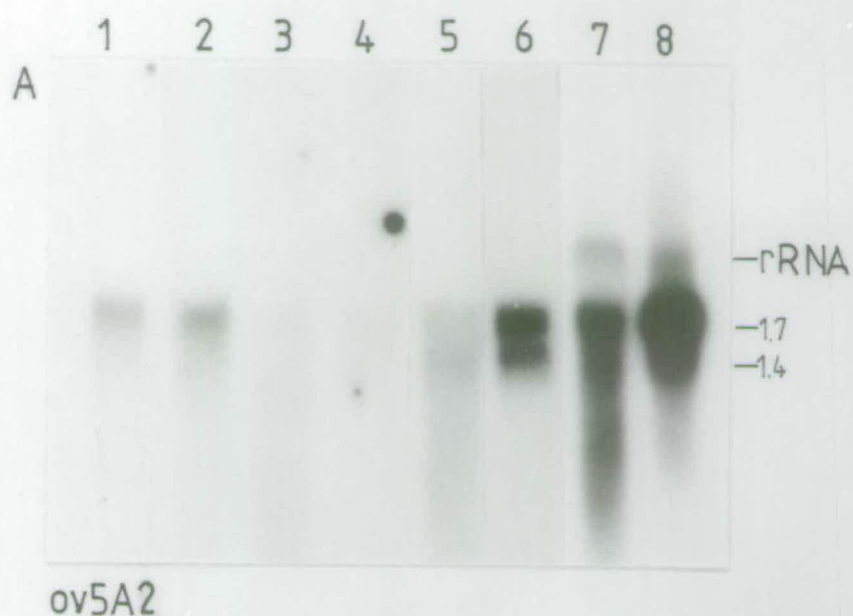
- 1 = embryos (0-24 hours)
- 2 = 1st instar larvae
- 3 = 2nd instar larvae
- 4 = 3rd instar larvae
- 5 = pupae
- 6 = adult males
- 7 = adult females
- 8 = ovaries
- (B only) 9 = ovaries

A: Autoradiograph of an RNA developmental profile probed with the ov5A2 cDNA probe. The transcription pattern in ovary RNA is shown in lane 8. Lanes 1-5, 4 day exposure; lanes 6-8, 18 hour exposure.

B: Autoradiograph of an RNA developmental profile probed with the ov5A5 cDNA probe. The transcription pattern in ovary RNA is shown in lanes 8 and 9. Lanes 1-5, 4 day exposure; lanes 6-8, 18 hour exposure; lane 9, 36 hour exposure (intended to highlight the 0.2 kb ovary specific transcript).

C: The Northern blots in A and B were loaded with identical amounts of poly (A)⁺ RNA. The Northern blot (B) was stripped and reprobed with the *Drosophila Adh* sequences. This autoradiograph represents an 18 hour exposure.

Sizes of transcripts (in kb) detected by each probe are indicated.



the 1.4 kb transcript extended into ov5B but it seemed more likely that ov5B encoded an unrelated 1.4 kb ovary specific transcript (discussed in Part Four).

Expression Of Ovary Enriched Transcripts During Development

Each cDNA ov5A1, ov5A2 and ov5A5 was used to probe developmental profiles of poly (A)⁺ RNA extracted from eggs (0-24 hour embryos), 1st, 2nd and early third instar larvae; male and female adults. A similar pattern of transcript detection during development was obtained with the ov5A2 and ov5A5 cDNA probes (Figure 4.4, panels A and B). ov5A1 gave the same result as ov5A2 (data not shown). The 1.7 kb transcript was detected in embryos and 1st instar larvae, apparently absent in 2nd and 3rd instar larvae, then re-expressed in pupae at a low level. In adults the 1.7 kb transcript was detected in males and females (Figure 4.4, Panels A and B, lanes 6 and 7). The 1.4 kb transcript was detected in the adult stages and possibly at a low level in pupae. The ov5A2 probe detected the 1.4 kb transcript in male and female poly (A)⁺ RNA. The ov5A5 probe did not detect the 1.4 kb transcript in female poly (A)⁺ RNA (Figure 4.4, Panel B, lane 7), but did detect this transcript at a low level in male poly (A)⁺ RNA (Figure 4.4, Panel B, lane 6). The 0.8 kb and 0.2 kb transcripts were detected only in ovary RNA extracts and not at any other developmental stage (Figure 4.4, Panel B, lanes 8 and 9).

Discussion: ov5A Encodes Overlapping Transcripts

When the ov5A genomic fragment was used to screen an ovarian cDNA library, three positive cDNA isolates were identified - λ ov5A1, λ ov5A2 and λ ov5A5. When each cDNA was used to probe ovary poly (A)⁺ RNA at least two transcripts of approximately 1.7 kb and 1.4 kb in length were detected. In the case of the ov5A5 cDNA, a third ovary specific transcript, approximately 0.8 kb in length and possibly another 0.2 kb ovary specific transcript were identified.

The 1.7 kb and 1.4 kb transcripts must overlap since all of the cDNA probes detect both. The cDNAs ov5A1 and ov5A2 may represent copies of one or both transcripts. Since the ov5A5 cDNA probe detected 0.8 kb and 0.2 kb transcripts it may be distinctly different from ov5A1 and

ov5A2. Overlapping transcripts can be generated by a number of different mechanisms, such as the use of different promoters, polyadenylation signals, alternative splice sites, or different lengths of poly (A) tail. Another possibility is that the overlapping transcripts are the products of overlapping genes.

In order to investigate how the overlapping transcripts are generated and to study their organisation within the ov5 genomic fragment, three sets of experiments were carried out. Firstly comparative restriction enzyme mapping of the ov5A1, ov5A2 and ov5A5 cDNAs was carried out as it should point to the nature of the mechanism(s) involved in generating the transcripts. Differences at the 5' or 3' ends of the overlapping transcripts may result in different restriction enzyme site locations in the corresponding cDNAs. Alternative splicing may result in different restriction enzyme sites, or in different lengths of restriction fragments. Secondly the cDNAs, were mapped within the ov5A genomic fragment by cross-hybridisation to restriction enzyme digests of pGem-ov5A. Thirdly, to complement these studies, transcript mapping using heteroduplex analysis was carried out using the ov5A1 and ov5A2 cDNA sequences with each other and with the corresponding ov5A genomic fragment.

Investigation Of Transcript Organisation

1: Comparison of Restriction Enzyme Maps Of Each cDNA ov5A1, ov5A2 And ov5A5

The cDNA sequences ov5A1 and ov5A2 were sub-cloned into the Gemini plasmid vector. The resulting plasmids, named pGem-ov5A1 and pGem-ov5A2 were digested with a variety of restriction enzymes. The restriction enzyme maps obtained for each cDNA and how their maps compare are illustrated in Figure 4.5. Restriction mapping data suggests that ov5A1 and ov5A2 represent transcripts which differ at initiation or polyadenylation sites. Alternatively, ov5A1 may be a 5' truncated cloning artefact. If ov5A1 and ov5A2 represent different transcripts, the data appear to rule out an alternative splicing mechanism as a means of transcript generation. The shortest length cDNA, ov5A5, was not individually restriction mapped but it was used to probe Southern blots of restriction enzyme digests of pGem-ov5A2 and pGem-ov5A2 to locate the cross-

Figure 4.5

Comparative Restriction Mapping : ov5A1, ov5A2 and ov5A5 cDNAs

The ov5A1 and ov5A2 cDNAs were sub-cloned into pGemini-1 vectors and individually restriction mapped.

The ov5A5 insert was used to probe Southern blots of restriction enzyme digests of pGem-ov5A1 and pGem-ov5A2 to determine the cross-hybridising sequences.

pGem-ov5A1 no sites for HindIII, Sall, BamHI (except in the plasmid
pGem-ov5A2 polylinker sequence)

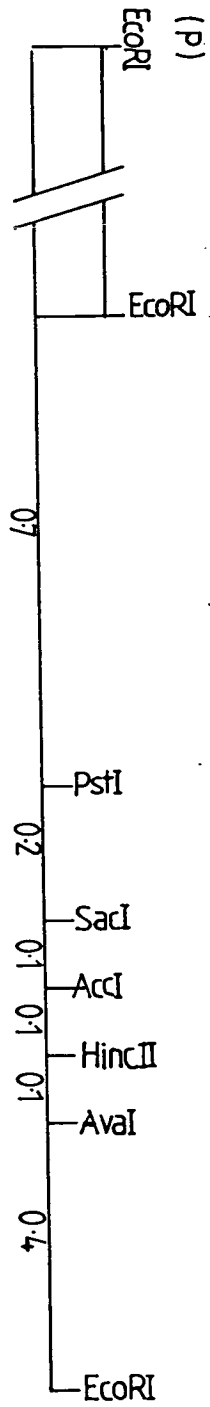
(P) = Polylinker multiple cloning site in the pGemini vector

 = pGemini vector sequences

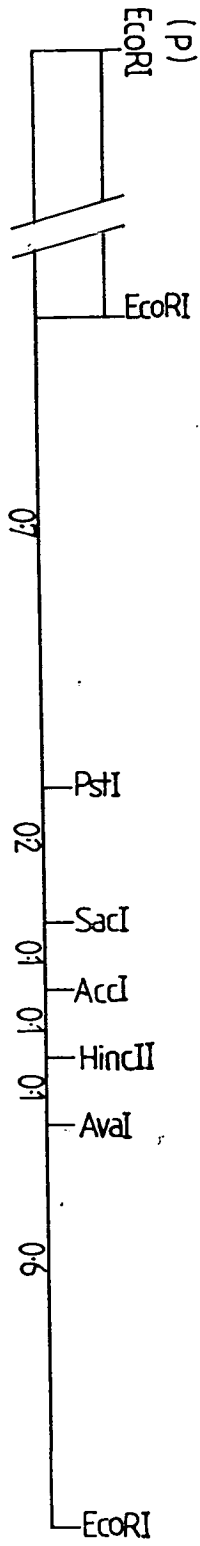
 = λgt11 vector arms - hatched lines indicate that the
orientation of the right and left arms
of the vector have not been
determined with respect to the insert.

Distances between each restriction site (in kb) are indicated.

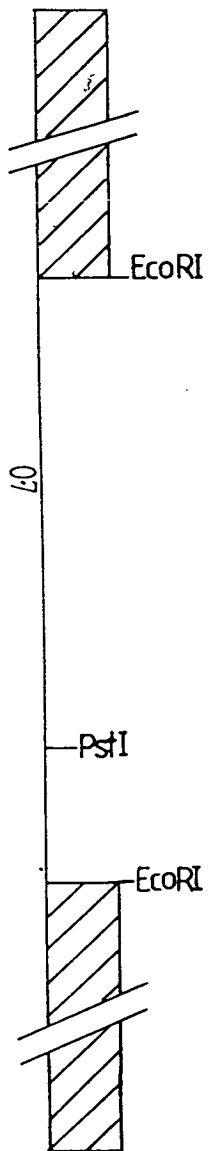
pGem-ov5A1



pGem-ov5A2



λ ov5A5



0.2 kb

hybridising fragments. This was found to be the easiest method of mapping ov5A5 since it was difficult to obtain a good DNA yield from λ ov5A5 preparations. Although the ov5A5 sequences hybridised at one common end of ov5A1 and ov5A2, within a 0.9 kb sized EcoRI/SacI DNA fragment (Figure 4.5), it seems likely that ov5A5 contains sequences unrelated to ov5A1 and ov5A2 since it detected two additional transcripts.

2: Location Of ov5A1, ov5A2 And ov5A5 cDNAs Within The Corresponding ov5A Genomic Fragment

Southern blots of restriction enzyme digests of pGem-ov5A were probed with each cDNA ov5A1, ov5A2 and ov5A5 to determine the organisation of the cDNAs within the corresponding genomic DNA fragment. Each cDNA hybridised within a 1.4 kb sized EcoRI/AvaI genomic DNA fragment (Figure 4.6). There appears to be no homology between the ov5A1 and ov5A2 cDNAs beyond the genomic AvaI site, so there must be an intron. The similarity in the position of the AvaI site may just be fortuitous.

3: Transcript Mapping; Heteroduplex Analysis

Introduction

The restriction enzyme mapping and cross-hybridisation experiments suggested that the cDNAs ov5A1, ov5A2 and ov5A5 differed only in length. In order to confirm this interpretation and to reveal any introns, transcript mapping experiments using heteroduplex analysis was performed. Each cDNA was denatured and allowed to renature with the genomic ov5A fragment. The renatured cDNA - genomic DNA heteroduplexes were then visualised using electron microscopy. This technique has been applied to the study of other transcripts and in the case of the *Drosophila* yolk protein genes (Hung *et al.*, 1982) loop structures corresponding the introns in genomic DNA were visualised. The results obtained when pGem-ov5A1 and pGem-ov5A2 were annealed with pGem-ov5A are illustrated in Figure 4.7, panels A and B respectively. The ov5A1 and ov5A2 cDNAs were simultaneously denatured and allowed to renature to see if they were

Figure 4.6

Transcript Mapping : Location Of ov5A1, ov5A2 And ov5A5 cDNAs Within The Corresponding ov5A Genomic Sub-Fragment

Each cDNA ov5A1, ov5A2 and ov5A5 was labelled with [^{32}P]dCTP and used individually to probe Southern blots of restriction enzyme digests of the pGem-ov5A plasmid, which contains the corresponding ov5A genomic fragment.

This figure illustrates the restriction map of each cDNA ov5A1, ov5A2 and ov5A5 and shows their location within a 1.4 kb EcoRI/AvaI fragment from ov5A.

Broken lines indicate sequences within ov5A1 and ov5A2 which are located outwith the ov5 genomic region.

(P) = Polylinker multiple cloning site in the pGemini vector

 = pGemini vector sequences

Distances between each restriction site (in kb) are indicated.

1 kb

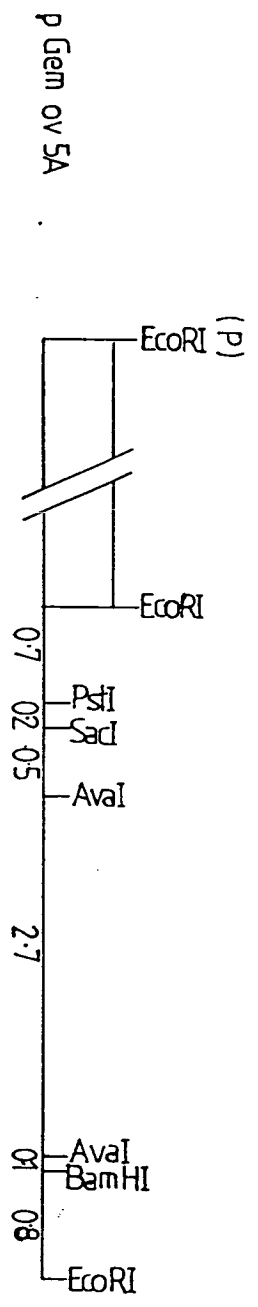


Figure 4.7

Transcript Organisation:

Mapping The ov5A1 and ov5A2 cDNA Sequences With Each Other And With The Corresponding ov5A Genomic Fragment Using Heteroduplex Formation

In order to determine if there were any introns within the ov5A genomic fragment, and show whether the ov5A1 and ov5A2 cDNAs were composed of the same or different exons, the following experiments were performed.

In A and B the ov5A1 and ov5A2 cDNAs were denatured and allowed to renature with the ov5A genomic fragment, and the resulting heteroduplexes visualised using electron microscopy.

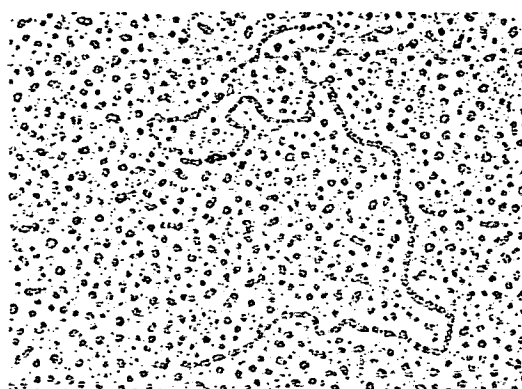
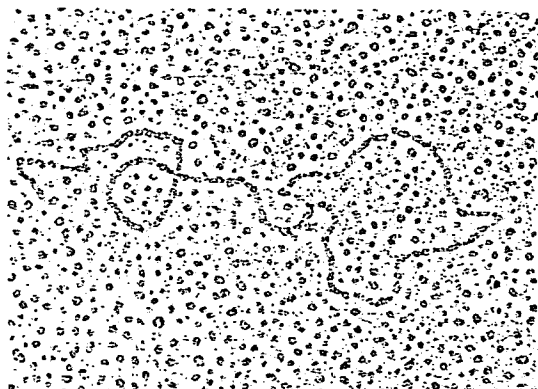
In C, the ov5A1 and ov5A2 cDNA molecules were denatured, allowed to form heteroduplexes and visualised using electron microscopy.

