EVOLUTION AND REGULATION OF EXPRESSION
OF THE GENES CODING FOR DROSOPHILA
YOLK PROTEINS

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### Abbreviations and definitions

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APS</td>
<td>ammonium persulphate</td>
</tr>
<tr>
<td>bisacrylamide</td>
<td>N,N'-methylene-bis-acrylamide</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumen</td>
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<tr>
<td>°C</td>
<td>degrees centigrade</td>
</tr>
<tr>
<td>Ci</td>
<td>curie(s)</td>
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<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>DENHARDTS</td>
<td>0.02% BSA, 0.02% Ficoll, 0.02% polyvinylpyridine</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>ERE</td>
<td>D. erecta</td>
</tr>
<tr>
<td>FUN</td>
<td>D. funebris</td>
</tr>
<tr>
<td>g</td>
<td>gram(s)</td>
</tr>
<tr>
<td>$^3$H</td>
<td>tritium (BETA emitting isotope of Hydrogen)</td>
</tr>
<tr>
<td>HBIOI</td>
<td>E. coli K.12 x E. coli B Hybrid</td>
</tr>
<tr>
<td>$^{125}$I</td>
<td>radioactive isotope of iodine</td>
</tr>
<tr>
<td>JH</td>
<td>juvenile hormone(s)</td>
</tr>
<tr>
<td>K</td>
<td>x1000 revolutions per minute</td>
</tr>
<tr>
<td>Kb</td>
<td>kilobases</td>
</tr>
<tr>
<td>LAMBDA</td>
<td>phage C1857 unless otherwise stated</td>
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<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mA</td>
<td>milliampere(s)</td>
</tr>
<tr>
<td>MAU</td>
<td>D. mauritiana</td>
</tr>
<tr>
<td>MEL</td>
<td>D. melanogaster</td>
</tr>
<tr>
<td>mg</td>
<td>milligram(s)</td>
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ml millilitre(s)
mm millimeter(s)
nM millimolar
mRNA messenger ribonucleic acid
ORE D. orena
$^{32}\text{p}$ radioactive (BETA particle emitting) isotope of phosphorous
% per cent
pH $-\log_{10}$ (hydrogen ion concentration)
PMSF phenylmethylsulphonyl fluoride
PPO 2,5-diphenyloxazole
RNA ribonucleic acid
$^{35}\text{S}$ radioactive (BETA particle emitting) isotope of sulphur
$[^{35}\text{S}]$methionine $^{35}\text{S}$-labelled solution of methionine
SDS sodium dodecyl sulphate
SEC D. sechellia
SIM D. simulans
SSC 0.15M NaCl, 0.015M sodium citrate
TCA trichloroacetic acid
TE 10mM Tris/HCl pH 7.5, 1mM EDTA
TEI D. teissieri
TEMED $N,N,N',N'$-tetramethylethylenediamine
Tris tris(hydroxymethyl)aminomethane
Triton X-100 iso-octylphenoxyethoxyethanol
$\mu$Ci microcurie(s)
$\mu$g microgram(s)
$\mu$l microlitre(s)
VIR D. virilis
distilled water

D. yakuba

yolk protein (either vitellin or vitellogenin)

1 kb Hind III/Bam HI Fragment of 5' coding region of YP1 gene cloned into phage M13.
Three yolk polypeptides (YPs), coded by three genes (YP1, 2, 3) located on the x chromosome, have been previously identified in D. melanogaster. These genes are expressed in a tissue, temporal and sex-specific manner, in the fat body and follicle cells of the female soon after eclosion. Although males do not normally produce yolk polypeptides it is shown that synthesis can be induced by steroid treatment (20-hydroxyecdysone). YP-transcripts are detected a few hours after hormone injection, they accumulate to a peak after about 16 hours and then decline in number until about 24 hours. Yolk polypeptides also accumulate and decline over a similar period but the peak is after about 12 hours, suggesting that post-transcriptional controls may act on these genes. In vitro translation studies suggest the reason for the post-transcriptional control does not lie within the structure of the induced transcripts. Northern analysis does not detect differences between female and male induced YP-transcripts. It is shown that 20-hydroxyecdysone can induce synthesis of YPs and YP-transcripts in other species of Drosophila even outside the Melanogaster Group Species.

By Southern blotting experiments three putative yolk protein genes have been identified in five of the D. melanogaster sibling species, (D. erecta, D. yakuba, D. teissieri, D. orena and D. sechellia) and at least two identified in the two remaining species, D. mauritiana and D. simulans. All these species appear to have three yolk polypeptides. One YP-gene and yolk polypeptide has been identified in D. funebris and two in D. virilis.

