CONTROL OF OVARIAN AND FAT BODY EXPRESSION OF THE DROSOPHILA YOLK PROTEIN 3 GENE

SIMONE FRANCES HUTSON

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Of course there is one person who has really transformed the last four years for me, in so many ways. Darren, it wouldn’t have been the same without you.
DECLARATION

I declare that the composition of this thesis, and the work presented in it are my own, unless otherwise stated.

Simone Frances Hutson

December 1998
ABSTRACT

In *Drosophila* the three *yolk protein* (*yp*) genes are expressed in the adult female fat body and ovary. The yolk proteins (YPs) are stored in the oocyte for utilisation during embryogenesis. Within the intergenic region of *yp1* and *yp2*, a fat body enhancer (FBE) and two ovarian enhancers have been found to direct YP1 and YP2 tissue- and sex-specific expression. The male and female forms of Doublesex protein (*DSX*\(^M\) and *DSX*\(^F\) respectively) have been found to bind to the FBE and are thought to confer the sex-specificity of *yp* expression to the fat body of females only. Other possible *trans*-acting factors have also been shown to bind to the FBE *in vivo*, these include AEF-1 and C/EBP proteins. The *dsx* gene is at the end of the sex determination pathway, other genes that operate from this branch of the pathway include *hermaphrodite* (*her*) and *intersex* (*ix*), and it is possible that HER and IX proteins could also regulate *yp* gene expression.

Upstream of *yp3* are sequences that direct female-, fat body-specific *yp3* expression (FBE3) and ovarian-specific *yp3* expression (OE3). This project involved analysis of FBE3 and OE3 to gain further understanding of the regulation of *yp3* expression. The role of DSX in the regulation of *yp3* has been investigated; the FBE3 was divided into smaller subfragments and it was determined that one of these fragments can bind DSX *in vitro*. These subfragments were cloned into reporter gene constructs to test their capabilities in directing correct *lacZ* expression pattern *in vivo*, using P-element mediated transformation. This revealed that the fragment capable of binding DSX *in vitro*, could direct *lacZ* expression in fat body cells, but this expression was not sex-specific, both male and female fat bodies showed reporter gene expression. C/EBP was reported to bind to the FBE *in vitro* and considered to be a potential activator of *yp* gene expression. From C/EBP mutant analysis, there was no observed difference from wild type in the level of YP expression *in vivo*; thus it is unlikely that C/EBP has a regulatory role in *yp* gene expression in the fat body.
*In situ* hybridisations were performed using *yp* probes on *Drosophila* ovaries, this revealed that the *yps* are expressed in the ovarian follicle cells during stages 8-10b of oogenesis. OE3 was found to contain a sequence conserved between several Dipteran insects, a 150bp PCR fragment containing this sequence is able to confer ovarian expression. Candidate *trans*-acting factors for the regulation of ovary-specific *yp* expression were investigated to determine if they are present at the correct developmental stages by *in situ* hybridisation.
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<td>amp</td>
<td>ampicillin</td>
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<td>calf intestinal alkaline phosphatase</td>
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<td>phenylmethysulphonyl fluoride</td>
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<td>(superscript) resistance/resistant</td>
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<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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<td>ss</td>
<td>single-stranded</td>
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<td>yp</td>
<td>yolk protein gene</td>
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<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
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CHAPTER 1

INTRODUCTION

The yolk protein genes of Drosophila melanogaster
Sexual differentiation in *Drosophila melanogaster* is the result of differential activation and expression of a hierarchy of sex determination genes. The understanding of the mechanisms involved is of great importance in developmental biology. The sex determination pathway has been well characterised at both genetic and molecular levels. However, only one direct link between the sex determination genes and the downstream sexual differentiation genes has been found; this is the regulation of the *yolk protein* (*yp*) genes.

The yolk proteins are synthesised in the fat body and ovarian follicle cells of the adult female and are subsequently transported to the oocyte during oogenesis, where they are stored for utilisation during embryogenesis. The expression of the yolk proteins is not only controlled by the sex determination hierarchy, but also the nutritional status of the fly and the insect hormones: ecdysone and juvenile hormone. The interactions between all these factors are responsible for the co-ordinated expression of the *yolk protein* genes in the right sex, in specific tissues and under the appropriate environmental conditions.

There are three *yolk protein* genes in *Drosophila* (*yp1*, *yp2* and *yp3*), and most investigations into the regulation of the *yolk protein* genes have concentrated upon *yp1* and *yp2*. Consequently, in comparison to *yp1* and *yp2*, very little is known about the regulation of *yp3*.

The aim of this PhD project was to further characterise *yp3* cis-acting DNA sequences and to identify trans-acting factors involved in directing *yp3* gene expression in the ovary and the fat body.
1.1 OOGENESIS

The adult female *Drosophila melanogaster* contains two ovaries, each consisting of 15-20 ovarioles organised in parallel (Figure 1.1A). Each ovariole contains a row of developmentally ordered egg chambers which can be divided into two sections: the germarium and the vitellarium. In the germarium cell proliferation and differentiation produces the three main cell types in the ovary; the oocyte, the nurse cells and the follicle cells. At the anterior tip of the germarium (Figure 1.1C), a small number of germ line stem cells (Weischaus and Szabad, 1979) divide to produce two daughter cells; another stem cell and a cystoblast. The cystoblast undergoes 4 mitotic divisions with incomplete cytokinesis, which gives rise to a cluster of 16 cells connected by intercellular bridges called ring canals. Only two of the cells have maximum four ring canals and it is always one of these cells which becomes the oocyte (Koch *et al.*, 1967), the other 15 cells will become the nurse cells. Before the cluster of cells exit the germarium and enter the vitellarium they are surrounded by small follicle cells to form the egg chamber. Unlike the oocyte and nurse cells, the follicle cells are somatic cells derived from the mesoderm. The egg chamber undergoes significant changes in morphology upon entering the vitellarium; these have been classified into 14 distinct stages by King (1970).

The vitellarium contains a series of progressively older egg chambers in varying developmental stages, connected by follicular stalk cells, some of these stages are shown in Figure 1.1B. Stages 1-7 are termed the previtellogenic stages, and during this period the oocyte and the nurse cells are roughly equal in size, and the oocyte is always the most posterior germ line cell in the egg chamber.

During stages 8-10 vitellogenesis proceeds; the oocyte undergoes rapid growth as yolk proteins, synthesised in the fat body and the follicle cells, are taken up into the oocyte by receptor-mediated endocytosis and accumulated as yolk granules. At the same time in the vitellarium, the 15 nurse cells enlarge and become polyploid (Jacob...
Figure 1.1

*Drosophila melanogaster* ovaries and the developmental sequence of oogenesis.

A) Dorsal view of the internal reproductive system of an adult female *Drosophila* (adapted from Mahowald and Kambysellis, 1980). Two ovarioles have been pulled loose from the right ovary.

B) Diagram of an adult wild type ovariole (adapted from Peifer *et al.*, 1993).

C) Diagram of a germarium (adapted from Mahowald and Kambysellis, 1980).

ant, anterior; bc, border cells; fc, follicle cells; nc, nurse cells; oc, oocyte; pc, polar cells; post, posterior; ppc, posterior polar cells; sc, stalk cells; ST, stage.
and Stirling, 1959), which enables them to synthesise large quantities of proteins and RNAs which are transferred into the oocyte at a later stage. Most of the RNA synthesised is ribosomal (Mahowald and Tiefert, 1970), some is mRNA which is involved in determining egg polarity and subsequent aspects of embryonic development (reviewed by Lasko, 1994). The internal layers of the vitelline membrane are also synthesised by the follicle cells during this time. The follicle cells also undergo a series of migrations during stages 8-10. The border cells, a group of 6-10 follicle cells located at the anterior of the egg chamber, migrate in a posterior direction between the nurse cells until they reach the nurse cell-oocyte interface. During stage 10 they migrate dorsally along the interface until they are located opposite the oocyte nucleus, at a later stage the border cells are involved in creating the micropylar pore. The follicle cell monolayer covers the entire egg chamber until stage 9 when the anterior follicle cells overlaying the nurse cells migrate posteriorly to surround the oocyte. By stage 10A the nurse cells have very few follicle cells associated with them and the oocyte is covered by a layer of thick columnar follicle cells.

During stages 10B-13 the majority of the nurse cell cytoplasm is quickly transferred into the oocyte via the ring canals. Following the completion of their migration at stage 11, the follicle cells synthesise large amounts of chorion proteins to form the eggshell. As the chorion is deposited, other structures are also assembled; the micropylar apparatus through which sperm entry takes place, and the dorsal appendages used for embryonic respiration are formed. When the eggshell is completed, the nurse cells and the follicle cells degenerate and die, leaving behind the mature egg.

The follicle cells are particularly relevant to this project because they are the site of yolk protein synthesis in the *Drosophila* ovary. The follicle cells differentiate from the somatic profollicle cells that surround the nurse cells and oocyte in the germarium (reviewed by Margaritis et al., 1980). During stages 2-5 the follicle cells proliferate to
produce approximately 1000 follicular epithelial cells which differentiate to form all the follicle cell subtypes, shown in Figure 1.2. The division of these precursor cells ceases by stage 6, when they differentiate into three follicle cell subtypes: border cells, cells overlying the nurse cells, and columnar cells. By stage 11, these cells undergo differentiation into three additional cell types: anterior pole cells, posterior pole cells and columnar main body cells.

1.2 THE YOLK PROTEINS

There are three yolk proteins (YPs) synthesised in *Drosophila*, and their approximate sizes are: 47 kDa (YP1), 46 kDa (YP2) and 45 kDa (YP3). The proteins are produced in the female fat body (a tissue considered to be analogous to the vertebrate liver) and the follicle cells surrounding oocytes undergoing vitellogenesis (Bownes and Hames, 1978; Brennan et al., 1982). Yolk proteins synthesised in the fat body are secreted into the haemolymph (Bownes, 1986), and those made in the follicle cells are secreted unidirectionally towards the oocyte membrane (Butterworth et al., 1992). The YPs are taken up into the oocyte by receptor-mediated endocytosis and assembled into yolk storage granules until they are required (Giorgi et al., 1979).

The site of yolk protein synthesis does not appear to confer any differences in their biochemical properties. However, there does appear to be differences in the rates of YP synthesis between the two tissues. In the fat body cells they are all present in equal amounts, but in the ovary YP3 synthesis is reduced by approximately 25% compared to YP1 and YP2 (Brennan et al., 1982; Isaac and Bownes, 1982). The transcription rates were found to be the same for all three yolk protein genes (*yp* genes) but the levels of mRNA were different, with *yp3* being less (Isaac and Bownes, 1982). The *yp3* transcripts were found to be less stable in the ovary and thus much less YP3 is produced by this tissue (Williams and Bownes, 1986).

The function of the yolk proteins is to provide a nutritional supply of amino acids for utilisation during embryonic development. The YPs have also been shown to
Figure 1.2

The differentiation lineage of *Drosophila* ovarian follicle cells (Logan and Wensink, 1980).

Each differentiation lineage is indicated by arrows. The approximate developmental stage of appearance (s) and the approximate number of cells (n) are listed under each cell type name.
Follicular Epithelial Cells (fe)
s6, n1000

Border Cells (bc)
s7, n6-10

Columnar Cells (cc)
s8-9, n900

Cells Covering Nurse Cells (cn)
s8-9, n50-100

Anterior Pole Cells (ap)
s10-11, n300

Columnar Main Body Cells (cmb)
s10, n500

Posterior Pole Cells (pp)
s?, n30

Main Body Cells (mb)
s11, n500
carry ecdysteroid hormones, in an inactive form, into the oocyte for release during embryogenesis (Bownes et al., 1988).

1.3 THE YOLK PROTEIN GENES

The yolk protein genes are a small family encoded by single copy genes on the X chromosome (Barnett et al., 1980). The genomic arrangement of the three yp genes is shown in Figure 1.3. yp1 and yp2 are located at position 8F-9A and are divergently transcribed with each containing one intron, they are separated by a shared intergenic region of 1226bp. yp3 is located at position 12BC, isolated from yp1 and yp2, and contains two introns (Garabedian et al., 1987). The first intron of yp3 is located at a similar position to those of yp1 and yp2, relative to the transcriptional start site of each gene. Single transcripts are detected for yp1 and yp3 which are estimated to be approximately 1.60 kb and 1.54 kb respectively. Two transcripts are detected for yp2 of approximately 1.67 kb and 1.60 kb, these are found to differ at their 3' ends (Hung et al., 1982). There is a high level of sequence similarity between the nucleotide sequences of these three genes, which is highest at the 3' translated regions. Between yp1 and yp2, and yp1 and yp3 there is 53% identity, and there is 48% identity between yp2 and yp3. Due to the similarities in gene structure and sequence identities, it is considered that these genes may have arisen from duplication events during evolution (Yan et al., 1987).

1.4 CIS-ACTING SEQUENCES FOR THE YOLK PROTEIN GENES

Cis-acting sequences governing yp gene expression have been identified using fragments of DNA flanking the yp genes fused to reporter genes. After P-element mediated germ line transformation of these constructs, any regulatory effects due to the yp flanking sequences can be determined by analysis of the transgenic flies produced.
Figure 1.3

Genomic arrangement of the three yolk protein genes.

The location on the X chromosome is indicated. The clear boxes represent the yp coding sequences, and the hatched boxes represent introns.

Transcripts are represented by arrows, with the arrowhead showing the direction of transcription, and spaces the location of the introns. The molecular weights of the mature polypeptides are given.
Location on X chromosome

12BC     8F-9A

Transcripts

1.54 kb  1.6 kb  1.67 kb

Protein products

45 kDa  47 kDa  46 kDa
In the 1226bp intergenic region of \textit{yp1} and \textit{yp2}, several \textit{cis}-acting sequences have been identified which are shared between the two genes, these are shown in Figure 1.4A. One of these sequences, the fat body enhancer (FBE), was localised to a 125bp fragment, sufficient for fat body, female-specific \textit{yp} expression (Garabedian \textit{et al}, 1986). However, the FBE is not essential for the fat body, female-specific \textit{yp} expression, and deletion of the FBE does not disrupt the expression pattern. Another region, located either within the upstream region conferring ovarian expression or within the \textit{yp2} coding sequence, has been identified to govern fat body, female-specific expression (Abrahamsen \textit{et al.}, 1993). Thus there appear to be multiple sites, flanking \textit{yp1} and \textit{yp2}, which can confer fat body expression in females, but not in males. It is not known whether these sites normally provide independent regulation of \textit{yp1} and \textit{yp2}, or if these genes both use the same enhancers for female-specific fat body expression. There are also two ovarian enhancers, OE1 and OE2, that are necessary for the correct expression of \textit{yp1} and \textit{yp2} in the ovary. OE1 is 301bp and is located 43bp upstream of \textit{yp2}, this fragment can be subdivided into two regions, both of which are needed to direct the timing of \textit{yp1} and \textit{yp2} expression to the correct stages of oogenesis in the follicle cells. OE2 is 105bp and is located within the \textit{yp2} coding region, OE2 enhances levels of ovarian \textit{yp1} transcripts but cannot regulate the expression to the correct stages of oogenesis (Logan \textit{et al.}, 1989; Logan and Wensink, 1990). Therefore, to provide the correct levels and pattern expressions of \textit{yp1} and \textit{yp2}, both OE1 and OE2 are required.

For \textit{yp3}, two \textit{cis}-acting sequences, located 5' of the transcription start site, were found to be necessary for the ovarian and sex-specific fat body expression of the \textit{Drosophila yp3} gene. A 328bp ovarian region (OE3) is sufficient to limit reporter gene expression to the correct follicle cell types at the expected stages 8-10 of oogenesis. OE3 was only tested in the forward orientation with its native \textit{yp3} promoter, and thus cannot formally be named an enhancer since it was not tested in the reverse orientation. A neighbouring 419bp sequence, termed FBE3, is able to direct reporter gene expression, from a heterologous promoter, in the fat body cells of
A) The *yp1* and *yp2* regions that contain transcription enhancer sequences.

The genomic arrangement of *yp1* and *yp2* is shown, with horizontal arrows representing the length and direction of transcripts, introns are indicated by spaces. Boxes show the location and sizes of the fat body enhancer (FBE), ovarian enhancer 1 (OE1) and ovarian enhancer 2 (OE2).

B) The *yp3* gene, showing the identified ovary and fat body *cis*-regulatory regions. Nucleotide positions of restriction enzyme sites are given in relation to the *yp3* transcription start site.

Not drawn to scale.
females, but not males (Ronaldson and Bownes, 1995). The position of these fragments relative to the \textit{yp3} transcription start site is shown in Figure 1.4B. No other sequences in the upstream, downstream or coding regions were found to capable of regulating \textit{yp3} expression. It was noticed that some reporter constructs were subject to chromosomal position effects, and these effects were less frequent with fragments that directed transcription from the native \textit{yp} promoter. This indicates that the \textit{hsp70} promoter (used as a heterologous promoter in some reporter constructs where a fragment is lacking the native promoter) may be more susceptible to the influences of nearby enhancers than the \textit{yp3} promoter, or differences may occur due to the organisation of sites in artificially created fusions (Ronaldson and Bownes, 1995).

1.5 FACTORS REGULATING YOLK PROTEIN GENE EXPRESSION

The regulation of the yolk protein genes is complex because the adult female fly must relate egg production to environmental conditions. The factors involved in controlling \textit{yp} gene expression are: the nutritional status of the fly, the hormones ecdysone and juvenile hormone, the sex determination hierarchy and the tissue-specific factors required to give expression in the correct tissues at the correct developmental stages.

1.5.1 Nutrition

In a nutritionally deficient environment female flies lay fewer eggs, and when starved, after previously being well fed, the uptake of YPs ceases. This leads to a decrease in egg production which eventually stops; the oocytes no longer enter vitellogenesis and this causes a reduction in the number of mature eggs.

Starved female \textit{Drosophila} have reduced levels of \textit{yp} mRNA in the fat body, however, this is not a general effect upon transcription rates because other genes, for example \textalpha-tubulin, do not have their mRNA levels affected by starvation (Bownes et
Control of yp transcript levels in the fat body does not seem to be mediated via the ovary; the presence or absence of vitellogenic ovaries has little effect on levels of yolk protein synthesis in the fat body (Bownes and Reid, 1990). The levels of ecdysone and juvenile hormone are similar in starved and fed flies, suggesting that these two hormones are not involved (Bownes, 1989). Both egg laying and yp gene transcription are resumed if the flies are returned to a protein-rich diet (Bownes and Blair, 1986).

This response to a protein diet in Drosophila may be similar to the induction of vitellogenesis by protein rich food seen in other Diptera: Aedes (Raikhel et al., 1990), Musca (Adams and Gerst 1992, 1993) and Phormia (Zou et al., 1988). The biological significance of this food response in flies could be a mechanism for conserving components for egg development when food is limited, or a means of ensuring a food supply required by developing larvae.

Several regions located within the 1226bp intergenic region of yp1 and yp2 were shown independently of each other to confer nutritional regulation on the expression of yp1 and yp2. These regions could interact with DNA binding proteins to provide the correct regulation of these genes, and nutrition may modulate the level of a trans-acting factor in the fat body (Søndergaard et al., 1995).

1.5.2 Hormones

Juvenile hormone (JH) and ecdysone are two insect hormones which regulate moulting and metamorphosis, many experiments also indicate that these hormones are involved in yp gene regulation.

The ability of ecdysone to regulate yp gene expression was first noted in abdomen isolation experiments, where levels of YP synthesis were low and could be increased by injection of 20-hydroxyecdysone (20E), the active form of ecdysone (Jowett and
Postlethwait, 1980). Injection of 20E into starved females produced a similar effect; the synthesis of YPs in the fat body, but not the ovary, appeared to increase (Bownes and Blair, 1986). The 20-hydroxyecdysone acts at the level of transcription, since in males it can initiate transient expression of the yp genes (Postlethwait et al., 1980). The expression is observed only in the fat body and not in any other of the adult tissues (Bownes et al., 1983), this will still occur in flies mutant in the sex determination pathway, and flies mutant in doublesex (dxx) or transformer (tra) can be induced to make YPs (Bownes and Nöthiger, 1981). From analysis of the effects of 20E on various yp1, yp2 and yp3 reporter constructs, it appears that complex interactions occur between 20E response elements and repressor sites to produce the response to this hormone (Bownes et al., 1996).

Juvenile hormone has also been implicated in the regulation of the yp genes. When the synthetic analogue of JH, methoprene, is applied topically to a female abdomen containing reduced yp expression levels, for example by isolating the abdomen or starvation, the level of transcripts increases (Jowett and Postlethwait, 1980). The level of yp transcripts from the fat body also increases with methoprene application in normally fed flies (Bownes et al., 1987). The effects of JH are subtle, resulting in stimulation of yp transcript levels up to twofold. Treatment with methoprene also leads to an increase in the level of yp transcripts in the ovary. However, since the methoprene stimulates uptake of yolk and the passage of oocytes through vitellogenesis, this effect could be due to genuine stimulation of yp gene expression or a consequence of the presence of vitellogenic oocytes leading to increased yp transcription. In experiments to test reporter gene constructs (containing various regions of yp1, yp2 and yp3) for their inducibility with methoprene, it was found that methoprene upregulation of YPs was only observed using native yp genes as reporters (Bownes et al., 1996). It was suggested that JH may act on intron sequences or yp coding sequences, or possibly by influencing stability of the yp mRNA.
A recent paper (Soller et al., in press) suggests that the balance between ecdysone and juvenile hormone regulates whether oocytes will progress through a control point at stage 9, or undergo apoptosis and resorption.

1.5.3 The sex determination hierarchy

The sex of the fly is a primary factor in determining YP synthesis in Drosophila since YP expression is female-specific. The X chromosome to autosome ratio is the initial determinant of sexual phenotype. A ratio of 1 (XX:AA) results in female differentiation and a ratio of 0.5 (XY:AA) results in male differentiation. From genetic studies it is known that sexual dimorphism in Drosophila is governed by a hierarchy of somatic sex determination genes (reviewed in MacDougall et al., 1995; Ryner and Swain, 1995). Figure 1.5 shows diagrams of sex determination in female and male somatic cells. The X:A ratio acts to make the first gene in the hierarchy, Sex-lethal (Sxl), active in females or inactive in males. A number of maternally supplied and zygotic products are also required to initiate the correct activation of Sxl. Sxl has several critical functions in somatic cells, one of these is the repression, in females, of the male-specific lethal (msl) genes required in males for dosage compensation of X-linked genes. In males, which have only one X chromosome, these msl genes activate the genes on the X chromosome to become hyperactive, thus producing dosage compensation (Lucchesi and Manning, 1987). The second major function of Sxl is the control of other regulatory genes in the sex determination pathway, including: transformer (tra), transformer-2 (tra-2), doublesex (dsx), intersex (ix) and hermaphrodite (her). When these genes are appropriately expressed they bring about the correct regulation of the downstream sexual differentiation genes, including the yp genes.

In females, the Sxl transcript is spliced in a female-specific manner to produce a functional protein which then confers female-specific splicing of tra. This female-specific tra transcript produces a functional protein which, together with tra-2,
Figure 1.5

Diagrams of sex determination in female and male somatic cells.

X:A, X chromosome to autosome ratio; SXL, Sex-lethal transcript is spliced in female mode and makes a functional protein; TRA, transformer transcript is spliced in female mode in the presence of SXL protein and encodes a functional protein; TRA-2, transformer-2 product is required; DSX^F, doublesex transcript is spliced in female mode in the presence of TRA and TRA-2 proteins and makes a female-specific protein which regulates the expression of downstream genes; DSX^M, in the absence of TRA protein, doublesex transcript is spliced in the male mode to produce a male-specific protein which regulates the expression of downstream genes; IX, intersex product interacts with DSX^F to regulate downstream genes in females and affects courtship behaviour in males; HER_ZYG, the zygotic product of the hermaphrodite gene is required for female differentiation independently of DSX. SOV, the product of the small ovaries gene is essential for ovarian and female germline development.

Arrows represent interactions shown experimentally. Arrows with question marks demonstrate possible interactions.