In each of A, B and C linearised plasmids containing the ov5A1, ov5A2 or ov5A DNA sequences were used. The 'loop' structure formed in each example shown in A, B and C resulted from the annealing of polylinker sequences present at the end of each molecule (explained in Figure 4.8)

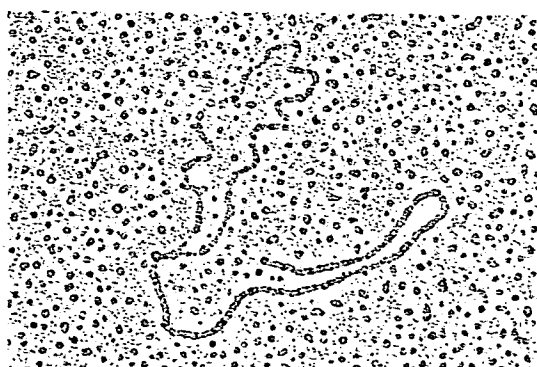
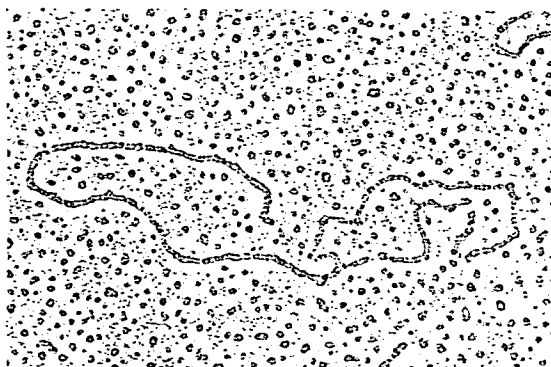
Heteroduplexes are as follows:

- A: pGem-ov5A1 (linearised with BamHI) with pGem-ov5A (linearised with HindIII)
- B: pGem-ov5A2 (linearised with BamHI) with pGem-ov5A (linearised with HindIII)
- C: pGem-ov5A1 with pGem-ov5A2 (linearised with BamHI in each case)

A



B



C

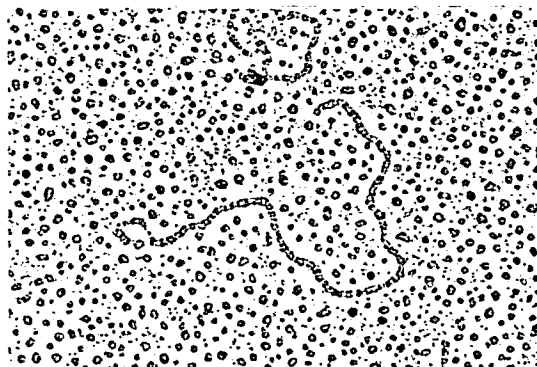
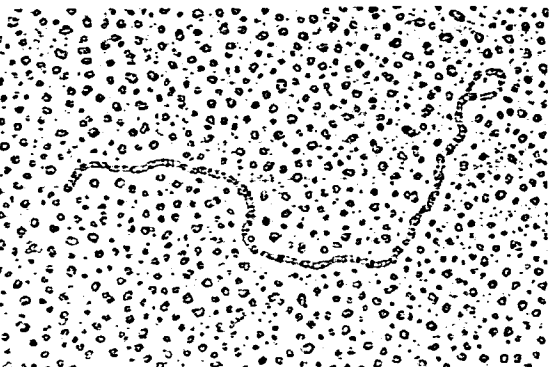


Table 4.2

Lengths of pGem-ov5A1, pGem-ov5A2 and pGem-ov5A DNA molecules

Plasmid	Estimated Plasmid Size (Restriction Mapping)	Estimated Plasmid Size (Electron Microscopy)
pGem-ov5A1	4.5 kb	4.46 kb
pGem-ov5A2	4.7 kb	4.79 kb
pGem-ov5A	7.9 kb	8.02 kb

colinear or if any structural differences between them (reflecting different exon composition) could be detected (Figure 4.7, panel C).

Results

In the following experiments linearised plasmids containing ov5A1, ov5A2 and ov5A DNA sequences were used. The electron microscopy was performed by Pam Beattie who provides this service for the department. Within the limits of resolution of the electron microscope there do not appear to be introns present in the genomic DNA sequences present within ov5A (Figure 4.7, A and B). The regions of ov5A1 and ov5A2 (~0.4 kb and ~0.6 kb in length respectively) sequences predicted to lie outwith the ov5A fragment (from restriction mapping data) did not form detectable unpaired regions when pGem-ov5A1 and pGem-ov5A2 were allowed to form heteroduplexes with pGem-ov5A. It is worthwhile to note that the single stranded unpaired ov5A sequences form a loop structure. This can be explained by the annealing of short lengths of polylinker sequences at the ends of the linearised pGem-ov5A1, pGem-ov5A2 and pGem-ov5A5 plasmids. This is explained and illustrated in Figure 4.8.

Heteroduplex mapping of the ov5A1 and ov5A2 sequences with each other confirms the restriction mapping data obtained for each cDNA, as ov5A1 and ov5A2 appear to be colinear, with ov5A2 appearing to be a longer length copy of ov5A1 by approximately 0.2 kb (Figure 4.7, Panel C). The loop structure formed by the single stranded ov5A2 sequences can be explained by the annealing of plasmid polylinker sequences (explained in Figure 4.8).

The length of each linearised plasmid was measured using electron microscopy (carried out by Pam Beattie). Double stranded M13 DNA was used as a standard. Estimates of plasmid sizes measured by restriction mapping and electron microscopy are listed in Table 4.2. Attempts to use λ ov5A5 in heteroduplex mapping experiments were unsuccessful. The heteroduplex mapping results suggest that there are no detectable introns in ov5A within the region encoding the overlapping ovary transcript family.

Figure 4.8

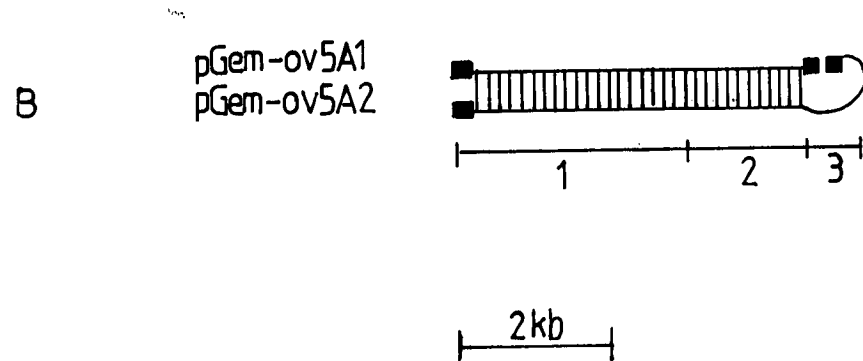
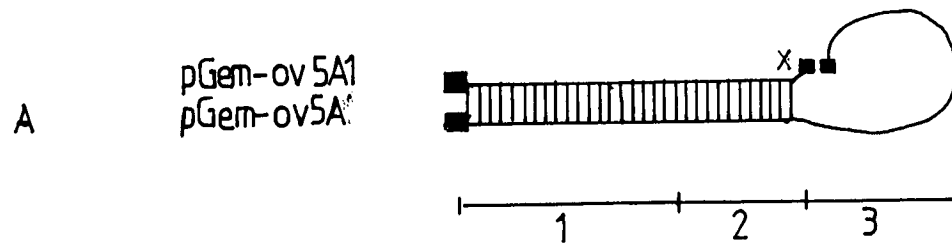
Heteroduplex Mapping - Formation of Loop Structure Due To Annealing of Plasmid Polylinker Sequences

In each of the heteroduplex molecules shown in Figure 4.7 A, B and C a loop structure forms as a result of the annealing of complementary plasmid polylinker sequences at the end of each DNA molecule. This figure illustrates a drawing of the pGem-ov5A1/pGem-ov5A and pGem-ov5A1/pGem-ov5A2 heteroduplexes shown in Figure 4.7 A and C respectively.

- A: pGem-ov5A1 with pGem-ov5: Region 1 represents the annealing of plasmid sequences; Region 2 represents the annealing of homologous sequences in the ov5A1 cDNA with the ov5A 1.4 kb EcoRI/AvaI fragment; X represents the single stranded ov5A1 sequence which is not homologous with ov5A; Region 3 represents the single stranded ov5A tail.
- B: pGem-ov5A1 with pGem-ov5A2: Region 1 represents the annealing of plasmid sequences; Region 2 represents the annealing of ov5A1 and ov5A2 cDNA sequences; Region 3 represents the single stranded ov5A2 cDNA sequence.

In each case ■ = Polylinker Sequence

■ ■ = Annealing of polylinker sequences at ends of each molecule,
which results in the single stranded tail forming a loop.



Conclusions: Transcript Organisation Within ov5A

When the ov5A genomic fragment was used to probe ovary and male poly (A)⁺ RNA extracts, two ovary enriched transcripts were detected. The 1.7 kb sized transcript appeared to be ovary specific and the 1.4 kb sized transcript appeared to be ovary enriched (Figure 4.2, panels A and B). The ov5A genomic fragment was used to screen an ovary cDNA library and three clones λ ov5A1, λ ov5A2 and λ ov5A5 were isolated. Comparison of the cDNAs revealed that they cross-hybridise, and restriction mapping data suggested that ov5A1 and ov5A2 differ at their transcript initiation sites or polyadenylation sites. Alternatively, ov5A1 may be a truncated copy of ov5A2. The shortest cDNA, ov5A5, mapped within one common end of ov5A1 and ov5A2 (Figure 4.5). Each cDNA detected at least two ovary enriched transcripts in ovary poly (A)⁺ RNA (Figure 4.4) indicating that the 1.7 kb and 1.4 kb transcripts are overlapping. The ov5A5 cDNA also hybridised with 0.8 kb and 0.2 kb ovary specific transcripts indicating that ov5A5 has sequences not present in ov5A1 or ov5A2. However, restriction mapping data so far indicates that ov5A1, ov5A2 and ov5A5 are colinear.

When the ov5A1 (1.8 kb), ov5A2 (1.6 kb) and ov5A5 (0.9 kb) sequences were mapped within the ov5A sub-fragment they hybridised within a 1.4 kb sized EcoRI-AvaI fragment (Figure 4.6) which clearly shows that some of the ov5A1 and ov5A2 sequences are located outwith ov5A. The best fit of the restriction data for λ ov5 (Figure 4.1) places the ov5A1, ov5A2 and ov5A5 cDNAs at the end of the ov5 region. There must be an intron (greater than 9.3 kb in length) extending outwith ov5 as the ov5B fragment does not cross hybridise with ov5A1, ov5A2 or ov5A5. This intron was not detected with the transcript mapping experiments using heteroduplex analysis as the cDNAs ov5A1 and ov5A2 were mapped only within the ov5A sub-fragment. Comparative restriction mapping of the cDNAs and heteroduplex analysis did not reveal any introns within the region of ov5A encoding the ovary enriched transcripts. The anomalies in this data and the mechanism(s) involved in transcript generation, will be resolved by DNA sequencing of the cDNAs and the corresponding genomic region.

The ov5A2 cDNA sequences predicted to lie outwith ov5 may be used as a primer for the polymerase chain reaction to obtain the corresponding sequences from genomic DNA extracts.

Restriction mapping will determine the size of the genomic fragment containing all the coding sequences present within ov5A2.

Once the DNA sequence of ov5A2 has been determined it can be used to search the most recent data bases for similarity with any other known sequence. This can be done at both the DNA sequence and amino acid sequence levels. Any significant level of similarity with a well characterised gene or protein may help to elucidate the function of the ovary enriched transcript encoded within ov5A2 during oogenesis.

Discussion:

Developmental Regulation Of Ovary Enriched Transcripts Detected By The ov5A1, ov5A2 and ov5A5 cDNA Probes

As far as can be detected on poly (A)⁺ profiles using the ov5A1, ov5A2 and ov5A5 cDNA probes, the 1.4 kb ovary enriched transcript is exclusive to the adult stage although it may be expressed at a low level in pupal RNA (Figure 4.4). The 1.7 kb ovary enriched transcript was detected in embryonic RNA and in 1st instar larval RNA extracts. It was not detected in 2nd or 3rd instar larval RNA but was found at a low level in pupal RNA. However, this apparent result may be due to underloading of these samples on the developmental profiles shown in Figure 4.4 (refer to loading check using the *Adh* probe, panel C). In adults, the 1.7 kb transcript was detected in males, but the expression level was higher in adult females. (This will be discussed in the next section.) The 0.8 kb and 0.2 kb transcripts detected by the ov5A5 cDNA were detected only in ovary poly (A)⁺ RNA, indicating that they function exclusively during oogenesis. The 1.7 kb transcript may be the product of a maternally expressed gene since it was detected in the ovary and the embryo. If this is the case, it will be interesting to determine if transcription from the zygotic genome occurs during embryogenesis. This is possible since the 1.7 kb transcript was detected in 1st instar larvae. The 1.7 kb transcript may have different functions at different developmental stages.

Discussion: Expression Of Ovary Enriched Transcripts In Adult Males

Each of the 1.7 kb and 1.4 kb ovary enriched transcripts were detected at a low level in adult males when the cDNA probes ov5A1, ov5A2 and ov5A5 were used. This was an unexpected result since the 1.7 kb transcript had never been previously detected in adult male poly (A)⁺ RNA using the ov5A genomic fragment probe. Until recently, it had been thought that genes specific for the functions of oogenesis would be transcribed only in the adult female. The precedents for this idea were that the yolk proteins (Barnett *et al.*, 1980) and the egg shell proteins, (Spradling *et al.*, 1980a) which are required specifically during oogenesis are transcribed only in females. Before the ov5A fragment was used to screen the ovary cDNA library, it was thought that the sequences encoding the 1.7 kb "ovary-specific" transcript might fall into such a class of "female-specific" genes expressed exclusively during oogenesis. However, recent reports suggest that at least some genes whose function in the ovary has been established are also transcribed in the adult testes. This is the case for at least two maternal-effect loci *exuperentia* and *vasa* (Hay *et al.*, 1988; Lasko and Ashburner, 1988). Each of these genes were originally isolated due to the female-sterile effects of mutations at each locus. A strict maternal-effect mutation affects the development of the egg and/or early embryo, with the fertility of the adult male being unaffected. Therefore when the loci responsible for the female sterility were cloned, they were not expected to be transcribed in adult males. Most of the reports on the cloning and transcription patterns of the female sterile loci so far have not indicated the pattern of transcription in adult males. It has been shown that the product of the *exuperentia* locus is required for the correct localisation of the maternally expressed *bicoid* transcript during oogenesis (discussed in Chapter 1; Frohnhöfer and Nüsslein-Volhard, 1986). The *exuperentia* (*exu*) locus has now been cloned and has been shown to encode overlapping male and female specific transcripts. Furthermore, experiments have been carried out to show that *exu* functions in the male germline as well as the female germline (T. Hazelrigg, University of Utah, personal communication).

The *vasa* gene has now been cloned and sequenced (Hay *et al.*, 1988; Lasko and Ashburner, 1988). The *vasa* gene product has been shown to be required for pole cell formation during embryogenesis. It is also required at a very early stage of germline development. Females

homozygous for a deletion of *vasa* exhibit aberrant oocyte differentiation (R. Lehmann, unpublished). Females homozygous for weak alleles of *vasa* develop eggs but the resulting embryos lack pole cells and show abnormal posterior development. Males homozygous for the *vasa* deletion or weaker alleles are fertile and viable and indistinguishable from wild type males (Lasko and Ashburner, 1988). This indicated that *vasa* functioning was specific to the female germline. However by using antibodies to the protein product of the *vasa* gene, it was shown that the *vasa* protein was detected during oogenesis and in embryonic pole cells as well as at the tip of the adult testes (Hay *et al.*, 1988; Lasko and Ashburner, 1990). The presence of the *vasa* protein product in the testes was unexpected from the observed normal viability and fertility of males carrying a homozygous deficiency or weak alleles of *vasa*. Lasko and Ashburner therefore suggest that *vasa* may not have a functional role in the testes.

It is possible that other transcripts expressed during oogenesis will also be expressed during spermatogenesis as the genes involved, for instance, in regulating germ cell differentiation in both sexes may be the same or related. It is also possible that other maternally-expressed genes whose products are contributed to the egg may also be expressed in males but do not function in males or perform an unrelated function to their role in females. In this respect, it may be that the ovary enriched transcripts detected by the *ov5A1*, *ov5A2* and *ov5A5* cDNAs are non-functional in adult males. Alternatively, these transcripts may have multiple functions, one of which is common to the differentiation of germline cells or gonadal tissue in both sexes. Northern blots containing male carcass and male testes RNA will be probed with the *ov5A2* and *ov5A5* cDNAs to determine the distribution of the 1.7 kb and 1.4 kb transcripts.

The potential functions of the 1.7 kb and 1.4 kb ovary enriched transcripts are considered in Chapter 6.

Table 4.3**ov5B Selected cDNAs**

cDNA name	Length of cDNA insert	BamHI/EcoRI cDNA fragments
λ ov5B1	*1.35 kb	0.85 kb, 0.5 kb
λ ov5B2	1.5 kb	0.85 kb, 0.65 kb
λ ov5B3	1.5 kb	0.85 kb, 0.65 kb
λ ov5B4	1.5 kb	0.85 kb, 0.65 kb
λ ov5B5	1.5 kb	0.85 kb, 0.65 kb
λ ov5B6	0.7 kb	0.7 kb

* ov5B1 cDNA has been sub-cloned into the pGemini-1 vector

Part 4: Characterisation Of An Ovary Specific Transcript Encoded Within ov5B

Introduction

At the beginning of this project when ov5B (5.7 kb genomic EcoRI fragment, Figure 4.1) was used to probe Northern blots containing female and ovary poly (A)⁺ RNA, a 1.4 kb sized ovary specific transcript was detected (Figure 4.2, panel C). In accordance with the proposed model (Figure 4.3) it was assumed that this transcript was likely to be the same 1.4 kb ovary transcript detected by ov5A on Northern blots (Figure 4.2, panel A and B). However, it was found that the cDNAs ov5A1, ov5A2 and ov5A5 (detected by ov5A) hybridised with a 1.4 kb ovary transcript which had no homology with the ov5B sequences. It seemed more likely that ov5B encoded a 1.4 kb ovary specific transcript unrelated to the 1.4 kb transcript detected by ov5A. Subsequently, the ov5B genomic fragment (refer to Figure 4.1) was used to screen an ovary λ gt11 cDNA library.

Screening An Ovary cDNA Library With The ov5B Genomic Fragment

Initially 50,000 plaques from an ovary cDNA library were screened with the ov5B probe and approximately twenty primary positive signals were obtained. Six primary positives were re-screened which led to the isolation of six cDNA size classes hybridising specifically with the ov5B probe, these were called λ ov5B1 - λ ov5B6. DNA prepared from each of λ ov5B1 - λ ov5B6 was digested with EcoRI and insert cDNA lengths ranging in size from 0.7 kb - 1.5 kb were observed (Table 4.3). Each cDNA was digested with BamHI and EcoRI and in the cases of λ ov5B1 - λ ov5B5 a 0.85 kb sized fragment was produced plus another fragment which varied in length according to the overall insert size (Table 4.3; Figure 4.9) indicating that the ov5B1 - ov5B6 cDNAs are colinear. The ov5B6 cDNA EcoRI insert is 0.7 kb in length. When the ov5B6 sequence was used to probe Southern blots of BamHI/EcoRI digests of the λ ov5B1 - λ ov5B6 cDNA isolates, it hybridised with itself and the 0.85 kb sized BamHI/EcoRI fragment common to the ov5B1 - ov5B5 cDNA sequences (data not shown). It was concluded from this experiment that the ov5B6 cDNA sequence encodes a 5' truncated cDNA copy of ov5B1 - ov5B5 cDNAs.