By in situ hybridization the putative YP-genes have been assigned x chromosomal locations. Very close linkage between YP1 and YP2-like genes is suggested by Southern blotting for all the sibling species, except D. yakuba, while in situ hybridization experiments are consistent with this notion for all the sibling species. Thus, a possible duplication event that resulted in the formation of the YP1 and YP2-like genes probably occurred before the divergent evolution of the D. melanogaster sibling species.
The Yolk Protein Genes:

Yolk proteins (YPs) have been defined as female specific proteins which are found in the haemolymph and are sequestered in large amounts by the oocyte during oogenesis. Vitellogenesis is the process whereby vitellogenins are selectively taken up by the oocyte and accumulated there. They are then termed vitellins. Since vitellogenin and vitellin in Drosophila appear to be equivalent these will be referred to as yolk proteins. In Drosophila melanogaster three genes have been identified which code for yolk proteins (YP1, YP2 and YP3) (Barnett et al, 1980). Each gene is present as a single copy, extending for less than 2kb, on the x-chromosome, YP1 and YP2 at position 9A, and YP3 at 12 B/C as determined by in situ hybridization to polytene chromosomes (Barnett et al, 1980; Ridden et al 1981; Redfern and Bownes, unpublished; see figs 3:1 and 4:1). The two locust vitellogenin genes are also X linked (Bradfield and Wyatt, 1983). D. melanogaster YP1 and YP2 genes are orientated in an inverted repeat fashion as has been observed for silk-moth chorion genes, and Drosophila histone and heat shock (87A7) genes.

Hung et al (1982) and Hovemann and Galler (1982) identified single introns for YP1 (75bp) and YP2 (456P), and two introns for YP3 (about 65-75 bp each). The YP1 and YP2 genes and intergenic region have been sequenced by Hung and Wensink (1984) and Hovemann et al (1981) who identified consensus sequences for TATA boxes and capping sites, at the 5' ends, and polyadenosine addition sites at the 3' ends of the genes. Hydrophobic amino terminal (5') leader sequences were identified where eleven out of the first seventeen amino acids are predicted to be conserved between YP1 and YP2. In the intergenic region a thirteen nucleotide sequence similar to a
proposed consensus sequence for a progesterone receptor binding site (Mulvihill et al 1982), was found.

The nucleotide sequences of the YP1 and YP2 genes are 63% homologous in the coding region while proposed amino acid homology is only 53%. The 5' and 3' boundary sequences of the YP1 intron have homology with the flanking sequences from two D. melanogaster actin gene introns (Fryberg et al, 1981).

In Xenopus laevis four vitellogenin (yolk protein) genes have been identified (Wahli and David, 1979 and 1980) which are all large (A1 is 21kb and A2 is 16kb) and contain many introns. So far only one vitellogenin gene has been identified in the chicken (Cozens et al, 1980) although others coding for less abundant yolk proteins are likely to be found. Germond et al (1984) and Walker et al (1984) have compared the Xenopus and avian yolk protein genes. 5' upstream conserved sequences were found; about -350bp a common palindromic sequence was identified with a 10bp consensus sequence; TATA boxes were identified; and two other conserved A + T rich regions. About ten pseudo YP genes have been identified in Xenopus but none have been found in D. melanogaster. The two coding regions of locust yolk protein genes extend over 10-12kb with several introns, but the genes show no cross-hybridization with each other (Wyatt et al, 1984).

Expression of the YP-Genes, and the Yolk Polypeptides

The yolk protein genes of D. melanogaster are expressed in the fat body cells and follicle cells of the ovary in females soon after eclosion. (Bownes, 1980A; Brennan et al, 1982; Isaac and Bownes, 1982). Labelling RNA in vivo shows that in adult females, these genes are being continuously transcribed (Bownes et al, 1984). However, transcripts from the three genes are not always present in equal quantities. For instance, the ovary contains a much reduced level of YP3-coding mRNA compared with YP1 and YP2-coding mRNA's. Thus the ovary contributes about 35% of the fly's total YP1 and YP2 mRNA but only about 10% of its YP3 mRNA. The rest is synthesized by
the fat body cells (Isaac and Bownes, 1982; Brennan et al, 1982). YP1-transcripts are about 1.6 kb and YP3 about 1.54 kb while YP2 produces two transcripts in equimolar amounts of 1.59 and 1.67 kb sizes due to two 3' termination signals (Barnett and Wensink, 1981). The YP1 and YP2 genes are transcribed in opposite directions. Transcription of these genes has also been achieved in vitro (Gergen, 1982). Alpha-amanitin, which inhibits RNA polymerase II mediated transcription, has been used to study the yolk protein system. However, alpha-amanitin fails to affect YP transcript accumulation at the time when abnormalities in egg chambers are first observed. So defective uptake of yolk proteins by the oocytes (vitellogenesis) is unlikely to result from the effect of alpha-amanitin on YP-gene transcription (Bownes et al, in preparation). Using in vitro translation and in vivo labelling studies Warren et al (1979) showed that a polypeptide sequence (presumably the leader sequence) is removed from each of the immature vitellogenin polypeptides, and for YP1 a second processing step results in an apparent increase in molecular weight before the polypeptide is exported from the fat body cells.