(Adapted from MacDougall et al., 1995)
SEX DETERMINATION IN FEMALE SOMATIC CELLS

\[ X: A = 1 \]

- Courtship behaviour
- \( \text{TRA-2} \)
- \( \text{TRA} \)
- \( \text{SXL} \)
- \( \text{DSX} \)
- \( \text{HER}_{Y} \)
- \( \text{SOV} \)
- Interaction with germ cells
- Repression of \( Y_P \) expression in females
- Cell divisions in neurons
- Continuous repression of male genital primordia, derepression of female genital development.
- Determination and maintenance of determination by repressing male development of discs, histoblasts and gonad.
- Dosage compensation pathway OFF

Female differentiation and behaviour

SEX DETERMINATION IN MALE SOMATIC CELLS

\[ X: A = 0.5 \]

- Dosage compensation pathway ON
- Male specific lethals and maleless - hyperactivation of genes on X chromosome
- \( \text{DSX}^M \)
- Repression of \( Y_P \) in adult fat body
- General and specific cell divisions in male neurons
- Continuous repression of female genital primordia, derepression of male genital primordia.
- Promotion of male development in leg discs?
- SIZE - limited growth of imaginal cells

Male differentiation and behaviour
directs female-specific splicing of \textit{dsx}. The female-specific \textit{dsx} transcript produces the female form of Doublesex protein, DSX\textsuperscript{F}, which is functional (Burris and Baker, 1989). In males, \textit{Sxl} and \textit{tra} are spliced to generate non-functional proteins, since the mRNA open reading frames are truncated due to the inclusion of male-specific exons. This leads to the default splicing of the \textit{dsx} transcript, which produces the functional male-specific Doublesex protein DSX\textsuperscript{M} (Burris and Baker, 1989). Also at the bottom of the \textit{dsx} branch of the somatic sex determination hierarchy are the \textit{her} (Pultz and Baker, 1995) and \textit{ix} (Chase and Baker, 1995) genes. DSX\textsuperscript{F} acts together with \textit{her} (Pultz and Baker, 1995) and \textit{ix} (Erdman \textit{et al}., 1996) gene products to inhibit male differentiation and activate female differentiation in females. Conversely, DSX\textsuperscript{M} acts to inhibit female differentiation and activate male differentiation in males (reviewed in Burris, 1993).

1.5.4 Genetic evidence for the control of \textit{yp} gene expression by the sex determination genes

Analysis of \textit{Drosophila Sxl, tra, tra-2} and \textit{dsx} mutants initially demonstrated that the genes in the sex determination hierarchy directly influence \textit{yp} gene expression and subsequent YP synthesis (Postlethwait \textit{et al}., 1980; Bownes and Nöthiger, 1981). Chromosomally female flies (XX) that are mutant at \textit{tra} or \textit{tra-2} do not express the \textit{yp} genes and develop morphologically as males, these are termed pseudomales. Chromosomally female flies mutant at \textit{dsx} develop as intersexes and display both male and female characteristics, these express the YPs at levels reflecting the degree of male or female characteristics displayed, for example flies with a predominantly male phenotype have the lowest levels of YP expression. Chromosomally male flies (XY) mutant at \textit{dsx} also develop as intersexes and are able to express the YPs in the fat body.

In the fat body, the correct \textit{yp} expression pattern is continuously dependent upon the correct expression of the somatic sex determination genes. This can be
demonstrated with experiments using tra-2ts (temperature sensitive) mutants (Belote, et al., 1985). XX tra-2ts flies are female when reared at 16°C, but are transformed into pseudomales when reared at 29°C. The female flies reared at 16°C express the YPs in the fat body, if these flies are shifted to 29°C their YP expression becomes repressed. Returning the flies to 16°C restores the expression of YPs in the fat body (Bownes et al., 1987).

To determine if the somatic sex determination genes are required for ovarian yp gene expression, XX tra-2ts flies were reared at 16°C to give females and then shifted to 29°C, at this restrictive temperature YP synthesis continued in the ovarian follicle cells. Despite YP expression being repressed in the fat body at 29°C, the ovaries can synthesise enough YPs to produce a few mature oocytes (Bownes et al., 1990). Thus ovarian yp gene expression is not under the continuous control of the sex determination genes, but depends upon tissue-specific factors, perhaps expressed in the follicle cells. The sex determination genes are necessary to determine the development of the ovaries in females, but once formed, they are no longer required to monitor sex-specific gene expression in the mature tissue.

Recently a paper has been published showing that her is a regulator of yp gene expression (Li and Baker, 1998). Temperature sensitive her' mutants were used in the experiments, raised at 18°C they are morphologically normal and have wild type viability, and raised at 25°C they are intersexual and have severely reduced viability (Pultz et al., 1994). her females raised at 25°C show a 10-fold reduction of yp transcript levels compared to wild type females and their her'/+ sisters. These results were comparable to those obtained with dsx mutant females. The level of yp transcript can be restored to wild type levels in her mutant females raised at 25°C when they carry a transgene containing a wild type copy of the her gene. In her males, the yp transcript level remains unchanged, this is in contrast to dsx males.
where the yp level is increased 20-fold compared to that of wild type males and dsx/+ brothers. It is proposed that HER protein is required, like DSXF, for the activation of the yps in female fat body cells. But, in contrast to DSXM, HER is not required for the inhibition of yp expression in males.

1.5.5 Trans-acting factors regulating the yps in the fat body

The 125bp FBE from the intergenic region of yp1 and yp2 has been used in footprinting assays to identify several transcriptional regulatory proteins implicated in the control of fat body-specific yp gene expression. These transcriptional regulatory proteins are: the adult enhancer factor-1 (AEF-1), the box B-binding factor-2 (BBF-2) and the CCAAT/enhancer-binding protein (C/EBP). Initially, these proteins were found to interact with the adult enhancer of the alcohol dehydrogenase (Adh) gene in Drosophila to control Adh gene expression in the adult fat body. BBF-2 is a member of the CREB/ATF family of transcriptional regulatory proteins and is an activator of Adh gene transcription in the Drosophila fat body (Abet et al., 1992). C/EBP is a transcriptional activator of the Adh gene originally identified in rat liver cells, and a member of the bZIP family of transcription factors, the Drosophila homologue of this protein (DmC/EBP) has been identified and is encoded by the slow border cells (slbo) gene (Montell et al., 1992). AEF-1 was isolated from Drosophila and found to inhibit the binding of C/EBP, the AEF-1 binding site is thought to function as a negative regulatory element for the Adh gene in Drosophila (Falb and Maniatis, 1992b). These three proteins were found to have binding sites within the FBE of yp1 and yp2 (Abel et al., 1992; Falb and Maniatis, 1992a), such that C/EBP and AEF-1 have overlapping recognition sites (Figure 1.6). They are thought to bind competitively creating a possible activation (C/EBP)/repression (AEF-1) mechanism, since AEF-1 can inhibit binding of C/EBP and thereby repress its activating potential (Falb and Maniatis, 1992a). It should be noted that the effects of these proteins on yp gene expression in vivo are unknown.
The FBE of \textit{yp1} and \textit{yp2} (Garabedian \textit{et al.}, 1986) is located between nucleotide positions -196 and -323, relative to the \textit{ypl} transcription start site. The symbols superimposed on the FBE sequence identify the sequences protected from DNaseI digestion by the corresponding transcription factors shown.

Footprint sequences for the transcriptional regulatory proteins AEF-1, C/EBP and BBF-2 (Abel \textit{et al.}, 1992; Falb and Maniatis, 1992a, 1992b) and four DSX binding sites (Burtis \textit{et al.}, 1991) are shown.

Nucleotide positions are given relative to the \textit{yp1} transcription start site.
The two forms of Doublesex protein, DSXM and DSXF, have been shown to bind to four sequences in the 125bp FBE of yp1 and yp2 (Burtis et al., 1991). Three footprints, named A, B and C, were identified in binding assays with the FBE and DSXM and DSXF. The footprints identified for DSXM were indistinguishable from those identified for DSXF, these binding sites are shown in Figure 1.7. Footprint A was found to have the highest affinity for the DSX proteins, and footprint C has the least. A comparison of the three footprints revealed a potential 9bp consensus recognition sequence, CTACAAAGT. Four sequences within the binding sites match this consensus, with two copies occurring in the larger footprint B, indicating that it may be composed of two binding sites. The homologies of each binding site to the consensus sequence is shown in Figure 1.7. It can also be seen that the consensus sequences are located at similar positions within the binding sites, four nucleotides from the 5' ends and five to six nucleotides from the 3' ends of the footprints. The location of the four DSX binding sites, in relation to the binding sites of AEF-1, C/EBP and BBF-2, are shown in Figure 1.6. Footprint A overlaps with the shared AEF-1 and C/EBP binding sites and footprint C overlaps with the BBF-2 binding site. If one site contains AEF-1 and C/EBP binding sites to confer fat body-specificity and DSX binding sites to confer the sex-specificity of yp1 and yp2 expression, this could explain why the sex- and fat body-specificity of yp gene expression has never been separated in the reporter constructs used to identify the FBE.

The DSXM and DSXF proteins can provide a mechanism for conferring sex-specificity. Sex-specific dsx transcripts produced by alternate splicing encode DSX proteins with identical N-termini comprising of 397aa, and sex-specific C-termini of either 152aa (DSXM) or 30aa (DSXF). The N-terminal region common to both proteins contains a zinc finger-related DNA binding domain (Erdman and Burtis, 1993), and thus the DSXM and DSXF proteins have the same binding sites within the FBE in vitro. The sex-specific C-termini of the DSX proteins could confer sex-
Figure 1.7

The four protected footprint sequences from binding of DSX proteins to the FBE of \( yp1 \) and \( yp2 \) (adapted from Burtis et al., 1991). Homologies to the proposed consensus binding sequence are underlined and the first nucleotide positions indicated. The number of matched nucleotides to the consensus in each footprint are also given.
<table>
<thead>
<tr>
<th>Footprint</th>
<th>Protected Sequence</th>
<th>Match</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>ACAACTACAATGGTGCAAT</td>
<td>8/9</td>
</tr>
<tr>
<td>B</td>
<td>GAGCCTACAAAGTGATTACAAATTAAAATA</td>
<td>9/9 7/9</td>
</tr>
<tr>
<td>C</td>
<td>GGTGCTGCTAAGTCATCA</td>
<td>7/9</td>
</tr>
</tbody>
</table>
specific expression of their target sex differentiation genes by causing different interactions between regulatory factors for these genes and DSXM or DSXF.

The DSXM and DSXF proteins were found to directly link the sex determination pathway and the sex-specific transcriptional regulation of yp1 and yp2 via the 125bp FBE (Coschigano and Wensink, 1992). Mutating the A and B dsx-binding sites within the FBE substantially reduces binding by both DSX proteins in vitro and also eliminates almost all of the sex-specific regulation by the FBE in vivo. Using dsx mutants, it was also demonstrated that both DSXM and DSXF require the A and B dsx-binding sites to regulate the sex-specificity of transcription in vivo. Both DSX proteins were found to bind to the same sites in the FBE, but they have opposite effects on reporter gene transcription. DSXM was found to be strong repressor of transcription and DSXF activates transcription. The observation that DSXM represses transcription is consistent with previous genetic results, which showed that in the absence of DSX proteins, YPs were expressed, but the presence of DSXM yp gene expression was repressed (Bownes and Nöthiger, 1981). In contrast, the observation that DSXF activates FBE-directed transcription is inconsistent with the simplest interpretation of previous genetic results: that DSXF is a repressor of male specific genes (Baker and Ridge, 1980). Although these experiments have demonstrated the role of DSXM and DSXF in linking the sex determination pathway to target gene regulation, it is possible that the DSX proteins act in conjunction with one or more other proteins to accomplish the observed fat body and sex-specific yp gene regulation.

Further experiments investigating the FBE-directed regulation of yp1 and yp2 identified a simple sex- and tissue-specific enhancer from within the FBE sequence (An and Wensink, 1995a). This enhancer, named o-r, is organised into two enhancer elements, o and r. The o element contains overlapping binding sites for DmC/EBP and the two DSX proteins. In the o element these binding sites are referred to as: bzip1 and dsxA, with bzip1 being the binding site of DmC/EBP. This element
corresponds to the highest affinity DSX footprint A overlapping with the shared AEF-1 and C/EBP binding sites previously mentioned (shown in Figure 1.6), but the AEF-1 binding site (aef1) was not included because mutations in aef1 were found to have no effect on the sex-and tissue-specific enhancer o-r. The r enhancer element is proposed to contain a binding site, named refl, for an unidentified positive regulatory factor. The elements o and r have no enhancer activity by themselves, but when combined are sufficient for the tissue- and sex-specific expression observed with the whole FBE. The o-r enhancer was characterised by site-directed mutagenesis, protein binding and germ line transformation experiments, to propose the following model.

A two-level model is proposed for regulation by o-r (An and Wensink, 1995a). At the first level, tissue-specific activation in fat bodies requires strong co-operative activation by two proteins, one bound to refl and the other bound to bzip1. At the second level, DSX proteins at dsxA modify this tissue-specific activation by their effect on a bZIP protein at bzip1. In females, DSX^f activates indirectly by keeping an active bZIP protein at the bzip1 site. It may do this by either increasing the activity of the bound protein or by increasing the binding between the bZIP protein and the bzip1 site. In males, DSX^M inactivates by blocking the synergy between bzip1 and refl, it does this by reducing the activity of the bZIP protein at the bzip1 site. A mechanism for DSX^M to achieve this is by sterically hindering the binding of bZIP or by inactivating this protein. If steric hindrance is involved, DSX^F may not be capable of excluding bZIP binding to bzip1 in females due to its shorter C-terminus compared to DSX^M (Burtis and Baker, 1989).

The exception to this model is the activation seen in female fat bodies when bzip1 is mutated. It is speculated that DSX^F gives sufficient activation to bZIP despite the lower affinity of the mutated bzip1 site. There is no evidence that DmC/EBP is a regulator of yp1 and yp2 gene expression, nor have any DmC/EBP transcripts been identified in adult fat bodies (Rørth and Montell, 1992), the DmC/EBP binding site is called bzip1 because the bZIP protein actually activating from this site is unknown.
1.5.6 Recent models proposed for the female, fat body regulation of yp1 and yp2

An and Wensink (1995b) investigated the o-r enhancer further, and in these experiments, an o element was used that contained the aef1 site which binds AEF-1 protein. A tetramer of the o enhancer element is found to be sufficient to activate female-specific reporter gene transcription in fat bodies. This shows that the dsxA, bzip1 and aef1 sites can be sufficient to direct the yp gene expression pattern, but require arrangement into a tetramer to be capable of doing so. A tetramer of the r element was found to direct reporter gene expression in all nongonadal tissue, and thus, is not responsible for either the sex or fat body-specificity of o-r. Mutated binding sites within the o tetramer were used in protein binding, germ line transformation and genetic experiments to examine both the independent and combined activities of the proteins and their binding sites. This led to the proposal of another model for the regulation of yp1/2 by the o-r enhancer, a summary diagram is shown in Figure 1.8.

In this model, in female fat bodies there is synergism between DSXF and bZIP activator possibly by co-operative binding or by altering bZIP activity upon its binding to the site. The bZIP also acts cooperatively with an activator at the refl site to yield gene expression in the female fat body. The ability of bZIP proteins to interact with other transcriptional factors has been well documented (for review, see Nolan, 1994). The second positive transcriptional effect of DSXF in female fat bodies is to interfere with the binding of AEF-1 and thus prevent it repressing transcription.

In male fat bodies, DSXF is not present and therefore prevents the bZIP activator from binding co-operatively. Instead, DSXM is present and binds to the dsxA site, because the DSXM protein has a bulkier male-specific carboxy-terminal domain it can sterically hinder activation by bZIP protein. Alternatively, bZIP may be inactivated by DSXM, this is not shown in Figure 1.8. The elimination of bZIP activation
Figure 1.8

Model for transcriptional regulation by \( o-r \).

**Top**
Activation in female fat bodies resulting from synergy among the \( DSX^F \), bZIPa and R proteins. Curved arrows indicate activation, and the arc between bZIPa and R represents positive cooperativity. These three proteins are shown bound to their cognate sites on \( o-r \) DNA (solid lines). The AEF-1 repressor is shown unbound, excluded from its DNA site by \( DSX^F \). Broken lines represent DNA between \( o-r \), and the promoter (right angle arrow).

**Middle**
Repression in male fat bodies resulting from \( DSX^M \). \( DSX^M \) is shown bound to DNA, excluding the other two proteins from binding.

**Bottom**
Inactivation in ovaries and tissue specificity. The absence of dsx proteins allows AEF-1 to bind, excluding activators from the bZIP site. The bZIPa and bZIPb proteins activate from the bzipl site but have different tissue distributions.

(adapted from An and Wensink, 1995b)
removes synergy with an activator at the ref1 site and will not yield gene expression in the male fat body. It is thought that AEF-1 is unlikely to repress in male fat bodies through o-r because of steric hindrance by DSX^M.

In ovaries it is proposed that due to the absence of DSX proteins, the AEF-1 repressor binds and excludes activators from the bZIP site. The bZIPa and bZIPb (proposed tissue-specific bZIP in the ovary) activate from the bzip1 site but have different tissue distributions.

Another paper has been published recently, Li and Baker (1998), describing the role of the hermaphrodite (her) gene and dsx in the regulation of the yps. It is proposed that they control the expression of the yp genes in the fat body by two separate pathways for the activation of the yps. One pathway is the female-specific activation of the yps, which is DSXF dependent. The other pathway is the non-sex-specific activation of the yps, which is HER-dependent, DSXF-independent and inhibited by DSX^M. The combination of these effects, in wild-type females, results in both HER and DSXF contributing independently to produce high level expression of the yps. In wild-type males, DSXM inhibits yp expression, which can override the activation function of HER. The 125bp FBE of yp1 and yp2 (position -196 to -322) was not found to be sufficient to confer her responsiveness, and the major her responsive element is located outside of FBE. HER is a zinc finger protein and is thought to regulate the yps directly. The her responsive region, termed HRR, is located between nucleotide positions -322 to -1225 in the intergenic region of yp1 and yp2. The fat body-specific expression of the yps is not specified by dsx since, in dsx mutant flies of both sexes, the expression of yp-reporter genes is still restricted to fat body cells. HER is also not considered to be necessary for fat body-specific expression of the yps because loss of her does not affect fat body-specific expression of a transgene, and the FBE-reporter construct can confer fat body-specific expression without the HRR present. An unknown fat body-specific factor, termed FBF, binding possibly to the bZIP site in the FBE is thought to confer tissue-specificity to the fat body.
only. The HRR is necessary for \textit{her} function but is not thought to be sufficient for \textit{yp} expression, it is deemed likely that the FBF is also necessary for HER to direct the \textit{yps}. Besides \textit{her} and \textit{dsx}, the \textit{intersex (ix)} gene also acts in the \textit{dsx} branch of the sex determination pathway. It has been observed that \textit{ix} is also required for the transcriptional activation of the \textit{yp} genes in females, but is not required for their repression in males. Genetic studies indicate that \textit{ix} interacts with \textit{her} and with \textit{dsx} in regulating the \textit{yps} in females (Baker, unpublished data). It is thought that \textit{ix} may participate both in the HER-dependent non-sex-specific activation pathway and in the DSX\textsuperscript{F}-dependent female-specific activation pathway. It is postulated that a candidate DNA site in the FBE through which IX might function is the "ref1" site, shown to synergistically function with the DSX binding site, dsxA (An and Wensink, 1995b).

Considerably more research is required in this area in order to fit these two models together and determine exactly how \textit{yp1} and \textit{yp2} are regulated in the female fat body. It should also be noted that the FBE is not the only sequence within the intergenic region of \textit{yp1} and \textit{yp2} capable of conferring female, fat body expression. The FBE can be deleted without preventing female, fat body \textit{yp} expression, thus suggesting there are more sites to be located.

1.5.7 \textit{Trans-acting factors regulating the yps in the ovary}

No regulatory proteins have yet been identified for the direction of \textit{yp} tissue-specific ovarian expression. For \textit{yp1} and \textit{yp2}, the mechanism of ovary-specific regulation is likely to be complex to resolve due to these genes having shared promoter sequences in the intergenic region, and there being two enhancers, OE1 and OE2, required for correct ovarian \textit{yp} expression. For \textit{yp3}, a 328bp ovarian enhancer (OE3) has been found to bind in gel retardation assays to a protein present in ovarian nuclear extracts, but the identity of this protein is unknown (E. Ronaldson, unpublished data).
Several years ago, the yolk protein factor 1 (YPF1) protein was isolated from tissue culture cells (KcO cells, an embryonic cell line derived from first instar larvae), and was also found to be present in ovarian and early embryonic extracts. YPF1 was thought to be a potential regulatory factor for ovarian yp expression because it was found to bind to a 31bp sequence located within the ypI coding sequence, close to the transcription start site (Mitsis and Wensink, 1989a; 1989b). Computer sequence comparisons revealed a similar sequence (21/31bp match) in a position in yp3. However, since this was in the yp3 coding sequence it is not known if the similarity is for YPF1 binding or a requirement for the structure or function of the yolk proteins themselves. Deletion of the binding site was found to reduce ypI transcript levels (Mitsis and Wensink, 1989b), but this could have been due to transcript instability instead of the loss of binding by a transcriptional activator protein. YPF1 has now been identified as a Drosophila homologue of the small (70 kDa) subunit of the heterodimeric Ku DNA-binding protein, a human DNA-dependent protein kinase (Jacoby and Wensink, 1994). Ku and YPF1 are thought to be involved in the initiation of transcription, and could be involved in bringing about high levels of yp transcription during oogenesis. YPF1 is expressed at low levels throughout Drosophila development, with high levels of transcripts found in the oocyte and early embryo, which is consistent with the proposed function for YPF1. It is unlikely that YPF1 has a specific role in regulating ypI gene expression in the ovary, but is more likely to have a general role in transcription initiation.

Genetic experiments have demonstrated that ovarian yp expression is not under the continuous control of the sex determination hierarchy, and it is therefore presumed that yp expression in the ovary depends upon tissue-specific factors, expressed in the follicle cells. It is possible that factors regulating fat body-specific expression of the ypIs could also be regulators of ovarian yp expression, however, this is entirely speculative.
1.6 THE YOLK PROTEIN GENES IN OTHER DIPTERAN INSECTS

In addition to *Drosophila melanogaster*, yolk protein encoding genes have also been isolated from the Mediterranean fruit fly *Ceratitis capitata* (Rina and Savakis, 1991), the bluebottle *Calliphora erythrocephala* (Martinez and Bownes, 1994) and the common housefly *Musca domestica* (White and Bownes, 1997). Compared with *Drosophila* yolk proteins, their expression profiles and polypeptide sequences are highly conserved. The restriction of yp expression to the adult female fat body and to the follicle cells at specific stages of oogenesis is conserved in all these species. Thus, it seems possible that the yp genes in these species share conserved regulatory sequences and mechanisms to achieve a similar pattern of gene expression.

To study conservation of yp regulation in dipteran insects, 5' flanking regions from one *Musca yp* gene and one *Calliphora yp* gene were tested for enhancer functions in *Drosophila* (Tortiglione and Bownes, 1997). A 823bp fragment from *Musca* and a 1046bp fragment from *Calliphora yp* genes are able to direct correct expression of a reporter gene in the ovarian follicle cells of transformed *Drosophila*. Reporter gene expression was also observed in the border cells, in contrast to the endogenous host *Drosophila yp* genes. This suggests that a repressor of border cell expression has evolved in *Drosophila*, although the reason for this happening is unknown. These fragments are unable to confer sex-specific reporter gene expression in the fat body, and expression was observed in the fat body cells of both males and females. This suggested that in *Drosophila*, DSX protein (diverged during evolution) would not recognise the enhancer binding sites between species, or by another mechanism. However, by *in vitro* DNA/protein interaction assays, a 248bp DNA region from the *Musca yp* enhancer was able to bind specifically to the *Drosophila* DSX protein, which in *Drosophila* directs the sex-specific expression of yp1 and yp2. It is therefore speculated that in *Musca* and *Calliphora* adult females, the sex determining pathway is not directly involved in yp regulation, but instead hormonal controls are used to achieve the sex-specific expression of the yp genes.
One aspect of development which is different between the species is the synchrony of oogenesis. In the more primitive species, a batch of eggs develops synchronously after feeding, whereas in *Drosophila* eggs of all stages are present in the ovaries of fed flies (King, 1970). In *Calliphora* and *Musca*, the fat body produces YPs in cycles related to feeding (Adams and Gerst, 1992; 1993).

1.7 THE REGULATION OF YP3 EXPRESSION IN *DROSOPHILA*

Although the regulation of *yp1* and *yp2* gene expression has been investigated in some detail, very little is known about the regulation of *yp3* in the ovary and the female fat body. For *yp3*, two cis-acting sequences, located 5' of the transcription start site, have been found to direct ovarian and sex-specific fat body expression of the *Drosophila yp3* gene. A 328bp ovarian region (OE3) is sufficient to limit reporter gene expression to the correct follicle cell types at the expected stages 8-10 of oogenesis., and an adjacent 419bp sequence, termed FBE3, is able to direct reporter gene expression, from a heterologous promoter, in the fat body cells of females, but not males (Ronaldson and Bownes, 1995).