Figure 4.9

Comparative Restriction Mapping ov5B1 - ov5B6 cDNAs

This figure shows the comparative restriction maps of the ov5B1 - ov5B6 cDNA isolates obtained using the restriction enzymes BamHI and EcoRI. The ov5B6 cDNA was mapped within the 0.85 kb EcoRI/BamHI fragment common to ov5B1 - ov5B6 by cross-hybridisation experiments (detailed in text). The ov5B1 - ov5B6 cDNAs are colinear and represent cDNA copies of an adult ovary specific transcript (Figure 4.10).

pGem-ov5B1 = The ov5B1 cDNA (1.35 kb in size) was sub-cloned into the pGemini-1 vector and further restriction mapped. There are no sites for SmaI except in the plasmid polylinker sequence.

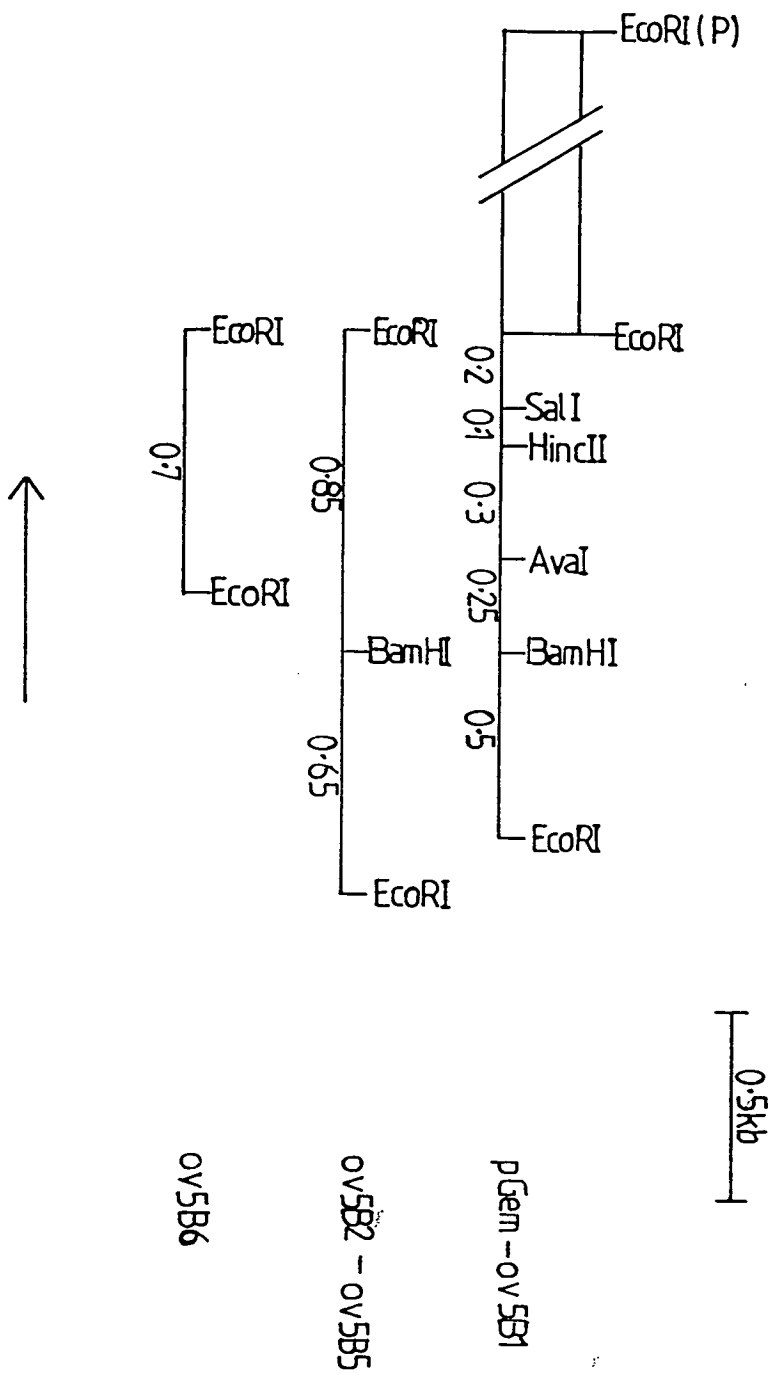
ov5B2-ov5B5 = Each cDNA represents a full length copy of the adult ovary specific transcript detected on Northern blots (Figure 4.10).

ov5B6 = 5' truncated cDNA, 0.7 kb in length.

= Probable direction of transcription

 = Plasmid sequences (pGemini vector)

(P) = Polylinker multiple cloning site in the pGemini vector



The ov5B1 - ov5B6 cDNAs did not cross hybridise with the ov5A fragment or any of the ov5A1, ov5A2 or ov5A5 cDNAs which proved that the transcript encoded within ov5B is unrelated in sequence to the transcripts detected by ov5A. This data confirmed that the initial model of transcript organisation with λ ov5 was incorrect. When the ov5B1 - ov5B6 cDNAs were isolated from a λ gt11 cDNA library, attempts were made to sub-clone each sequence into the p-Gemini vector. The ov5B1 sequence was successfully sub-cloned into pGemini-1 and the resultant recombinant was called pGem-ov5B1 (refer to Figure 4.9). The ov5B1 cDNA was digested with a series of restriction enzymes and a restriction map was constructed (Figure 4.9).

Detection Of An Ovary Specific Transcript In Adults With The ov5B1 cDNA Sequence

The ov5B1 cDNA detected a 1.4 kb ovary specific transcript in adults (Figure 4.10). This transcript was detected in ovary total RNA extracts using the ov5B1 cDNA probe. It was not detected in males (Figure 4.10, panel A, lane 7; panel B, lane 1) or female carcasses (Figure 4.10, panel A, lane 10). The ov5B6 cDNA also detected the 1.4 kb ovary specific transcript in adults confirming that it represents a 5' truncated copy of the 1.4 kb ovary specific transcript (data not shown).

Developmental Regulation Of The Transcript Detected By ov5B1

A Northern blot loaded with total RNA samples extracted from eggs (0-24 hour embryos); 1st, 2nd, early and late third instar larvae; pupae; male and female adults was probed with the ov5B1 cDNA sequence. A transcript approximately 1.4 kb in size was detected in 1st and early 3rd instar larvae and female adults (Figure 4.10; lanes 2, 4 and 8 respectively). This transcript is the same size as the 1.4 kb ovary specific (in the adult stage) transcript. A similar pattern of transcription of the 1.4 kb transcript during development was obtained with the ov5B1 sequence with poly (A)⁺ RNA extracts (data not shown).

Figure 4.10

Detection Of An Ovary Specific Transcript In Adults Using The ov5B1 cDNA Probe;

A Similar Sized Transcript Is Also Detected In 1st and Early Third Instar Larvae

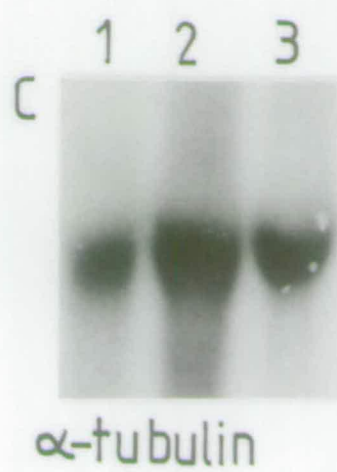
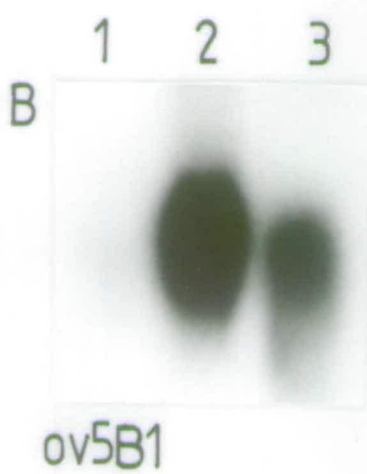
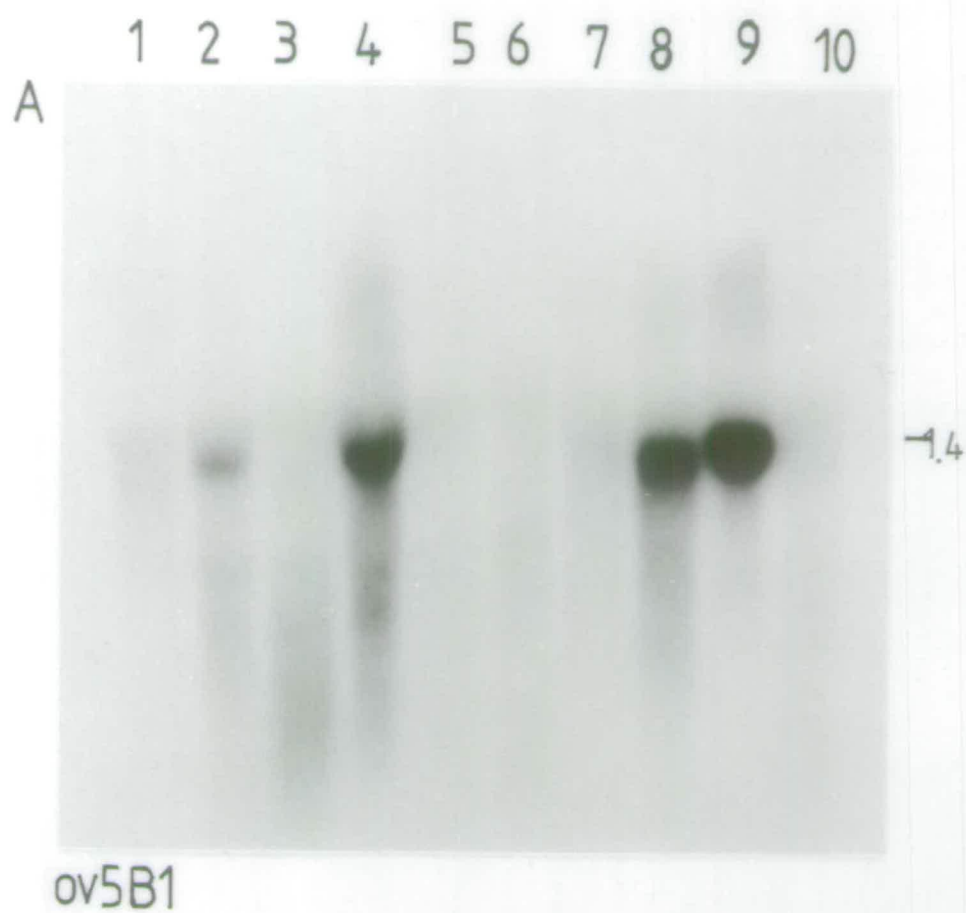
In the Northern blots A and B, 10 μ g of total RNA from the following samples were loaded:

A: 1 = embryos (0-24 hours)
 2 = 1st instar larvae
 3 = 2nd instar larvae
 4 = early third instar larvae
 5 = late third instar larvae
 6 = pupae
 7 = males
 8 = females
 9 = ovaries
 10 = female carcasses

B: 1 = males
 2 = ovaries
 3 = females

The figure opposite shows autoradiographs of Northern blots A and B probed with the ov5B1 cDNA probe (panels A and B respectively). Panel C shows an autoradiograph of Northern blot B which was stripped and reprobed with α -tubulin sequences.

Exposure Lengths: A : 18 hours
 B : 2 days
 C : 18 hours



Organisation Of The ov5B1 cDNA Within The Corresponding ov5B Genomic Fragment

A variety of restriction enzymes were used to digest pGem-ov5B. The digested DNA fragments were Southern blotted and probed with the ov5B1 cDNA sequences. The ov5B1 cDNA was located within a 2.4 kb sized PstI/SalI internal DNA fragment (Figure 4.11).

Discussion

ov5B Encodes A 1.4 kb Sized Ovary Specific Transcript Unrelated To The Transcripts Encoded Within ov5A

When the ov5B genomic DNA fragment was used to screen an ovary cDNA library, six related cDNA sequences were isolated called ov5B1 - ov5B6. The ov5B1 - ov5B6 cDNA sequences represent copies of the same transcript. The ov5B1 cDNA detected a transcript in ovary RNA extracts estimated to be 1.4 kb in length. This transcript is more likely to be about 1.5 kb in length in accordance with the 1.5 kb cDNA size class (ov5B2 - ov5B5, Figure 4.9). For ease of reference and consistency in this thesis the transcript detected by ov5B1 will be referred to as the 1.4 kb ovary specific transcript. The cDNA sequences encoded within ov5A1, ov5A2 and ov5A5 are unrelated to the cDNA sequences encoded within ov5B1 - ov5B6 since they do not cross hybridise. It follows that the overlapping ovary enriched transcripts detected by the ov5A genomic fragment are unrelated in sequence to the ovary specific transcript encoded within the ov5B genomic fragment.

Consideration Of The Possible Roles Of The Transcript Detected By ov5B

The 1.4 kb transcript detected with the ov5B1 cDNA sequence was not detected in adult males or female carcasses (i.e. tissues remaining after removal of the ovaries) (Figure 4.10, A and C). This transcript may therefore belong to a class of genes which function specifically during oogenesis in the adult. Since it was not detected in embryos, this gene product might be involved in a process specifically concerned with the development of the egg rather than being a maternally expressed gene whose product is required for embryogenesis. This possibility will be considered again in Chapter Six. It is interesting that this transcript is also developmentally regulated during

Figure 4.11

Transcript Organisation

Mapping The ov5B1 cDNA Within The Corresponding Genomic ov5B Sub-Fragment

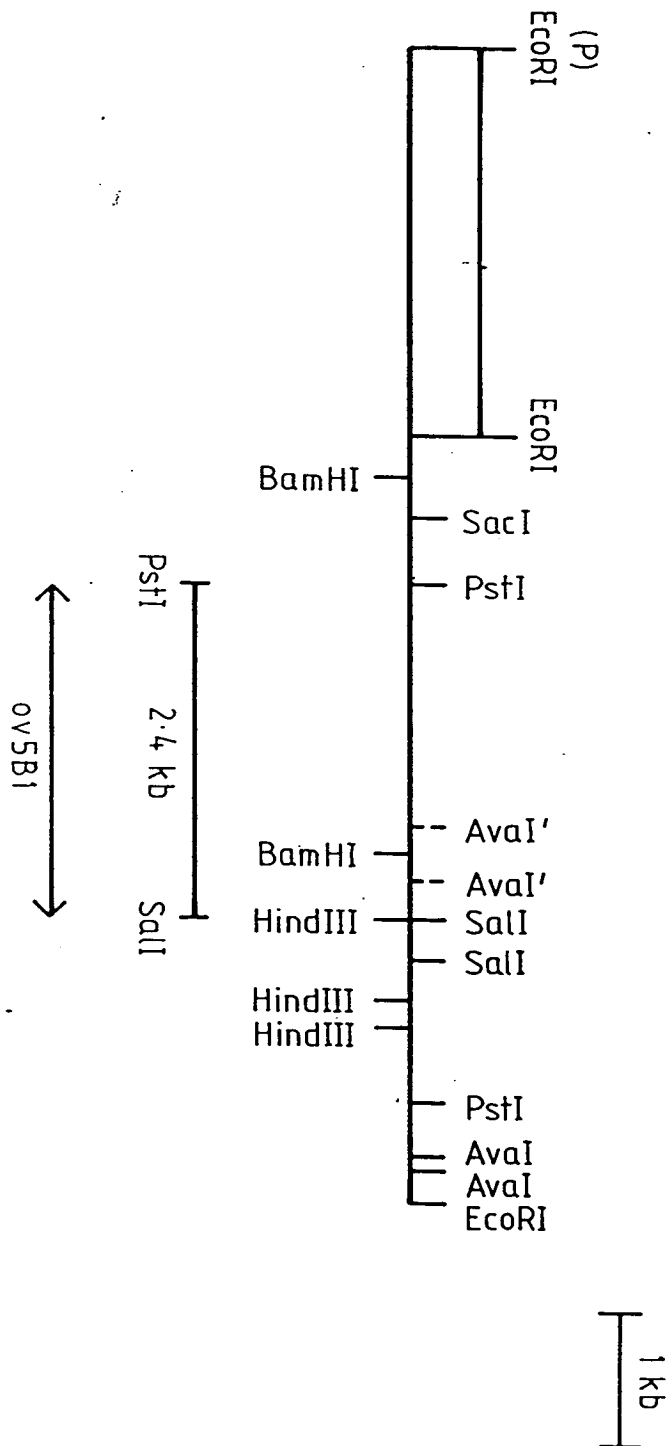
The ov5B1 cDNA was labelled with [^{32}P]-dCTP and used to probe a Southern blot containing restriction enzyme digests (using SacI, PstI, AvaI and SalI) of pGem-ov5B, which contains the corresponding ov5B genomic fragment. The ov5B1 cDNA hybridised within a 2.4 kb PstI/SalI fragment from ov5B. This relationship of ov5B1 with ov5B is illustrated opposite.

pGem-ov5B = pGemini vector containing the ov5B sub-fragment.

(P) = polylinker multiple cloning site in the pGemini vector.

 = pGemini vector sequences

pGem-ov5B



the larval stages and appears to be expressed only in 1st and early 3rd instar larvae. Perhaps the function of this transcript during larval development is different to its role in the adult ovary.

Part Five

In situ Hybridisation To Third Instar Salivary Gland Polytene Chromosome Preparations

In order to locate the *ov5* genomic sequences on the cytological map, third instar salivary gland polytene chromosome squashes were probed with the *ov5A* fragment. The *ov5A* DNA fragment was labelled with [³H]-dCTP and the hybridisation signal visualised by the appearance of silver grains on a layer of photographic emulsion. Figure 4.12 provides an example of the chromosomal hybridisation signal obtained with the *ov5A* probe. It was assumed that *ov5B* also hybridises at the same genomic location as *ov5A*. It was suggested that *ov5A* and *ov5B* may in fact represent two *EcoRI* fragments from different parts of the genome that somehow became fortuitously linked together during the preparation of the original genomic library (Maniatis *et al.*, 1978). During the construction of the library DNA fragments 10-20 kb in size were selected after random shearing of genomic DNA. *EcoRI* linkers were then added and the DNA fragments subsequently cloned into an *EcoRI* site in the λ Charon 4A vector. Due to the size selection of genomic insert lengths I think it is reasonable to assume that the 10.7 kb genomic DNA fragment within λ *ov5* most likely contains a single contiguous region of genomic DNA and therefore *ov5A* and *ov5B* will hybridise at the same genomic location. Unfortunately attempts to locate the *ov5B* fragment by *in situ* hybridisation to salivary gland polytene chromosome preparations were unsuccessful. The *ov5A* probe was found to hybridise at a single genomic location, at position 88 B/C on the right arm of the 3rd chromosome (3R).