Cleavage of signal peptides has been observed in many proteins prior to export (Davis and Tai, 1980), although others contain internal signals for export which are not cleaved, such as in ovalbumen (Lingappa et al, 1979). Jost and Seldran (1984) have found that in chickens, prior to vitellogenin mRNA synthesis, only transcriptionally active vitellogenin genes are associated with the nuclear matrix.

Bownes and Hames (1977) detected three yolk polypeptides in D. melanogaster which have molecular weights of 47,500 (YP1), 46,000 (YP2) and 44,500 (YP3) determined by denaturing polyacrylamide gel electrophoresis (Mintzas and Kambysellis, 1982). The polypeptides are fairly rich in acidic amino acids and have isoelectric points between 6.5 and 7.0. Non denaturing gels suggest YP2 vitellins may be present as trimeric aggregates. This protein appears also to contain carbohydrates which have not been detected for YP1 and YP3 (Mintzas and Kambysellis, 1982).
Variants of D. melanogaster and D. simulans have been isolated in which mobility of yolk polypeptides and polyacrylamide gels are altered, although this does not appear to affect the 'fitness' of the offspring (Postlethwait and Jowett, 1980). D. simulans appears to contain three yolk polypeptides, D. funebris one and D. virilis two or three (Bownes, 1980B; Srdic et al, 1979). Different Drosophila species seem to contain yolk polypeptides of approximately the same size as has been found for D. melanogaster.

Four vitellogenins have been found in Acheta domesticus (Bradley and Edwards, 1978) and Phormia terra-novae (De loof and Huybrechts, 1982) although Sarcophaga bullata, Calliphora vicina and Lucilia caesar appear to have three vitellogenins, again, of similar sizes to those of Drosophila species (De loof and Huybrechts, 1982).

Four yolk polypeptides are found in C. elegans but two of these appear to be derived by cleavage of a single, large precursor (Sharrock, 1984).

In vertebrates, such as Xenopus, vitellogenin cleavages occur only after uptake by the oocyte. A putative major yolk protein precursor has also been detected in the sea urchin, although it is present in males also (Harrington and Easton, 1982).

The site of vitellogenin synthesis in Xenopus is the liver, which is analoguous to the Drosophila fat body. In Xenopus paevis (African Clawed Toad), a large protein precursor is synthesised in the liver, transported by the blood, and taken up by the oocytes where it is cleaved and converted to phosvitin and lipovitellin (Wallace and Berguik, 1974).

In Locusta migratoria, two polypeptides are synthesized in the fat body, but they are cleaved to eight smaller polypeptides (52,000 - 140,000 daltons) before being secreted (Chen et al, 1976; Wyatt, 1980). A similar phenomenon is seen in the cockroach Leucophaea maderae (Koeppe and Ofengard, 1976). In the nematode, C. elegans,
yolk proteins are synthesized in the intestine (Kimble and Sharrock, 1983).

Vitellogenesis and Function of the Yolk Proteins

Vitellogenesis in *D. melanogaster* occurs during stages 8 - 12 of oogenesis (King, 1970), shown in Fig 1:1. Meiosis of germ cells gives rise to an oocyte plus 15 adjacent nurse cells, the whole complex becoming enveloped by somatic follicle cells, which synthesize some of the yolk proteins in *Drosophila* and also the membranes that surround and protect each oocyte (Waring and Mahowald, 1979).

Ruddell and Jacobs-Lorena (1983) demonstrated that vitellogenesis is a rapid process in *Drosophila*, 50% of the mature egg proteins being accumulated within four hours. They also showed that the polysome content of egg chambers remains high, even at stage 12 of oogenesis, when no detectable protein accumulation occurs. This supports the contention that late in oogenesis, the efficiency of translation drops dramatically in the egg chamber.