Genetic analysis has shown that the sex-specificity of *yp3* expression is under the control of the sex determination hierarchy (Bownes and Nöthiger, 1981; Li and Baker, 1998), but no investigations into the direct binding of DSX or HER proteins with *yp3* sequences have been reported. Due to the expression patterns of *yp1*, *yp2* and *yp3* being so highly co-ordinated, it is likely that the factors involved in the transcriptional regulation of all three *yp* genes are the same, or very similar. The cis-acting sequences involved in regulating *yp3* expression may be less complex than those shared between the divergently transcribed *yp1* and *yp2*.
The aim of this PhD project was to further characterise the FBE3 and OE3 sequences by dividing them into subfragments and testing their ability to direct reporter gene expression in the correct yp3 expression pattern. Also to identify trans-acting factors involved in directing yp3 gene expression in the ovary and the fat body. The trans-acting factors involved in yp1 and yp2 are also candidates for the regulation of yp3, therefore, comparisons can be made between the factors involved in the regulation of all three yolk protein genes.

CHAPTER ONE: INTRODUCTION
CHAPTER 2

MATERIALS AND METHODS
2.1 MEDIA

2.1.1 Bacterial Media

All media were made up in distilled water and sterilized by autoclaving (15psi/20mins). Ampicillin was added to media and plates to a final concentration of 100μg/ml when required.

Luria Broth (LB) 10g Difco Bacto Tryptone, 5g Difco Bacto Yeast Extract, 5g NaCl. Made up to 1 litre with H₂O and adjusted to pH 7.2.

Luria Agar As LB with the addition of 15g Difco Bacto Agar per litre.

2.1.2 Drosophila Media

Staffan Food 250g cornflour, 175g yeast pellets, 100g agar dissolved in 10 litres H₂O and boiled. Nipagin was added to 4.5μg/l when cooled to 60°C and the food was then poured into bottles and vials.

Apple Juice Media 9g Difco Bacto Agar and 10g sucrose was dissolved in 300ml H₂O by microwaving, 100ml apple juice was added when the solution had cooled to 60°C and the media was poured into petri dishes.

Tomato Juice Media 4g Difco Bacto Agar was dissolved in 165ml H₂O by microwaving, 40ml tomato juice and 0.5ml 10% Nipagin was added when the solution had cooled to 60°C and the media was poured into petri dishes.
2.2 BACTERIAL, PLASMID AND DROSOPHILA STOCKS

Table 2-1: Bacterial Stocks

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>XL1-Blue</td>
<td>recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac[F', proAB, lac^ZΔM15, Tn 10 (tet')]</td>
<td>Bullock, Fernandez and Short, 1987</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>F-, ompT, hsdS^B, (r^B-,m^B-), dcm, gal, λ(DE3)</td>
<td>Studier and Moffat, 1986</td>
</tr>
</tbody>
</table>

Table 2-2: Plasmids for subcloning

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescript</td>
<td>Amp'</td>
<td>Vector used for subcloning</td>
<td>Stratagene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>yp3 sequences</td>
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</tr>
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Table 2-3: Plasmids for P-element mediated germ line transformation

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pER1</td>
<td>Amp'</td>
<td>Used to test the effects of yp3 constructs lacking the yp3 promoter on lacZ reporter gene expression</td>
<td>Ronaldson and Bownes, 1995</td>
</tr>
<tr>
<td>pΔ2-3</td>
<td>Amp'</td>
<td>Helper plasmid for P-element transformation, encodes transposase</td>
<td></td>
</tr>
<tr>
<td>Plasmid</td>
<td>Genotype</td>
<td>Comments</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------</td>
<td>----------</td>
<td>----------------------------------------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>pGEX-KG</td>
<td>Amp'</td>
<td>GST-DSX binding domain fusion protein expression vector</td>
<td>Erdman and Burtis, 1993</td>
</tr>
<tr>
<td>bsφ35</td>
<td>Amp'</td>
<td>pBluescript SK containing AEF-1 cDNA</td>
<td>T. Maniatis, personal communication</td>
</tr>
<tr>
<td>DmC/EBP</td>
<td>Amp'</td>
<td>pT5 expression vector containing DmC/EBP</td>
<td>Rorth and Montell, 1992</td>
</tr>
<tr>
<td>yp3/9</td>
<td>Amp'</td>
<td>419bp FBE3 fragment in pBluescript</td>
<td>E. Ronaldson, personal communication</td>
</tr>
<tr>
<td>yp3/5</td>
<td>Amp'</td>
<td>328bp OE3 fragment in pCaSpeR-AUG-βgal vector</td>
<td>E. Ronaldson, personal communication</td>
</tr>
<tr>
<td>pGEM YP1A</td>
<td>Amp'</td>
<td>pGEM vector containing yp1</td>
<td></td>
</tr>
<tr>
<td>pGEM YP2B</td>
<td>Amp'</td>
<td>pGEM vector containing yp2</td>
<td></td>
</tr>
<tr>
<td>pGEM YP3</td>
<td>Amp'</td>
<td>pGEM vector containing yp3</td>
<td></td>
</tr>
</tbody>
</table>
## Table 2-5: *Drosophila* stocks

<table>
<thead>
<tr>
<th>Name</th>
<th>Relevant Features</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oregon R (OrR)</td>
<td><em>A Drosophila melanogaster</em> wild type strain</td>
<td>Lindsley and Grell, 1968</td>
</tr>
<tr>
<td>wr</td>
<td><em>white-</em> (white eyed)</td>
<td></td>
</tr>
<tr>
<td>l(2)slbo&lt;sup&gt;57b&lt;/sup&gt;</td>
<td><em>silbo</em> deletion, homozygous lethal</td>
<td>Montell <em>et al.</em>, 1992</td>
</tr>
<tr>
<td>fs(2)slbo&lt;sup&gt;598&lt;/sup&gt;</td>
<td><em>rosy</em>+ insertion, female sterile</td>
<td>Montell <em>et al.</em>, 1992</td>
</tr>
</tbody>
</table>
2.3 OLIGONUCLEOTIDES

Oligonucleotides were obtained from Oswel and were supplied dissolved in ~1ml sterile distilled water. Oligonucleotides used are detailed in Table 2.6.

Table 2-6 Oligonucleotides used in this work

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5’ to 3’</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3</td>
<td>AAT TAA CCC TCA CTA AAG</td>
<td>General sequencing primer</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td></td>
</tr>
<tr>
<td>T7</td>
<td>GTA ATA CGA CTC ACT ATA</td>
<td>General sequencing primer</td>
</tr>
<tr>
<td></td>
<td>GGG C</td>
<td></td>
</tr>
<tr>
<td>Adh</td>
<td>ATC GAA AGA GCC TGC TAA</td>
<td>pERI sequencing primer</td>
</tr>
<tr>
<td></td>
<td>AGC</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>TTC AAT GAT GTC CAG TGC TAA</td>
<td>pERI sequencing primer</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td></td>
</tr>
<tr>
<td>825X</td>
<td>TTT CCC TTG ACT TGC ACT T</td>
<td>PCR amplification of 150bp OE3 fragment</td>
</tr>
<tr>
<td>826X</td>
<td>TCG CGT GAC GGT CGC TAA</td>
<td>PCR amplification of 150bp OE3 fragment</td>
</tr>
<tr>
<td>P4048</td>
<td>GGA ATT CAA GGG ATC TGC ACA AGT TG</td>
<td>PCR amplification of 246bp FBE3 subfragment</td>
</tr>
<tr>
<td>P4049</td>
<td>GGA ATT CAA CCT CTT CAC ACT TGG C</td>
<td>PCR amplification of 246bp FBE3 subfragment</td>
</tr>
<tr>
<td>CEBPRF2</td>
<td>CGA TGC CGT TCA GAC ATT</td>
<td>DmC/EBP forward primer</td>
</tr>
<tr>
<td>W2495</td>
<td>GG</td>
<td></td>
</tr>
<tr>
<td>CEBPRR2</td>
<td>ATG TCG CTG AAG ATG CCC AG</td>
<td>DmC/EBP reverse primer</td>
</tr>
<tr>
<td>W2496</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P5699</td>
<td>GTG CAC AAC TAC AAT GTT GCA ATC AGC GG</td>
<td>FBE yp1/2: -312 to -284 coding strand</td>
</tr>
<tr>
<td>P5700</td>
<td>CCG CTG ATT GCA ACA TTG TAG TTG TGC AC</td>
<td>FBE yp1/2: -312 to -284 non coding strand</td>
</tr>
</tbody>
</table>
2.4 SOLUTIONS

Chemicals were obtained from Sigma, BDH, Aldrich, Fison Radioisotopes [32]dCTP and [35S]dATP, and Hybond-C were obtained from Amersham.

Enzymes were supplied by Boehringer Mannheim, Gibco BRL, NBL, NEB, Pharmacia, Promega or USB.

Digoxygenin labelling and detection kit was purchased from Boehringer Mannheim.

Standard solutions were made using sterile distilled water and sterile, baked glassware. Most solutions were sterilized by autoclaving (15 psi/15 min) or by passing through a 0.45μm nitrocellulose filter.

TAE 40mM Tris-acetate, 1mM EDTA, pH 7.8

TBE 89mM Tris-HCl, 89mM boric acid, 2.5mM EDTA, pH 8.3

TE 10mM Tris-HCl, 1mM EDTA, pH 8.0

10X DNA Loading Buffer

0.25% Bromophenol Blue, 0.25% Xylene Cyanol FF, 30% glycerol in H2O.

PBS (phosphate buffered saline)

8g NaCl, 0.2g KCl, 1.44g Na2HPO4, 0.24g KH2PO4 dissolved in 800ml H2O, pH adjusted to 7.4, made to 1 litre and sterilized by autoclaving.

PBT PBS with 0.1% (v/v) Tween-20

CHAPTER TWO: MATERIALS AND METHODS
Ringer’s Solution 6.5g NaCl, 0.14g KCl, 0.2g NaHCO₃, 0.12g CaCl₂, 0.01g NaH₂PO₄ per litre, sterilized by autoclaving.

2.4.1 Competent Cell Solutions

TSS 10% (w/v) PEG (Mr 3350 or 8000), 20-50mM MgCl₂ in LB media. pH adjusted to 6.5-6.8 and filter-sterilized. An aliquot of DMSO was added to give a final concentration of 5% (v/v) and the solution was stored at 4°C.

TFB1 30mM potassium acetate, 10mM CaCl₂, 50mM MnCl₂, 100mM RbCl, 15% glycerol. pH adjusted to 5.8 with 1M acetic acid. Filter-sterilized and stored at room temperature.

TFB2 10mM MOPS or PIPES (pH 6.5), 75mM CaCl₂, 10mM RbCl, 15% glycerol. pH adjusted to 6.5 with 1M KOH. Filter-sterilized and stored at room temperature.

2.4.2 DNA Preparation Solutions

P1 3.03g Tris, 1.86g EDTA, pH adjusted to 8.0 and made up to 500ml. Stored at 4°C.

P2 200μl 10M NaOH, 1ml 10% SDS, volume adjusted to 10ml. Made fresh each time used.

P3 147.23g potassium acetate dissolved in 250ml H₂O, adjusted to pH 5.5 with glacial acetic acid (approximately 55ml), made up to 500ml with H₂O. Stored at 4°C.
2.4.3 Sequencing Gel Solutions

Sequencing Gel Acrylamide Stock

Long Ranger™ (AT Biochem), 50% acrylamide stock solution

6% Sequencing Gel Solution

21g Urea, 3ml 10x TBE, 6ml Long Ranger dissolved by warming gently, then cooled and made up to 50ml with deionized water. Polymerised with 250µl 10% APS and 25µl TEMED.

2.4.4 Protein Gel Solutions

2x Polypeptide Sample Buffer

1.25ml 1M Tris-HCl pH 6.8, 2ml glycerol, 5ml 10% SDS, 0.5ml 0.1% Bromophenol Blue. Made up to 9ml with H₂O, then added 1ml 2-mercaptoethanol.

Protein Gel Acrylamide Stock

Protogel (National Diagnostics) 30% acrylamide solution, 2.7% crosslinked with methylene bisacrylamide (37.5:1 ratio), in distilled H₂O.

10% Separating Gel Solution

6.66ml Protogel, 2.5ml 3M Tris pH8.8, 200µl 10% SDS, 10.42ml H₂O. Polymerised with 200µl 10% APS and 20µl TEMED.
4% Stacking Gel Solution

650μl Protogel, 650μl 0.5M Tris pH6.8, 50μl 10% SDS, 3.59ml H₂O. Polymerised with 50μl 10% APS and 10μl TEMED.

10x Running Buffer

30g Tris, 144g glycine, 10g SDS. Made up to 1 litre with H₂O.

2.4.5 Protein Purification Solutions

Z-100

100mM KCl, 25mM HEPES pH7.9, 12.5mM MgCl₂, 1mM DTT, 0.1% NP-40, 10% glycerol, 1mM PMSF

Z-50

50mM KCl, 25mM HEPES pH7.9, 12.5mM MgCl₂, 1mM DTT, 0.1% NP-40, 10% glycerol, 1mM PMSF

2.4.6 Bradford Assay Solutions

0.5 mg/ml bovine serum albumin (BSA)

the concentration of BSA is determined using the $A_{280} = 6.6$ for a 10mg/ml solution of BSA (1cm pathlength).

Coomassie Brilliant Blue Solution

100mg Coomassie Brilliant Blue G250 was dissolved in 50ml of 95% ethanol. 100ml 85% phosphoric acid was added and the solution was made up to 1 litre with H₂O. The solution was then filtered through Whatman No. 1 filter paper and stored at 4°C.
2.4.7 β-galactosidase Staining Solutions

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

X-gal Staining Buffer Solution A
0.5M Na₂HPO₄/NaH₂PO₄

Solution B
10mM Solution A, 150mM NaCl, 1mM MgCl₂, 7mM Potassium Ferrocyanide, 7mM Potassium Ferricyanide. Stored in a dark container at 4°C. Immediately prior to use, X-gal was added to a concentration of 0.2% (w/v).

2.4.8 Gel Retardation Assay Solutions

5X Tris-glycine 30.28g Tris, 142.7g glycine, 3.92g EDTA. Made up to 1 litre with H₂O, approximately pH 8.5.

High Ionic Strength Gel Mix
6ml 5X Tris-glycine, 4.8ml Protogel, 1.5ml 50% glycerol, 17.7ml H₂O. Polymerised with 250μl 10% APS and 34μl TEMED.

5X Z-50 Incubation Buffer
250mM KCl, 125mM HEPES pH7.9, 62.5mM MgCl₂, 5mM DTT, 0.5% NP-40, 50% glycerol, 5mM PMSF

High Ionic Strength Electrophoresis Buffer 1X Tris-glycine
5X Gel Shift Loading Buffer

8ml 5X Tris-glycine, 2ml glycerol, 0.01g Bromophenol Blue.

2.4.9 Western Blot Solutions

Transfer Buffer

25mM Tris pH 8.3, 192mM glycine, 20% methanol

Ponceau S Staining Solution

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

2.4.10 HRP Western Detection Solutions

10x Milk Buffer

12.1g Tris, 57.7g NaCl, dissolved in 800ml H₂O and pH adjusted to 8.1. 5ml Tween-20 was added and the volume made up to 1 litre.

Developer

60mg 4-chloro-1-napthol dissolved in 20ml methanol. 100ml 1X milk buffer and 100μl hydrogen peroxide was added prior to use.

2.4.11 ECL Solutions

PBS-T

PBS with 0.1% (v/v) Tween-20

1.250mM Luminol

0.44g Luminol dissolved in 10ml DMSO, stored at 4°C in the dark

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2.90mM p-Coumaric acid

0.15g p-Coumaric acid dissolved in 10ml DMSO, stored at 4°C in the dark

Solution A

1ml Luminol stock, 0.44ml p-Coumaric acid stock, 10ml Tris-HCl (pH 8.5) and made up to 100ml with water. Stored at 4°C in the dark

Solution B

10ml Tris-HCl (pH 8.5), 61μl 30% H2O2, and made up to 100ml with water. Stored at 4°C in the dark, and made weekly.

ECL detection solution

Solutions A and B mixed 1:1, just before use

2.4.12 In situ Hybridisation Solutions

pp (fix)

2g paraformaldehyde was dissolved carefully in 40ml H2O (in 65°C water bath) by adding 30μl 10M NaOH and shaking occasionally. At high pH the paraformaldehyde dissolves easily. 5ml 0.5M PIPES pH7.0, 100μl 0.5M EGTA pH8 and 100μl 1M MgSO4 was then added and the pH checked as ~6.8. The volume was adjusted to 50ml with water and stored at 4°C for up to 24 hours.

Hybrix

50% formamide, 5x SSC, 100μg/ml sonicated, heat denatured salmon sperm DNA (ssDNA), 50μg/ml heparin, 0.1% Tween-20.
NMTT  100mM NaCl, 50mM MgCl₂, 100mM Tris (pH 9.5), 0.1% Tween-20, 1mM Levamisol (fresh).

NBT  75mg/ml NBT in 70% dimethylformamide

X-phosphate  50mg/ml X-phosphate in 100% dimethylformamide

2.5 METHODS

The spectrophotometer used was a Perkin-Elmer, Lambda 15, UV/VIS Spectrophotometer.

2.5.1 Microbiological Techniques

2.5.1.1 Growth of E. coli Bacterial Cultures

Liquid cultures of E. coli were grown in LB, containing antibiotic if necessary, at 37°C with shaking. Cultures greater than 10mls were grown in conical flasks with a capacity of 5-10X that of the culture volume to allow proper aeration during growth. Cultures were also grown on the surface of Luria agar plates at 37°C.

2.5.1.2 Storage of E. coli Bacterial Cultures

For short term storage (4-6 weeks) bacteria were streaked onto Luria agar plates, and stored at 4°C. For longer term storage, 500μl of a fresh culture, grown overnight in LB, was mixed with 500μl glycerol, and frozen at -70°C. Cells were recovered by scraping the surface of the frozen culture with a sterile inoculating loop and streaking onto a Luria agar plate.
2.5.1.3 Preparation and Transformation of Competent Cells

The *E. coli* strains XL1-Blue and BL21 (DE3) were made competent using either of the following methods.

2.5.1.3.1 DMSO method

LB media was inoculated with an overnight culture of bacteria, to give a 1:100 dilution of the culture. The bacteria were then grown at 37°C, with shaking (225 rpm), to the early exponential phase (A_600 0.3-0.4). The cells were pelleted by centrifugation at 1000xg for 10 minutes at 4°C and the pellet resuspended in ice-cold TSS in one-tenth of the original culture volume. The cells were pipetted into 200μl aliquots, quick-frozen in a dry ice/isopropanol bath and stored at -70°C until they were needed.

2.5.1.3.2 RbCl method

5ml LB medium was inoculated with a single colony from an LB plate and incubated overnight at 37°C with shaking (approx. 225 rpm). On the following day, 250ml LB medium, containing 20mM MgSO_4, was inoculated with 2.5ml of the overnight culture and grown in a 1 litre flask until the A_600 reached 0.4-0.6. The cells were centrifuged at 4,500xg for 5 minutes at 4°C. The cell pellets were gently resuspended in 100ml of ice-cold TFB1, and all subsequent steps were performed on ice. The resuspended cells were incubated on ice for 5 minutes, then pelleted by centrifugation at 4,500xg for 5 minutes at 4°C. The cells were gently resuspended in 10ml of ice-cold TBF2 and incubated on ice for 15-60 minutes. Then the cells were divided in to
200μl aliquots, quick-frozen in a dry ice/isopropanol bath and stored at -70°C until they were needed.

2.5.1.3.3 Transformation of Competent Cells

Aliquots of competent cells were thawed on ice and used immediately upon thawing. Transformation was carried out by mixing approximately 50ng DNA with 100μl cells followed by incubation on ice for 30 minutes. For the RbCl method, the cells were heat shocked at 42°C for 1 minute in a water bath. The DMSO method does not require heat shock. LB media (900μl) was then added and the cells incubated at 37°C for 1 hour with shaking, to allow expression of antibiotic resistance. The culture was then spread onto antibiotic plates to select for transformants. Where appropriate, 30μl of 2% (w/v in dimethylformamide) X-Gal and 20μl of 100mM IPTG were also spread over the surface of the plate to enable blue-white colour selection of colonies containing recombinant plasmids.

2.5.1.4 Small Scale Preparation of Plasmid DNA

2.5.1.4.1 Plasmid DNA minipreps

A modification of the method described by Ish-Horowicz and Burke (1981) was used to prepare amounts of plasmid DNA up to 10μg. Bacteria were grown overnight at 37°C in 5ml LB supplemented with ampicillin to a final concentration of 100μg/ml. 1.5ml of the culture was transferred to a microfuge tube, and the cells pelleted by centrifugation for 5 minutes. The pellet was resuspended in 200μl of solution P1, and the cells lysed by addition of 200μl of solution P2 with 5 minutes incubation at room temperature. 200μl of cold solution P3 was added, with gentle mixing, and the tube incubated on ice for 15 minutes. Precipitated chromosomal DNA, SDS and protein
were sedimented by centrifugation for 30 minutes at 4°C, and the supernatant, 
containing the plasmid DNA, removed to a fresh tube. Plasmid DNA was 
precipitated by the addition of 1ml isopropanol. Following 15 minutes 
centrifugation, the nucleic acid pellet was washed with 70% (v/v) ethanol, air dried, 
resuspended in 20μl H2O and stored at -20°C.

2.5.1.4.2 Promega minipreps

The Promega Wizard™ Plus Minipreps DNA Purification System was used to 
prepare DNA for automated sequencing. Procedures detailed in the manufacturer’s 
handbook were followed, an initial culture volume of 5ml was used and 50μl H2O 
was used to elute the final DNA sample.

2.5.1.5 Large Scale Preparation of Plasmid DNA

For general stocks of plasmid DNA, the Hybaid kit was used. The CsCl2 method and 
Qiagen kit were used to prepare plasmid DNA for microinjection of Drosophila 
embryos.

2.5.1.5.1 CsCl2 Method

A bacterial colony, containing the plasmid of interest, was picked using a sterile 
toothpick and inoculated into 2ml LB media with 100μg/ml ampicillin and incubated 
at 37°C for 5 hours with shaking. The 2ml culture was transferred into 300ml LB 
media with 100μg/ml ampicillin and incubated at 37°C overnight with shaking. The 
cells were pelleted by centrifugation at 6,000 rpm for 10 minutes, and the pellet 
resuspended in 4ml TGE buffer by vortexing. 8ml 0.2N NaOH/1% SDS (made fresh) 
was added and incubated on ice for 15 minutes, then 6ml 3M potassium acetate pH 
5.0 was added and incubated on ice for 15 minutes. The sample was then centrifuged
at 13,000 rpm for 10 minutes and the supernatant transferred to a new tube. Plasmid DNA was precipitated by the addition of 10ml isopropanol, incubation at room temperature for 10 minutes and then centrifuging at 13,000 rpm for 10 minutes. The DNA pellet was dissolved in 8.6ml TE buffer at 4°C overnight.

To perform the CsCl₂ purification, 8.6g CsCl₂ and 520μl ethidium bromide (10mg/ml stock) was added at room temperature and centrifuged at 5,000 rpm to remove any protein-EtBr complexes that had formed. The supernatant was transferred to a Ti50 ultracentrifuge tube, and all tubes were balanced carefully and sealed before centrifuging at 45,000 rpm for at least 36 hours in an ultracentrifuge. Following centrifugation, the band of DNA in the tube was visualised with UV light and removed using a needle and syringe (after puncturing the top of the tube with another needle, to give air exchange). The DNA was extracted with water saturated butanol to remove the ethidium bromide, an equal volume of butanol was added, mixed with the DNA and then removed. This process was repeated until all of the ethidium bromide had been removed and the DNA solution was colourless. The volume of the sample was made up to 5ml with TE buffer, and the DNA precipitated by the addition of 13ml 100% ethanol. If the sample volume was larger than 5ml, sodium acetate was added with the ethanol to raise the salt concentration. The DNA was pelleted by centrifugation at 5,000rpm for 5 minutes, the DNA pellet was washed with 70% ethanol and dried at 37°C for 5 minutes. The DNA was then dissolved in 0.5ml TE buffer.

To prepare the DNA for microinjection, the DNA was extracted twice with phenol-chloroform and once with chloroform only, then precipitated with 50μl sodium acetate and 350μl isopropanol and centrifuged at 13,000 rpm for 10 minutes. The DNA pellet was washed with 70% ethanol and dried at 37°C for 5 minutes. Finally the DNA was resuspended in 250μl buffer.
2.5.1.5.2 QIAGEN QIAfilter™ Plasmid Midi Kit

The QIAGEN QIAfilter™ Plasmid Midi Kit was found to give an acceptable transformation frequency when used for P-element mediated transformation, and had the advantage of taking very little time to perform compared with the standard CsCl₂ method. Phenol-chloroform extraction, prior to use in microinjection procedures, was also found to be unnecessary. Procedures detailed in the manufacturer’s handbook were followed with the exception of the initial culture volume used; instead of the recommended 25ml culture, a volume of 15ml was used to ensure that the column was not overloaded and DNA purity would be optimal.