The *Drosophila* Information Service (DIS) provides lists of mutants and cloned genes that have been mapped cytologically. At the time this *in situ* hybridisation result was obtained, there were no known female sterile mutations in this region, or cloned sequences with expression patterns similar to those obtained with either *ov5A* or *ov5B*. However, more recently at a

Figure 4.12

In situ Hybridisation To Third Instar Salivary Gland Polytene Chromosome Preparations
Using The ov5A Genomic Probe

The ov5A genomic fragment from λ ov5 was labelled with [^{3} H]-dCTP and used to probe third instar salivary gland polytene chromosomes prepared from a *Drosophila melanogaster* wild type strain (OrR). ov5A hybridises at 88B/C on chromosome 3R.

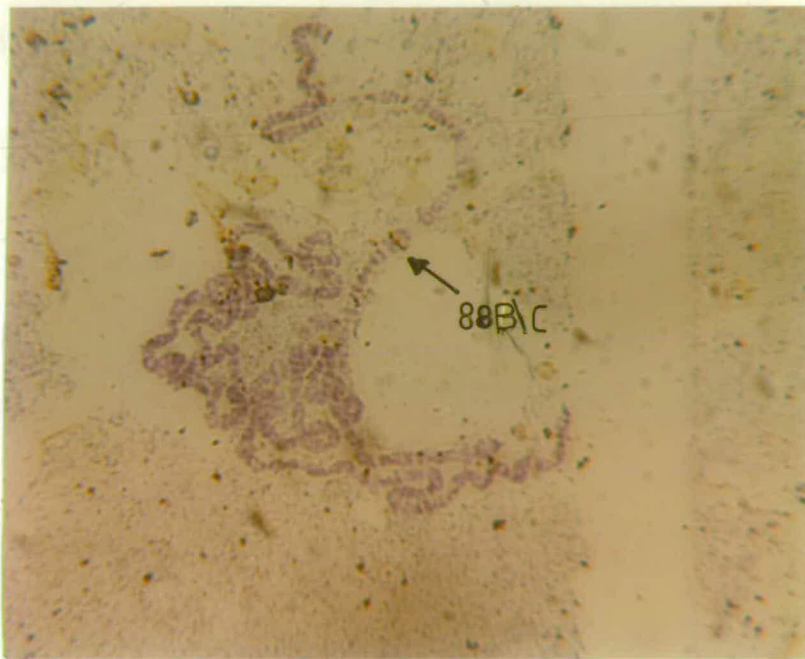
A: X40 Magnification

B: X100 Magnification

The hybridisation signal is indicated by an arrow

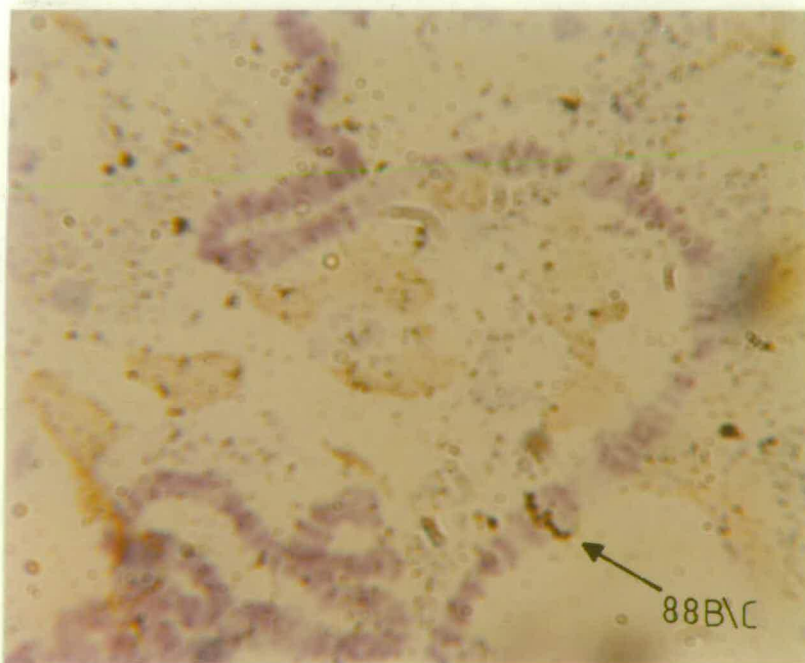
ov5A

A



X100

B



X100

research meeting, two female sterile mutations mapping within the 88 region were reported (S. Parkhurst, University of Oxford). One of these mutations is called *Spindle-B* (Tearle and Nusslein-Volhard, 1987) and the other, *fs293^{Y5}* (R. Kelley, University of Texas, Austin). Experiments with a set of deficiencies spanning the 88 region were carried out to find out if the *ov5* sequences mapped within deficiencies which uncover these female sterile mutations. These experiments are discussed Chapter Six of this thesis.

Part Six

Summary: λ ov5 Encodes Multiple Ovary Enriched Transcripts

When the *ov5A* and *ov5B* genomic fragments were used separately to screen an ovary cDNA library, two sets of cDNA clones were isolated. The *ov5A* genomic fragment detected three cDNA clones called *ov5A1*, *ov5A2*, *ov5A5* which each hybridised with at least two ovary enriched transcripts, 1.7 kb and 1.4 kb in length. The *ov5A5* cDNA also hybridised with 0.8 kb and 0.2 kb ovary specific transcripts. Therefore within the *ov5A* region of the chromosome there must be either a single genetic unit which generates overlapping transcripts, or overlapping genes whose related products give rise to different sized mRNAs. In the adult, the *ov5B* genomic fragment detected a 1.4 kb ovary specific transcript which is unrelated to the 1.4 kb ovary enriched transcript detected by the *ov5A* probe. Six cDNA clones (λ ov5B1 - λ ov5B6) were isolated from a cDNA library using the *ov5B* probe, and at least one of these cDNA clones is likely to encode a full length copy of the 1.4 kb ovary specific transcript. These results proved to be different from the original model of predicted transcript organisation within λ ov5 as described at the beginning of this chapter in Figure 4.3. Figure 4.13 illustrates how the cluster of ovary enriched and ovary specific transcripts are organised within λ ov5, according to the results obtained when cDNAs were used to probe Southern blots of restriction enzyme digests of the *ov5A* and *ov5B* sub-fragments.

It was exciting to find within a 10.7 kb (*ov5*) genomic region, multiple transcription units whose products were enriched in the adult ovary and, in some cases, the embryonic stages. It will

Figure 4.13

Summary

Organisation Of Ovary Enriched Transcripts Within The ov5 Genomic Region

The ov5 genomic region contains two distinct coding regions.

The ov5B1 cDNA (which detects a 1.4 kb sized adult ovary specific transcript) hybridises within a 2.4 kb PstI/SalI fragment, which is indicated on the figure.

The other coding region within ov5, which encodes overlapping ovary enriched (1.7 kb and 1.4 kb in length) and ovary specific (0.8 kb and 0.2 kb in length) transcripts is located within a 1.4 kb EcoRI/AvaI fragment, within the ov5A sub-fragment.

Broken lines indicate sequences within ov5A1 and ov5A2 which are located outwith the ov5 genomic region.



= λ Charon 4A vector arms

AvaI'

= An AvaI' site is located at one of these two positions.

PstI'

= There are a further four PstI sites in ov5 whose positions have still to be determined.

L-arm

= Left arm of λ Charon4A vector (19.8 kb)

R-arm

= Right arm of λ Charon4A vector (10.8 kb)

be interesting to determine a function(s) for these genes (this will be examined in the final chapter). There are other examples of tightly clustered genes whose products are expressed in the adult gonads and/or in early development. These gene clusters often contain overlapping genes or genes which encode overlapping transcripts. This will be discussed in detail in the final discussion.

CHAPTER FIVE

IDENTIFICATION OF MATERNALLY EXPRESSED TRANSCRIPTS, OVARY SPECIFIC TRANSCRIPTS AND A MALE SPECIFIC TRANSCRIPT WHICH ARE ENCODED WITHIN λ ov2

Table 5.1

λ ov2: Sizes Of EcoRI Fragments

19.8 kb		
10.8 kb		λ Charon 4A vector arms
* 4.2 kb	-	ov2B
* 3.25 kb	-	ov2A
2.0 kb		
1.9 kb		
1.4 kb		
0.75 kb		
0.2 kb		
13.7 kb	insert	
44.5 kb	total length of recombinant λ	
* EcoRI sub-fragments hybridising with only female cDNA (refer to Figure 3.4)		

Introduction

The recombinant bacteriophage λ ov2 was isolated from a differential screen (Chapter 3). Two EcoRI DNA sub-fragments from λ ov2 hybridised with radiolabelled female cDNA but not with radiolabelled male cDNA (Figure 3.3). The two sub-fragments ov2A (3.25 kb EcoRI fragment) and ov2B (4.2 kb EcoRI fragment) hybridised with a transcript approximately 4.0 kb in size in total RNA extracted from ovaries (Figure 3.4).

When the sensitivity of Northern analysis was improved, several maternally-expressed and ovary specific transcripts were detected using ov2A and ov2B probes. Furthermore, a male specific transcript was also detected by ov2A. This chapter describes the transcript patterns detected by ov2A and ov2B, and the location of these sub-fragments on the *Drosophila* salivary gland polytene chromosome map.

Results

Restriction Site Information: λ ov2

Attempts were made to construct a restriction map of λ ov2. The ov2 genomic sequence was found to be 13.7 kb in length and has seven EcoRI restriction fragments. Their sizes are listed in Table 5.1. Due to this complexity it was difficult to construct a reliable order of restriction enzyme sites and this piece of work remains to be completed.

Improving Transcript Detection With The ov2A And ov2B Fragments

Each of the EcoRI sub-fragments ov2A and ov2B which hybridised with the female cDNA probe (Figure 3.3) were used separately to probe RNA developmental profiles. When these experiments were carried out it was found that by changing the Northern blotting procedure from Ammonium Acetate 'dry blotting' (as recommended by the manufacturer) to the standard transfer of RNA to membrane filters using 20X SSC 'wet blotting' (described in Chapter 2), the sensitivity of transcript detection was greatly increased.

Two Maternally Expressed Transcripts And A Male Specific Transcript Are Detected By ov2A

When the ov2A fragment (3.25 kb EcoRI sub-fragment) was used to probe total RNA extracted from embryos (0-24 hours), 1st, 2nd and 3rd instar larvae, pupae, sexed adults and ovaries, three transcripts were detected (Figure 5.1, panel A). In female adults, ovary and embryo RNA two transcripts approximately 4.0 kb and 3.0 kb in size were detected (Figure 5.1, panel A, lanes 8, 9, and 1 and 10, respectively). The 4.0 kb and 3.0 kb sized transcripts were not detected in the larval or pupal stage (Figure 5.1, panel A, lanes 2-7) or in female carcass RNA (data not shown). The ov2A fragment also detected a transcript of approximately 1.6 kb in length expressed in adult males (Figure 5.1, panel A, lane 7). The 1.6 kb adult male specific transcript was also observed at a low level in early and late third instar larvae and pupae (Figure 5.1, lanes 4, 5 and 6).

Detection of Two Ovary Specific Transcripts Using The ov2B Probe

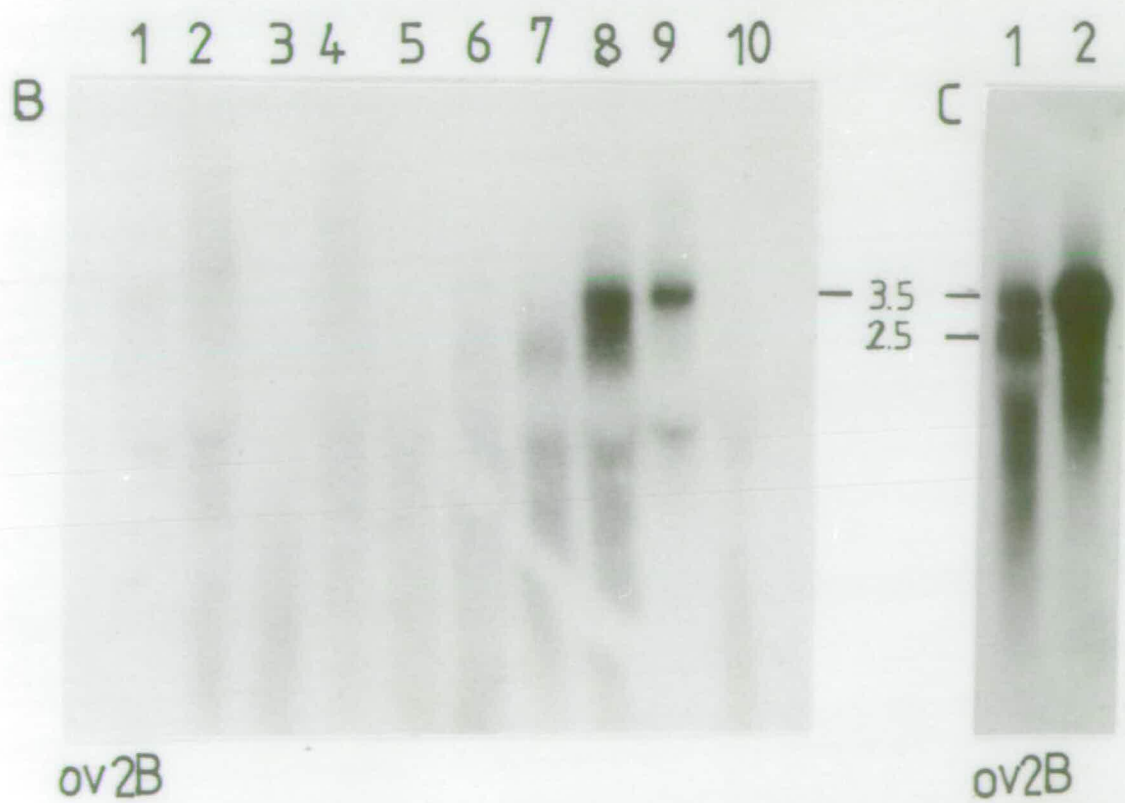
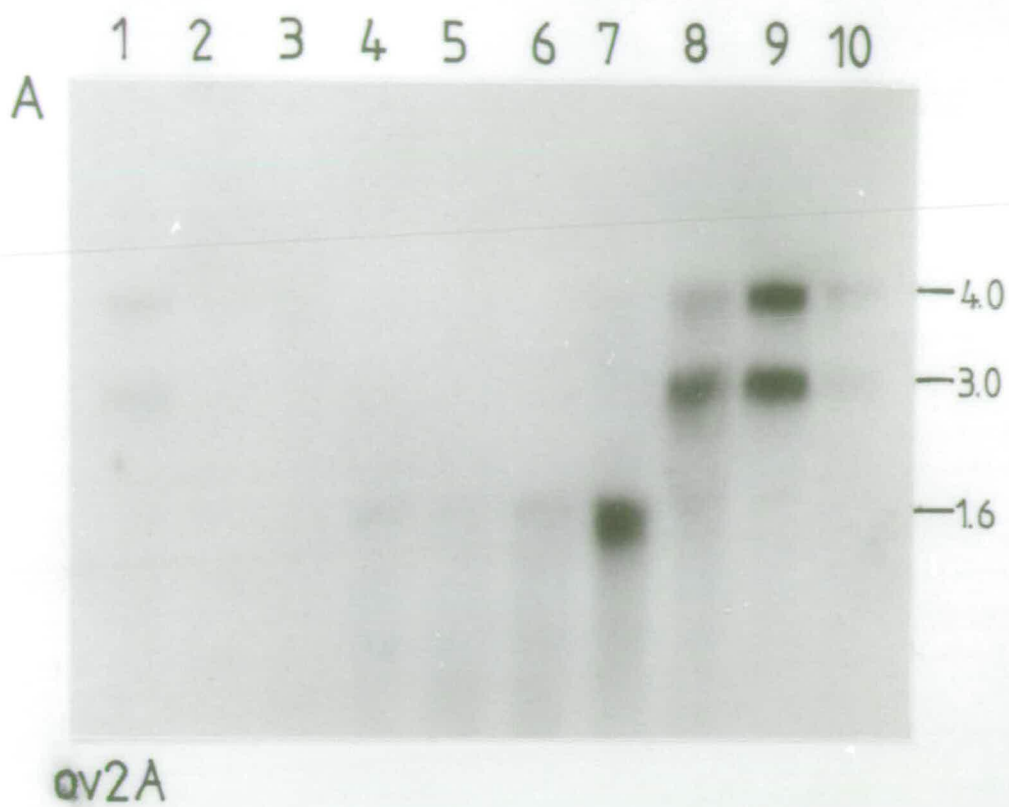
The ov2B sub-fragment from λ ov2 was used to probe a Northern blot containing total RNA extracted from embryos; 1st, 2nd and 3rd instar larvae; adult males and females, and female carcasses. A transcript approximately 3.5 kb in size was detected in the ovary RNA sample (Figure 5.1, panel B, lane 9). In poly (A)⁺ RNA extracted from ovaries and adult females, two transcripts estimated to be 3.5 kb and 2.5 kb in size were detected with the ov2B probe (Figure 5.1, Panel C, lanes 1 and 2 respectively). Presumably the 2.5 kb transcript is expressed at a lower level than the 3.5 kb transcript since it was not detected in total ovary RNA samples.

Figure 5.1

Expression Of Ovary Enriched Transcripts Detected By ov2A And ov2B And Their Developmental Regulation

- A: 10 μ g of total RNA prepared from 0-24 hour embryos (lane 1), 1st instar larvae (lane 2), second instar larvae (lane 3), early third instar larvae (lane 4), late third instar larvae (lane 5), pupae (lane 6), adult males (lane 7), adult females (lane 8), ovaries (lane 9), 0-24 hour embryos (lane 10) was Northern blotted and probed with the ov2A sub-fragment. This autoradiograph was exposed for 7 days.
- B: Autoradiograph of a Northern blot containing the same samples indicated in blot A (lanes 1-9). Lane 10 contains 10 μ g of female carcass total RNA. The blot was probed with the ov2B sub-fragment. This autoradiograph was exposed for 8 days.
- C: Autoradiograph of a Northern blot containing 2 μ g of poly (A)⁺ RNA prepared from ovaries (lane 1) and adult females (lane 2) was probed with the ov2B sub-fragment. This autoradiograph was exposed for 8 days.