Transplantation experiments by Srdic et al (1979) and Bownes (1980B) suggest that the ovary of *D. melanogaster* does not export yolk polypeptides into the haemolymph *in vivo*. Whereas in *D. melanogaster* synthesis and secretion of yolk polypeptides from the fat bodies occurs soon after eclosion, in *Hyalophora cecropia* the yolk polypeptides are found in the haemolymph at the time of the larval-pupal moult. And in the American cockroach (*Periplaneta americana*) yolk polypeptides are not synthesized until five days after eclosion (Pan *et al*, 1969). In *D. melanogaster* yolk proteins are taken up into the developing oocytes by pinocytosis, where the vesicles appear to fuse giving rise to yolk platelets (Giorgi and Jacob, 1977A) also known as yolk spheres (Cohn and Brown, 1968). A variety of markers were observed by Mahowald (1972) to be taken up from the haemolymph, so from this it appears that protein uptake into the oocyte is non-specific, although there may be a specific degradation of non-vitellogenic proteins (Giorgi and Jacob, 1977B).
However, there is evidence from inter-specific ovary transplant experiments between different *Drosophila* species to suggest that yolk protein uptake into the oocyte is of a specific nature (Bownes, 1980B).

In *Blatella germanica* an oligosaccharide is found associated with the vitellogenin which appears to help stabilize the protein as a trimer (Kunkel et al, 1984). Schjerde et al (1963) claimed to find specific uptake of alligator yolk protein into chicken oocytes although they did not control for non-specific uptake. Also Kunkel and Pan (1976) working with *H. cecropia* and *B. germanica*, found that the vitellogenins of these two species, despite appearing similar, will not support vitellogenesis in each others oocytes, suggesting a selective uptake or degradation mechanism.

The function of vitellins is unknown although, as they tend to disappear during embryogenesis, it is thought they may be simply a food store (Isaac, 1982). In the nematode, *C. elegans*, the yolk proteins do not disappear until the larval stages (Sharrock, 1983). Hahn et al (1984) found ecdysteroids bound to yolk proteins in eggs of *Carausus moronus* so one function may be to store maternal hormones. In the cricket, *A. domesticus*, Nicolaro and Bradley (1980) found a differential distribution of two vitellins established during oogenesis between the posterior and anterior halves. So it is possible that they may play a part in the formation of a gradient which is important for cell determination.
Fig 1:1


g = germarium; f = follicle cells; fs = follicular stalk; o = oocyte; on = oocyte nucleus; nc = nurse cell; ncn = nurse cell nucleus; bc = border cells; dnc = degenerating nurse cells; ca = chorionic appendage.
1:2 Hormonal Regulation of the Vitellogenin Genes

In insects two hormones, Juvenile hormone (JH) and a steroid hormone 20-OH ecdysone (see Fig 1:2), appear to play important roles in the regulation of the vitellogenin genes and vitellogenesis, although there appear to be few general modes of action. Postlethwait and Weiser (1973) and Jowett and Postlethwait (1980) showed that, for D. melanogaster, JH, but not 20-OH ecdysone, can cause yolk proteins to be sequestered from the haemolymph into the oocytes, and also cause the ovary to synthesize yolk proteins. Furthermore ovaries of the apterous " (ap4) mutant (which appears to be deficient in JH) will take up yolk proteins from the haemolymph if a JH analogue (ZR515) is added (Gavin and Williamson, 1976). Also there is a cave-dwelling species of Drosophila which enters diapause with yolk proteins in the haemolymph but lacks vitellogenic oocytes. JH terminates the diapause and causes vitellogenesis to occur (Kambysellis and Heed, 1974). It was later found that JH or 20-OH ecdysone causes an increase in yolk protein synthesis, after about 1½ hours, in female abdomens isolated and kept in culture soon after eclosion (Handler and Postlethwait, 1978). JH analogues stimulate the synthesis of YP1 and YP2 to a greater extent than YP3 (Jowett and Postlethwait, 1980), but it is not known whether JH is acting at the level of transcription or translation in these isolated abdomen experiments. These induced yolk proteins are also exported into the female haemolymph (Jowett and Postlethwait, 1980). So it appears as if JH stimulates YP synthesis in the fat bodies and ovaries as well as permitting YP uptake into the developing oocyte, while 20-OH ecdysone only causes YP synthesis in the fat body cells.

20-OH ecdysone treatment will induce YP synthesis also in male flies, while JH does not (Postlethwait et al, 1980). This is the best evidence that 20-OH ecdysone is the hormone involved in the primary activation of the YP genes in the fat body. The response of males to 20-OH ecdysone is dose dependent, there is no increased secondary response, and long-term treatment does not lead to permanent activation of the YP genes. In contrast, mature female
fat bodies can produce YPs in vitro for several days without requiring added hormone (Bownes, 1982). Shirk et al. (1983) showed that 20-OH ecdysone stimulates the accumulation of YP gene transcripts in adult males. We have also shown (Bownes et al., 1983A) that the response, in males and females, to 20-OH ecdysone is tissue specific, only the fat body cells being responsive. Pseudomales (mutants which despite having two x chromosomes have a male phenotype) can also be induced to synthesize YPs with 20-OH ecdysone (Bownes and Nothiger, 1981).