2.5.1.5.3 HYBAID RECOVERY™ Plasmid Midi Prep Kit

The HYBAID RECOVERY™ Plasmid Midi Prep Kit was used for general plasmid preparations. The protocol detailed in the manufacturer’s handbook was followed and usually the DNA in the final step was eluted with 250μl H₂O and stored at -20°C.

2.5.2 Manipulation and Detection of Nucleic Acids

2.5.2.1 Estimation of Plasmid DNA Yields

DNA samples were diluted in 1ml H₂O and the absorbance(A) at 260 and 280nm was measured in a spectrophotometer. Double stranded DNA of concentration 50μg/ml has an A₂₆₀ = 1.0. The ratio A₂₆₀/A₂₈₀ gives an estimate of nucleic acid purity. For DNA, a value of around 1.8 indicates that the preparation is not significantly contaminated with protein or phenol. Estimates of DNA concentrations
were also obtained by visual comparison of DNA samples to a known quantity of λDNA on an agarose gel stained with ethidium bromide.

### 2.5.2.2 Phenol-Chloroform Extraction

Phenol-chloroform extraction is used to remove protein from DNA solutions. Phenol was equilibrated with Tris-HCl pH 8 as described by Sambrook et al. (1989) then mixed in a ratio 25:24:1, phenol : chloroform : iso-amyl alcohol respectively, and stored at 4°C. DNA was extracted by adding an equal volume of phenol-chloroform and mixed by vortexing, or by gentle inversions if the DNA fragments were large. The two phases were separated by centrifugation at 12,000 xg for 5 minutes, and the upper, aqueous phase was removed to a fresh tube. If necessary, the extraction was repeated, and to increase recovery, the organic phase could be back-extracted with a small volume of water or TE buffer. A final extraction with an equal volume of chloroform was performed to ensure complete removal of phenol.

### 2.5.2.3 Precipitation of DNA

DNA was precipitated by adding 0.1 volume of 3M sodium acetate, pH 5.2, and 2 volumes of 100% ethanol. The sample was then incubated at -20°C for at least 30 minutes, and the DNA pelleted by centrifugation at 12,000 xg for 15 minutes, or 30 minutes for small fragments. The supernatant was carefully removed and the pellet washed with 200μl 70% ethanol, to remove any residual salts, and centrifuged at 12,000 xg for 5 minutes. The ethanol was removed and the DNA pellet air dried and dissolved in an appropriate solution and volume for further manipulation.
2.5.2.4 Restriction Endonuclease Digestion of DNA

DNA was digested with 5 units of the required endonuclease per μg of DNA, using buffer and temperature conditions recommended by the manufacturer. For double digests involving enzymes with different buffer requirements, reactions were either carried out under intermediate buffer conditions, or DNA was digested in two sequential steps separated by a DNA purification procedure. Reactions were halted by addition of 10X DNA loading buffer or by heating at 65°C for 10 minutes.

2.5.2.5 Agarose Gel Electrophoresis of DNA

DNA fragments were separated on 0.8-2% (w/v) electrophoresis grade agarose gels containing 0.5μg/ml ethidium bromide and 1X TAE. DNA samples were mixed with 1/10 volume of 10X DNA loading buffer prior to loading. Gel tanks were supplied by Bioscience Services, NBL and Bio-Rad. Mini-gels were run at 50-100V for 1-2 hours and midi-gels were run at 100-150V for 1-2 hours. After electrophoresis, DNA was visualised by ultra-violet illumination.

2.5.2.6 Molecular Weight Markers

The sizes of DNA restriction fragments were determined by comparing the distance migrated in the gel to that of known molecular weight markers, a 1 kilobase ladder (1 kb ladder from Gibco BRL) was used.

2.5.2.7 Recovery of DNA from Agarose Gels

Two methods were used to purify DNA fragments from agarose gels: the QIAGEN QIAquick and QIAEX II kits. The protocols detailed in the manufacturer’s
handbooks were followed for each procedure. Usually DNA was eluted in 20µl H₂O with the QIAEX II kit and 30µl H₂O with the QIAquick kit.

2.5.2.8 Ligation of DNA Molecules

2.5.2.8.1 Dephosphorylation

Calf intestinal alkaline phosphatase (CIAP) was used to dephosphorylate linearised plasmid vectors, prior to insert ligation, to prevent vector recircularisation and increase the frequency of recombinant plasmids produced. Approximately 1µg of DNA, in 90µl H₂O, was mixed with 10µl of 10X CIAP buffer and 1 unit of CIAP. The reaction mix was incubated for 30 minutes at 37°C, following which the DNA was purified to remove all traces of the enzyme.

2.5.2.8.2 Creation of blunt ends

Klenow Polymerase was used to convert a 5' overhang to a blunt end terminus. Approximately 1µg of DNA was incubated in 1X Klenow buffer containing 40µM of each dNTP and 1 unit of Klenow Polymerase, in a total reaction volume of 50µl. The reaction mix was incubated for 30 minutes at room temperature, following which the DNA was purified to remove all traces of the enzyme.

2.5.2.8.3 Ligation

After the vector and insert DNA had been prepared for ligation, the concentration of each was estimated by agarose gel electrophoresis along with DNA standards of
known concentration. The vector and insert DNA ratio used was 1:3 molar ratio of vector: insert, this was calculated according to the following equation:

$$\text{ng of vector} \times \frac{\text{kb size of insert}}{\text{kb size of vector}} \times \frac{\text{molar ratio of insert}}{\text{vector}} = \text{ng of insert}$$

Ligation reactions were set up with 100ng vector DNA and the appropriate amount of insert DNA in a total volume of 10μl, also containing 1μl 10x Ligase Buffer and 1u T4 DNA Ligase (Weiss units). The reactions were incubated at 18°C overnight, then used to transform competent cells of a suitable bacterial host strain.

### 2.5.2.9 Polymerase Chain Reaction

Ready-To-Go™ PCR beads (Pharmacia Biotech) were used for PCR reactions. When brought to a final volume of 25μl, each reaction contained ~1.5 units of Taq, 10mM Tris-HCl, 50mM KCl, 1.5mM MgCl₂, 200μM of each dNTP and stabilisers, including BSA. Approximately 100ng of DNA was used per reaction, along with 1ng of each primer and sterile distilled H₂O was added to a final volume of 25μl. PCR reactions were overlaid with a drop of mineral oil if the thermal cycler did not have a heated lid. Cycle conditions varied for different primer pairs, but denaturation was generally carried out at 95°C for 1 minute, followed by 1 minute at an appropriate annealing temperature, and synthesis at 72°C for 1 to 2 minutes. 30-35 cycles were carried out for most PCR reactions. All experiments were carried out using an Omnigene thermal cycler (Hybaid Ltd.).
2.5.2.10 DNA Sequencing

Two methods were used for DNA sequencing; initially manual sequencing was performed, until automated sequencing facilities became available. All sequencing was carried out using the cloning vector pBluescript (Stratagene) and the P-element transformation vector pERI. The T3 and T7 primers were used for pBluescript sequencing, and the primers Adh and White were used for pERI sequencing.

2.5.2.10.1 Manual Sequencing

The dideoxy chain termination method of Sanger et al. (1977) was used in the form of a Sequenase™ Version 2.0 kit (United States Biochemical). Double stranded DNA was sequenced according to the kit manual, labelling the sequenced DNA with $\alpha^{35}$S dATP. The sequencing reactions were run on a 6% polyacrylamide gel in TBE buffer containing 7M urea, with TBE as electrophoresis buffer. Gels were electrophoresed at a constant power setting of 40W for 40cm gel size for the required length of time. The gels were transferred to filter paper and dried under vacuum for 1-2 hours at 80°C. The dried gels were exposed to X-ray film at room temperature for 1-3 days depending on the amount of radioactive signal present.

2.5.2.10.2 Automated Sequencing

DNA sequencing was performed using an ABI PRISM dye terminator cycle sequencing reaction ready kit (Perkin-Elmer Corporation), on an Omnigene thermal cycler (Hybaid Ltd.) and extension products separated on an ABI PRISM 377 DNA sequencer (Perkin-Elmer Corporation). Approximately 0.4μg of double stranded DNA template was mixed with 3.2pmoles of an appropriate oligonucleotide primer and 8μl of terminator ready reaction mix in a
final volume of 20μl. The mixture was overlaid with a drop of mineral oil, and subjected to thermal cycling as follows: 96°C for 30 seconds, 50°C for 15 seconds, 60°C for 4 minutes, repeated for 25 cycles. Extension products were purified away from unincorporated terminators by addition of 2.0μl 3M sodium acetate pH 4.8, 50μl absolute ethanol, and incubation on ice for 10 minutes followed by 15 minutes centrifugation. The resulting pellet was washed with 70% (v/v) ethanol, air dried, and stored dry at 4°C prior to electrophoresis.

2.5.2.11 Sequence analysis

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

2.5.3 Manipulations of Drosophila Stocks

2.5.3.1 Establishment of Transgenic Lines by P-Element Transformation

P-element mediated germ line transformation (Rubin and Spradling, 1982) of wr Drosophila embryos was performed with DNA prepared by either the CsCl2 method or the QIAGEN QIAfilter method. The transformation construct to be tested was mixed with the helper plasmid pΔ2-3 at a ratio of 4:1, then ethanol precipitated and resuspended in injection buffer (5mM KCl, 0.1mM NaPO4 pH6.8) to give a final concentration of 400μg/ml of transformation construct and 100μg/ml of helper
plasmid. The DNA was centrifuged for 1 minute in a microfuge to spin down any debris, taken up into a microloader (Eppendorf) and then loaded into an injection needle (Eppendorf). The needle was then mounted in the micromanipulator and stored under Kel-F oil when not in use to prevent drying.

Embryos were collected every 45-60 minutes from 4-10 day old wr flies kept in a population cage containing a detachable apple juice plate to allow easy removal of newly laid embryos. Embryos were dechorionated manually by gently rolling across double sided adhesive tape (Scotch brand 3M No.666) mounted on a microscope slide. The dechorionated embryos were lined up on the edge of a coverslip, on a narrow strip of tape, with their posterior ends positioned just over the edge of the coverslip. The embryos were desiccated by leaving the microscope slide under the light of the microscope for 5-7 minutes (depending on the humidity and temperature of the room, and how long it took to dechorionate them). The embryos were then covered with Kel-F halocarbon oil and mounted on the stage of the micromanipulator. DNA was carefully injected into the posterior pole of preblastoderm embryos prior to pole cell formation, or stage 1 and 2 embryos (Bownes, 1975). Embryos at later stages of development were destroyed by tearing the vitelline membrane or injecting a fatally large quantity of DNA. Injected embryos were incubated on tomato juice plates with a small spot of yeast paste at 25°C for 24-36 hours, and larvae collected as they emerged. Larvae were placed in vials of fly food and incubated at 25°C. When adult flies eclosed, they were individually mated with 2-3 wr virgins and the progeny examined for a red eye phenotype. Individual transformants were crossed with 2-3 wr virgins and homozygous lines established by sibling matings with the progeny from these crosses. Stably transformed lines were used for histochemical staining.

2.5.3.2 Histochemical Staining of Transformed Drosophila

Flies were dissected in Ringer's Solution and stained for β-galactosidase activity overnight at 4°C in 100μl of X-gal Staining Buffer. β-galactosidase activity
represents lacZ reporter gene expression, indicated by blue colouration appearing over several hours.

2.5.3.3 Extraction of Haemolymph Samples from Drosophila

Glass needles were pulled from 25μl Drummond glass microcapillaries using a vertical pipette puller (Model 700C, David Kopf Instruments, Tujunga, California). Flies were lightly etherised and attached by their wings to a microscope slide using double sided adhesive tape. The haemolymph was collected with a glass needle through the throat of each fly and pooled in Ringer’s solution incubated on ice, then stored at -20°C, until ready for SDS-PAGE analysis (section 2.5.4.7).

2.5.4 Protein Expression and Purification

The methods for protein expression and purification have been adapted from Erdman and Burtis (1993).

2.5.4.1 Expression

A single colony of E. coli BL21 (DE3) cells containing a recombinant pGEX plasmid was used to inoculate 50ml LB media with 100μg/ml carbenicillin. This culture was incubated overnight at 37°C with shaking, and subsequently used to inoculate 450ml LB media containing 100μg/ml carbenicillin, in a 2 litre flask. The culture was grown for 1 hour at 37°C with shaking, and was then induced with 0.1mM isopropyl-β-D-thio-galactopyranoside (IPTG) for 4 hours, after which the cells were harvested by centrifugation at 5000 rpm for 5 minutes. A control culture of E. coli BL21 (DE3) cells without a pGEX plasmid was produced at the same time.
2.5.4.2 Preparation of glutathione-agarose

Approximately 1g glutathione-agarose beads were put into a 15ml tube and washed 3X with 10ml PBS, using 1 minute spins at 1000 rpm in a centrifuge to collect the beads at the bottom of the tube. Then the glutathione-agarose beads were resuspended in an equal volume of PBS and left to hydrate and swell overnight at 4°C. Then the beads were equilibrated in Z-100 buffer and finally resuspended in an equal volume of Z-100, to give 50% glutathione-agarose beads.

2.5.4.3 Purification

Harvested cells were resuspended in 20ml cold Z-100 buffer containing lysozyme and incubated on ice for 15 minutes. The cell suspension was sonicated on ice for 2 minutes and the lysate centrifuged at 10000 rpm for 10 minutes at 4°C. The supernatant was removed to a 15ml tube and 1ml 50% glutathione-agarose beads were added and incubated at 4°C with gentle shaking for 30 minutes, to bind the GST-fusion protein to the beads. After washing twice with 10ml cold Z-100, proteins were eluted from the beads twice in 2ml cold Z-100, 10mM reduced glutathione, 5mM NaOH, and stored at 4°C until dialysed.

The control extract supernatant was stored at 4°C after lysate centrifugation, without undergoing GST purification. 200µl samples were taken at each stage to allow the purification stages to be monitored.
2.5.4.4 Dialysis

2.5.4.4.1 Preparation of Dialysis Tubing

Dialysis tubing was prepared as described by Sambrook et al. (1989), the tubing was cut into pieces of convenient length, approximately 10-20cm. The pieces of dialysis tubing were boiled for 10 minutes in a large volume of 2% (w/v) sodium bicarbonate 1mM EDTA (pH 8.0), and then rinsed thoroughly in distilled water. The tubing was boiled for a further 10 minutes in 1mM EDTA (pH 8.0) and then allowed to cool and stored at 4°C, with the tubing submerged in the solution. Before use, the tubing was washed inside and out, and boiled briefly for 5 minutes in distilled water.

2.5.4.4.2 Dialysis

Purified GST-fusion protein was pipetted into a piece of dialysis tubing and sealed with plastic clips, leaving room for possible expansion of the sample during dialysis. The tubing was suspended over a 2 litre beaker containing 1.8 litres of Z-50 or PBS buffer at 4°C, using a glass rod and elastic band, so that the entire piece of tubing was submerged. The protein sample was dialysed overnight at 4°C with gentle stirring. This was repeated the next day with fresh Z-50 or PBS buffer for at least 6 hours, then the sample was removed from the tubing, and stored at -20°C.

2.5.4.5 Estimation of Protein Concentration

Protein concentrations were estimated using the Bradford method (Bradford, 1976). The Bradford method depends on quantitating the binding of a dye, Coomassie

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

Duplicate amounts of 0.5mg/ml BSA (5, 10, 15 and 20μl) were measured into microfuge tubes and made up to 100μl with 0.15M NaCl. Two blank tubes contained 100μl 0.15M NaCl. 1ml Coomassie Brilliant Blue solution was added to each tube and vortexed, then allowed to stand 2 minutes at room temperature. The A_595 was measured using a 1cm pathlength cuvette and a standard curve constructed by plotting absorbance at 595nm versus protein concentration. The standard curve can then be used to determine the protein concentration of the unknown sample, by determining the unknown absorbance. This method can be used to quantify 1 to 10μg of protein.

2.5.4.6 Concentration of Protein Samples

The purified GST-fusion protein samples were often not concentrated enough for use in assays and for antibody production. The samples were concentrated using an Ultrafree-4 centrifugal filter unit (Millipore), the sample was pipetted into the unit and then placed in a 15ml disposable centrifuge tube. This was centrifuged at 5,000 rpm at 4°C until the sample occupied the desired volume, then the sample was recovered from the unit with a pipette and stored at -20°C.

2.5.4.7 SDS-Polyacrylamide Gel Electrophoresis of Proteins (Laemmli, 1970)

Protein gel plates were washed and assembled according to the manufacturer's instructions. The separation mix was poured between the plates, overlaid with a small volume of H_2O and allowed to set for approximately 30 minutes. The H_2O was poured off and stacking gel mix was poured between the plates before the comb was inserted in the top of the gel. When set, the gel was fixed into the gel tank (supplied
by ATTO) and 1X running buffer was poured into the reservoirs in the tank. Protein samples and Drosophila tissues used for electrophoresis were mixed with an equal volume of 2X sample buffer, these samples were boiled for 10 minutes and briefly centrifuged before loading onto the gel. 15μl pre-stained markers (low range, supplied by Bio-Rad) were boiled for 3 minutes prior to loading on the gel. Electrophoresis was generally carried out overnight at 35-50V.

After electrophoresis gels were fixed in 12% (w/v) TCA for 1 hour at room temperature, and stained using the ISS Pro-Blue Staining System. Initially the gel was incubated in 100ml pretreatment solution (Reagent A diluted 1:5 with deionised water) for 1 hour at room temperature. The gel was then incubated in stain solution (16ml Reagent A, 64ml H2O, 1.6ml Reagent B and 20ml methanol) for 3-16 hours at room temperature. The gel was destained with 25% methanol at room temperature, with shaking, to obtain a clear background.

2.5.5 Antibody Production and Immunostaining

2.5.5.1 Protein Preparation for Injection

Protein was expressed and purified as described in section 2.5.4. The initial purified protein sample was in Z-50 buffer, which is not suitable for injection into animals. The protein sample was dialysed into PBS, and the resulting concentration of the sample was measured. The protein sample was concentrated using an Ultrafree-4 centrifugal filter unit (Millipore) until the protein concentration was approximately 1μg/μl. A sample of the protein was checked by SDS-PAGE prior to injection into mice. The research adjuvant TiterMax®Gold was used to provoke an immune response to the antigen in mice. A mouse can be injected with a total volume of 100μl, and the antigen was mixed with adjuvant at a 50:50 ratio, the two components were mixed by vortexing until an emulsion formed.
2.5.5.2 Antibody Production in Mice

Five MF1 mice were anaesthetised with Halothane and each bled from the eye (10 drops of blood) to give a sample of pre-immune serum. The mice were then injected with 100\(\mu\)l of a stable emulsion of antigen-TiterMax\(^{\text{R}}\)Gold, which contained 50\(\mu\)l of antigen (1\(\mu\)g/\(\mu\)l protein) and 50\(\mu\)l TiterMax\(^{\text{R}}\)Gold. The injections were given subcutaneously at the base of the tail. Primary bleeds were taken after 4 weeks by bleeding from the eye and a boost injection of antigen (50\(\mu\)l of 1\(\mu\)g/\(\mu\)l protein) in PBS was given after 6 weeks. Secondary bleeds were taken after a further 3 weeks and the mice were bled out after another 16 weeks. All the mouse handling and serum preparation was performed by Kay Samuel (ICAPB, Edinburgh).

2.5.5.3 Western Blotting

Proteins were transferred from gels onto Hybond C-super membrane by electroblotting using a Trans-Blot SD semi-dry transfer cell (Bio-Rad). The gel was equilibrated in transfer buffer for 10 minutes. Three pieces of blotting paper, soaked in transfer buffer, were placed on the bottom electrode plate of the unit, one at a time ensuring no air bubbles remained trapped between layers. On top of this was laid the membrane, the gel, and three further pieces of blotting paper soaked in transfer buffer, again checking for air bubbles. The other electrode plate was positioned on top to complete the circuit, and proteins were transferred at 15V for 3 hours. In order to confirm that complete transfer had occurred, the membrane was stained in Ponceau S solution for 2 minutes and the transfer checked, then destained with H\(_2\)O.
2.5.5.4 Immunostaining

Two different methods were used for immunodetection depending upon the sensitivity required; the ECL method gives much greater sensitivity than the HRP detection method.

2.5.5.4.1 HRP Method for Immunodetection

Following Ponceau S destaining the Western filter was incubated for 1 hour at 37°C in 15ml 3% milk (fat free powdered milk dissolved in 1X milk buffer), in a plastic bag, with shaking. The milk solution acts as a blocking reagent to minimise nonspecific binding of antibodies during subsequent stages of immunodetection. The filter was then placed in a fresh bag and incubated in 15ml 3% milk, containing 1° antibody, for 2 hours at 37°C with shaking. The 1° antibody was removed and stored at 4°C for future use. The filter was washed 5x 5 minutes in 1X milk buffer at room temperature with shaking. The filter was then placed in a fresh bag and incubated in 15ml 3% milk, containing 2° antibody (horseradish peroxidase, HRP, conjugated), for 1 hour at 37°C with shaking. The 2° antibody was discarded and the filter was washed 5x 5 minutes in 1X milk buffer at room temperature with shaking. The filter was placed in developing solution and incubated at room temperature in the dark, with shaking. When the staining had reached the desired intensity on the filter, the filter was washed with H₂O, dried, and stored in the dark to prevent the stain from fading. It is not possible to reprobe filters with this detection method.
2.5.5.4.2 ECL Method for Immunodetection

Following Ponceau S destaining the Western filter was incubated overnight at 4°C (or 1 hour at room temperature with shaking) in 5% blocking reagent (fat free powdered milk) in PBS-T. The filter was rinsed twice in PBS-T, washed once for 15 minutes and twice for 5 minutes with PBS-T at room temperature, with shaking. The filter was placed in a plastic bag and incubated with 1° antibody, diluted in PBS-T, for 1 hour at room temperature. The filter was rinsed twice in PBS-T, washed once for 15 minutes and twice for 5 minutes with PBS-T at room temperature, with shaking. The filter was then placed in a fresh bag and incubated with 2° antibody (HRP conjugated), for 1 hour at room temperature with shaking. The 2° antibody was discarded and the filter was rinsed twice in PBS-T, washed once for 15 minutes and four times for 5 minutes with PBS-T at room temperature, with shaking. For the antibody detection solution, equal volumes of solutions 1 and 2 were mixed, in sufficient volumes to cover the filter. The filter was drained of PBS-T and placed protein side facing up on a piece of SaranWrap, the detection solution was added to cover the entire surface of the filter and incubated for 1 minute at room temperature without agitation. Excess detection solution was drained from the filter, and the filter was wrapped in SaranWrap, any air bubbles were smoothed out. The filter was placed protein side up in a film cassette and exposed to film (in the dark) for 15 seconds and the film developed. Depending upon the intensity of the signal the filter was exposed to film until the desired exposure was achieved. It is possible to reprobe filters several times with this detection method.
2.5.6 Gel Retardation Assays

2.5.6.1 Radioactive End-labelling of Probe

200ng of digested, gel-purified DNA fragment was incubated in a total volume of 30μl with 3μl 10x Klenow Buffer (supplied with Klenow enzyme), 3μl 20mM nucleotide solution (dATP, dGTP and dTTP), 2μl (20μCi) α-32P[dCTP] and 1μl of *E. coli* DNA polymerase I Klenow fragment (5u/μl). The labelling reaction was allowed to proceed for 30 minutes at room temperature, after which 5μl 5mM dCTP was added and the reaction incubated for a further 30 minutes at room temperature. The reaction volume was made up to 50μl with TE buffer, and the radiolabelled fragments separated from unincorporated nucleotides by purification through a Sephadex G-50 NICK™ Column (Pharmacia Biotech). The column was rinsed and equilibrated with 3ml TE buffer, and the labelled DNA was added to the column. 400μl TE buffer was then added and passed through the column, and a further 400μl TE buffer was used to elute the labelled DNA from the column. The 400μl eluted probe was ethanol precipitated overnight at -20°C and resuspended in 20μl TE buffer to give a probe concentration of approximately 10ng/μl and 10-30cps/μl radioactivity.

2.5.6.2 DNA-binding Reactions

GST-DSX binding domain protein (approximately 2μg protein) was incubated in a total volume of 20μl with 4μl 5x Z-50 buffer, 10ng 32P end-labelled DNA probe and varying amounts of specific or non-specific (poly[dIdC]) competitor DNA. The binding reactions were incubated at room temperature for 30 minutes, then 5μl 5x gel shift loading buffer was added and the samples immediately loaded onto nondenaturing polyacrylamide gels for electrophoresis.