The estimated sizes of transcripts (in kb) are indicated.



DISCUSSION

λ ov2 Encodes Maternally Expressed Transcripts, Ovary Specific Transcripts And A Male Specific Transcript

The ov2 genomic fragment contains two sub-fragments which code for at least five transcripts. The ov2A sub-fragment detected two transcripts 4.0 kb and 3.0 kb in size which have transcription patterns expected of maternally expressed gene products. The 4.0 kb and 3.0 kb transcripts were detected exclusively in the ovary and embryo RNA extracts. The ov2A sub-fragment also encodes a 1.6 kb adult male specific transcript. It is probable that there is some overlap between the 4.0, 3.0 and 1.6 kb transcripts since they are all detected by a 3.25 kb (ov2A) genomic fragment.

The ov2B sub-fragment is 4.2 kb in length and detected two transcripts expressed exclusively in the ovary. The 3.5 kb ovary specific transcript detected by ov2B appears to be more abundant than the 2.5 kb ovary specific transcript as the 2.5 kb transcript was detected only in poly (A)⁺ RNA.

It will be interesting to determine the function of these transcripts during oogenesis, for example to see if they perform related functions, and to carry out a molecular study of the organisation of these transcripts within the ov2 region. The detection of a male specific transcript with the ov2A fragment was unexpected, as this fragment did not hybridise with the male cDNA probe (Figure 3.4). Very few male-specific transcripts have been isolated to date (Schafer, 1986a, 1986b; DiBeneditto *et al.*, 1987) so this transcript will be interesting to characterise further.

Genomic Location - λ ov2

Third instar salivary gland polytene chromosomes were prepared from a wild type *Drosophila melanogaster* strain (OrR). Each of the ov2A and ov2B sub-fragments from λ ov2 were labelled with [³H]-dCTP and used to probe the polytene chromosome preparations. Each fragment hybridises at 89B on chromosome 3R (confirmed by M. Ashburner). Figure 5.2 shows a

Figure 5.2

In situ Hybridisation To Third Instar Salivary Gland Polytene Chromosome Preparations

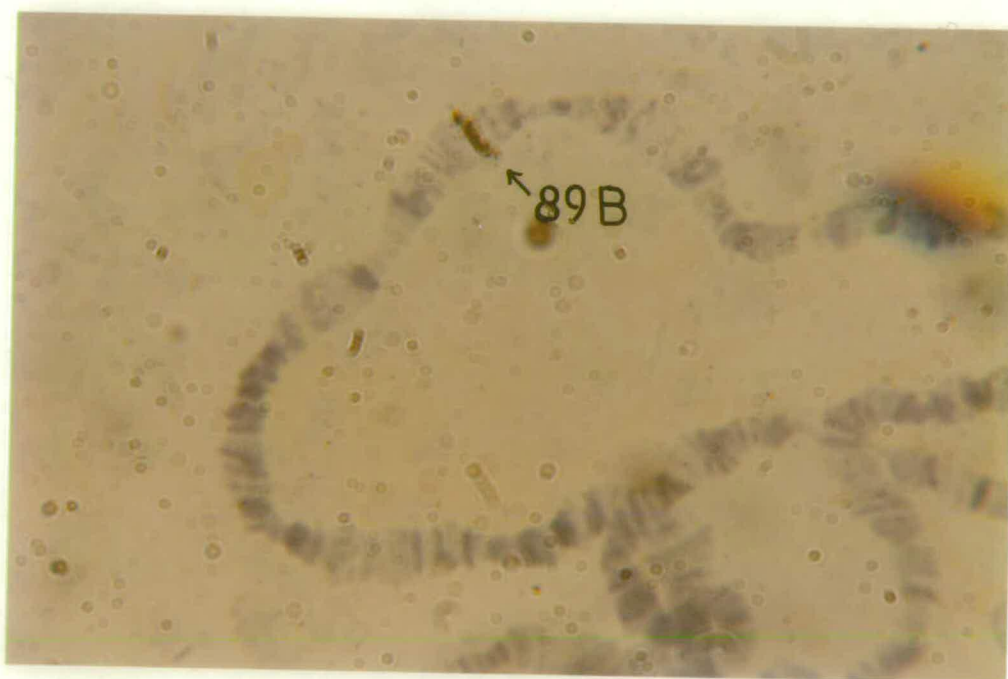
Using the ov2B Genomic Probe

The ov2B genomic sub-fragment from λ ov2 was labelled with [^{3} H]-dCTP and used to probe *Drosophila melanogaster* (Oregon R) third instar larval salivary gland polytene chromosomes.

The ov2B sequences hybridise at 89B on the right arm of chromosome 3.

X100 magnification. The hybridisation signal is indicated by an arrow.

ov 2B



X100

photograph of the hybridisation signal obtained with the ov2B probe. It was of interest to note that ov2 maps closely on the chromosome map to ov5 (Chapter 4, Figure 4.12) which hybridises at 88B/C. The ov2A and ov2B fragments both hybridise to the same single chromosomal location and to only a single band on genomic Southern blots containing EcoRI digested DNA extracted from OrR (Figure 6.3, panels B and C) therefore it is very unlikely that the transcripts detected on Northern blots are encoded by other genes. If the ov2A and/or ov2B fragments hybridised to more than one location in the genome, or to multiple fragments in genomic Southern blots, then it would be possible that the transcripts detected on Northern blots would represent transcripts encoded by related genes.

CHAPTER SIX

TOWARDS A FUNCTION: TRANSCRIPTS EXPRESSED IN THE OVARY

Part One: Towards A Function

ov2 and ov5 Map Close To Two Known Female Sterile Mutations

Introduction

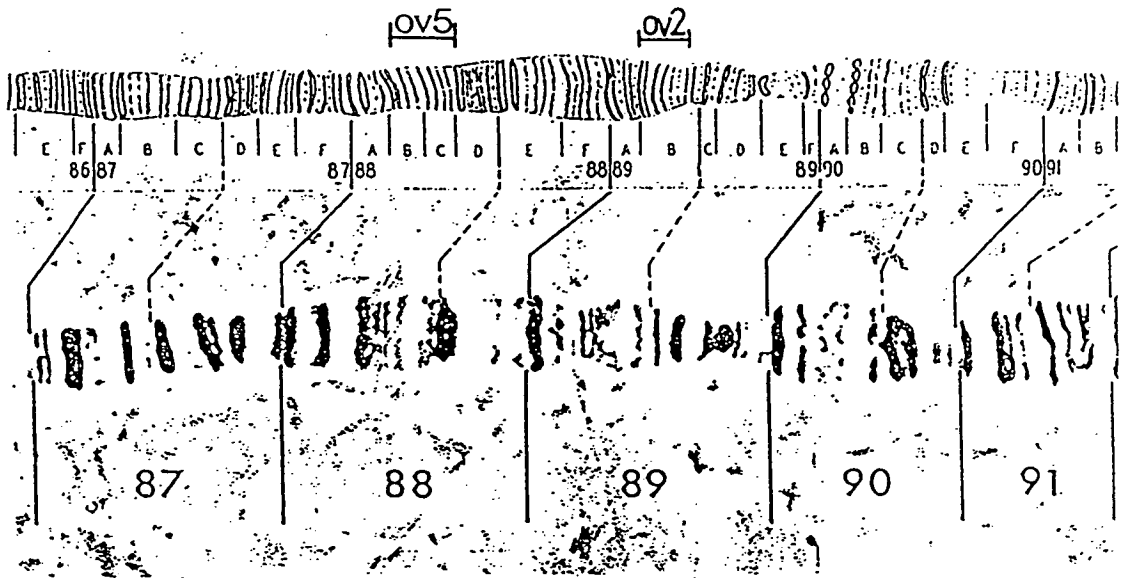
Chapters 4 and 5 described two genomic sequences, λ ov2 and λ ov5, which were found to encode ovary enriched transcripts. Ultimately it is necessary to determine a function(s) for these transcripts during oogenesis and, where appropriate, during embryogenesis. λ ov2 and λ ov5 map close together on chromosome 3R at positions 89B and 88B/C respectively. Within this cytological region two female sterile mutations have been mapped (S. Parkhurst, University of Oxford, personal communication). Figure 6.1 provides a map of deficiencies in the 88 region and the location of the female sterile mutations *Spindle-B* and *fs293^{Y5}* are indicated (S. Parkhurst supplied this map and the deficiency stocks). The female sterile mutation, *Spindle-B* was isolated by C. Nüsslein-Volhard in a genetic screen (Tearle and Nüsslein-Volhard, 1987; Nüsslein-Volhard, 1990). The eggs of homozygous *Spindle-B* mothers are always unfertilised and have an abnormal elongated shape. *Spindle-B* mutations are uncovered by the *Df(3R)red^{3L}* and *Df(3R)red^{P93}* deficiencies but not by *Df(3R)red^{P52}*. The mutation causing female sterility in *fs293^{Y5}* maps within the *Df(3R)293^{Y5}* deficiency and is being studied by R. Kelley, University of Texas, Austin (personal communication, S. Parkhurst).

Since these two *fs* mutations mapped nearby the ov2 and ov5 sites of chromosomal hybridisation two types of experiment were carried out to try and localise ov5 and ov2 more precisely with respect to these mutations. In the first set of experiments genomic DNA was extracted from the seven stocks with a deficiency around the cytological region 88 on chromosome 3R (Figure 6.1), and from the wild type, Oregon R strain. Equal amounts of genomic DNA from each sample were digested with EcoRI, electrophoresed on an agarose gel and Southern blotted. As an internal control the blots were probed with the *Drosophila Adh* sequences which are present in two copies in each sample as these sequences lie outwith the *Df(3R)* deficiency region studied. The *Adh* signal can be compared directly in each sample with the ov2 or ov5 hybridisation signal

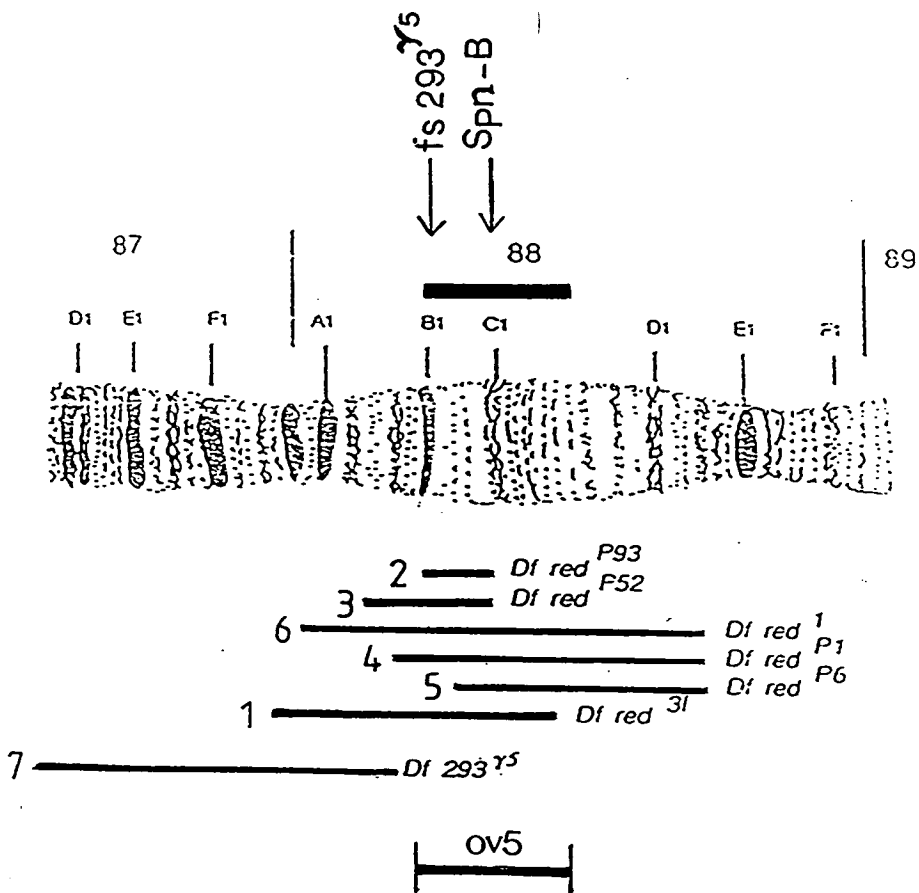
Figure 6.1

- A: Schematic illustration (above) and photocopy of photographs of the cytological region 87-91 of Chromosome 3R of *Drosophila melanogaster* (adapted from genetic and physical maps contained in "The Genetics and Biology of *Drosophila*", Volume 1a, Editors Ashburner and Novitski). The map positions of *ov5* (88B/C) and *ov2* (89B) were determined by *in situ* hybridisations to polytene chromosomes and their location on 3R is indicated.
- B: Drawing of 87D-89A on Chromosome 3R from *Drosophila melanogaster* (reference in Figure 6.1 A). Two female sterile mutations called *fs293^{y5}* and *Spindle-B (Spn-B)* have been mapped genetically within the 88 region using the set of deficiencies indicated below the illustration (nos. 1-7). The map position of *ov5* is indicated.
- Note: The numbers allocated to the deficiency stocks correspond to those used in lanes 1-7 of the Southern blots shown in Figures 6.2 and 6.3.

A



B



thus compensating for any loading differences between each track. The EcoRI digested genomic DNA extracts from the deficiency stocks were probed with each of the ov5A, ov5B, ov2A and ov2B genomic fragments. Any of these sequences mapping within a particular deficiency would be present in only one copy (i.e. on the balancer chromosome). Alternatively, if the ov2 and ov5 sequences tested lie outwith a particular deficiency then one copy of the cognate sequence would be present on each chromosome, giving a signal of similar intensity to the control *Adh* signal. This technique has been used successfully to identify the copy number of other *Drosophila* sequences in genomic DNA, for example in the estimation of the number of I-element sequences per genome (Bucheton *et al.*, 1984).

Results

Probing Genomic DNA Extracted From Strains Carrying Deficiencies Around Region 88 On Chromosome 3R With ov5A

In the first set of experiments equal amounts of EcoRI digested genomic DNA from the *Df(3R)88* stocks and Oregon R were loaded onto two gels and Southern blotted. The first Southern (A) was probed with the ov5A fragment and the second Southern (B) was probed with the *Adh* gene (1.75 kb XbaI fragment). These results are illustrated in Figure 6.2, panels A and B respectively.

The *Adh* fragment hybridised with a 5.0 kb EcoRI DNA fragment, and also very faintly with a 3.0 kb sized DNA fragment (indicated by an arrow; Figure 6.2, Southern B). The ov5A EcoRI fragment hybridised with a 22.5 kb genomic EcoRI DNA fragment. It is important to note here that the insert within λ ov5 was generated by random shearing of *Drosophila* genomic DNA followed by the addition of EcoRI linkers before subsequent cloning into the λ Charon 4A vector (Maniatis *et al.*, 1978). Thus, the EcoRI sites at the cloning sites of λ ov5 are not the endogenous EcoRI sites. Therefore in the case of ov5A the endogenous EcoRI site is at a distance of 17.5 kb from the cloning EcoRI site. The signal intensity in each track of Southern A and Southern B was measured using computerised scanning densitometry. The ratio of the ov5A signal/*Adh* signal should be approximately 1.0 if there are the same number of ov5A and *Adh* sequences in each track. The

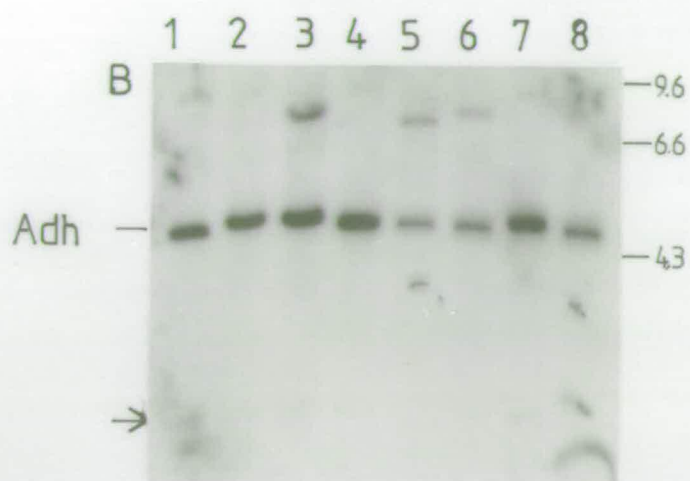
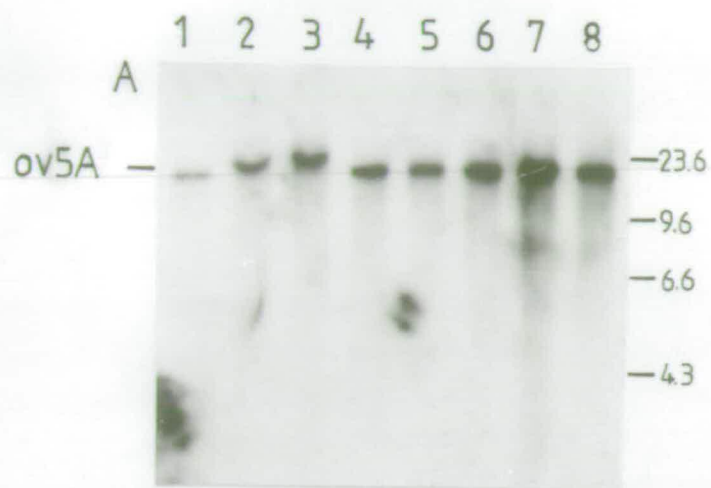
Figure 6.2

Autoradiographs of identical Southern blots A, B and C, containing 2 μ g of EcoRI digested genomic DNA in each track extracted from the deficiency (3R) stocks 1-7 (listed in Figure 6.1) and Oregon R, probed with *ov5A* and *Adh* sequences.