One model to explain the sex-limited synthesis of YPs is that males and females contain different titres of 20-OH ecdysone, the active form of ecdysone. However, so far no differences in titre have been detected (Hodgetts et al., 1977; Handler, 1982; Bainbridge, 1984). In insects the prothoracic gland (ring gland) produces ecdysteroids (Bownes and Redfern, 1983) before pupal stages, although there are other sites of synthesis as well. In several insects, such as the mosquito, the ovary secretes 20-OH ecdysone (Hagedorn et al., 1975) and certain cells associated with the fat body, the oenocytes, appear to synthesize ecdysteroids (Huybrechts and de Loof, 1977; Ruhland and Romer, 1977). Furthermore, little is known about steroid metabolic inactivation, excretion, or binding to haemolymph proteins (Koolman, 1983) which will influence titres. The existing model for steroid action suggests that they regulate specific gene expression through a direct interaction of the hormone-receptor complex with DNA sequences in and around the induced genes (see for instance Geisse et al., 1982). As the D. melanogaster YP1 - YP2 intergenic sequence contains a consensus sequence similar to one for progesterone - receptor binding (Hung and Wensink, 1984; Mulvihill et al., 1982; Compton et al., 1983) it is likely that steroid action plays an important part in yolk protein gene regulation. It could be that it is 20-OH ecdysone receptor levels or affinities which are different between male and female Drosophila and could explain the sex-limited nature of yolk protein synthesis. Unphysiologically high doses of steroid given to males in experiments could simply upset the normal operating system. Even the classical "two-step" hypothesis for steroid action where bound cytoplasmic receptors move
to the nucleus (Toft et al, 1966; Jensen et al, 1968) has been opposed by results from King and Green (1984) and Welshons et al (1984) who appear to have found steroid receptors in the nucleus at all times.

Males of S. bullata, C. vicina, P. terrae-novae, and L. caesar can be induced to produce YPs with 20-OH ecdysone but not JH (De Loof and Huybrechts, 1982). In C. vicina the induction in males occurs at least partly at the transcriptional level, and in females the response to 20-OH ecdysone appears to be tissue specific, only affecting the fat body cells (Huybrechts et al, 1984), as is seen in D. melanogaster (Bownes et al, 1983A). However, in the cockroach, D. punctata, males are only induced to synthesize YPs using JH, and not ecdysteroids (Mundall et al, 1983), although females are more responsive to JH than males and so this could explain the sex-specificity. The secondary response to JH has a shorter lag period but otherwise is similar to the primary response.

The male mosquito also does not appear to respond to 20-OH ecdysone (Hagedorn et al, 1984), while in the female there is a peak of YP synthesis 20 hours after a blood meal, possibly mediated by an increase in 20-OH ecdysone. However, it is also possible that the response is mediated by an increase in other hormones synthesized by the gut (Brown and Lea, 1984). JH causes the growth of previtellogenic oocytes and may also affect fat body development. Ecdysone is secreted from the ovary in response to the blood meal, after which it is converted to the active form 20-OH ecdysone, which is thought to cause the fat body to synthesize and export yolk polypeptides.

In the locust, as in other insects, JH is synthesized in the corpora allata, being controlled from neurosecretory brain cells (Applebaum et al 1983 and 1984), and the JH causes YP synthesis in the fat body and also allows vitellogenesis. Applebaum et al (1984) also detected two protein inhibiting factors which cause a general reduction of fat body protein synthesis, including that of the YPs.
More is known about hormonal control in vertebrate systems. In *Xenopus* estrogen appears to play the main role in YP gene action. Males can be induced to synthesize YPs using estrogen and, in contrast to *Drosophila*, secondary stimulation is 30 times faster than a primary one (Ryffel et al, 1977; Searle and Tata, 1981). In male induced culture systems actinomycin D causes an inhibition of YP synthesis if administered within 8 hours of estrogen stimulation. Cyclohexamide also causes the reduction of yolk proteins (Cameron-Clarke, 1980) which together suggest that there is a transcriptional turn on in *Xenopus* males, as is seen in *D. melanogaster*, and that continual translation is required.

Gerber-Huber et al (1981) showed that the YP A1 and A2 *Xenopus* genes are more DNase 1 sensitive after estrogen treatment in male hepatocytes. Hayward (1982) found that de novo protein synthesis is not required for the initiation of YP gene transcription, after estrogen induction, which supports the idea that vitellogenin synthesis is a direct response to estrogen.