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2.5.6.3 Nondenaturing Polyacrylamide Gel Electrophoresis of Protein-DNA Binding Reactions

1mm, 4% nondenaturing high ionic strength polyacrylamide gels were pre-run, in high ionic strength electrophoresis buffer, for 1-2 hours at 150V. The entire volume of the binding reactions were loaded onto the gels and run at 150V. The length of electrophoresis depended upon the size of the probe, ~200bp probes were run for 2 hours and ~400bp probes for 3 hours. Following electrophoresis the gels were transferred onto dampened blotting paper and dried under vacuum at 80°C for 1-2 hours. The dried gels were either autoradiographed at -20°C or exposed in a phosphorimager cassette overnight, or longer if required.

2.5.6.4 Autoradiography

Radioactive signals from dried DNA sequencing gels and dried protein gels were detected using blue sensitive X-ray film (Genetic Research Instrumentation) and cassettes with intensifying screens at -70°C for $^{32}$P, and room temperature for $^{35}$S. The same X-ray film and cassettes were used for ECL immunodetection. Films were developed in an X-ograph CompactX2 automatic film processor.

2.5.6.5 Phosphorimagery

Phosphorimagery was performed using a storage phosphor screen (Molecular Dynamics). After exposure of the screen, signals were visualised through a Molecular Dynamics phosphorimager, and analysed with ImageQuant software.

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2.5.7 In situ Hybridisation to Drosophila ovaries

2.5.7.1 Preparation of digoxygenin labelled DNA

2.5.7.1.1 DNA probes

150ng DNA, dissolved in 19μl water, was denatured by boiling in a waterbath for 5 minutes and snap frozen in a dry-ice/ethanol bath. While still frozen, 2.5μl hexanucleotides and 2.5μl DIG DNA labelling mix (Boehringer Mannheim) was added. Once thawed, 1μl (1 unit) Klenow polymerase was added and the reaction incubated at 37°C overnight. The reaction was stopped by adding 1μl 0.5M EDTA, and the labelled DNA was precipitated by adding 2μl 4M LiCl and 60μl prechilled 100% ethanol, incubated at -70°C for at least 30 minutes and centrifuging at 17,000 rpm for 15 minutes at 4°C. The DNA pellet was washed with 70% ethanol, air dried and resuspended in 150μl hybris. Immediately prior to use, the labelled DNA probe was denatured by boiling for 5 minutes and snap freezing in a dry-ice/ethanol bath.

2.5.7.1.2 PCR probes

DNA probes labelled with the PCR DIG labelling mix (Boehringer Mannheim) can give increased sensitivity compared to the standard DNA probes. This is particularly useful for low copy transcripts where there is a risk of high background.

The PCR conditions for the PCR DIG labelling mix are the same compared to conditions established for a defined primer/template pair. The reaction components, shown in Table 2.7, were added to a sterile, microcentrifuge tube at 4°C:
Table 2-7 Reaction mixes for PCR DIG probes

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
<th>Final concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile, deionised water</td>
<td>variable</td>
<td>-</td>
</tr>
<tr>
<td>PCR buffer, 10x conc., 15mM MgCl₂</td>
<td>10μl</td>
<td>1.5mM MgCl₂</td>
</tr>
<tr>
<td>PCR DIG labelling mix</td>
<td>10μl</td>
<td>200μM dNTP</td>
</tr>
<tr>
<td>Primer 1</td>
<td>1μl</td>
<td>0.1-1μM</td>
</tr>
<tr>
<td>Primer 2</td>
<td>1μl</td>
<td>0.1-1μM</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.2-1μl</td>
<td>1-5U/100μl</td>
</tr>
<tr>
<td>Template DNA</td>
<td>variable</td>
<td>variable</td>
</tr>
<tr>
<td>Total</td>
<td>100μl</td>
<td></td>
</tr>
</tbody>
</table>

2.5.7.2 In situ Hybridisation

Ovaries from female OrR Drosophila were dissected in Ringer’s solution and transferred into 1ml pp (fix) and incubated at room temperature for 1-1.5 hours with gentle mixing (on a whirly wheel). The ovaries were washed 3x 10 minutes in PBT to remove the fix, then incubated in 9:1, methanol:EGTA (0.5M, pH 8) for 10 minutes, and washed again 3x 10 minutes in PBT. This was followed by proteinase K (Sigma, 100μg/ml in PBT) treatment for 1 hour at 18°C, which was stopped by 2x 5 minute washes in PBT. The ovaries were then post-fixed for 20 minutes in pp and washed 6x 5 minutes with PBT.

The ovaries were equilibrated for 10 minutes in 200μl 1:1, PBT:hybrix and prehybridised with 100μl hybrix for at least 1 hour at 45°C (in a heating block). The prehybridisation step can be extended to reduce background if necessary. Following this, the ovaries were hybridised with the digoxygenin labelled probe, dissolved in hybrix, at 45°C overnight.
After hybridisation, the ovaries were washed (with pre-warmed wash solutions) 2x 20 minutes at 45°C with 500µl hybrix, then 3x 20 minutes at 45°C with 500µl 1:1, PBT:hybrix, 3x 20 minutes at 45°C with 500µl PBT and finally 5x 5 minutes at room temperature with PBT and gentle shaking. Signals were detected by incubation with 1ml Alkaline phosphatase (AP) conjugated anti-digoxygenin antibody (antibody diluted 1:1000 in PBT, and preabsorbed before use with post-fixed ovaries overnight at 4°C) at room temperature for 1.5 hours. Excess antibody was removed by washing the ovaries 3x 20 minutes in PBT with gentle shaking. If necessary the ovaries were stored overnight at 4°C before developing.

The ovaries were equilibrated by washing 3x 5 minutes in NMTT (made fresh). The ovaries were incubated in the dark in 1ml NMTT containing 3.5µl X-phosphate and 4.5µl NBT. The signal was developed for 15 minutes to several hours, depending on the staining intensity. The colour reaction was stopped by 3 rinses in PBT, the ovaries were mounted and observed under the microscope.

Unless stated otherwise the steps were carried out at room temperature.
CHAPTER 3

RESULTS

Regulation of yp3 in the fat body
3.1 INTRODUCTION

Although yp1 and yp2 gene expression has been investigated in some detail, this is not the case for yp3. Due to the expression patterns of yp1, yp2 and yp3 being so highly co-ordinated it is likely that the factors involved in the transcriptional regulation of all three yp genes are very similar. A 419bp DNA fragment, termed FBE3, located 5' of the transcription start site of Drosophila yp3, was found to direct lacZ expression, in the fat body of females, but not males (Ronaldson and Bownes, 1995). This fragment was used for further investigation: to identify trans-acting factors binding to the FBE3, to narrow the size of the region down and to consider its transcriptional regulation of yp3.

3.2 ANALYSIS OF CIS-ACTING FRAGMENTS REQUIRED FOR FEMALE-, FAT BODY-SPECIFIC EXPRESSION

3.2.1 The pERI Transformation vector

DNA fragments were cloned into the transformation vector pERI; a map of the vector is shown in Figure 3.1 and construction of the vector is reported in Ronaldson and Bownes (1995). The pERI vector is a modification of the pCaSpeR-AUG-βgal vector and contains a Drosophila hsp70 promoter fragment. This allows fragments lacking the yp3 promoter to be tested for their effects on reporter gene activity. Unique restriction sites in pERI for cloning constructs to be tested were EcoRI and XhoI positioned 5' of the hsp70 promoter, and BamHI positioned 3' of the promoter. A diagram of the pERI transformation vector is shown in Figure 3.1.
Figure 3.1

Structure of the pERI transformation vector. The pCaSpeR-AUG-βgal vector was modified to contain a 282bp hsp70 promoter fragment. Unique cloning sites are EcoRI (E) and XhoI (Xh) upstream of the hsp70 sequence, and BamHI (B) downstream of the hsp70 promoter. The size of the vector is 12.5kb. The vector construction is detailed in Ronaldson and Bownes, 1995.

Unshaded boxes at the HindIII sites (H) indicate P-element ends. The white gene is transcribed in the opposite direction to the lacZ reporter gene, arrows show the direction of transcription. A trailer region derived from SV40 provides the lacZ polyadenylation signal.
3.2.2 Construction and Analysis of the Reporter Constructs

The yp3 regions tested for the presence of enhancer elements are illustrated in Figure 3.2. A pBluescript (Stratagene) plasmid, containing the 419bp yp3 upstream fragment found to direct \textit{lacZ} expression in the fat body of females, was used to generate fragments for subcloning. The 419bp DNA fragment, termed FBE3, had been cloned into the \textit{EcoRI} site in pBluescript by the addition of \textit{EcoRI} linkers, named yp3/9 (E. Ronaldson, personal communication).

A 246bp subfragment from FBE3, spanning nucleotides -528 to -282, was isolated using the polymerase chain reaction (PCR), and this PCR product had been designed to give \textit{EcoRI} restriction sites at each end. The PCR product was digested with \textit{EcoRI} and cloned into pERI cut with \textit{EcoRI} and CIAP treated (to prevent vector religation). This allowed the 246bp fragment to be cloned in both forward and reverse orientations, which were verified by sequencing. The sequence of the PCR product was also checked to confirm that no mutations had been introduced by the PCR reaction. Construct A was the 246bp PCR fragment in the forward orientation and construct B the reverse orientation. Constructs C and D contained a smaller upstream region of 177bp (-704 to -527), in forward and reverse orientations respectively. The 177bp fragment was isolated by a \textit{EcoRI} and Sau3AI digestion, then cloned into pBluescript cut with \textit{EcoRI} and \textit{BamHI}. The fragment was excised with \textit{XbaI} and \textit{XhoI}, Klenow treated to create blunt ends and cloned into pERI, which had been cut with \textit{EcoRI}, Klenow treated to create blunt ends and CIAP treated. Constructs E and F were created by digesting yp3/9 with RsaI to yield a blunt ended 250bp fragment, which was cloned into pBluescript cut with \textit{EcoRV}. The fragment was then excised by digestion with \textit{EcoRI} and cloned into pERI, which had been cut with \textit{EcoRI} and CIAP treated. This generated constructs with the inserts in both orientations, however, when sequenced the inserts were found to have varying lengths; some were 206bp and others 190bp in length. Single copies of the fragment in pERI were only found in the forward orientation for the 206bp insert (construct E) and the reverse orientation for the 190bp insert (construct F). It is not known why the procedure
Figure 3.2

A summary of the \( yp3 \) constructs tested for transcription enhancer elements regulating \( yp3 \) fat body expression. At the top is a schematic diagram of the \( yp3 \) gene showing the 419bp region found to be capable of directing female- and fat body-specific \( yp3 \) expression.

The hatched boxes represent introns within the \( yp3 \) coding sequence. Transcripts and their direction of transcription are represented by arrows. The sizes (in bp) of the \( yp3 \) fragments used in reporter constructs are indicated below the constructs, the nucleotide positions in relation to the \( yp3 \) transcription start site and the orientation of the fragments are indicated above.
ATG

-704  419bp  -285
SspI  Asp700

yp3

-528  -282  hsp70-lacZ
246bp

-282  -528  hsp70-lacZ
246bp

-704  -527  hsp70-lacZ
177bp

-527  -704  hsp70-lacZ
177bp

-704  -498  hsp70-lacZ
206bp

-514  -704  hsp70-lacZ
190bp
generated fragments of unequal length. All constructs were sequenced to check orientation, position and size, a summary of this information is shown in Table 3.1.

Table 3.1 Summary of constructs used to analyse yp3 fat body regulation

<table>
<thead>
<tr>
<th>Name</th>
<th>Size (bp)</th>
<th>Position relative to yp3 start site</th>
<th>Orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Start</td>
<td>Finish</td>
</tr>
<tr>
<td>A</td>
<td>246bp</td>
<td>-528</td>
<td>-282</td>
</tr>
<tr>
<td>B</td>
<td>246bp</td>
<td>-528</td>
<td>-282</td>
</tr>
<tr>
<td>C</td>
<td>177bp</td>
<td>-704</td>
<td>-527</td>
</tr>
<tr>
<td>D</td>
<td>177bp</td>
<td>-704</td>
<td>-527</td>
</tr>
<tr>
<td>E</td>
<td>206bp</td>
<td>-704</td>
<td>-498</td>
</tr>
<tr>
<td>F</td>
<td>190bp</td>
<td>-704</td>
<td>-514</td>
</tr>
</tbody>
</table>

All constructs were introduced into the Drosophila germline using standard transformation techniques (Rubin and Spradling, 1982), detailed in section 2.5.3.1. As many independently transformed lines as possible were assayed for each construct tested, a summary of the number of lines produced for each construct is shown in Table 3.2. Male and female fat bodies were dissected and used for histochemical staining to determine the ability of each construct for yp3 regulation. Reporter gene expression was detected by blue colouration produced by β-galactosidase activity and the effects of each construct were analysed. All body parts were used for staining to compare position effects between constructs (discussed in section 1.4). Because the transformed constructs were not used for quantitative assays, merely the presence or absence of β-galactosidase activity, the transformed lines were not analysed for the copy number of constructs they contained. If the transformed lines were used for quantitative assays in the future, the construct copy numbers would be analysed and single copy inserts used.
Table 3.2 The number of transformed lines obtained for each construct tested

<table>
<thead>
<tr>
<th>Construct</th>
<th>Number of transformed lines obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
</tr>
<tr>
<td>C</td>
<td>12</td>
</tr>
<tr>
<td>D</td>
<td>15</td>
</tr>
<tr>
<td>E</td>
<td>2</td>
</tr>
<tr>
<td>F</td>
<td>1</td>
</tr>
</tbody>
</table>

The 419bp FBE3 was found to direct reporter gene expression in a sex- and tissue-specific pattern; to the female fat body. Flies transformed with constructs containing the different fragments derived from the 419bp FBE3, were stained for β-galactosidase activity to identify which fragments were capable of directing this pattern of reporter gene expression. Table 3.3 shows a summary of the reporter gene expression patterns found in the fat body cells of each transformed line analysed.

Constructs A and B, containing the 246bp fragment in forward and reverse orientations, were found to be unable to direct reporter gene expression in the female fat body, shown in Figure 3.3.

Constructs C and D, containing the 177bp fragment in forward and reverse orientations, were found to give two different classes of transformant, these results are shown in Figure 3.4. In some lines the 177bp fragment was found to direct reporter gene expression in the fat body cells, but this was not limited to females only; both male and female fat bodies showed staining (Figure 3.4A and B). In transformed lines that showed reporter gene expression, staining was always in both sexes. Other lines showed no staining in the fat bodies of either sex, shown in Figure 3.4C and D.

Constructs E and F were created to extend the length of the 177bp fragment to determine if consistent female-fat body-specific expression could be resumed,
Photographs illustrating the \( \beta \)-galactosidase activity observed in *Drosophila* fat body cells transformed with constructs A and B containing 246bp of \( yp3 \) upstream sequence. Reporter gene expression, and thus \( \beta \)-galactosidase activity, is shown when tissues are stained with X-Gal and produces a blue colouration.

X-Gal stained female abdomen (A) and male abdomen (B) of line (SH3)2D4B, typical of lines transformed with constructs A and B, showing an absence of staining or reporter gene activity.

Constructs A and B are shown in diagram C, below the photographs.
C

-704
SstI
419bp
Asp700

+1

yp3

246bp

hsp70-lacZ

A

-528
-282

246bp

hsp70-lacZ

B

-282
-528

246bp

hsp70-lacZ
Figure 3.4

Photographs illustrating the β-galactosidase activity observed in *Drosophila* fat body cells transformed with constructs C and D containing 177bp of *yp3* upstream sequence.

Line (SH8)1D5A, transformed with the 177bp fragment, shows reporter gene expression in the fat bodies of females (A) and also males (B). Other independently transformed lines containing the 177bp fragment also showed the same expression pattern, but there were also lines that did not show reporter gene expression in either female (C) or male fat bodies (D), shown by line (SH8)51-2 in this example.

Expression in flies transformed with constructs C and D either had reporter gene expression in both sexes or neither sex.

Constructs C and D are shown in diagram E, below the photographs.
because there was a possibility that the isolation of the 177bp fragment could have disrupted any binding sites located at the 3' end, around position -527.

Of the two fly lines transformed with construct E, containing a 206bp fragment, one line showed reporter gene expression in the fat bodies of both males and females, and the other line gave no staining in the fat body of either sex (Figure 3.5). Only one transformed line was created containing construct F (190bp fragment) which showed reporter gene expression in the fat bodies of both males and females (Figure 3.6). The addition of 29bp and 13bp to the 177bp fragment does not appear to be sufficient to regain sex-specificity of the reporter gene expression.

**Table 3.3 Summary of the β-galactosidase staining patterns obtained with the transformed lines analysed**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Prefix code</th>
<th>Line numbers</th>
<th>Staining in fat body cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>SH3</td>
<td>1D2A, 2D4A, 2D4B, 2D4C, 3D5A</td>
<td>no</td>
</tr>
<tr>
<td>B</td>
<td>SH4</td>
<td>1D5A, 1D5B, 1D5C, 1D5D, 1D5E, 1D5F</td>
<td>no</td>
</tr>
<tr>
<td>C</td>
<td>SH8</td>
<td>1D4A, 48-2, 51-2, 51-4, 1D5A, 1D5B, 1D5C, 1D5D, 3D5A, 1-1, 26-1, 48-1</td>
<td>yes, in males and females</td>
</tr>
<tr>
<td>D</td>
<td>SH7</td>
<td>2-1, 5-3, 10-1, 10-6, 12-6, 44-1, 42-1, 3-1, 4-1, 5-1, 10-4, 11-1, 12-2, 18-2, 34-1</td>
<td>yes, in males and females</td>
</tr>
<tr>
<td>E</td>
<td>r5</td>
<td>2-1</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1-1</td>
<td>yes, in males and females</td>
</tr>
<tr>
<td>F</td>
<td>r2</td>
<td>1-1</td>
<td>yes, in males and females</td>
</tr>
</tbody>
</table>
Figure 3.5

Photographs illustrating the β-galactosidase activity observed in *Drosophila* fat body cells transformed with construct E containing 206bp of *yp3* upstream sequence.

Only two independently transformed *Drosophila* lines were obtained with construct E. Line (r5)1-1 showed reporter gene expression, and thus β-galactosidase activity, in the fat bodies of females (A) and also males (B). The other independently transformed line containing the 206bp fragment, line (r5)2-1, did not show reporter gene expression in either female (C) or male fat bodies (D).

Expression in flies transformed with construct E either had reporter gene expression in both sexes or neither sex.

Construct E is shown in diagram E, below the photographs.
Figure 3.6

Photographs illustrating the β-galactosidase activity observed in *Drosophila* fat body cells transformed with construct F containing 190bp of *yp3* upstream sequence.

Only one transformed *Drosophila* line was obtained with construct F. This line (r2)1-1 showed reporter gene expression in the fat body cells of both males and females.

Construct F is shown in diagram C, below the photographs.
3.2.3 Conclusion

A 246bp fragment, spanning nucleotides -528 to -282, was unable to direct reporter gene expression in the female fat body when transformed into flies.

A 177bp fragment, spanning nucleotides -704 to -527, was found to be capable of directing reporter gene expression in fat body cells, but expression was not sex-limited and occurred in both males and females, or neither sex.

Increasing the 177bp fragment to 206bp did not restore the reporter gene expression in the fat body to females only, expression was either in both sexes or not at all, and the one transformed line containing a 190bp fragment gave staining in both sexes.

3.3 DOUBLESEX GEL RETARDATION ASSAYS

Sex determination in Drosophila involves a hierarchy of regulatory genes, the expression of which depends upon the ratio of X chromosomes to autosomes. The doublesex gene is the final gene in the regulatory pathway and encodes both male-specific and female-specific polypeptides, whose synthesis is regulated by alternative sex-specific splicing of the primary dsx transcript. The only target genes shown to be under direct control of the sex determination hierarchy are the yolk protein genes, specifically yp1 and yp2 (Burtis et al., 1991).

The male-specific and female-specific forms of the doublesex polypeptide, DSXM and DSXF respectively, have amino acid sequences common to both. They have identical N-termini of 397 amino acids which contain a zinc finger-related DNA binding domain (Erdman and Burtis, 1993) and it has been established that the DSX proteins can act as DNA binding transcription factors. The C-termini are different due to the alternative splicing of the dsx transcript and are either 152aa (DSXM) or 30aa (DSXF).

Both DSXM and DSXF are able to bind in vitro to the 125bp FBE of yp1 and yp2 (Burtis et al., 1991). The DSX proteins bind to four sites within the FBE. DSXM represses and DSXF activates transcription from the two strongest binding sites.
Genetic evidence shows that the DSX proteins confer the sex-specificity of the yolk protein gene expression such that there is expression in the fat body of females, but not males.

One of the aims of this project was to determine if the DSX proteins could bind to the 419bp FBE3 found to direct lacZ expression in the fat body of females, and smaller subfragments of this region.

The DNA-protein binding assay is a sensitive method for the detection of sequence-specific DNA-binding proteins. Proteins that bind specifically to a radioactive end-labelled DNA fragment retard the mobility of the fragment during polyacrylamide gel electrophoresis, the positions of which can be viewed on an autoradiograph of the gel.

### 3.3.1 Preparation of DSX protein extracts

A pGEX T7 expression vector containing the N-terminal DNA binding domain common to both DSX\textsuperscript{m} and DSX\textsuperscript{f} proteins (amino acids 1-397) was used to express this domain as a glutathione-S-transferase (GST) fusion protein, for use in gel retardation assays. The vector, named pGEX-KG, was a gift from K. Burtis (University of California). The method for DSX DNA binding domain protein expression and purification was adapted from Erdman and Burtis (1993), and is detailed in section 2.5.4, materials and methods. The purified GST-DSX was dialysed in Z-50 buffer for use in the gel retardation assays. An example of the protein purification stages run on SDS-PAGE is shown in Figure 5.1 in Chapter 5.

### 3.3.2 Gel retardation assays with 419bp FBE3

Gel retardation assays were carried out with the 419bp FBE3 and purified DSX DNA binding domain protein extracts. The 419bp fragment was excised from yp3/9 by an XbaI and XhoI restriction digest and end-labelled with \(^{32}\text{P}\), using Klenow to
make a probe and purified prior to use. The experimental procedures used for the gel retardation assays were modified from Burtis et al., 1991.

Preliminary experiments were carried out to determine the levels of non-specific competitor DNA polydIdC required to eliminate excess binding of protein to the probe. An example of these results for the 419bp FBE3 is shown in Figure 3.7A. In the gel retardation assays with the FBE3, 10ng of probe and ~2μg purified DSX were used in each reaction, and the amount of polydIdC varied between 100ng and 2μg, lanes 3 to 7. A control reaction was included that contained a protein extract from E. coli transformed with the pGEX vector without the dsx insert. The reactions were incubated at room temperature for 30 minutes and under these binding conditions, the presence of polydIdC eliminated any non-specific binding to give a mobility shift of the FBE3 with the DSX binding domain protein. 250ng polydIdC was enough to give a clear gel shift band, the position of which is shown by an arrow in Figure 3.7A, and further increases in polydIdC did not appear to have any further effect.

To demonstrate the specificity of interactions between the FBE3 and the DSX binding domain protein, gel retardation assays were performed including a synthetic 29bp competitor oligonucleotide, containing the highest affinity binding site for the DSX protein, “dsx A” in the FBE (An and Wensink, 1995a). The sequence of this oligonucleotide is GTG CAC AAC TAC AAT GTT GCA ATC AGC GG. The reactions were based on those above, but reactions other than the free probe contained 200ng polydIdC. An example of these results is shown in Figure 3.7B. The 419bp FBE3 does not shift with the E. coli control extract, lane 2, but will shift with DSX in the presence of 2μg and 10μg polydIdC, molar excesses of 2900X and 14500X respectively, lanes 3 and 4 (gel shift position marked with an arrow). Competition by the “dsx A” oligonucleotide, at molar excesses of 2900X and 14500X, led to the disappearance of the protein-DNA complexes, lanes 5 and 6, confirming the presence of specific DSX binding sites within the 419bp FBE3 fragment.

Sometimes extra bands could be seen in the end labelled probes, it is not known exactly what these bands are, but they could be more than one fragment joined
Figure 3.7A
Autoradiograph of gel retardation assays with 419bp FBE3.
Lane 1 represents free probe; unbound DNA. A control was performed using an *E. coli* cell extract, lane 2. The probe was incubated with DSX binding domain protein and increasing amounts of polydIdC, lanes 3 to 7. The gel shift position is marked with an arrow.