In each case: Lane 1 = *Df(3R)red^{3l} / MKRS, M $\bar{e}$*
 2 = *Df(3R)red^{P93} Sb / P18e, M $\bar{e}$*
 3 = *Df(3R)red^{P52} / TM1, M $\bar{e}$*
 4 = *Df(3R)red^{P1} X α / TM1, M $\bar{e}$*
 5 = *Df(3R)red^{P6} X α / TM1, M $\bar{e}$*
 6 = *Df(3R)red¹ / MKRS, M $\bar{e}$*
 7 = *Df(3R)293^{v5} / TM6B, Tb*

Molecular Weight markers are listed on the right hand side of each blot. Sizes are in kb.

- A: *ov5A* fragment detects a 22.5 kb sized EcoRI DNA fragment (16 hour exposure)
- B: *Adh* sequences detect a major band 5.0 kb in size along with a minor band approximately 3.0 kb in size indicated by an arrow (-). (16 hour exposure)
- C: A mixed *ov5A/Adh* probe was used on Southern blot C to allow the intensity of the *ov5A* and *Adh* signals to be compared directly in each lane. The arrow (-) indicates an *Adh* 3.0 kb minor hybridisation signal. (16 hour exposure)



ratios observed when the *ov5A* and *Adh* signal intensity in each sample were compared and are illustrated in Figure 6.4. The results indicate that the ratio in lane 5 (*Df(3R)red^{P6}*) may be significantly lower than the OrR ratio. In order to try to reproduce this tentative result, the experiment was repeated using a mixed *Adh/ov5A* probe. The results obtained are illustrated in Figure 6.2, panel C. The signal intensity in each track including *Df(3R)red^{P6}* looks similar for the *Adh* and *ov5A* signals. Again, densitometry was used to measure the ratio of the *Adh* : *ov5A* signals in each track and all the ratios lie within the range 0.96 - 1.40 except for lanes 6 and 7 (Figure 6.5). Lane 7 on Southern C which contains DNA from the *Df(3R)293^{Y5}* stock has an *Adh* : *ov5A* ratio of 2.48. However, although this is a markedly higher *Adh* : *ov5A* signal ratio than was obtained with other DNA samples it is probably due to star activity of the *EcoRI* enzyme in the *ov5A* genomic DNA sequence in the *Df(3R)293^{Y5}* DNA. In other *EcoRI* digested *Df(3R)293^{Y5}* DNA samples probed with the *ov5A* sequence, only a single 22.5 kb genomic DNA fragment was detected on Southern blots. In lane 7 in blot C, two additional smaller bands were also observed (possibly due to star activity of the enzyme), and only one of the 22.5 kb bands was measured and compared with the *Adh* signal. The apparent difference between the *Adh* and *ov5A* signals in lane 7 is therefore likely to be an artefact. In lane 6, the *Adh* : *ov5A* signal ratio was 1.86 which also appeared to be markedly raised compared to the ratio obtained with Oregon R (0.91).

Discussion

When Southern blots of *EcoRI* digested genomic DNA extracted from a set of deficiency stocks were probed with the *ov5A* fragment, a 22.5 kb sized DNA fragment was detected. The signal intensity in each track with the *ov5A* probe was compared to the signal intensity obtained when the control *Adh* probe to determine if any difference in *ov5A* sequence copy number between the deficiency stocks and the wild type (OrR) could be detected. The results of the experiments however seem to be inconclusive. When the *ov5A* : *Adh* signal ratios were compared for Southern A and Southern B, it was indicated that the *Df(3R)red^{P6}* stock might contain only one copy of the *ov5A* sequence (Figure 6.2, lane 5) as it was found to have an *ov5A* : *Adh* signal ratio of 0.38 compared to 0.75 obtained with OrR. If this was a genuine result then it would have been expected that the *Df(3R)red^{P1}* and *Df(3R)red¹* deficiency stocks would have similar *ov5A* : *Adh* signal ratios

Figure 6.3

Autoradiographs of duplicate Southern blots containing 2 μ g of EcoRI digested genomic DNA per track, extracted from the deficiency (3R) stocks illustrated in Figure 6.1 and OrR, probed with (A) a mixed ov5B/*Adh* probe and (B) the ov2B fragment. The Southern blot shown in B was stripped and reprobed with the ov2A fragment (C).

In each case: Lane 1 = *Df(3R)red*³¹ / *MKRS*, *M $\bar{e}$*
 2 = *Df(3R)red*^{P93} *sb* / *P18e*, *M $\bar{e}$*
 3 = *Df(3R)red*^{P52} / *TM1*, *M $\bar{e}$*
 4 = *Df(3R)red*^{P1} *X α* / *TM1*, *M $\bar{e}$*
 5 = *Df(3R)red*^{P6} *X α* / *TM1*, *M $\bar{e}$*
 6 = *Df(3R)red*¹ / *MRS*, *M $\bar{e}$*
 7 = *Df(3R)293r*⁵ / *TM6B*, *Tb*

- A: A mixed ov5B/*Adh* probe was used to allow the signal intensity of each to be compared directly in each lane. The *Adh* sequences hybridise with a 5.0 kb sized EcoRI fragment and to a lesser extent with a 3.0 kb sized fragment which is indicated by an arrow (-). The ov5B fragment hybridises with an 8.5 kb sized EcoRI fragment (except in lane 3, where it also recognises a 6.5 kb fragment).
 16 hour exposure.
- B: The ov2B fragment hybridises with a 4.8 kb sized fragment in each lane. In lane 3 (*Df(3R)red*^{P52}) an additional fragment of 4.5 kb also hybridises with the ov2B probe (16 hour exposure).
- C: The Southern blot used in (B) was stripped and reprobed with the ov2A fragment. A single band approximately 5.0 kb in size was observed in each lane (5 days exposure).

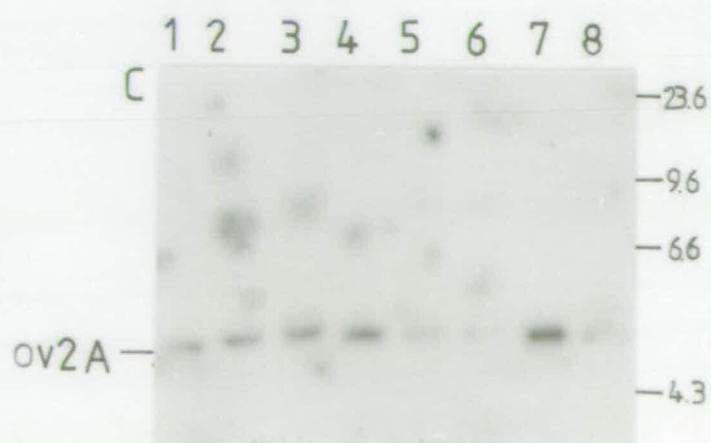
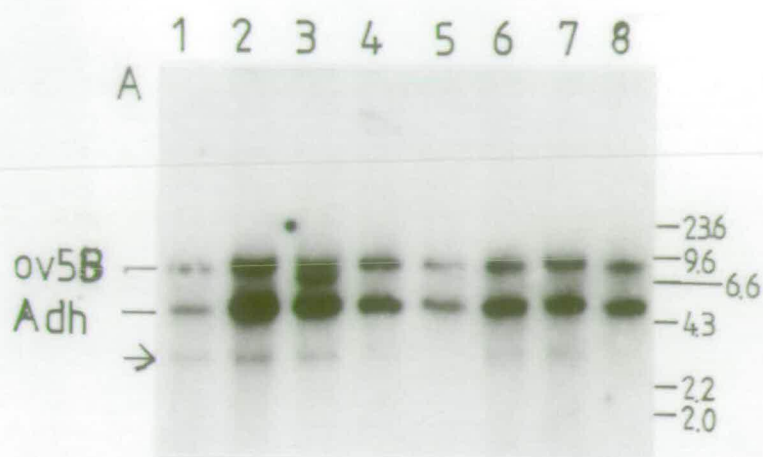


Figure 6.4

Adh: ov5A Signal Ratio (1)

Comparison of The ov5A and *Adh* Signals Obtained On Duplicate Southern Blots

(Shown in Figure 6.2, Panels A and B)

This bar chart illustrates the signal intensity ratios obtained when the ov5A and *Adh* signals were measured and compared using computerised scanning densitometry. On the x-axis the lane numbers from Southern blots A and B in Figure 6.2 are indicated. The y-axis indicates the *Adh* : ov5A signal intensity ratio.

Adh : ov5A Signal Ratio (1)

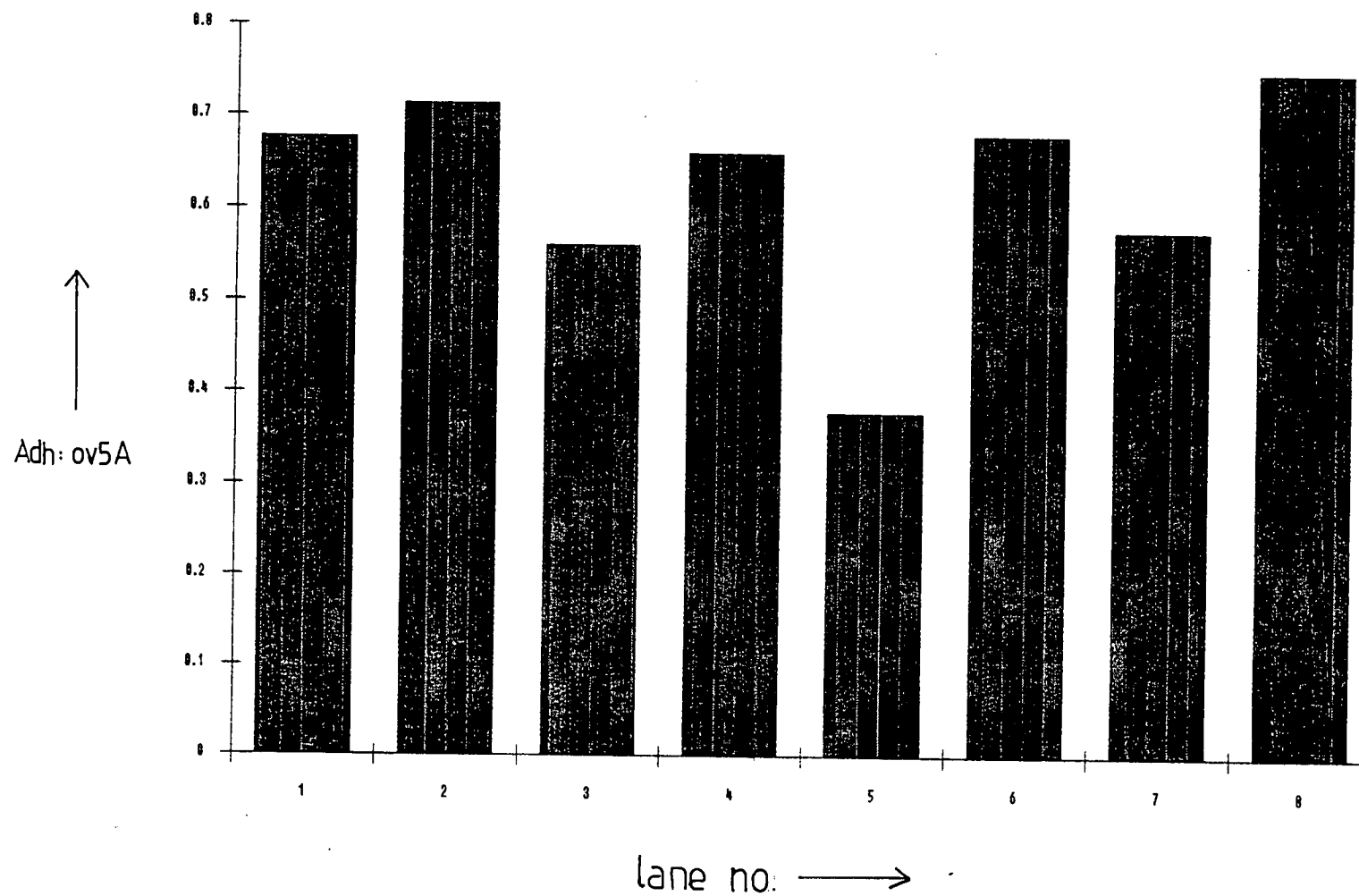


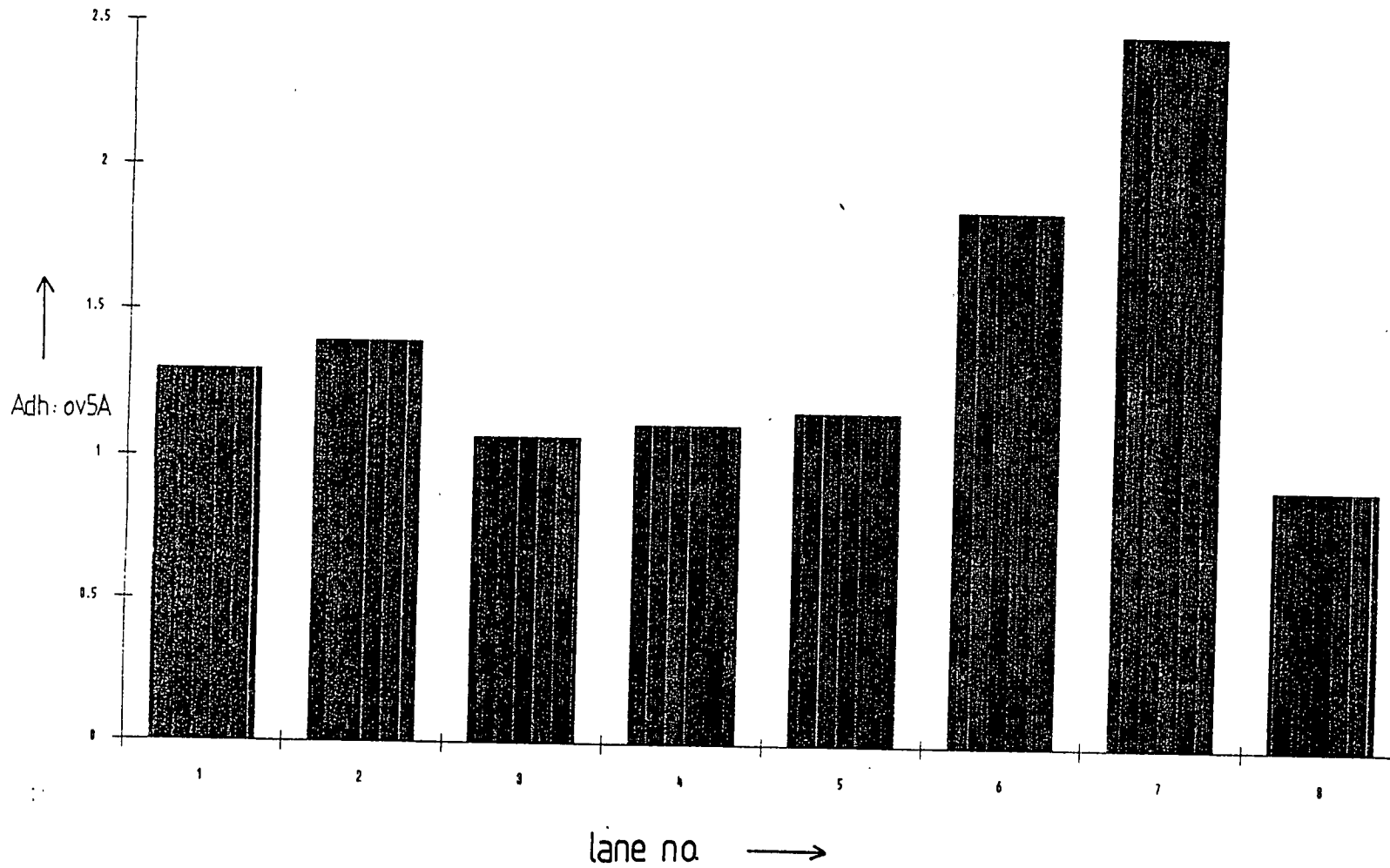
Figure 6.5

Adh : ov5A Signal Ratio (2)

Comparison Of The ov5A And *Adh* Signals Obtained Using A Mixed Probe On Southern Blot C, Figure 6.2

This bar chart illustrates the signal intensity ratios obtained when the ov5A and *Adh* signals were measured and compared when a mixed ov5A/*Adh* probe was used on a Southern blot (shown in Figure 6.2, panel C). The lane numbers from this blot are indicated on the x-axis. The y-axis charts the *Adh* and ov5A signal ratios obtained when the *Adh* and ov5A hybridisation signals were compared directly in each lane using computerised scanning densitometry.

Adh:ov5A Signal Ratio (2)



since each of these deficiencies encompass the *Df(3R)red*^{P6} deficiency. However when the experiment was repeated using a mixed *ov5A/Adh* probe on Southern blot C, the data obtained conflicted with the *ov5A : Adh* ratios obtained in the first set of experiments for the *Df(3R)red*¹ and *Df(3R)293*^{Y5} stocks. The results indicate that using this technique to assess the number of copies of the *ov5A* sequence within a particular deficiency was unreliable and inconsistent. This may be due to the technique not being sensitive enough to detect a quantitative difference in the number of *ov5A* sequences (expected to be one or two in number) compared to the number of *Adh* sequences (two per diploid genome). This quantitative Southern transfer technique has been used successfully in the past to estimate the number of transposable element sequences in different *Drosophila* strains. For example, Bucheton *et al.* (1984) estimated that there are 1-3 copies of the I-factor sequence per haploid genome in 'reactive' strains and about 15 copies per haploid genome in 'inducer' strains. However, their experiments did not require the resolution of a difference of one copy versus two copies of the I-sequence per genome in the strains studied.

Df(3R)88 Stocks: Experiments with *ov5B*

Attempts were also made to map the *ov5B* sequence within the 3R deficiency stocks alongside the experiments with the *ov5A* sequence described in the previous section. Despite the limitations of this experiment two useful pieces of information were obtained.