Estrogen can induce vitellogenin synthesis in all stages of *Xenopus* after metamorphosis, but not before (Knowland, 1983). He has found that non-inducible tadpoles contain receptors but synthesis of this receptor cannot be induced by estrogen, while at inducible stages estrogen causes an increase in estrogen receptors as well as vitellogenin synthesis. Furthermore, in contrast to the situation in *D. melanogaster*, the sex-specificity in vitellogenin synthesis can be explained by different estrogen levels in male and female *Xenopus*. The increased effect on secondary stimulation can be explained because primary estrogen treatment causes new synthesis of nuclear receptors which are stable for several months (Knowland, 1983). After primary stimulation the four vitellogenin genes are not turned on in a co-ordinated manner (B genes are transcribed before A genes), whereas they are after secondary stimulation. This is the case for male and female hepatocyte cultures (Pearlman et al, 1983). As already mentioned Walker et al (1984) have found a consensus sequence upstream from the four *Xenopus* and one chicken vitellogenin gene which may play a part in estrogen-receptor DNA binding. However, I have not been able to detect this consensus
sequence in the D. melanogaster YP1 and YP2 gene sequences by computer search.

As in Xenopus, chicken vitellogenin is synthesized in the liver in response to estrogen (Cozens et al., 1980; Wang, 1981). Also, again, the response is not in a co-ordinated fashion with respect to the vitellogenin I and II genes. The induction of vitellogenin genes in the rooster appears to be controlled at the transcriptional level (Protter, 1983). Estrogen stimulation causes the association of the vitellogenin gene with the nuclear matrix (Jost and Seldran, 1984), which precedes transcription. An estrogen-receptor complex binding site has been detected upstream from the vitellogenin II gene (Jost et al., 1984).

My aims were to study the levels of control of expression of the YP genes, in response to 20-OH ecdysone, in D. melanogaster males and to determine whether the response could be detected in other species of Drosophila.
Fig. 1:2 (a). Structures of Ecdysone (IV), 20-Hydroxyecdysone (V), Makisterone A (VI), Podecdysone A (VII), 20-Deoxymakisterone A (VIII), and 26-Hydroxymakisterone A (IX).

JH-O \( R' = R'' = R''' = C_2H_5 \)
JH-I \( R' = R'' = C_2H_5 \), \( R''' = CH_3 \)
JH-II \( R' = C_2H_5 \), \( R'' = R''' = CH_3 \)
JH-III \( R' = R'' = R''' = CH_3 \)

Fig. 1:2 (b). Structures of Juvenile Hormones O, I, II, and III.
Evolution of the Melanogaster Group Species

The evolutionary position of Drosophila species within the Diptera is described by Wheeler (1980) and Throckmorton (1975). Within the Melanogaster Group species of Drosophila, as defined by Sturtevant (1942), the Melanogaster Subgroup (or Melanogaster sibling species) was later defined (Bock and Wheeler, 1972). The sibling species are all medium brown flies with clear wings, and the males are black distally and have sex-combs. Other distinguishing features are described by Bock and Wheeler (1972). The two best known species, D. melanogaster and D. simulans, are both cosmopolitan; four of the remaining species have been found only in regions of Africa (D. yakuba, D. erecta, D. teissieri and D. orena); D. mauritiana is found on the island of Mauritius and D. sechellia on the Seychelles Islands. The species, except for D. melanogaster, D. simulans and D. yakuba, have only been discovered during the last twenty years. These species can only be reliably separated by comparing genitalia of the males (Bock and Wheeler, 1972).

As well as studying the morphology of these species, a variety of methods have been employed to construct phylogenetic trees. The generally accepted phylogeny is shown in Fig 1:3. It should be remembered that "phylogenetic analysis to date have almost always suffered by being incapable of defining a direction of evolution within the phylad of interest" (Giddings and Templeton, 1983). Work on the Melanogaster sibling species is no exception.

Behavioural studies have suggested that D. melanogaster, D. mauritiana and D. simulans are especially closely related as they can be crossed although most of the offspring are sterile (Sanchez, 1982). An extensive study comparing the polytene chromosome banding sequences by Lemeunier and Ashburner (1976) gave the first phylogeny of the siblings, except D. orena and D. sechellia, again suggesting that D. melanogaster, D. mauritiana and D. simulans are most closely related. Variations in electrophoretic migrations of polypeptides, especially enzymes (Eisses et al 1979; Gonzalez et al, 1982) have
been used. Ohnishi et al (1983) used 2D electrophoretic gels while Kabisch et al (1982) compared cross reactivity of monoclonal antibodies prepared against non-histone chromosomal proteins. These workers came to the same essential conclusions as Lemeunier and Ashburner.