The components of each reaction (buffers and reaction volumes are detailed in section 2.5.6) are shown in the following table:

<table>
<thead>
<tr>
<th>Lane</th>
<th>Probe</th>
<th>Amount of DSX protein</th>
<th>Amount of <em>E. coli</em> extract</th>
<th>polydIdC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10ng</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>10ng</td>
<td>-</td>
<td>2μg</td>
<td>250ng</td>
</tr>
<tr>
<td>3</td>
<td>10ng</td>
<td>2μg</td>
<td>-</td>
<td>100ng</td>
</tr>
<tr>
<td>4</td>
<td>10ng</td>
<td>2μg</td>
<td>-</td>
<td>250ng</td>
</tr>
<tr>
<td>5</td>
<td>10ng</td>
<td>2μg</td>
<td>-</td>
<td>500ng</td>
</tr>
<tr>
<td>6</td>
<td>10ng</td>
<td>2μg</td>
<td>-</td>
<td>1μg</td>
</tr>
<tr>
<td>7</td>
<td>10ng</td>
<td>2μg</td>
<td>-</td>
<td>2μg</td>
</tr>
</tbody>
</table>

Figure 3.7B
Autoradiograph of gel retardation assays with 419bp FBE3.
Lane 1 represents free probe; unbound DNA. A control was performed using an *E. coli* cell extract, lane 2. Lanes 3 and 4 contained increasing amounts of non-specific competitor, polydIdC. Lanes 5 and 6 contained increasing amounts of specific competitor, the synthetic oligonucleotide for the DSX binding site, “dsxA”. The gel shift position is marked with an arrow.

The components of each reaction (buffers and reaction volumes are detailed in section 2.5.6) are shown in the following table:

<table>
<thead>
<tr>
<th>Lane</th>
<th>Probe</th>
<th>Amount of DSX protein</th>
<th>Amount of <em>E. coli</em> extract</th>
<th>polydIdC</th>
<th>Synthetic Oligonucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10ng</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>10ng</td>
<td>-</td>
<td>2μg</td>
<td>2μg</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>10ng</td>
<td>2μg</td>
<td>-</td>
<td>2μg</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>10ng</td>
<td>2μg</td>
<td>-</td>
<td>10μg</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>10ng</td>
<td>2μg</td>
<td>-</td>
<td>2μg</td>
<td>2μg</td>
</tr>
<tr>
<td>6</td>
<td>10ng</td>
<td>2μg</td>
<td>-</td>
<td>2μg</td>
<td>10μg</td>
</tr>
</tbody>
</table>
together, possibly created during the labelling reaction. In Figure 3.7B, the free probe (lane 1) has an extra band which is larger than the 419bp fragment, this fragment also appears to bind DSX protein and forms two DNA-protein complexes, located at the top of the gel in lanes 3 and 4. These complexes are also competed away by the addition of “dsxA” oligonucleotide, lanes 5 and 6. If the extra fragment is a multiple of the 419bp fragment it will also have more than one DSX binding site, and the presence of two DNA-protein complexes could be due to differing numbers of DSX protein molecules binding to this fragment.

3.3.3 Gel retardation assays with 177bp and 250bp FBE3 subfragments

The 419bp FBE3 was divided into two smaller fragments; a 177bp fragment (-704 to -527) and a 246bp fragment (-528 to -282), these are also the same fragments tested for their yp3 enhancer capabilities in section 3.2.2. These fragments were cloned into pBluescript and excised for use as gel shift probes with an XbaI and XhoI restriction digest.

Reaction conditions used in the gel retardation assays with the FBE3 were used again for the smaller subfragments, 10ng of probe and ~2µg purified DSX were used in each reaction. Preliminary experiments with the 177bp fragment showed a gel shift with the DSX binding domain protein, marked with an arrow in Figure 3.8A, which remained as the level of polydIdC was increased from 100ng to 2µg, lanes 3-8. Lane 2 is a control reaction with an E. coli protein extract that does not contain the dsx insert, and thus does not give a gel shift.

The gel shift was shown to be a specific interaction between the 177bp fragment and the DSX binding domain by specific competition with the “dsx A” synthetic oligonucleotide. An example of these results is shown in Figure 3.8B. The gel shift was competed away with the addition of the “dsx A” oligonucleotide, at molar excesses of 1200X and 6100X, lanes 5 and 6, thus confirming the 177bp fragment contains a specific DSX binding site. The addition of non-specific competitor,
Figure 3.8A
 Autoradiograph of gel retardation assays with 177bp fragment of FBE3. Lane 1 represents free probe; unbound DNA. A control was performed using an *E. coli* cell extract, lane 2. The probe was incubated with DSX binding domain protein and increasing amounts of polydIdC, lanes 3 to 8. The gel shift position is marked with an arrow.

The components of each reaction (buffers and reaction volumes are detailed in section 2.5.6) are shown in the following table:

<table>
<thead>
<tr>
<th>Lane</th>
<th>Probe</th>
<th>Amount of DSX protein</th>
<th>Amount of <em>E. coli</em> extract</th>
<th>polydIdC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10ng</td>
<td>-</td>
<td>-</td>
<td>250ng</td>
</tr>
<tr>
<td>2</td>
<td>10ng</td>
<td>-</td>
<td>2µg</td>
<td>100ng</td>
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<tr>
<td>3</td>
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<td>-</td>
<td>500ng</td>
</tr>
<tr>
<td>4</td>
<td>10ng</td>
<td>2µg</td>
<td>-</td>
<td>1µg</td>
</tr>
<tr>
<td>5</td>
<td>10ng</td>
<td>2µg</td>
<td>-</td>
<td>1.5µg</td>
</tr>
<tr>
<td>6</td>
<td>10ng</td>
<td>2µg</td>
<td>-</td>
<td>2µg</td>
</tr>
</tbody>
</table>

Figure 3.8B
 Autoradiograph of gel retardation assays with 177bp fragment of FBE3. Lane 1 represents free probe; unbound DNA. A control was performed using an *E. coli* cell extract, lane 2. Lanes 3 and 4 contained increasing amounts of non-specific competitor, polydIdC. Lanes 5 and 6 contained increasing amounts of specific competitor, the synthetic oligonucleotide for the DSX binding site, “dsxA”. The gel shift position is marked with an arrow.

The components of each reaction (buffers and reaction volumes are detailed in section 2.5.6) are shown in the following table:

<table>
<thead>
<tr>
<th>Lane</th>
<th>Probe</th>
<th>Amount of DSX protein</th>
<th>Amount of <em>E. coli</em> extract</th>
<th>polydIdC</th>
<th>Synthetic Oligonucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10ng</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>10ng</td>
<td>-</td>
<td>2µg</td>
<td>2µg</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>10ng</td>
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</tr>
<tr>
<td>4</td>
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<td>2µg</td>
<td>-</td>
<td>10µg</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>10ng</td>
<td>2µg</td>
<td>-</td>
<td>2µg</td>
<td>2µg</td>
</tr>
<tr>
<td>6</td>
<td>10ng</td>
<td>2µg</td>
<td>-</td>
<td>2µg</td>
<td>10µg</td>
</tr>
</tbody>
</table>
A  non-specific competitor  

B  specific competitor  

pdIdC  

synthetic oligonucleotide  

1  2  3  4  5  6  

1  2  3  4  5  6
polydIdC, to molar excesses of 1200X and 6100X had no effect upon the DNA-protein complexes, shown in lanes 3 and 4.

Preliminary experiments with the 246bp fragment revealed that it does not appear to have DSX binding capabilities. Reactions contained 10ng of probe and ~2μg purified DSX, and the amount of polydIdC varied between 100ng and 2μg. The results for this experiment are shown in Figure 3.8C. As the amount of polydIdC increases non-specific protein binding is eliminated, but there is no specific binding occurring with DSX to give a gel shift. With the 419bp FBE3 and the 177bp fragment, 250ng polydIdC was sufficient to show a clear gel shift, for the 246bp fragment, 250ng polydIdC gives a smeared lane on the gel (lane 4) as a result of non-specific DNA-protein binding.

3.3.4 Conclusion

The 419bp FBE3 was found to be capable of forming a specific complex with DSX binding domain protein in vitro. This 419bp fragment was divided into two smaller subfragments of 177bp and 246bp. The 177bp fragment was also found to bind specifically to the DSX binding domain protein in gel retardation assays. The 246bp fragment was not capable of binding to DSX binding domain protein in vitro.

These results indicate that the 419bp FBE3 contains a DSX binding site and this binding site is located between positions -704 to -527.

It should also be noted that there may be more than one DSX binding site located within the 177bp fragment, considering that three sites were found in the FBE of yp1 and yp2. However, if there are multiple sites, they all appear to bind to the same number of DSX molecules in the assays because multiple gel shift bands are not observed for single copies of the 419bp and 177bp fragments.
Figure 3.8C

Autoradiograph of gel retardation assays with 246p fragment of FBE3. Lane 1 represents free probe; unbound DNA. A control was performed using an *E. coli* cell extract, lane 2. The probe was incubated with DSX binding domain protein and increasing amounts of polydIdC, lanes 3 to 8.

The components of each reaction (buffers and reaction volumes are detailed in section 2.5.6) are shown in the following table:

<table>
<thead>
<tr>
<th>Lane</th>
<th>Probe</th>
<th>Amount of DSX protein</th>
<th>Amount of <em>E. coli</em> extract</th>
<th>polydIdC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10ng</td>
<td>-</td>
<td>-</td>
<td>250ng</td>
</tr>
<tr>
<td>2</td>
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</tr>
<tr>
<td>8</td>
<td>10ng</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
3.4 THE EFFECT OF C/EBP MUTANTS ON YP PROTEIN EXPRESSION

The 125bp FBE from the intergenic region of yp1 and yp2 has been used in footprinting assays to identify transcriptional regulatory proteins implicated in the control of fat body-specific yp gene expression (Abel et al., 1992; Falb and Maniatis, 1992). One of these was the CCAAT/enhancer-binding protein (C/EBP) from rats, which was found to have an overlapping recognition site with the adult enhancer factor-1 (AEF-1), which has been isolated from Drosophila. They are thought to bind competitively creating a possible activation (C/EBP)/repression (AEF-1) mechanism, since AEF-1 can inhibit binding of C/EBP and thereby repress its activating potential (Falb and Maniatis, 1992).

The Drosophila homologue of C/EBP (DmC/EBP) has since been identified and is encoded by the slow border cells (slbo) gene (Montell et al., 1992). The slbo gene encodes a CCAAT/enhancer-binding protein, a basic region-leucine zipper transcription factor, which is a member of the bZIP family of proteins. The slbo gene was identified as a result of a screen for P-element insertion mutations that specifically blocked border cell migration during oogenesis. Deletion of the slbo gene results in late embryo lethality; embryos that lack C/EBP die just before or upon hatching. P-element insertion in slbo delays border cell migration, but all other aspects of oogenesis appear normal and the observed female sterility is caused by eggs remaining unfertilised. The micropyles of these slbo eggs appeared to lack a pore canal and their tips were defective and misshapen, resulting in the slbo eggs being unfertilised (Rorth and Montell, 1992).

Mutations in the slbo gene had been produced by P-element insertion and also by the subsequent excision of these P-elements (Montell et al., 1992), and these mutants were used in this project to investigate their effect on YP protein expression. Specifically to determine if Drosophila C/EBP is the bZIP protein responsible for activating YP protein expression in the female fat body, by comparing the YP protein levels between heterozygote and homozygote slbo mutants.
Two *slbo* mutants were used:

l(2)slbo⁷ᵇ  
slbo deletion, homozygous lethal

fs(2)slbo⁹ᵇ  
rosy+ insertion, female sterile

Heterozygous virgins, with the curly wing marker (CyO), were collected for each mutant and crosses set up between opposite sexes of each line.

cross:  
l(2)slbo⁷ᵇ  
CyO

X

fs(2)slbo⁹ᵇ  
CyO

trans-heterozygotes:  
l(2)slbo⁷ᵇ  
fs(2)slbo⁹ᵇ

The progeny from these crosses were collected daily and aged for three days, for vitellogenesis to be occurring, before haemolymph samples were taken. The haemolymph contains YPs synthesised in the fat body and thus can be used to assess the levels of Yps produced. Samples were taken from heterozygous males, heterozygous females and trans-heterozygous females (straight wing phenotype). Approximately 20-30 flies were used for each sample, the haemolymph was pooled in 25µl Ringer’s solution on ice, then stored at -20°C. The protein concentration of each sample was measured using the Bradford assay, then approximately 8µg of protein for each sample was used for SDS-PAGE analysis. Proteins were visualised using the ISS Pro-Blue staining system, an example of which is shown in Figure 3.9. These protein gels were scanned using a Shimadzu dual-wavelength chromato scanner (CS-930), the three YP protein bands plus two other bands in each sample were scanned to obtain values for the percentage of the sample each protein occupied. The percentage of YP proteins were then compared between heterozygote and trans-
Figure 3.9

Polyacrylamide gel stained with ISS Pro-Blue. Haemolymph samples were taken from heterozygous \textit{slbo} males (lane 1), heterozygous \textit{slbo} females (lane 2) and homozygous \textit{slbo} females (lane 3). Each lane contained approximately 8\(\mu\)g of protein. The positions of the YPs are indicated with arrows.
heterozygote \textit{slbo} mutants. This experiment was repeated three times to obtain average values for the YP percentages and the 95\% confidence limits were calculated:

\begin{align*}
\text{\textit{slbo} heterozygous mutants} & \quad 65.3 \pm 7.75 \\
\text{\textit{slbo} trans-heterozygous mutants} & \quad 72.9 \pm 2.88
\end{align*}

This indicates that the 95\% confidence limits overlap between the two samples and thus the true means for each sample may not be significantly different, this was also confirmed by a t-test.

\subsection*{3.4.1 Conclusion}

Trans-heterozygote mutants in the \textit{slbo} gene (\textit{Drosophila} C/EBP) were not found to have significantly different levels of the YPs in their haemolymph when compared to heterozygote \textit{slbo} mutants. These results indicate that \textit{Drosophila} C/EBP is unlikely to be an activator of \textit{yp} expression in the female fat body. It would be expected that the loss of an activator of the \textit{yps} would decrease transcriptional activation resulting in less YP protein expression, but this observation was not made. However, it should be noted that this experiment has limitations to its interpretation because the degree of penetrance for each of the \textit{slbo} mutants is not known and there are also steps within the procedure at which variability may occur.
3.5 IDENTIFICATION OF PUTATIVE TRANSCRIPTION FACTOR BINDING SITES

Since the \( yps \) are coordinately transcribed, it is likely that the mechanisms and factors involved will be the same for all three genes. The footprint sequences identified for AEF-1, C/EBP and DSX in the FBE of \( yp1 \) and \( yp2 \) were used in computer searches to determine their putative binding sites in the FBE3 sequence, comparisons were also made between the \( yp1 \) and \( yp2 \) FBE and the FBE3 of \( yp3 \). The Bestfit and Gap search programs (University of Wisconsin Computer Genetics Group (GCG) software, Devereux et al, 1984) were used to identify regions of sequence homology. The results of the searches are shown in Figure 3.10A. The best sequence similarity was found to be with the AEF-1 and C/EBP overlapping footprint contained within the 125bp FBE (Falb and Maniatis, 1992), the \( yp3 \) sequence has 75% identity to a 24 nucleotide footprint sequence, including a gap of 4 nucleotides. If the gap is excluded the similarity is 90% between the matched sequences. The \( yp3 \) region -504 to -523 containing this homology is present in the 206bp \( yp3 \) fragment in construct E, but is not contained within the 177bp fragment in constructs C and D. Figure 3.11 shows how the regions of sequence similarity are located relative to constructs A to F. A 9bp DSX consensus binding site sequence (reverse complement) was found to give a 7 nucleotide match to a region of \( yp3 \), shown in Figure 3.10B. This putative DSX binding site is located within the 177bp \( yp3 \) fragment that was found to gel shift with the DSX binding domain, this was also the nearest site to the overlapping AEF-1 and C/EBP region of homology (Figure 3.11). However, many putative DSX binding sites are possible because the consensus sequence is short and several \( yp3 \) regions show homologies with the computer search.
Figure 3.10A

Region of the 419bp yp3 FBE3 with sequence similarity to the AEF-1 and C/EBP binding sites located in the FBE of yp1 and yp2. The vertical lines represent nucleotide identities, and the degree of similarity between the two sequences is also indicated.

Figure 3.10B

The computer sequence homology in the 419bp yp3 FBE3 to a 9bp DSX consensus binding site sequence (reverse complement).

Nucleotide positions are given in relation to the transcription start site of the yp3 gene.
### A

**yp1/2 FBE**

<table>
<thead>
<tr>
<th>-311</th>
<th>AEF-1</th>
<th>C/EBP</th>
<th>-288</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGCACA</td>
<td>AACTAC</td>
<td>AATGTTGCAATCA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>18/24nt</td>
</tr>
<tr>
<td>TGCACAAGT</td>
<td>TGTTC</td>
<td>CAATCA</td>
<td></td>
</tr>
<tr>
<td>-523</td>
<td></td>
<td></td>
<td>-504</td>
</tr>
</tbody>
</table>

**yp3 FBE**

### B

**yp3 FBE**

<table>
<thead>
<tr>
<th>-556</th>
<th>-548</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAACAA</td>
<td>ACT</td>
</tr>
<tr>
<td></td>
<td>7/9nt</td>
</tr>
<tr>
<td>CTACA</td>
<td>AAGT</td>
</tr>
<tr>
<td>1</td>
<td>9</td>
</tr>
</tbody>
</table>

DSX consensus binding site sequence
Figure 3.11

Diagram to show the location of sequence similarities in the 419bp yp3 FBE3 to transcription factor binding sites previously identified within the yp1 and yp2 FBE.

The locations of these sequence similarities are shown in relation to constructs A to F.
Constructs A and B

246bp
-528 -285

Constructs C and D

177bp
-704 -527

Construct E

206bp
-704 -498

Construct F

190bp
-704 -514
3.6 SUMMARY AND DISCUSSION

The 419bp FBE3 of yp3 was subdivided into smaller fragments and the following observations were made:

A 246bp fragment, spanning nucleotides -528 to -282, was unable to direct reporter gene expression in the female fat body when transformed into flies, and did not give a gel shift with DSX binding domain protein.

A 177bp fragment, spanning nucleotides -704 to -527, was found to be capable of directing reporter gene expression in fat body cells, but expression was not sex-limited and occurred in both males and females, or neither sex. This 177bp fragment was found to bind specifically to the DSX binding domain protein in gel retardation assays.

Increasing the 177bp fragment to 206bp did not restore the reporter gene expression in the fat body to females only, expression was either in both sexes or not at all. The 29bp addition to the 177bp fragment (to give 206bp) contained a region of homology, identified by computer searches, to the footprints of AEF-1 and C/EBP for the FBE of yp1 and yp2. The one transformed line containing a 190bp fragment gave staining in both sexes.

The role of Drosophila C/EBP as an activator of yp expression was investigated. Trans-heterozygote mutants in the slbo gene (Drosophila C/EBP) were not found to have significantly different levels of the YPs in their haemolymph when compared to heterozygote slbo mutants.

It is useful to compare these results to the work that has already been performed with the FBE of yp1 and yp2 (previously detailed in Chapter 1); it has always been predicted that all three yolk protein genes would share similar, if not identical, regulatory mechanisms.
A model was proposed for the regulation of \( yp1/2 \) in the female fat body (An and Wensink, 1995b). A small enhancer, \( o-r \), located in the intergenic region of \( yp1 \) and \( yp2 \), was found to direct female- and fat body-specific transcription in a similar pattern to the \( yp \) genes (An and Wensink, 1995a). The \( o-r \) enhancer contains four protein binding sites positioned in two enhancer elements; the \( o \) element consists of three overlapping binding sites: \( dsxA \) which binds the female (DSXF) and male (DSXM) forms of Doublesex protein, the \( aef1 \) site which binds the AEF-1 repressor and the \( bzip1 \) site which is capable of binding DmC/EBP. The \( r \) element is a single site, named \( refl \), which is postulated to bind an unidentified activator. Germline transformation experiments showed neither \( o \) nor \( r \) can activate transcription by themselves, but together in single copy they direct transcription in female fat bodies only. The \( o-r \) enhancer was manipulated to create multimeric and mutated binding sites for each of the four protein binding sites, which were used in protein binding, germline transformation and genetic experiments to examine both the independent and combined activities of the proteins and their binding sites. This led to the proposal of a model for the regulation of \( yp1/2 \) by the \( o-r \) enhancer, a summary diagram is shown in Figure 3.12.

Model for the regulation of \( yp1/2 \) by the \( o-r \) enhancer:

In female fat bodies, the DSXF and bZIP proteins activate synergistically, possibly by co-operative binding or by altering bZIP activity upon its binding to the site. The bZIP also acts cooperatively with an activator at the \( refl \) site to yield gene expression in the female fat body. DSXF also interferes with the binding of AEF-1 and prevents it repressing transcription in female fat bodies.

In male fat bodies, DSXM is present and binds to the \( dsxA \) site, and because the DSXM protein has a bulkier male-specific carboxy-terminal domain it can sterically hinder activation by bZIP protein. Alternatively, bZIP may be inactivated by DSXM, this is not shown in Figure 3.12. DSXF is not present and therefore cannot bind...
Figure 3.12

Model for transcriptional regulation by o-r.

*Top*
Activation in female fat bodies resulting from synergy among the DSX^F, bZIPa and R proteins. Curved arrows indicate activation, and the arc between bZIPa and R represents positive cooperativity. These three proteins are shown bound to their cognate sites on o-r DNA (solid lines). The AEF-1 repressor is shown unbound, excluded from its DNA site by DSX^F. Broken lines represent DNA between o-r, and the promoter (right angle arrow).

*Middle*
Repression in male fat bodies resulting from DSX^M. DSX^M is shown bound to DNA, excluding the other two proteins from binding.

*Bottom*
Inactivation in ovaries and tissue specificity. The absence of dsx proteins allows AEF-1 to bind, excluding activators from the bZIP site. The bZIPa and bZIPb proteins activate from the bzip1 site but have different tissue distributions.
FEMALE
FAT BODY

MALE
FAT BODY

OVARY

\[\text{AEF-1} \quad \text{DSX}^F \quad \text{bZIPa} \quad \text{R} \]

\[\text{aef-1} \quad \text{dsxA} \quad \text{bzip1} \quad \text{ref1} \]

\[\text{AEF-1} \quad \text{DSX}^M \quad \text{bZIPa} \quad \text{R} \]

\[\text{aef-1} \quad \text{dsxA} \quad \text{bzip1} \quad \text{ref1} \]

\[\text{AEF-1} \quad \text{bZIPb} \quad \text{R} \]

\[\text{aef-1} \quad \text{dsxA} \quad \text{bzip1} \quad \text{ref1} \]
cooperatively with the bZIP activator. The elimination of bZIP activation removes
synergy with an activator at the refl site and will not yield gene expression in the
male fat body. It is thought that AEF-1 is unlikely to repress in male fat bodies
through o-r because of steric hindrance by DSXm.

In ovaries it is proposed that due to the absence of DSX proteins, the AEF-1
repressor binds and excludes activators from the bZIP site. The bZIPa and bZIPb
(proposed tissue-specific bZIP in the ovary) activate from the bzip1 site but have
different tissue distributions.

Some points to note about these experiments and the subsequent model:

Enhancers are expected, by definition (Serfling et al., 1985), to activate transcription
independent of orientation. In the experiments using o-r (An and Wensink, 1995a, An
and Wensink, 1995b) the constructs do not appear to have been tested in the reverse
orientation.

There also seems to be some degree of confusion about the role of bZIP activators in
the ovary. The model states that the AEF-1 repressor binds and excludes activators
from the bZIP site, and that the bZIPa and bZIPb activate from the bzip1 site but
have different tissue distributions, suggesting that bZIPb activates in ovaries. If the
AEF-1 repressor excludes activators from the bZIP site in ovaries, then surely a
bZIPb protein cannot activate from the bzip1 site? If bZIP proteins really are
activating expression in the ovary from bzip1, they do not appear to be activating yp
expression because the expression is not in the correct cells, the yps are expressed in
the follicle cells surrounding the oocyte during stages 8–10b of oogenesis (see Chapter
4). The ovarian enhancers OE1 and OE2 have been found to be necessary for yp
expression in the ovary (Logan et al., 1985 and Logan and Wensink, 1990).

Another paper has been published recently, Li and Baker (1998), describing the role
of the hermaphrodite (her) gene and dsx in the regulation of the yps. It is proposed
that they control the expression of the \( yp \) genes in the fat body by two separate pathways for the activation of the \( yps \). One pathway is the non-sex-specific activation of the \( yps \), which is HER-dependent, DSX\(^F\)-independent and inhibited by DSX\(^M\). The other pathway is the female-specific activation of the \( yps \), which is DSX\(^F\) dependent. The combination of these effects, in wild type females, results in both HER and DSX\(^F\) contributing independently to produce high level expression of the \( yps \).