Results

A Southern blot containing *EcoRI* digested genomic DNA extracted from the deficiency stocks 1-7 (listed in Figure 6.1) and Oregon R was probed with a mixed *ov5B/Adh* probe (Figure 6.3, panel A). The *ov5B* sequence (5.7 kb *EcoRI* genomic fragment) hybridised with an 8.5 kb genomic *EcoRI* fragment in OrR and the deficiency stocks (except *Df(3R)red*^{P52}), indicating that the endogenous *EcoRI* site is at a distance of 2.8 kb from the *EcoRI* linker site at the end of the *ov5B* fragment. The *ov5B* probe recognised two *EcoRI* fragments in the *Df(3R)red*^{P52} stock (Figure 6.3, panel A, lane 3) 8.5 kb and 6.5 kb in size. Possibly this is a genuine result since the distribution of

the signal over the two bands is similar. It may be that the ov5B sequence crosses a breakpoint in the *Df(3R)red*^{P52} deficiency chromosome or that there is a polymorphism in this strain at the ov5B sequence. The *Adh* probe recognised a 5.0 kb EcoRI genomic DNA fragment. A smaller faint band approximately 3.0 kb in length was also detected by the *Adh* probe and is indicated by an arrow. The ov5B and *Adh* signals in each track look to be in similar proportions. Densitometry was not carried out in this case.

Discussion

The ov5B genomic fragment recognised an 8.5 kb genomic EcoRI fragment with a similar intensity to the signal obtained with the control *Adh* probe in genomic DNA prepared from the *Df(3R)* stocks 1-7 (Figure 6.1) and Oregon R (with the exception of *Df(3R)red*^{P52}). It is possible that the ov5B fragment crosses a deficiency breakpoint in the *Df(3R)red*^{P52} chromosome since it recognises two genomic fragments, 8.5 kb and 6.5 kb in size, with equal intensity. This may be the case since the ov5B fragment is expected to hybridise at 88B/C (discussed in Chapter 4) and one breakpoint of the *Df(3R)red*^{P52} deficiency lies within 88C. Alternatively there may be a restriction fragment length polymorphism within the ov5B sequence in the *Df(3R)red*^{P52} stock.

Attempts To Locate Ov5A Sequences Within The *Df(3R)red*¹ Deficiency By *in situ* Hybridisation To Polytene Chromosomes

Introduction

The ov5A genomic DNA fragment maps at 88B/C on Chromosome 3R. A stock carrying the *Df(3R)red*¹ deficiency was crossed to a wild type, Oregon R strain. The *Df(3R)red*¹ deficiency spans the 88B/C region, and it was the aim of this experiment to probe chromosome preparations carrying the *Df(3R)red*¹/+ chromosome combination. The *Df(3R)red*¹ stock was chosen because it contains the longest deficiency in the stocks provided and should uncover the ov5A sequences. It was also expected to be visible morphologically. Where the wild type chromosome cannot pair with the deficiency chromosome a 'loop' structure is formed. If the ov5A sequence maps within

the *Df(3R)red¹* deficiency region then it would be expected to hybridise within the wild type unpaired chromosome region.

Results

When chromosome preparations were made from the salivary glands of larvae from the *Df(3R)red¹* x OrR cross, two types of chromosome spreads were observed. Chromosome preparations with the balancer (*MRS*)/wild type chromosome constitution were easy to recognise as the balancer chromosome contains many inversions and does not pair well with the wild type chromosome. This results in poorly spread chromosomes with many broken chromosome arms. Chromosome preparations with the *Df(3R)red¹*/wild type chromosome constitution spread well and looked similar to wild type chromosome preparations. It was difficult to determine the presence of the predicted wild type chromosome loop at the *Df(3R)red¹* chromosomal location as the banding pattern at this region is faint. Nevertheless, putative *Df(3R)red¹/+* chromosome preparations were probed with the *ov5A* fragment. The chromosomal signal obtained is illustrated in Figure 6.6. From the hybridisation signal obtained in the nucleus in photograph A it can be seen that the *ov5A* fragment hybridised with both chromosomes indicating that it is present in two copies in this putative *Df(3R)red¹/+* chromosome preparation. In the example shown in photograph B, the chromosomal morphology also looks to be wild type in the region of the *ov5A* hybridisation signal.

Discussion

The experiment with the putative *Df(3R)red¹/+* heterozygote chromosome preparation suggested that the *ov5A* sequence was present on each chromosome. On further examination of the chromosome preparations used to obtain this result, it was questioned whether or not the *Df(3R)red¹* stock used for this experiment indeed carries the *Df(3R)red¹* deficiency. The hybridisation signal obtained for *ov5A* shown in photographs A and B (Figure 6.6) seems to indicate that in the 3R-88 region there was no evidence for any disturbance in the normal chromosomal morphology. After this experiment further *Df(3R)red¹/+* chromosome squashes were prepared and the banding pattern in this region indicated that the *Df(3R)red¹* deficiency was not present. It was

Figure 6.6

In situ Hybridisation To Third Instar Salivary Gland Chromosome Preparations From Putative *Df(3R)red¹/+* Heterozygotes, Using The ov5A Probe

Wild type *Drosophila melanogaster* (OrR) males were crossed to putative *Df(3R)red¹/MRS* virgin females. Salivary gland chromosome preparations from larva with the putative *Df(3R)red¹/+* constitution were probed with [³H]-dCTP labelled ov5A fragment to determine if the ov5A sequences mapped within this deficiency.

- A: X100 magnification. The hybridisation signal is indicated by an arrow. The hybridisation signal was present on each chromosome at 88B/C which is clearly visualised in this photograph where the chromosomes have separated.
- B: X100 magnification. The hybridisation signal is indicated by an arrow (88B/C). The morphology indicated that the *Df(3R)red¹* deficiency chromosome was not present in this salivary gland chromosome preparation.

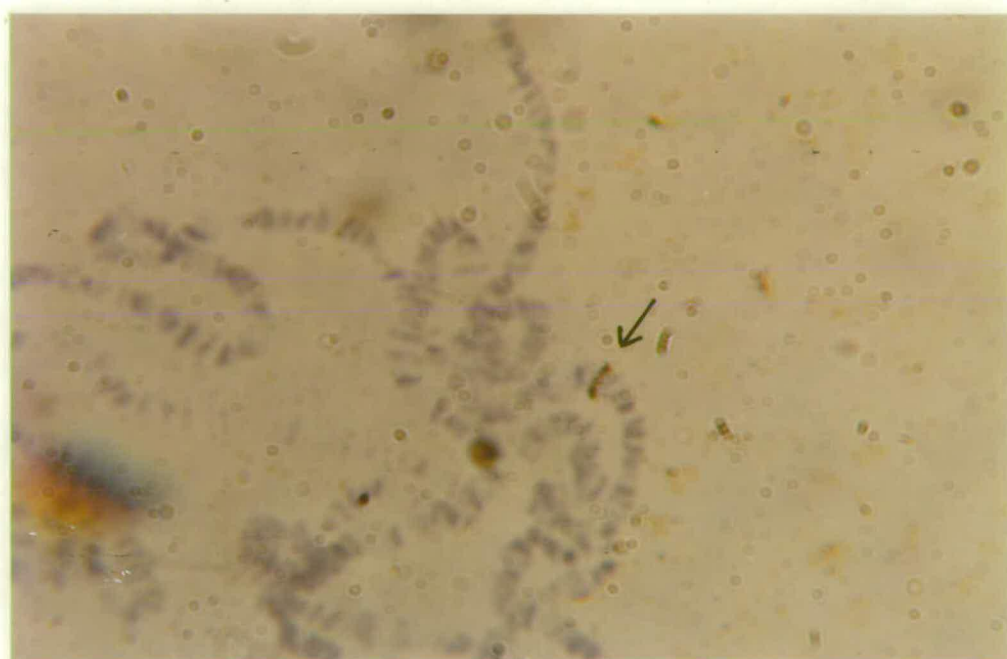
ov5A

A



X 100

B



X 100

later confirmed by M. Ashburner (University of Cambridge) that the *Df(3R)red*¹ deficiency chromosome was definitely not present in the chromosome preparations used in the *in situ* hybridisation experiment shown in Figure 6.6. The *Df(3R)red* stocks will be tested genetically by crossing each *Df(3R)red* stock to a mutant line carrying a *red* mutation. If the *Df(3R)red* stock carries the deficiency then 50% of the progeny will have *red* eyes (mutant red colour) and the other 50% will have wild type eye pigmentation. If there is no deficiency at the *red* locus then all the progeny will have the wild type eye colour. Deficiency stocks will be obtained from other sources, checked genetically and then this experiment will be repeated.

Final Comments: Organisation Of The ov5A And ov5B Fragments In The *Drosophila* Genome And Future Experiments With The Deficiency Stocks

The ov5A and ov5B fragments each recognised single EcoRI fragments in Oregon R genomic DNA (Figure 6.2, panel A, lane 8; Figure 6.3, panel A, lane 8 respectively). The signals obtained were of a similar intensity to the control *Adh* probe, indicating that the ov5A and ov5B sequences are unique and unrelated to other *Drosophila* DNA sequences. This confirms that the transcripts recognised by the ov5A and ov5B fragments must derive from the ov5A and ov5B sequences.

The experiments with ov5A and ov5B fragments with each of the deficiency (3R)88 stocks were intended to locate each sequence to within one or several deficiencies. It was hoped that the Southern blot experiments designed to assess the sequence dosage of the ov5A and ov5B fragments in each stock would act as a guide to which deficiency stock(s) would uncover each fragment. Further chromosome *in situ* hybridisation experiments with the deficiency stocks which uncover the *Spindle-B* and *fs293*^{Y5} mutations together with Northern analysis of *Spindle-B* and *fs293*^{Y5} mutants will help determine if any of these mutant phenotypes result from disruption of the ov5 sequences. It would be exciting if this was found to be the case, or the ov5 sequences were found to be functionally associated with another known ovarian mutant phenotype.

Clone λ ov2: Estimation Of The Size Of The ov2A And ov2B EcoRI Fragments In The *Drosophila* Genome And Attempts To Estimate The ov2a And ov2B Sequence Copy Number Within The Deficiency Stocks.

Introduction

The ov2A and ov2B subfragments from λ ov2 each hybridised at 89B (Chapter Five). It was decided to probe the genomic blots of EcoRI digested DNA from the *Df(3R)* stocks and OrR. It was thought that the ov2A and/or ov2B fragments may have been more precisely localised using this technique, on the same principle as the experiments carried out with ov5A and ov5B. However, for the reasons described in the previous section, this experiment did not provide such information. Nevertheless, it was shown that the ov2A and ov2B EcoRI fragments hybridised with larger size genomic EcoRI fragments which provided some information about the location of the ov2A and ov2B fragments within λ ov2.

Results

ov2B

The ov2B (4.2 kb) EcoRI fragment was used to probe EcoRI digested genomic DNA extracted from the deficiency (3R) stocks (as listed in Figure 6.1), and Oregon R. The ov2B fragment hybridised with a 4.8 kb fragment in each track (except for *Df(3R)red^{P52}*) with a similar signal intensity as the 5.0 kb fragment recognised by the control *Adh* probe (Figure 6.3, panel B and Figure 6.2, panel B, respectively). In Figure 6.3, panel B, lane 3 (which contains DNA from the *Df(3R)red^{P52}* stock), the ov2B fragment detected two bands 4.8 kb and 4.5 kb in size. This is likely to be due to a restriction enzyme polymorphism in the ov2B sequence in the *Df(3R)red^{P52}* stock, since the ov2B fragment maps outwith this deficiency. In all other tracks, the ov2B sequence detected a 4.8 kb genomic fragment. Since this is 0.6 kb greater in length than the ov2B fragment, it indicates that ov2B EcoRI fragment is located at one end of the ov2 genomic insert within λ ov2 and that the endogenous EcoRI site is at a distance of 0.6 kb from the linker EcoRI site (see library construction, described in the first discussion in this chapter).

ov2A

The ov2A (3.25 kb) EcoRI fragment hybridised with a 5.0 kb DNA fragment in all 8 tracks of EcoRI digested genomic DNA extracted from the *Df(3R)88* stocks and OrR (Figure 6.3, panel C). This indicated that the endogenous EcoRI site is at a distance of 1.25 kb from the linker EcoRI site in the ov2A fragment. This would imply that the ov2A sub-fragment is positioned at an end of the ov2 genomic fragment.

Discussion

The results are consistent with the ov2A and ov2B sequences being present in two copies in each deficiency stock studied. However, it is important to be cautious in the interpretation of these results in view of the unreliability of the technique indicated in earlier discussions. Each of the ov2A and ov2B fragments hybridised with genomic EcoRI DNA fragments of longer length. This indicated that the ov2A and ov2B sub-fragments may be located at opposite ends of the ov2 insert.

Since the ov2A and ov2B fragments each recognised one band in Southern blots of EcoRI digested genomic DNA extracted from Oregon R, it is unlikely that the genes present within these fragments are related to other *Drosophila* genomic sequences. Therefore, the ov2A and ov2B fragments represent unique sequences in the *Drosophila* genome.

Part Two: Towards A Function

In situ Hybridisation Of cDNA Probes (ov5A2 and ov5B1) To Whole Mounts Of *Drosophila* Ovaries.

Introduction

As described in the introduction in Chapter I, there are somatic and germline derived cells in the ovary. The ovarian follicle cells are mesodermal in origin. The nurse cells and the oocyte are derived from the germline cells. The nurse cells synthesise most of the components within the oocyte which will be utilised by the developing embryo. The ovarian follicle cells manufacture yolk proteins and secrete the chorion. The temporal and spatial expression patterns of various maternally expressed genes have been determined using *in situ* hybridisation to sectioned ovaries (e.g., with *bicoid* (Berleth *et al.*, 1988) and *vasa* (Lasko and Ashburner, 1990)).

A recent method published by Tautz and Pfeifle (1989) utilised digoxigenin labelling and subsequent antibody detection of DNA probes to examine the expression pattern of the maternally and zygotically expressed *hunchback* gap gene in *Drosophila* embryo whole mounts. In order to determine the spatial and temporal distribution of the ovary enriched transcripts within λ ov5, the ov5A2 and ov5B1 cDNAs were labelled with digoxigenin and used to probe whole mounts of *Drosophila* ovaries. The preliminary results obtained are described in this section with a view to determining the possible function(s) of the ovary enriched transcripts during oogenesis.

Results

In situ Hybridisation To Whole Mounts of *Drosophila* Ovaries Using The ov5A2 cDNA Probe

The ov5A2 cDNA detected two ovary enriched transcripts 1.7 kb and 1.4 kb in length on Northern blots (Figure 4.4). Presumably the hybridisation signal detected by ov5A2 directly in the ovary whole mount preparations reflects the distribution of both transcripts. Therefore, in this experiment it is not possible to determine whether or not the 1.7 kb and 1.4 kb transcripts are co-regulated or independently regulated during oogenesis. The preliminary results obtained

when the ov5A2 probe was used to detect transcripts in ovary whole mounts are shown in Figure 6.7. The hybridisation signal appeared deep purple/brown in colour when viewed using light microscopy. However in photographs A and B in Figure 6.7 the hybridisation signal appears to be more brown in colour. The ov5A2 probe detects transcripts in stage 7 egg chambers (Figure 6.7; Photograph A). The hybridisation signal appears to increase through subsequent developmental stages. There is a marked increase in signal detection between stages 10A and 10B. In stage 10B, staining is strongest in the nurse cell cytoplasm and there is also staining in the oocyte. The follicle cells do not appear to be expressing the transcripts detected by ov5A2 as there was no staining in this cell type (Figure 6.7, photograph B). By stage 12 there is a strong signal in the oocyte and a weaker signal in the degenerating nurse cells (Figure 6.7, photograph B).

Discussion

In this set of preliminary *in situ* hybridisation experiments, the 1.7 kb and 1.4 kb ovary transcripts detected by the ov5A2 cDNA probe appear to be expressed as early as stage 7. The hybridisation signal increased dramatically in the nurse cells of stage 10B egg chambers, and was also intense in the ooplasm of stage 13 oocytes. The transcripts appear to be synthesised in the nurse cells and transferred to the developing oocyte. The ov5A2 probe did not detect the transcripts in the follicle cells indicating that transcription is restricted to the germline derived nurse cells. The observed pattern of expression of the 1.7 kb and 1.4 kb transcripts during oogenesis is similar to that of other maternally expressed genes in that they are transcribed in the nurse cells and contributed to the developing oocyte. However, as it is not possible to distinguish between the 1.7 kb and 1.4 kb transcripts detected by the ov5A2 probe, it is not clear if both transcripts are contributed to the oocyte. Northern analysis has shown that the 1.7 kb transcript is enriched in ovary and embryo RNA. The 1.4 kb transcript was only detected in adults and enriched in the ovary. It is possible that the 1.4 kb transcript is rapidly translated or degraded in the embryo, or alternatively that it functions specifically during oogenesis and is not required by the embryo. It may be then that the strong hybridisation signal observed in mature

Figure 6.7

Detection Of Transcripts In The Ovary Using The ov5A2 cDNA Probe

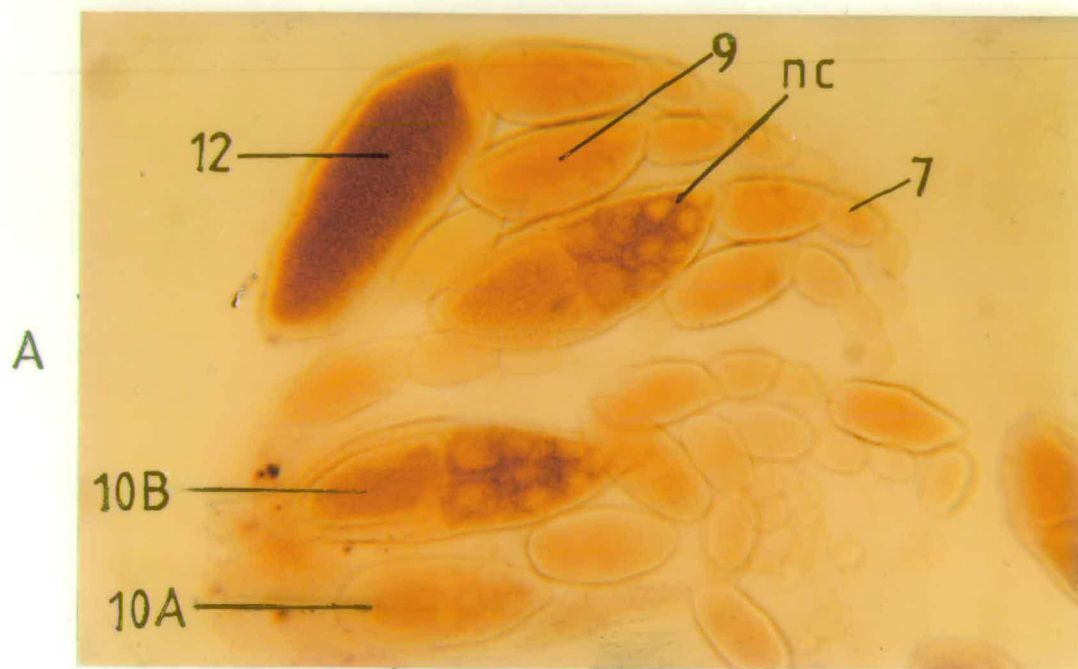
The ov5A2 cDNA fragment was labelled with digoxigenin-dUTP and used to probe whole mounts of *Drosophila* ovaries. Antibody to digoxigenin was detected using a colour reaction (Boehringer Mannheim Kit). The hybridisation signal appeared as a purple/brown colour.