The advent of molecular cloning, and protein and DNA sequencing methods, has led to rapid growth of information on *Drosophila* evolution. Zwievel et al (1982) found that, despite being very closely related, 35% of *D. melanogaster* single copy DNA fails to hybridize with *D. mauritiana* or *D. simulans* total DNA, which suggests that single copy DNA is rapidly evolving. The presence of repetitive dispersed DNA, including transposable elements, is varied for the sibling species, although "alpha" and "beta" middle repetitive sequences (complementary to a class of heat shock induced RNAs found at locus 87 CI) were detected in all the sibling series (Martin et al 1983; Dowsett, 1983), and "p" elements (derived from transposable elements, P factors, that are responsible for causing hybrid dysgenesis in *Drosophila*) have only been detected in *D. melanogaster* (Brookfield et al, 1984). Fauron and Wolstenholme (1980) found that the sibling species have similar restriction enzyme recognition sites in mitochondrial DNA (mt DNA), but found that A + T rich regions varied between species, except *D. melanogaster*, *D. simulans* and *D. mauritiana*, suggesting that some regions are evolving faster than others. A highly repetitive nucleotide sequence of *D. melanogaster* was also found in only *D. simulans* and *D. mauritiana* of the siblings and was found to be present on different chromosomes in these species (Cseko et al, 1979). Coen et al (1982) and Barnes et al (1978) compared ribosomal DNA, histone gene, and satellite-type DNA families in the sibling species which resulted in the same phylogenetic tree as is shown in Fig 1:3.

Much work has been carried out recently on the alcohol dehydrogenase gene (Adh). Zwiebel et al (1982) and Bodmer and Ashburner (1984) have cloned this gene for *D. mauritiana*, *D. simulans*, *D. orena* and *D. melanogaster* and the latter authors have sequenced and compared
these genes. The phylogeny of species based on nucleotide sequencing is again consistent with that shown in Fig 1:3, and they also showed that rates of change of nucleotides vary greatly in different functional domains of this gene, presumably as a result of differing selective forces. As expected, from work of Miyata et al (1980), nucleotide substitutions in coding regions rarely result in coding for a different amino acid. Furthermore, Dover et al (1984) have evidence for concerted evolution (higher nucleotide homology within species than between them) occurring in the sibling species.

Work on the evolution of the vitellogenin genes has been conducted for Xenopus tropicalis and Xenopus laevis (Jaggi et al, 1982) which suggests that the genes have originated by duplication events. Further comparisons of the four X. laevis and one chicken vitellogenin genes has been made by Germond et al (1984) who showed that all these genes have three similar exons, and again suggests that a gene duplication event followed by a chromosomal duplication event led to the existence of the four X. laevis vitellogenin genes.

For insects De Loof (1984) and Harnish and White (1982) point out that vitellogenins can be placed into three groups. Firstly, there is the largest group of insects (including H. cecropia and B. mori) which have vitellogenins synthesized as primary products of approximately 200,000 daltons. These are later cleaved to yield polypeptides of around 150,000 and 50,000 daltons. The second group of insects (including Aedi aegypti and Sphecid wasps) produce slightly smaller primary product proteins, of about 180,000 daltons, which are not cleaved prior to secretion. The third group of insects synthesize yolk polypeptides of about 50,000 daltons directly, and include species of Calliphora and Drosophila.

Some work has been done on the evolution of vitellogenins in Hawaiian Drosophila employing polyacrylamide gel electrophoresis, isoelectric focusing and V8 protease peptide mapping (Kambysellis et al, 1984). The only work on the Melanogaster sibling species has involved yolk polypeptide comparisons between a few of these species (Bownes, 1980B; Srdic et al, 1979). My aim was to compare the yolk
polypeptides for all the sibling species, using anti YP-antibody reactivity as a detection method, and gradient polyacrylamide gel electrophoresis for polypeptide separation. Conservation of restriction enzyme recognition sites in and around the YP-genes would be investigated for the sibling species by Southern blotting (Southern, 1975) of genomic DNA, and chromosomal locations would be determined by in situ hybridization (Pardue et al, 1970; Pardue and Gall, 1972). The logical progression would be to isolate, using cloning methods, DNA sequences containing yolk protein genes from the sibling species and compare the evolution of these genes between, and within, these species.
Fig. 1: THE PHYLOGENY OF THE DROSOPHILA MELANOGASTER SIBLING SPECIES

BASED ON CHROMOSOME BANDING PATTERNS (LEMEUNIER AND ASHEURNER, 1970).
CHAPTER TWO

YOLK PROTEIN INDUCTION IN MALES OF DROSOPHILA SPECIES

2.1 INTRODUCTION

Adult *D. melanogaster* males do not normally synthesize yolk polypeptides, although they can be induced to do so by either feeding or injecting flies with 20-OH ecdysone (Postlethwait et al., 1980). The response following hormone induction is a short burst of yolk polypeptide synthesis lasting about 24 hours, the maximum rate of synthesis being between 8 and 14 hours after hormone treatment (Bownes, 1982). 20-OH ecdysone treatment also leads to accumulation of yolk-protein transcripts (Shirras and Bownes, unpublished), the peak of which occurs 16 hours after induction. Therefore, it appeared that there may be a period when yolk protein gene transcripts were not being efficiently translated into yolk polypeptides *in vivo*. 
It is of interest to determine whether any biological phenomenon is particular to one species or whether it occurs in a variety of species. Although yolk polypeptides of several species of Drosophila had been identified (Bownes, 1980A; Kambysellis, 1977), it was not known whether their synthesis could be induced in males.