In wild type males, DSX\(^M\) inhibits \( yp \) expression, which can override the activation function of HER. HER is a zinc finger protein and is thought to regulate the \( yps \) directly. The 125bp FBE of \( yp1 \) and \( yp2 \) (position -196 to -322) was not found to be sufficient to confer her responsiveness, and the major her responsive element is located outside of FBE. The her responsive region, termed HRR, is located between nucleotide positions -322 to -1225 in the intergenic region of \( yp1 \) and \( yp2 \). \( dsx \) does not confer the fat body-specific expression of the \( yps \), because in \( dsx \) mutant flies of both sexes, the expression of \( yp \)-reporter genes is still restricted to fat body cells. HER is also not considered to be necessary for fat body-specific expression of the \( yps \) because loss of her does not affect fat body-specific expression of a transgene, and the FBE-reporter construct can confer fat body-specific expression without the HRR present. An unknown fat body-specific factor, termed FBF, binding possibly to the bZIP site in the FBE is thought to confer tissue-specificity to the fat body only. The HRR is necessary for her function but is not thought to be sufficient for \( yp \) expression, it is deemed likely that the FBF is also necessary for HER to direct the expression of \( yps \). In addition to her and dsx, the intersex (ix) gene also acts in the dsx branch of the sex determination pathway. It has been observed that ix is also required for the transcriptional activation of the \( yp \) genes in females, but is not required for their repression in males. Genetic studies indicate that ix interacts with her and with dsx in regulating the \( yps \) in females (Baker, unpublished data). It is thought that ix may participate both in the HER-dependent non-sex-specific activation pathway and in the DSX\(^F\)-dependent female-specific activation pathway. It is postulated that a
candidate DNA site in the FBE through which IX might function is the “refl” site, shown to synergistically function with the DSX binding site, dsxA (An and Wensink, 1995b).

It should also be mentioned that other sexual phenotypes were found in this study to be created by strong interaction between HER and DSX^2, for example the number and morphology of the last transverse row of bristles (LTRB) of the basitarsus (LTRB form sex combs in males) and the degree of pigmentation of tergites 5 and 6 (T5 and T6). The authors also acknowledge that the her mutants used in these studies have a low level of activity, to rescue lethality, and this might complicate the results observed, as different activations may have different thresholds.

Considering now the results for the FBE3 and its subfragments, the most intriguing observation is the loss of sex-specificity in fat body expression. All previous studies to identify tissue-specific enhancers associated with the yp genes found that the sex- and tissue-specificity of expression were never separated. Another interesting point about the sex-specificity is the role of DSX protein, the 177bp fragment that showed male and female fat body expression in the germline transformation experiments was also found to bind specifically to DSX binding domain protein in gel shift assays. It is considered that the action of DSX binding activates expression in females and represses expression in males, but this does not appear to be the case in this system. These two observations suggest that regulation of the yp3 gene may not occur in exactly the same way as for yp1 and yp2; the factors involved may be the same but the details of the mechanism or organisation of the interacting binding sites could be different.

There was a possibility that the isolation of the 177bp fragment could have disrupted any binding sites located at the 3' end (around -527 position), there could have been a dsx binding site responsible for conferring sex-specificity to female expression only, for example. To resolve this situation, the 177bp fragment was extended to 190bp
and 206bp. However, transformation of these constructs into flies showed that they exhibited the same expression patterns as the 177bp fragment; reporter gene expression was either found in both male and female fat bodies or in neither sex. The 246bp fragment adjacent to the 177bp fragment was not found to bind DSX binding domain protein in vitro, and it seems unlikely that further extension of the 206bp fragment will provide extra dsx sites to confer sex-specificity. It seems likely that for yp3 another factor, in addition to DSX, is required for sex-specificity, and that its binding site is present in the 246bp subfragment of FBE3, but not between nucleotides -528 and -498.

It was also considered that extension of the 177bp fragment might have eliminated the occurrence of transformed fly lines that did not give expression in either sex. The observed expression patterns found with the 177bp fragment could be explained by an activator and/or repressor binding site being lost in the isolation of the fragment. Computer searches revealed putative AEF-1 and C/EBP (bZIP) binding sites located in the 29bp sequence extended on the 177bp fragment to create the 206bp fragment, it seemed possible that this sequence could contain the missing sites needed to restore the correct yp expression pattern in every line transformed. However, addition of these putative binding sites did not appear to have any affect upon reporter gene expression, and there were still lines that did not show any expression in either sex.

The result that expression is not switched on in every line seems likely to be a position effect, and depends upon where in the Drosophila genome the P-element construct has inserted. If the 177bp fragment alone is not sufficient to direct fat body expression due a missing activator site, certain locations of insertion in the genome may place the fragment near an activator sequence, resulting in some lines being capable of expression. It may be that the action of the external activator, in the lines that give expression, does not provide sex-specificity resulting in expression in the fat body of both sexes. The missing activator site could bind a sex-specific activator.

CHAPTER THREE: REGULATION OF YP3 IN THE FAT BODY
or one that interacts with DSX<sup>F</sup> to give activation in females only. Conversely, if the 177bp fragment alone is sufficient to direct fat body expression, but not sex-specifically, the loss of expression in some lines could be due to location of the P-element in the genome so that the fragment is affected by silencers or repressors (for a review of silencers, see Ogbourne and Antalis, 1998). Silencers have been defined as sequence elements which are capable of repressing promoter activity in an orientation- and position-independent fashion, in the context of a native or a heterologous promoter (Brand et al., 1985). This theory does not explain the loss of sex-specificity, unless the silencing effect happens in conjunction with there being a sex-specific site missing from the 177bp fragment.

Considering the model proposed for yp1/2 regulation, it may be possible that a homologue of the refl site, binding the unknown activator R, is missing from the 177bp fragment and is located in the 246bp fragment. If this is the case, in the regulation of yp3 the activator R would need to be capable of conferring sex-specific expression, possibly by interaction with DSX<sup>F</sup> or by itself, to give expression in female fat bodies only.

If the regulation of yp3 is different to that of yp1 and yp2, it could be that sex-specificity is conferred by the interaction of her, dsx and ix and the HER and/or IX binding sites are missing from the 177bp fragment.

It is interesting to note that in Musca and Calliphora, upstream yp sequences were also unable to confer sex-specific reporter gene expression in the fat body, and expression was observed in the fat body cells of both males and females (Tortiglione and Bownes, 1997). The Musca upstream fragment was also found to be capable of binding DSX protein <i>in vitro</i>, but this was not sufficient to confer sex-specificity of expression <i>in vivo</i>. These results indicated that the regulation of the yps in Musca and Calliphora is different to that of yp1 and yp2 due to divergence between the species, and it was suggested that hormonal controls might be used to achieve the sex-specific expression of the yp genes in Musca and Calliphora. The results obtained in this
project for yp3 fat body regulation are very similar to those seen for the regulation of
the yps in Musca and Calliphora. It appears that yp regulation has diverged between
yp1/yp2 and yp3, but conserved between yp3 and the yps of Musca and Calliphora.

The results obtained for yp3 also suggested that DmC/EBP, encoded by the sibo gene
is not the bZIP protein required for activation of the yp genes. The levels of YP
protein in haemolymph, a measure of YPs produced by the fat body, showed no
significant difference in trans-heterozygous sibo mutants compared to their
heterozygous siblings. It would be expected that the loss of an activator of the yps
would result in less transcriptional activation and thus less YP protein expression,
but this effect was not observed. This result is consistent with the findings that the
DmC/EBP transcript was undetectable in adult fat bodies (Rorth and Montell, 1992),
which indicates that the DmC/EBP protein is not expressed in the right cells to be a
fat body-specific gene activator.

Future work on the regulation of yp3 is needed to determine what region of the 246bp
fragment of the FBE3 is capable of restoring the sex-specificity to female fat bodies
only. It would then be interesting to find out which protein(s) bind to this site to
direct sex-specificity. The DSX proteins could be used in footprinting assays to
locate their actual binding site within the 177bp fragment. There are many candidates
for yp3 regulators: AEF-1, bZIP, HER, IX, whose involvement could be investigated,
for example by identifying footprints and mutating binding sites for these proteins.
CHAPTER 4

RESULTS

Regulation of yp3 in the ovary
4.1 INTRODUCTION

Experiments to identify enhancers governing yp3 expression revealed a 328bp fragment immediately upstream of the yp3 coding sequence was sufficient to confer an ovarian yp expression pattern (Ronaldson and Bownes, 1995). The β-galactosidase activity was detected in ovarian follicle cells during stages 8-10 of oogenesis, and was termed OE3. A 150bp PCR fragment was also produced from this region (Figure 4.1), and found to bind specifically with components of ovarian nuclear extracts in gel retardation assays (Ronaldson and Bownes, unpublished data). The 150bp fragment was created to exclude the yp3 TATA and CAAT-box promoter elements, the reason for this was to exclude the binding of their associated proteins in ovarian nuclear extracts, and thus only show gel shifts with potential DNA binding proteins involved in ovarian yp3 regulation. This 150bp fragment was also isolated because it contained four sites of conserved sequence, revealed by computer similarity searches between OE1, OE2 and OE3. These sites include a consensus sequence, reported by Tortiglione and Bownes (1997), which is present in OE1 and OE3, but is also conserved between Musca and Calliphora yp upstream regions, shown in Figure 4.2. The presence of this sequence motif in all three species suggests a potential functional role as a binding site for ovarian-specific factors.

In this project the 150bp fragment was investigated further to determine if it was sufficient to direct yp ovarian expression, and possible candidates for trans-acting factors required to regulate the correct yp3 expression pattern in the ovaries were also investigated.

4.2 YP EXPRESSION IN THE OVARY

Original investigations into the yp expression pattern in the ovary utilised a crude in situ hybridisation method, using a 3H-labelled probe, that only showed roughly when expression was occurring and that the yps were expressed in the follicle cells (Brennan et al., 1982). To obtain a clear picture of the yp gene expression pattern, in
Figure 4.1

A diagram of the *Drosophila yp3* gene illustrating the location of the 150bp subfragment of OE3 used in reporter gene assays. It can also be seen that the 150bp fragment does not contain the *yp3* TATA and CAAT-box promoter elements that are present in the 328bp OE3 fragment. The positions of the identified enhancer regions, OE3 and FBE3, are also shown. All nucleotide positions are given relative to the *yp3* transcription start site.
-1822 -704 -285 +43 +1756 +2458

150bp PCR
Figure 4.2

Sequence similarities between *Drosophila* ovarian enhancers OE1 and OE3 and sequences from *Musca* (Mupro8) and *Calliphora* (Cepro1) constructs, located 5' of *Musca Mdypa* gene and *Calliphora CeypB* gene respectively (Tortiglione and Bownes, 1997).

The vertical lines indicate nucleotide identities. Nucleotide positions of OE1 and OE3 are given relative to the transcription start sites of *yp1* and *yp3* respectively. *Calliphora* and *Musca* nucleotide positions are relative to the *CeypB* and *Mdypa* genes respectively.

Underneath the sequences, percentage similarities relative to the OE1 sequence are shown.
-1120  GATCAGCACAA  -1110  OE1
    |    |    |   |   |   |  |
-163   GATCAGCAGAA  -153   OE3
    |    |    |   |   |   |  |
-186   ATCAGCAAAA  -178   Mupro8
    |    |    |   |   |   |  |
-307   ATCATCAAAAA  -298   Cepro1

OE1
OE3        10/11 nt  90%
Musca       9/10 nt  90%
Calliphora  8/10 nt  80%
situ hybridisations to whole mount Drosophila ovaries were carried out using the yp genes as digoxygenin DNA probes. This method allows much more precise observation of the cells expressing the yp genes.

The yp clones detailed in section 2.2 were used to generate yp fragments for the synthesis of digoxygenin DNA probes. The following probes were used: an 800bp yp1 probe (isolated by a restriction digest with BamHI), a 541bp yp2 probe (isolated by a restriction digest with HindIII and BglII) and a 1500bp yp3 probe (isolated by a restriction digest with HindIII and BglII). The yp probes were used for in situ hybridisation to whole mount Drosophila ovaries, these methods are detailed in section 2.5.7.

The results show that yp gene expression is switched on at stage 8 of oogenesis in the follicle cells surrounding the egg chamber, and their expression continues until stage 10b (Figure 4.3). During stages 8-10 the oocyte undergoes rapid growth as a consequence of the uptake of yolk proteins which are synthesised in the fat body and the follicle cells. At stage 8 the follicle cells form a monolayer surrounding the entire egg chamber. During stage 9 anterior follicle cells, which overlay the nurse cells, elongate and migrate posteriorly, by stage 10a the oocyte is covered by a sheet of thick columnar follicle cells and the nurse cells have very few follicle cells associated with them. This follicle cell migration can be observed in the pattern of yp gene expression, which moves down the egg chamber as the follicle cells expressing them migrate. This expression pattern was found to be the same for all three yp genes.

The follicle cells initially covering the nurse cells, which are programmed to migrate later to cover the oocyte, have the yp genes activated in advance of being adjacent to the oocyte. This means that the signal to activate the yp gene expression in the follicle cells does not originate in the oocyte. The yp gene expression is also not in all the follicle cells, there is a clear gap in the expression pattern in the anterior follicle cells when expression is first observed. This shows a subdivision in the follicle cells that was not previously described in relation to yp gene expression in the ovary.
Figure 4.3

*In situ* hybridisation of a *yp1* DNA probe to whole mount *Drosophila* ovaries.

Expression occurs in the ovary between stages 8 and 10b, specifically in the follicle cells surrounding the egg chamber, which migrate posteriorly to cover the oocyte during stage 9.

All three *yp* genes, *yp1*, *yp2* and *yp3*, gave the same expression pattern.
In summary, the three *yp* genes, *yp1*, *yp2* and *yp3*, are all expressed in the ovary between stages 8 and 10b. Specifically, the *yp* expression occurs in the follicle cells surrounding the egg chamber, which migrate posteriorly to cover the oocyte during stage 9. These results are also useful as a comparison for reporter gene expression patterns for constructs being tested for ovarian *yp3* enhancer capabilities.

4.3 ANALYSIS OF A CIS-ACTING FRAGMENT REQUIRED FOR OVARY-SPECIFIC EXPRESSION

4.3.1 Construction and Analysis of the Reporter Constructs

The 150bp subfragment of OE3, shown in Figure 4.1, was tested to determine if it was sufficient to direct *lacZ* reporter gene expression, in the correct pattern for *yp* ovarian expression, when transformed into *Drosophila*. The 328bp OE3 fragment cloned in the pCaSpeR-AUG-βgal vector, named *yp3/5*, was used as a template to synthesise a 150bp PCR product, which spanned nucleotides -197 to -47. The 150bp fragment lacked *yp3* promoter elements and therefore required cloning into the P-element transformation vector pERI, which contains a heterologous promoter, *hsp70*. The PCR product was purified and cloned into pERI cut with EcoRI, blunt ended and CIAP treated (to prevent vector religation). This allowed the 150bp fragment to be cloned in both forward and reverse orientations, which were verified by sequencing. The sequence of the PCR product was also checked to confirm that no mutations had been introduced by the PCR reaction. Construct G was the 150bp PCR fragment in the forward orientation and construct H two copies in the reverse orientation, diagrams of these reporter constructs are shown in Figure 4.4.

 Constructs G and H were introduced into the *Drosophila* germline using standard transformation techniques (Rubin and Spradling, 1982), detailed in section 2.5.3.1. At least three independently transformed lines were assayed for each construct tested. Ovaries were dissected from female transformants and used for histochemical staining.
Figure 4.4

A diagram of the yp3 constructs tested for transcription enhancer elements regulating yp3 ovarian expression. At the top is a schematic diagram of the yp3 gene showing the 328bp OE3 region found to be capable of directing ovary-specific yp3 expression.

The hatched boxes represent introns within the yp3 coding sequence. Transcripts and their direction of transcription are represented by arrows. The sizes (in bp) of the yp3 fragments used in reporter constructs are indicated below the constructs, the nucleotide positions in relation to the yp3 transcription start site and the orientation of the fragments are indicated above.
to determine the ability of each construct to direct ovarian *yp3* regulation. Reporter gene expression was detected by blue colouration produced by β-galactosidase activity and the effects of each construct were analysed. To compare position effects between constructs all body parts were used for staining (discussed in section 1.4). Because the transformed constructs were not used for quantitative assays, merely the presence or absence of β-galactosidase activity, the transformed lines were not analysed for the copy number of constructs they contained. If the transformed lines were used for quantitative assays in the future, the construct copy numbers would be analysed and single copy inserts used.

The 328bp OЕ3 was found to direct reporter gene expression in ovarian follicle cells during stages 8 to 10b, this fragment contained *yp3* promoter sequence and was transformed using the pCaSpeR-AUG-βgal vector (Ronaldson and Bownes, 1995). This meant that the 328bp OЕ3 fragment was tested in the forward orientation only, and its ability to act as an enhancer in the reverse orientation is unknown. Constructs G and H, containing the 150bp fragment in forward and reverse orientations, were also found to be capable of directing reporter gene expression in ovarian follicle cells during stages 8 to 10b, these results are shown in Figure 4.5. The majority of transformed *Drosophila* lines containing constructs G and H showed reporter gene expression in the *yp* expression pattern, however, a few lines did not show any staining patterns in the ovary and one line showed a modified expression pattern (Figure 4.6). In this line the expression pattern did not cover the entire oocyte; there is expression in the follicle cells around the anterior and posterior of the oocyte, but there is a band of staining missing around the centre of the oocyte.
Photographs illustrating the β-galactosidase activity observed in *Drosophila* ovaries transformed with constructs G and H containing 150bp of *yp3* upstream sequence. Reporter gene expression, and thus β-galactosidase activity, is shown when tissues are stained with X-Gal and produces a blue colouration.

X-Gal stained ovaries (A) of flies transformed with constructs G and H, showing reporter gene activity. The lines used for these examples were (51A)17-1 (top) and (51B)6-1 (bottom).

Constructs G and H are shown in diagram B, below the photographs.
Figure 4.6

An example of fly line (51B)45, transformed with construct H, which shows a modification of the yp expression pattern in the ovary.

In this line the X-Gal staining pattern did not cover the entire oocyte, a band of staining was missing around the oocyte, this is thought to be due to a chromosomal position effect. This area is marked by an arrow.
In summary, it appears that the 150bp subfragment of OE3 is capable of directing reporter gene expression in ovarian follicle cells during stages 8 to 10b, and may contain all necessary sequences to be the enhancer for yp3 expression in the ovary. The staining pattern for the 150bp fragment was also identical to the pattern observed for the 328bp OE3 in reporter constructs transformed into flies (Ronaldson and Bownes, 1995).

4.4 INVESTIGATION OF TRANSCRIPTION FACTOR CANDIDATES

Very little is known about the regulatory mechanisms involved in ovarian yp expression and no transcription factors regulating yp expression in ovaries have been identified to date. It is possible that the transcription factors identified for the regulation of yp1 and yp2 in the fat body could also be responsible for yp regulation in the ovary. Two of these factors (DSXF and DSXM) are products of the doublesex gene; the doublesex gene is the final gene in the sex determination pathway and encodes both male-specific and female-specific polypeptides, whose synthesis is regulated by alternative sex-specific splicing of the primary dsx transcript. The only target genes shown to be under direct control of the sex determination hierarchy are the yolk protein genes, specifically yp1 and yp2 gene expression in the female, fat body tissues (Burtis et al., 1991). Genetic evidence has indicated that dsx is not involved in regulating yp gene expression in the ovary, and that the expression of theyps in the ovary is not under the continuous control of the sex determination hierarchy (Bownes et al., 1990). To investigate if dsx is expressed in the ovary, and to verify the genetic data, it was decided the ovarian expression pattern of dsx would be determined by in situ hybridisation. Other transcription factors regulating yp1 and yp2 in the fat body include AEF-1 and DmC/EBP (sibo) proteins, and it was decided their possible role in regulating yp expression, in the ovary, would be investigated. Transcription factors regulating tissue-specific yp gene expression in the ovary are expected to be expressed at the same developmental stages and cells as the yps, to be capable of performing this function. It was therefore decided that the expression
patterns of \textit{aef-I} and \textit{slbo} would be determined by \textit{in situ} hybridisation to investigate their potential role in ovarian \textit{yp} regulation. The \textit{aef-I} gene encodes the Adult Enhancer Factor-1 protein, AEF-1, which is thought to be a transcription factor involved in the regulation of fat body expression of the \textit{yps} and the \textit{adh} gene. The \textit{Drosophila} homologue of C/EBP (DmC/EBP) is encoded by the \textit{slow border cells (slbo)} gene (Montell et al., 1992). The \textit{slbo} gene encodes a CCAAT/enhancer-binding protein, a basic region-leucine zipper transcription factor, which is a member of the bZIP family of proteins.

DNA probes were labelled with the PCR DIG labelling mix (Boehringer Mannheim) to give increased sensitivity compared to the standard DNA digoxygenin probes, the methods are detailed in section 2.5.7.1. Initial experiments were performed using standard DNA digoxygenin probes but they gave high background staining, these problems were resolved by using the PCR labelling method. The primers T3 and T7 were used to generate PCR probes for \textit{aef-I} from the plasmid \textit{bs035}, containing the \textit{aef-I} cDNA, and also \textit{dsx} from the plasmid \textit{pGEX-KG}. The \textit{dsx} probe contained the DSX DNA binding domain encoding sequence common to both male- and female-specific transcripts. The primers CEBPRF2 and CEBPRR2 were used to synthesise a \textit{slbo} probe by PCR from the plasmid named DmC/EBP. These probes were used for \textit{in situ} hybridisations with whole mount ovaries and the results are shown in Figure 4.7. All details of plasmids, primers and methods are contained in Chapter 2.

The \textit{aef-I} gene is expressed in the nurse cells from stage 8, expression levels increase to their maximum at stage 10, and by stage 11 expression is reduced to a low level and then ceases (Figure 4.7A). It is therefore, not possible for AEF-1 protein to be directing \textit{yp} expression, in the follicle cells surrounding the oocyte, from its location in the nurse cells.

The \textit{slbo} gene is expressed in just the border cells, which are initially located at the anterior end of the egg chamber. These break free from the follicular monolayer and migrate posteriorly between the nurse cells during stage 9, the expression pattern can
Figure 4.7

*In situ* hybridisations to *Drosophila* whole mount ovaries to investigate potential transcription factors for ovarian *yp* regulation.

The DNA probes used are:

A)  *aeff-1* gene, encodes AEF-1 protein

B)  *slbo* gene, encodes DmC/EBP
    the position of the border cells is marked by an arrow

C)  *dsx* gene, encodes the DSX DNA binding domain protein, common to male and female DSX proteins
be seen during border cell migration in Figure 4.7B. From observing this expression pattern it seems unlikely that \textit{slbo} expression in the border cells is capable of regulating transcription of the \textit{yps} in the ovary.

The \textit{in situ} hybridisations did not give any staining above background for the \textit{dsx} probe (Figure 4.7C), this indicates that \textit{dsx} is not expressed in the ovary and thus is not a regulator of ovarian \textit{yp} expression. This is consistent with previous genetic evidence against \textit{dsx} being a regulator of \textit{yp} gene expression in the ovary (Bownes et al, 1990).

In summary, it does not appear that either \textit{aef-1}, \textit{slbo} or \textit{dsx} are expressed in the right cells to be able to regulate transcription of the \textit{yps} in the follicle cells.

4.5 OTHER EXPERIMENTS

Attempts were made to mutate the first 4bp (by mismatching nucleotides: purines exchanged for pyrimidines, and vice versa) and also to delete the 10bp consensus sequence located within OE3 (Figure 4.2) by site-directed mutagenesis, but unfortunately these experiments were unsuccessful. The aim was to mutate and delete the consensus sequence from the 328bp OE3 and determine if these mutations had any affect upon ovarian reporter gene expression when transformed into flies. This would have indicated if the consensus sequence was involved in ovarian \textit{yp3} regulation, and also revealed if it were a potential binding site for a transcription factor regulating \textit{yp3} expression. These mutated sites could also have been used in gel retardation assays with ovarian nuclear extracts to find out if they disrupted the specific gel shift previously identified (E. Ronaldson, unpublished data).
4.6 SUMMARY AND DISCUSSION

In situ hybridisation of yp DNA probes to Drosophila whole mount ovaries showed that all three yp genes have the same expression pattern; they are expressed in the ovary between stages 8 and 10b, specifically in the follicle cells surrounding the egg chamber, which migrate posteriorly to cover the oocyte during stage 9.

The 328bp OE3 of yp3 was used to create a subfragment of 150bp which was found to be capable of directing reporter gene expression in ovarian follicle cells during stages 8 to 10b of oogenesis, in both forward and reverse orientations. This suggests that the 150bp fragment contains sequences responsible for the tissue-specific expression of yp3 in the ovary.

Investigation of potential transcription factors responsible for the regulation of yp expression by in situ hybridisation did not reveal any likely candidates. It is expected that a regulator of tissue-specific expression in the ovary, i.e. limited to certain follicle cells, is required to be expressed in those cells in order to perform regulatory functions directly. None of the genes tested, aef-1, slbo and dsx showed expression patterns in the follicle cells surrounding the egg chamber and then the oocyte during stages 8 to 10b.