A: X10 magnification. The hybridisation signal is strongest in the nurse cell cytoplasm of stage 10B egg chambers, and in stage 12 oocytes.

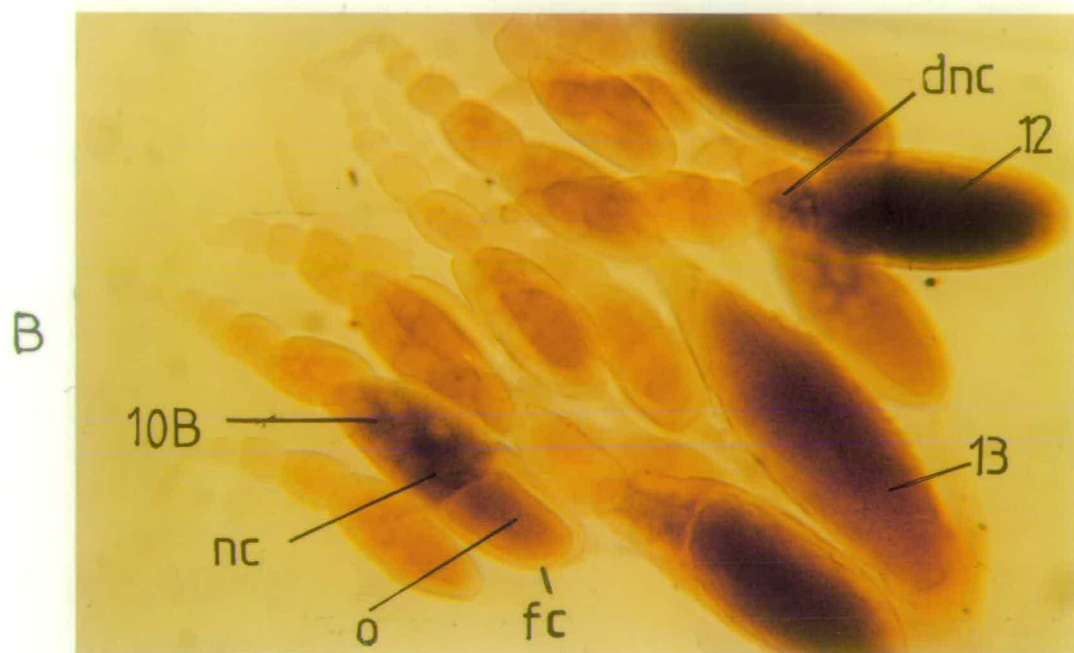
B: X10 magnification. The hybridisation signal is strongest in stage 10B nurse cell cytoplasm and mature oocytes (stages 12 and 13 are indicated).

7, 9, 10A, 12, 13	=	Egg Chamber Stages
nc	=	nurse cell
o	=	oocyte
fc	=	follicle cell
dnc	=	degenerating nurse cells

ov5A2



×10



×10

oocytes reflects the distribution of the 1.7 kb transcript.

It will be interesting to determine the transcript distribution during early embryonic development to see if there is any transcript localisation after fertilisation, indicating a role in pattern formation. This is the case with the maternally expressed *hunchback* transcript. It is uniformly distributed in the mature oocyte but becomes localised over the anterior two-thirds of the embryo soon after fertilisation (Tautz and Pfeifle, 1989). An alternative possibility is that the ovary transcripts encode a housekeeping function and their abundance in the oocyte could reflect the requirement for stored messages which will be used during the first few hours of embryogenesis.

In situ hybridisation experiments using the ov5A2 cDNA probe were carried out on isolated male testes and accessory glands. No hybridisation signal was detected (data not shown).

In situ Hybridisation To Whole Mounts of *Drosophila* Ovaries Using The ov5B1 cDNA Probe

The ov5B1 cDNA detected a 1.4 kb ovary specific transcript on Northern blots (Figure 4.10). Figure 6.8 (A and B) shows the results obtained when the ov5B1 cDNA probe was used to probe whole mounts of *Drosophila* ovaries. The 1.4 kb transcript is first detectable in the nurse cells of stage 8 egg chambers (Figure 6.8, photograph B). There was a strong signal observed in the nurse cells of stage 10B egg chambers, and in the degenerating nurse cells (Figure 6.8, A and B). It is probable that as the nurse cells decay this transcript becomes concentrated into a smaller area. In a few cases the transcript may be adhering to the chorionic appendages (Figure 6.8, photograph A).

Discussion

The ov5B1 cDNA probe detected the 1.4 kb ovary specific transcript in the nurse cells of whole mounts of *Drosophila* ovaries. This transcript may be expressed in stage 8 nurse cells. The hybridisation signal may also be detected during stages 9 and 10A, but the strongest signal was detected in stage 10B nurse cell cytoplasm and in the degenerating nurse cells. There was no

Figure 6.8

Detection Of Transcripts In The Ovary Using The ov5B1 cDNA Probe

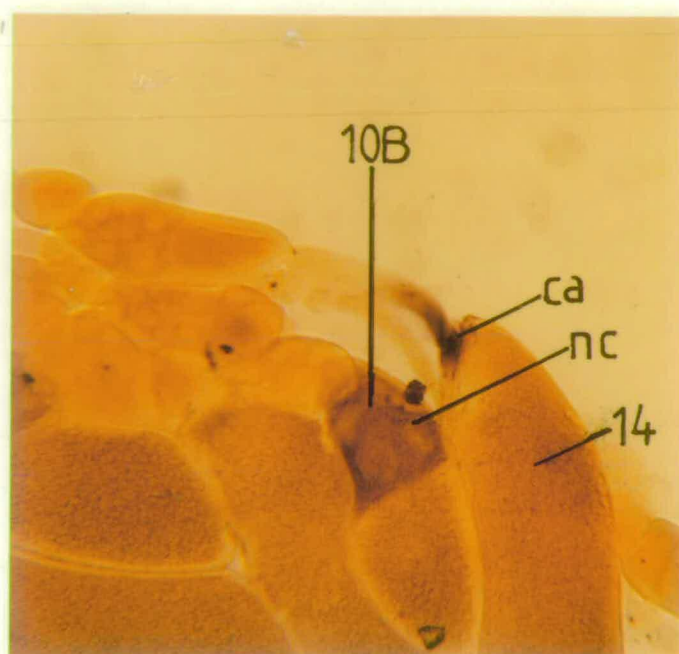
The ov5B1 cDNA fragment was labelled with digoxigenin-dUTP and used to probe whole mounts of *Drosophila* ovaries. The hybridisation signal appeared as a dark purple/brown colour in the photographed ovaries.

- A: X10 magnification. The ov5B1 cDNA probe detects transcripts in stage 10B nurse cells. The transcripts are strongly detected in the degenerating nurse cells and are probably adhering to the chorionic appendages.
- B: X10 magnification. The ov5B1 probe is hybridising strongly with the degenerating nurse cells of the stage 12 egg chamber indicated. Staining in the nurse cell cytoplasm may be present as early as stage 8.

8, 10B, 14	=	Egg Chamber Stages
nc	=	nurse cells
ca	=	chorionic appendages
dnc	=	degenerating nurse cells

ov5B1

A



x10

B



x10

signal detection in the oocyte indicating that the 1.4 kb ovary specific transcript is not contributed to the oocyte. This is in agreement with the Northern blot data shown in Figure 4.10 as the 1.4 kb transcript was not detected in embryonic RNA extracts. Since the chorionic appendages are secreted protein structures it is unlikely that the staining observed with the ov5B1 probe reflects the storage of RNA in this exterior egg structure. Given the pattern of staining detected by the ov5B1 probe it is possible to speculate that it may encode a protease involved in nurse cell degradation.

Control Experiments

Two additional sets of whole ovaries were fixed and subjected to the experimental procedure. One set was probed with the plasmid vector (pGemini-1) and in another set no probe was added. No hybridisation signal was detected in either of these control groups (data not shown). The *in situ* hybridisation results described in this section should be viewed as preliminary data as they have been carried out once only and will be repeated.

FINAL DISCUSSION AND COMMENTS

FINAL DISCUSSION AND COMMENTS

CHARACTERISATION OF NEWLY ISOLATED GENES EXPRESSED IN THE OVARY OF *DROSOPHILA MELANOGASTER*

In this thesis two genomic clones λ ov5 and λ ov2 have been described which contain genes that are expressed specifically or differentially in the ovary of *Drosophila melanogaster*. From the preliminary Northern data, it was not expected that either of λ ov5 or λ ov2 would be involved in encoding clusters of transcripts.

λ ov5 was found to be located on the right arm of the third chromosome at 88B/C and shown to include two distinct coding regions within the sub-fragments ov5A and ov5B. The ov5A fragment encodes a family of overlapping ovary enriched and ovary specific transcripts. These transcripts may be the products of a single gene which generates multiple transcripts differing in 5' initiation sites, 3' polyadenylation sites or length of the poly (A) tail. Restriction mapping and heteroduplex mapping data indicate that there are no introns in the ov5A genomic fragment. However, the data obtained from cross hybridising the ov5A1 and ov5A2 cDNAs with the corresponding ov5A fragment shows that some of the sequence encoding the 1.7 kb and 1.4 kb ovary enriched transcripts must be located outwith the ov5A region. This suggests there must be an intron extending beyond the ov5 region. Ultimately DNA sequencing will resolve these questions.

The ov5B sub-fragment contains a coding region which recognises a 1.4 kb ovary specific transcript in adults. This transcript is unrelated in sequence to the ovary enriched transcripts encoded within the ov5A sub-fragment.

λ ov2 hybridises at 89B on chromosome 3R. The ov2 genomic fragment contains two EcoRI sub-fragments which hybridise with female cDNA but not with male cDNA. The ov2A sub-fragment (3.25 kb) detected 4.0 kb and 3.0 kb maternally expressed transcripts and a 1.6 kb male specific transcript. It is almost certain that there is overlap of these different mRNAs. The ov2B sub-fragment (4.2 kb) hybridised with 3.5 kb and 2.5 kb ovary specific transcripts, which probably have common sequences. The nature of the mechanism(s) generating the putative

overlapping transcripts encoded by gene(s) present within the ov2A and ov2B sub-fragments will be determined by isolating cDNA copies of each transcript, cross hybridisation experiments and DNA sequencing analysis.

Most other genes which are specifically involved in oogenesis and that have been cloned are members of gene families, e.g. yolk protein genes (Barnett *et al.*, 1980) and vitelline membrane protein genes (Burke *et al.*, 1987; Gigliotti *et al.*, 1989). The chorion genes are located within two developmentally regulated gene clusters on the third and X chromosomes (Spradling *et al.*, 1980). These genes have been described in detail in Chapter One. Each chorion, yolk protein and vitelline membrane gene appears to encode a single transcript. However, there are other examples of genes which have been isolated using molecular methods that are specifically or differentially expressed in the ovary and/or embryo which are clustered and have a more complex gene organisation.

For example, using a differential screening technique, Ait-Ahmed *et al.* (1987) isolated a genomic fragment which hybridised with transcripts enriched in early embryos. Bidirectional chromosomal walks in this genomic region resulted in the isolation of a cluster of differentially expressed maternal genes now known as the *yema* region. From this region there are eight transcripts ranging from 2.6 kb to 9.5 kb in size, all of which are recognised by a 3.9 kb genomic fragment and are enriched in the ovary and/or early embryos.

When the gene encoding a protein inducible by ecdysone in *Drosophila* cell lines (*Eip28/29* gene) was investigated, it was found to be flanked by genomic sequences detecting embryo, ovary and testes specific transcripts (Schulz and Butler, 1989). At least seven distinct transcripts are derived from four overlapping genes which are located within a 4.5 kb genomic region called *z600-gonadal-Eip28/29*. At one end of the cluster there is a gene (*z600*) which encodes a 600 base pair transcript which is expressed predominantly during embryogenesis. This transcription unit overlaps with another gene called *gonadal (gdl)* which has two modes of expression in adults. In ovaries *gdl^f* expression results in two transcripts 1.3 kb and 1.0 kb in length, whereas in testes *gdl^m* expression leads to transcripts 1.5 kb and 1.2 kb in length. The *gdl* transcription unit overlaps distally with the *Eip28/29* gene.

The *serendipity* locus was first identified in a genomic clone which was originally isolated on the basis that it encodes the ribosomal protein, rp49. Sequences from the *serendipity* (*sry*) locus identified sex specific transcripts on Northern blots and subsequent analysis of the *sry* region revealed that it contains three genes *sryδ*, *sryα* and *sryβ* which encode five transcripts that are present during oogenesis, embryogenesis or both (Vincent *et al.*, 1984, 1985). Three of the mRNAs result from the expression of the three *sry* genes and two mRNAs are the products of readthrough transcription of two protein coding domains.

Within the 8 kb region at 99D which contains the *rp49* gene, and the *sryδ*, *sryα* and *sryβ* genes, a further coding region called *janus* has been identified (Yanicostas *et al.*, 1989). The *janus* locus is composed of two partially overlapping genes called *janA* and *janB*. *janA* encodes two non-sex specific mRNAs and a male specific mRNA. *janB* encodes a male specific transcript. The 5' end of *janB* is located within the 3' untranslated region of the *janA* coding region.

Of the other six clones isolated in the screen from which *lov5* and *lov2* were isolated, at least three encode multiple transcripts, some of which are ovary specific. The pattern of transcription detected by sub-fragments of each clone is complex as are the cross-hybridisation patterns between related cDNA isolates (C. Gilman, pers. comm.).

Thus the study of genes isolated using molecular methods which encode transcripts enriched in the ovary has revealed that at least some of these genes exist within densely transcribed regions of the genome. The organisation of these genes is often complex with examples of overlapping transcripts and overlapping genes being commonplace.

The large number of female sterile mutants generated from genetic screens have provided a valuable insight into the complexity of the processes involved in oogenesis. The cloning of many maternal effect loci involved in embryonic pattern formation has led to rapid progress in our understanding of the positional information contributed to the egg during oogenesis.

The P-element enhancer trap technique offers the potential to isolated many important ovarian genes (discussed in Chapter One). By analysing many enhancer trap lines with ovary-specific staining patterns, very localised areas of staining in morphologically indistinguishable cells in the soma and germline have been identified (Fasano and Kerridge, 1988; Grossniklaus *et al.*,

1989). In some cases the P-element insertion was associated with an observable ovarian defect. P-element screens have been carried out with the aim of identifying mutations which disrupt oogenesis (e.g. Galanopoulos *et al.*, 1989) or result in female sterility (e.g. Couderac *et al.*, 1990). The main advantage of this technique is that the mutated locus can be rapidly cloned (discussed in Chapter One).

However, despite all the information concerning oogenesis obtained so far from conventional genetic screens, P-element mutagenesis and the P-element enhancer trap technique, there are still many events occurring early in oogenesis (stages 0-7) which are not understood at the molecular level (discussed in Chapter One). Perhaps combining genetic, P-element and molecular searches for genes involved in oogenesis will lead to a better understanding of both the early and late events occurring during egg development.

In this respect it was interesting to find that two known female sterile mutations map close to the *ov2* and *ov5* genomic sequences which contain genes encoding ovary enriched transcripts.

The *Spindle-B* (*Spn-B*) mutation affects the formation of the egg shell and the dorso-ventral polarity of the egg and embryo (Tearle and Nüsslein-Volhard, 1987; Nüsslein-Volhard, 1990). The *fs293^{y5}* mutation causes female sterility but there is no information available at present as to which aspect(s) of oogenesis and/or early development are affected by this mutation.

Using *in situ* hybridisation to polytene chromosomes, attempts were made to map the *ov5* sequences to within one or more chromosomal deficiencies in the cytological region of 88 which were known to uncover the female sterile mutations *fs293^{y5}* and *Spn-B* (Figure 6.1). However, these experiments have so far been unsuccessful due to defective stocks and they will be repeated with a new set of deficiencies which will be checked genetically before use.

A further set of experiments was carried out with a view to identifying the functional role of the ovary enriched transcripts encoded within the *ov5* genomic region. Each of the *ov5A2* and *ov5B1* cDNAs was used to probe whole mounts of *Drosophila* ovaries to determine the spatial and temporal distribution of the ovary transcripts. The *ov5A2* cDNA detects transcripts from stage 7 onwards in the nurse cell cytoplasm and the oocyte. It will be interesting to determine the

distribution of the 1.7 kb transcript detected by ov5A2 in embryo whole mounts using this method.

The ov5B1 cDNA detected transcripts as early as stage 8 in the nurse cell cytoplasm, but the hybridisation was strongest in stage 10B nurse cells and in the degenerating nurse cells of stage 11 - 13 oocytes. The ov5B1 cDNA, which detects a 1.4 kb ovary specific transcript on Northern blots may therefore encode a protein which is specifically required in the nurse cells and could be involved in nurse cell degeneration.

It may also be possible to express the proteins encoded within ov5A2 and ov5B1 cDNAs in *E.coli* or in a *Drosophila* cell line and raise antibodies to them. The antibodies could then be used to examine the patterns of distribution of the proteins in the ovaries, which can be compared with the distribution of transcripts.

DNA sequencing of the ov5A2 and ov5B1 cDNAs and the corresponding genomic regions within ov5 is underway. Hopefully this information will provide a better understanding of how the ovary enriched transcripts within ov5 are organised. It will be possible to search the data bases both at the DNA sequence and amino acid sequence levels to find out if the transcripts encoded within ov5A2 and ov5B1 have any similarity to other known DNA sequences or polypeptides. Hopefully this data, together with the information obtained from ovary *in situ* hybridisation experiments, will give an insight into the role of the ovary enriched transcripts during oogenesis and broaden our understanding of the many processes which interact to form a fully mature egg ready for fertilisation.

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