Although computer searches have indicated there are regions of similarity between OE1, OE2, OE3 and Musca and Calliphora yp upstream regions, no transcription factors have been identified for the regulation of yp expression in the ovary. It is possible that regulation of ovarian yp3 expression is less complicated than for yp1 and yp2, as yp1 and yp2 share enhancers for their expression in the ovary. In the 1226bp intergenic region of yp1 and yp2, two ovarian enhancers (OE1 and OE2) were found to regulate the tissue-specific ovarian expression of yp1 and yp2. OE1 is 301bp in length and is situated 43bp upstream of yp2, OE2 is 105bp in length and is located within the yp2 coding region. OE1 directs the timing of yp1 and yp2 expression to the correct stages of oogenesis and limits expression to the ovarian follicle cells. OE2 enhances levels of ovarian yp1 transcripts and gives tissue-specificity but cannot
regulate the expression to the correct stages of oogenesis (Logan et al., 1989, Logan and Wensink, 1990). Therefore, to provide the correct levels and pattern expressions of yp1 and yp2, both OE1 and OE2 are required. For yp3 the 328bp OE3 and a 150bp subfragment appear to be capable of directing reporter gene expression to the correct stages and cells in the ovary.

The majority of transformed Drosophila lines containing the 150bp fragment showed reporter gene expression in the yp expression pattern, however, a few lines did not show any staining patterns in the ovary and one line showed a modified expression pattern. These exceptions to the yp expression pattern could be caused by position effects, depending upon where in the Drosophila genome they are located. This is a common problem with reporter constructs containing small enhancer elements without their native promoter. Some lines showed very faint reporter gene expression in the yp pattern, it is also possible that lines appearing not to show expression have levels too low to show visible staining with β-galactosidase.

Future experiments to investigate the regulation of yp3 would include the identification of the ovarian DNA binding protein(s) involved. The 150bp fragment could be used to screen an ovarian expression library to identify the cis-acting factors regulating yp ovarian expression. These factors could be used in DNaseI footprinting assays to identify their binding sites within OE3, and site-directed mutagenesis followed by P-element mediated transformation would reveal if these sequences were involved in enhancer activity.
CHAPTER 5

RESULTS

Production of Doublesex Antibodies
The *doublesex (dsx)* gene is the final gene in the *Drosophila* somatic sex determination pathway and thus regulates the genes responsible for sexual differentiation in the fly. The primary transcript of the *dsx* gene is alternatively spliced in males and females to yield sex-specific mRNAs which encode male-specific and female-specific polypeptides (Burtis and Baker, 1989). The two DSX proteins are identical for the first 397 amino acids (aa), but have unique carboxy termini of 152aa in males and 30aa in females. The male-specific doublesex polypeptide, DSXM is thought to repress the expression of female-specific genes in males, and conversely, the female-specific doublesex polypeptide, DSXF is thought to repress the expression of male-specific genes in females. The N-termini of 397 amino acids, common to DSXM and DSXF, contains a zinc finger-related DNA binding domain (Erdman and Burtis, 1993) and it has been established that the DSX proteins can act as DNA binding transcription factors.

Despite the extensive studies into the developmental consequences of *dsx* mutations, very little is known about the molecular interactions of DSX with its target sexual differentiation genes. The only target genes shown to be under direct control of the sex determination hierarchy are the yolk protein genes, specifically *yp1* and *yp2* (Burtis *et al.*, 1991).

In this project, DSX binding domain protein was found to bind specifically to a fragment located upstream of the *yp3* gene, which was also found to be involved in fat body-specific regulation of *yp3* (Chapter 3). The expression of *dsx* was also investigated in *Drosophila* ovaries, to determine if it was a candidate for a transcription factor regulating *yp* expression in the ovary (Chapter 4). No evidence was found by *in situ* hybridisation for *dsx* expression in the ovary, and there have never been reports of *dsx* transcripts found in the ovary. Very little is known about actual DSX protein expression in *Drosophila*, the DSXM and DSXF proteins have
been expressed in bacterial cultures to use in binding assays, but the localisation of DSX in tissues has not been reported.

Antibodies to DSX binding domain protein were made during this project to investigate DSX protein expression in male and female fat bodies, considering that they are proposed to be regulators of fat body-specific yp expression. It was also hoped that the DSX antibodies could finally determine if DSX is produced in the ovary. The antibodies could also be used to look at DSX expression and localisation throughout development. There are specific questions that could be answered, for example, the involvement of dsx in the development of the male foreleg. The male foreleg carries the sex comb and the pattern of neuronal axons differs between the male and female first legs. Experiments using ectopic expression of a dsx male-specific cDNA in flies showed that sex comb morphology could be induced in female forelegs, and also on the second and third legs of both sexes. This has led to the suggestion that DSXM positively promotes the development of male-specific structures of the foreleg (Jursnich and Burtis, 1993). However, dsx null mutants do not have a similar phenotype, and dsx mutants constitutively expressing DSXM do not have sex combs on other legs. It is possible that dsx is not normally expressed in these cells and high levels of expression may lead to new phenotypes by interactions with different combinations of tissue-specific transcription factors. The DSX antibodies could be used to identify if DSX is expressed in the male foreleg and the foreleg imaginal disc; this would determine if DSXM is present and therefore capable of having a role as a promoter of sex-specific gene activation.
A pGEX T7 expression vector containing the N-terminal DNA binding domain common to both DSXM and DSXF proteins (amino acids 1-397) was used to express this domain as a glutathione-S-transferase (GST) fusion protein in BL21 (DE3) cells. The vector, named pGEX-KG, was a gift from K. Burtis (University of California). The GST-DSX fusion protein was estimated to have an approximate molecular weight of 37.5kDa, this was calculated by adding together 26kDa for the GST protein and 11.5kDa for the DSX DNA binding domain protein (calculated from average amino acid molecular weight values). The method for DSX DNA binding domain protein expression and purification was adapted from Erdman and Burtis (1993), and is detailed in section 2.5.4. The GST-DSX was purified from the bacterial cell extract using glutathione-agarose beads to bind the GST-DSX, which was then eluted from the beads in Z-50 buffer. Samples were taken during the purification stages and analysed by SDS-PAGE, an example of this is shown in Figure 5.1. The GST-DSX eluted from the glutathione-agarose beads migrates at approximately 40kDa, shown in lanes 5 and 6, and it can be seen that the first elution releases more protein from the beads than subsequent elutions. It is also clear that the purification procedure has successfully removed other bacterial cell proteins from the sample. The Z-50 buffer was used because it contained protease inhibitors which minimise degradation of the protein sample, however, Z-50 buffer is unsuitable for injection into animals for antibody production. Therefore, the GST-DSX was produced in Z-50 buffer, checked on SDS-PAGE and then dialysed into phosphate buffered saline (PBS) which can be used for injection into animals. The protein concentration of the sample was determined by the Bradford assay and the sample was concentrated accordingly, using an Ultrafree-4 centrifugal filter unit (Millipore), to give a protein concentration of approximately 1µg/µl. A sample of the protein was checked again by SDS-PAGE prior to injection into mice, because the dialysis into PBS can result in slight degradation of the sample in the absence of protease inhibitors.
Figure 5.1

The expression and purification of DSX DNA binding domain protein.

The DSX DNA binding domain protein was expressed as a glutathione-S-transferase (GST) fusion protein in BL21 (DE3) cells. Samples were taken from bacterial cell extracts during the expression and purification procedure, separated by SDS-PAGE and stained with ISS-ProBlue.

Lane 1: pre-stained protein molecular weight markers (BioRad), the sizes are shown in kDa
Lane 2: bacterial cell extract taken from lysed cells
Lane 3: bacterial cell extract after incubation with glutathione-agarose beads
Lane 4: wash solution from the glutathione-agarose beads
Lane 5: first elution of GST-DSX from the glutathione-agarose beads
Lane 6: second elution of GST-DSX from the glutathione-agarose beads

The position of the ~40kDa GST-DSX protein is marked with an arrow.
Marker size (kDa)

<table>
<thead>
<tr>
<th>102</th>
<th>81</th>
<th>47</th>
<th>33</th>
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GST-DSX
5.3 ANTIBODY PRODUCTION

The research adjuvant TiterMax®Gold was used to provoke an immune response to the antigen (GST-DSX) in mice, the antigen was mixed with adjuvant at a 50:50 ratio, and the two components were mixed by vortexing until an emulsion formed. Five MF1 mice were anaesthetised with Halothane and each bled from the eye (10 drops of blood) to give a sample of pre-immune serum. The mice were then injected with 100μl of a stable emulsion of antigen-TiterMax®Gold, which contained 50μl of antigen (1μg/μl protein) and 50μl TiterMax®Gold. The injections were given subcutaneously at the base of the tail. Primary bleeds were taken after 4 weeks by bleeding from the eye and a boost injection of antigen (50μl of 1μg/μl protein) in PBS was given after 6 weeks. The GST-DSX protein used for the boost injection was prepared freshly and checked by running a sample on SDS-PAGE before injection into the mice. Secondary bleeds were taken after a further 3 weeks and the mice were bled out after another 16 weeks. All the mouse handling and serum preparation was performed by Kay Samuel (ICAPB, University of Edinburgh).

5.4 ANTIBODY RESULTS

Initially the five DSX antibody samples were tested with a BL21 (DE3) cell extract (without transformed construct), to look at their background cross-reactivity, and also with a sample of GST-DSX to check their reactivity. The protein samples to be tested were run on SDS-PAGE, along with pre-stained markers (BioRad), and the gel was used for Western blotting (detailed in section 2.5.5.3). The filter was stained with Ponceau S to check the transfer and then used for immunostaining. The HRP method for antibody staining was used and a sample of these results is shown in Figure 5.2. Lane 1 contains pre-stained protein markers (BioRad), lane 2 is a BL21 (DE3) cell extract and lane 3 is a sample of GST-DSX. The strong staining observed with GST-DSX in lane 3 shows that the antibody has good recognition and binding.
Figure 5.2

Western blot analysis of BL21 (DE3) cell extracts using DSX antibodies (number 8), and the HRP method of immunodetection.

Lane 1: pre-stained protein molecular weight markers (BioRad), the sizes are shown in kDa
Lane 2: bacterial cell extract taken from BL21 (DE3) cells without the pGEX-KG plasmid, i.e. not expressing GST-DSX protein
Lane 3: purified GST-DSX protein

The position of the ~40kDa GST-DSX protein is marked with an arrow.
with its antigen. The immunostaining also shows up degradation of the GST-DSX protein, as smaller bands appear under the main GST-DSX migrating at ~40kDa. On SDS-PAGE, stained with ISS Pro-Blue, the same GST-DSX sample appeared to have a strong band of intact GST-DSX with very slight degradation visible on the gel. It therefore seems that the immunostaining detects degradation products of GST-DSX more strongly than ISS Pro-Blue staining on a polyacrylamide gel. All of the GST-DSX antibodies, from all 5 mice, gave immunostaining with a GST-DSX protein sample. Against the BL21 (DE3) cell extract all five antibody samples brought up a large sized protein on the filter, this can be seen faintly in lane 2 of Figure 5.2. Three of the GST-DSX antibody samples also showed a reaction with another smaller protein in the BL21 (DE3) cell extract. The antibody used in Figure 5.2, named number 8, gave strong staining with GST-DSX protein and low background with the BL21 (DE3) cell extract, and was therefore used in subsequent immunostaining experiments.

The GST-DSX antibody (number 8) was then used for immunostaining of Western blot filters containing *Drosophila* tissue samples. Adult *Drosophila* were aged to 3 days, to ensure oogenesis was proceeding, before being dissected and the proteins separated by SDS-PAGE. The tissues used were: ovaries, testes, male carcass and female carcass (the carcass contains the fat body cells which cannot be isolated easily from the cuticle), a mixture of pupal stages were also used as a control because they are expected to contain both male and female forms of DSX protein. Initially, the HRP method of immunodetection was performed, but this did not produce any bands of staining on the filter even if high levels of primary (GST-DSX) antibody were used.

The next procedure used was the ECL method for immunostaining, which gives much greater sensitivity than the HRP method. The ECL method produces a chemiluminescent reaction which is detected by autoradiography and because it is also a very sensitive method high backgrounds are sometimes seen. Western filters
were produced containing proteins from pupae, ovaries, testes, male carcass and female carcass, these were used for ECL immunodetection using the GST-DSX antibody (number 8). Generally, the DSX antibodies were diluted 1:1000 in PBS-T, and the secondary anti-mouse HRP conjugated antibodies were diluted 1:5000 in PBS-T. An example of these results is shown in Figure 5.3. The strongest bands seen are in the male carcass and female carcass, lanes 3 and 4, these correspond to an approximate size of 40kDa. Another larger protein band comes up in the female carcass lane that is approximately 70-75kDa in size. No immunostaining is detected in testes or ovaries.

Predictions of DSX<sup>M</sup> and DSX<sup>F</sup> sizes calculated from the amino acid sequences were 58.5kDa (DSX<sup>M</sup>) and 45.8kDa (DSX<sup>F</sup>). The DSX polypeptides were then overexpressed in <i>E. coli</i> from pT7-7 constructs containing DSX<sup>M</sup> and DSX<sup>F</sup> encoding cDNAs, to give the DSX polypeptides as fusion proteins containing 10 additional amino acids. The migration of the male and female proteins on SDS-PAGE indicated molecular weights of 67 kDa for the female fusion protein and 52kDa for the male fusion protein (Burtis <i>et al.</i>, 1991). Apparently, results obtained from pT7-7 constructs containing DSX<sup>M</sup> and DSX<sup>F</sup> encoding cDNAs, to give the DSX polypeptides without any additional amino acids fused to them, gave identical results. From these results it seems that the DSX<sup>M</sup> and DSX<sup>F</sup> polypeptides are significantly larger than initially predicted.

The identity of the 40kDa protein seen in both males and females (Figure 5.3) is therefore unknown. The larger protein band observed in the female carcass lane could be DSX<sup>F</sup>, but more investigation of this would need to be made before drawing conclusions.

To check that the 40kDa protein was not a product of degradation and to verify its size, the filter was reprobed with YP antibodies. The YPs are 47kDa (YP1), 46kDa (YP2) and 45kDa (YP3), and are produced in females only. These results are shown...
Figure 5.3

Western blot analysis of *Drosophila* tissue samples using DSX antibodies and the ECL method of immunodetection.

Lane 1: pupae  
Lane 2: male carcass  
Lane 3: female carcass  
Lane 4: testes  
Lane 5: ovaries

The sizes (in kDa) and locations of protein molecular weight markers are indicated.

The arrow indicates the ~40kDa protein in the male and female carcass samples, and the star marks a ~70-75kDa protein in the female carcass sample.
in Figure 5.4, and the YPs can be seen clearly in the female carcass and ovary lanes. There appears to be slight degradation in the ovary sample, but not in the female carcass lane, this shows that there is plenty of undegraded protein in the ovary sample. This indicates that the lack of a signal in the ovary with DSX antibodies is not caused because the sample is degraded, but because DSX protein is not present in the ovary. Since the female carcass protein does not appear to be degraded, the 40kDa protein showing immunostaining with the DSX antibodies is not likely to be a degradation product. It can also be seen that the YPs are larger in size compared to the estimated 40kDa size protein, this estimate is therefore, a reasonable one when compared to the YPs.

It appears that despite the GST-DSX antibodies giving strong immunostaining to GST-DSX protein, the situation is more complex when the antibody is used with Drosophila tissues. A 40kDa protein is recognised by the DSX antibodies in males and females, and it is not known at this stage if this protein is a form of DSX or a protein cross-reacting with the antibody. It is possible that the DSX antibodies could cross-react with zinc-finger proteins other than DSX, if they are present in the tissues sampled.

5.5 SUMMARY AND DISCUSSION

DSX DNA binding protein was produced in E. coli BL21 (DE3) cells as a glutathione-S-transferase fusion protein, and then purified to give a sample containing only GST-DSX protein. This GST-DSX protein was used as an antigen to raise antibodies in mice. The resulting DSX antibodies were initially tested against a GST-DSX sample and were found to have good recognition and binding with their antigen. One of the DSX antibody samples, referred to as number 8, gave strong immunostaining with GST-DSX protein and low background with a BL21 (DE3) cell extract, this antibody was used for subsequent experiments. When the DSX antibodies were used for Western blot analysis of Drosophila tissue samples, an
Figure 5.4

Western blot analysis of *Drosophila* tissue samples using YP antibodies and the ECL method of immunodetection.

Lane 1: pre-stained protein molecular weight markers (BioRad), the sizes are shown in kDa
Lane 2: pupae
Lane 3: male carcass
Lane 4: female carcass
Lane 5: testes
Lane 6: ovaries

The positions of the 45, 46 and 47kDa yolk proteins (YPs) in the female carcass and ovary samples are indicated by an arrow.
approximately 40kDa protein in the male and female carcass samples gave the strongest signal. Another larger protein of approximately 70-75kDa gave a weaker signal in the female carcass sample. No immunostaining was detected in testes or ovaries.

These results show that the DSX antibodies react strongly to a non sex-specific protein of an approximate molecular weight of 40kDa. The identity of the 40kDa protein is unknown, the protein might be a form of DSX or a protein cross-reacting with the antibody. However, no non sex-specific dsx transcripts have been reported, but as very little is known about DSX proteins actually produced in vivo, the possibility that there non sex-specific DSX proteins cannot be ruled out. Experiments to investigate the potential role of the 40kDa protein could include the analysis of dsx mutants. Tissue samples from dsx mutants could be used in Western blot experiments to determine if the 40kDa protein is a form of DSX protein. If the 40 kDa protein is still present in dsx mutants, it is unlikely to be a DSX protein. Analysis of the Drosophila tissue samples using YP antibodies showed that the samples were not degraded, and therefore, the 40kDa protein is not a degradation product of DSX. The 40kDa protein could be cross-reacting to the DSX antibodies, possibly because it contains a similar protein motif as the DSX DNA binding domain, for example, a zinc finger binding domain.

Despite the DSX antibodies giving strong staining with GST-DSX, their affinity for DSX\textsuperscript{M} (67kDa) and DSX\textsuperscript{F} (52kDa) does not appear to be so strong. There are no obvious reactions between proteins of these sizes and the DSX antibodies in the Western blots analysed so far. The large protein band observed in the female carcass lane could be DSX\textsuperscript{F}, but the size for this protein was estimated to be 70-75kDa, compared to 52kDa for DSX\textsuperscript{F}. Therefore, more accurate estimations of the size of this protein need to be made in future experiments. It may be neccessary to affinity purify the DSX antibodies to improve their specificity in immunodetection studies.
CHAPTER 6

Final Discussion
6.1 THE REGULATION OF YP3 EXPRESSION IN THE FAT BODY

A 419bp DNA fragment, termed FBE3, located 5' of the transcription start site of *Drosophila yp3*, was previously found to direct lacZ expression, in the fat body of females, but not males (Ronaldson and Bownes, 1995). This fragment was investigated with the aim of identifying trans-acting factors binding to the FBE3 and localising the enhancer properties to smaller fragments within the 419bp FBE3. Various subfragments of the FBE3 were tested for their effects on lacZ reporter gene expression *in vivo* by P-element-mediated germ line transformation, and assaying for β-galactosidase activity by histological staining. Using this technique, a 177bp fragment was identified that was capable of directing tissue-specific expression in the fat body. However, this fragment was not sufficient to confer the sex-specificity of expression to females only, and the reporter gene was expressed in the fat body cells of both males and females. It was noted that the 177bp fragment only gave this expression pattern in 60% of transformed lines, and consequently this result is thought to be due to chromosomal position effects. The expression patterns observed from the 177bp fragment could be explained by an activator and/or repressor binding site being lost in the isolation of the fragment, and this being supplied externally as a position effect, depending upon where in the *Drosophila* genome the construct had inserted, resulting in lacZ expression in some lines and not others. The 177bp fragment was used in gel retardation assays and was found to bind specifically to DSX protein *in vitro*. However, this binding does not appear to be sufficient *in vivo* for conferring the sex-specificity of reporter gene expression. These results for fat body regulation of *yp3* are similar to observations made for the regulation of the *yps* in *Musca* and *Calliphora*, where *yp* enhancer fragments directed reporter gene expression in male and female fat bodies despite binding to DSX *in vitro* (Tortiglione and Bownes, 1997). This is in contrast to the results obtained for the FBE of *yp1* and *yp2*, where the sex- and tissue-specificity of expression were never separated, believed to be due to the trans-acting factors having overlapping binding sites within the FBE. These results suggest that regulation of the *yp3* gene, and also the *yps* in...
Musca and Calliphora, may not occur in exactly the same way as for yp1 and yp2; the factors involved may be the same, but the details of the mechanism or organisation of the interacting binding sites could be different.

Analysis of slbo mutants suggested that DmC/EBP protein is not involved in the regulation of the yp genes in Drosophila. The levels of YP protein in the haemolymph were not significantly different between homozygous slbo mutants and their heterozygous siblings. The levels of YP protein in the haemolymph provide a measure of the YPs produced by the fat body. From these results it seems very unlikely that DmC/EBP functions as an activator of yp gene expression in the fat body.

6.2 THE REGULATION OF YP3 EXPRESSION IN THE OVARY

The exact expression pattern of the yolk protein genes in the ovary was determined by in situ hybridisation. These experiments showed that all three yp genes have the same expression pattern; they are expressed in the ovarian follicle cells surrounding the egg chamber between stages 8 and 10b.

Candidates for the regulation of yp gene expression in the ovary are expected to be expressed in the follicle cells during stages 8 to 10b in order to be capable of performing regulatory functions directly. Investigation of potential transcription factors responsible for the regulation of yp expression by in situ hybridisation did not reveal any likely candidates. The ovarian expression patterns of the aef-1, slbo and dsx genes were investigated by in situ hybridisation, but none of these showed expression patterns in the follicle cells surrounding the egg chamber and then the oocyte during stages 8 to 10b. As yet, no transcription factors have been identified for the regulation of yp expression in the ovary.
Previous experiments to identify enhancers governing yp3 expression revealed a 328bp fragment, located immediately upstream of the yp3 coding sequence, sufficient to confer an ovarian yp expression pattern (Ronaldson and Bownes, 1995). A 150bp subfragment from this region was tested for its effects upon lacZ reporter gene expression in vivo by P-element mediated germ line transformation. This 150bp fragment was found to be capable of directing reporter gene expression in ovarian follicle cells during stages 8 to 10b of oogenesis, in both forward and reverse orientations. This suggests that the 150bp fragment contains sequences responsible for the tissue-specific expression of yp3 in the ovary.

6.3 THE PRODUCTION OF DOUBLESEX ANTIBODIES

Antibodies were made to the DSX DNA binding domain protein, which was synthesised in E. coli as a glutathione-S-transferase fusion protein. The DSX antibodies were produced with the aim of investigating DSX protein expression in male and female fat bodies, considering that the DSX proteins are proposed to be regulators of fat body-specific yp expression. It was also hoped that the DSX antibodies could verify that DSX protein is not present in the ovary. These DSX antibodies were used in preliminary experiments; they showed good recognition of GST-DSX, but the results obtained with Drosophila tissue samples were less conclusive. It may be necessary to affinity purify the DSX antibodies in order to increase their specificity in immunodetection studies.

6.4 CONCLUDING REMARKS

Initially, the yolk protein genes of Drosophila were considered to provide an ideal system to identify factors involved in the transcriptional control of sex- and tissue-specificity during highly co-ordinated developmental stages. As research projects have progressed, it has become clear that the regulation of the Drosophila yp genes is not as simple as initially thought. Like many other eukaryotic genes, multiple binding
sites for various different trans-acting factors have been identified. Although models have been proposed for the regulation of the yps, these studies have mainly been focused on a single enhancer element. This element is contained within the fat body enhancer (FBE) of yp1 and yp2, however, there are additional binding sites located within this enhancer, and the FBE can be deleted without preventing female, fat body yp expression, thus suggesting there are more sites to be located. Therefore, complete characterisation of gene regulation for yp1 and yp2 is still far from being achieved. The regulation of yp1 and yp2 has additional complications because the enhancer elements are shared between the two genes, this indicates that the regulation of yp3 may be simpler since the yp3 enhancer elements are not shared. However, results obtained in this project suggest that the regulatory mechanisms involved in regulating yp3 fat body expression may not be the same as for yp1 and yp2.

It would be interesting to identify all the trans-acting factors involved in the transcriptional regulation of all three yolk protein genes, and to determine if the mechanism or organisation of the factors is different for the fat body regulation of yp3 compared with yp1 and yp2.

An important discovery in this field would be the identification of ovarian trans-acting factors involved in the transcriptional regulation of the three yolk protein genes since none have been identified to date. The continuous action of the sex determination hierarchy is not involved in the regulation of the yp genes in the ovary, but the actual factors and mechanisms involved remain to be elucidated. It would also be interesting to find out if the ovarian regulation of yp3 is different to that of yp1 and yp2.
CHAPTER SEVEN

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CHAPTER SEVEN: REFERENCES