An attempt was made to determine the level or levels at which the hormone-induced expression of the yolk protein genes is regulated in D. melanogaster males, and to go some way towards explaining why the response to induction is so short-lived. The question of whether males of other Drosophila species could be induced to synthesize YP-transcripts and yolk polypeptides was also investigated.

Therefore, adult males were injected with 20-OH ecdysone and their responses were investigated. Analysis of yolk polypeptides and YP-transcripts from the same population of flies was necessary to avoid bias resulting from biological variation, which had been observed in induction experiments. Haemolymph polypeptides, sometimes radioactively
pulse-labelled, were separated by electrophoresis on gradient SDS polyacrylamide gels. Yolk polypeptides were identified by cross-reaction with anti-YP antibodies, raised using D. melanogaster yolk proteins injected into rabbits (Isaac and Bownes, 1982). Accumulation of YP transcripts was determined by measuring the hybridization of radioactively labelled cloned XP-genes (pYP1, pYP2, and pYP3) to total RNA samples bound to nitrocellulose. Yolk protein transcripts were further characterised by determining how total RNA directed the in\_vitro translation of yolk polypeptides.
2:2 MATERIALS AND METHODS

Maintenance of Drosophila Stocks

Fly stocks, except *D. orena*, were maintained on standard yeast, cornmeal, sugar and agar medium at 25°C. *D. orena* was kept on oatmeal, black treacle, yeast and agar medium at 18°C. All stocks were maintained with 0.15% nipagen (antifungal agent) and kept uncrowded.

Collection of Haemolymph Polypeptides

Pooled haemolymphs of each set of flies (10-15) were collected into 50μl Laemmli buffer (Laemmli, 1970), or antibody precipitation buffer (APB).

Treatment with Hormone

3 day old adults were lightly etherized and injected with 0.2μl of 10⁻²M 20-OH ecdysone in Ringers (Chan and Gehring, 1971). Control flies were injected with Ringers.

Isolation of RNA

Flies were homogenized on ice in 10 mM Tris/Hcl pH 7.3, 0.5% SDS, 2mM Mg Cl₂, phenol extracted once and phenol/chloroform extracted (and back extracted) four times. The aqueous layer was centrifuged at 5K for 5 minutes to remove debris, then precipitated using ethanol plus 0.3M Naacetate), and the pellet resuspended in 0.1M NaCl, 0.01 M Hepes and again precipitated with ethanol (without NaAcetate). The pellet was dried and resuspended in distilled water.

Translation of RNA in a Cell-free Translation System

One ml of rabbit reticulocyte lysate was added to 25μl haemin (1mM in 90% ethylene glycol, 20mM Tris/Hcl pH 7.2, 50mM KOH), 10μl
creatine Kinase (5mg/ml in 50% glycerol), 50μl amino acid mix minus methionine (each 50mM). For a background control 100μl lysate was mixed with 5 μl [35S]-methionine (40-80μCi). To the remaining 900μl, 9μl CaCl₂ (0.1M) and 5μl micrococcal nuclease (1mg/ml) was mixed on ice and incubated at 20°C for 15 minutes, followed by the addition of 9μl EDTA (0.2M neutralized with KOH). 100μl aliquots were each mixed with 5μl [35S]-methionine and 25μl aliquots were added to 5μg RNA (in 5μl H₂O) each of 5μl H₂O as controls. Tubes were mixed on ice for 2 minutes, vortexed and centrifuged briefly and incubated at 30°C for 30 minutes. 2μl samples were precipitated with TCA and collected onto Whatman glass fibre filters for scintillation counting, the rest stored under liquid nitrogen.

The method is essentially that described by Pelham and Jackson (1976).

Preparation of Rabbit Reticulocyte Lysate

The method followed was that of Jackson and Hunt (1983). The prepared lysate was aliquoted into eppendorf tubes and stored frozen in liquid nitrogen.

Precipitation with Anti-YP Antibody

Yolk polypeptides were precipitated as described by Isaac and Bownes (1982). Polypeptide containing solutions were made up to 1 x antibody precipitation buffer (APB) using 5 x APB (1 x APB is 0.1M Sodium phosphate buffer pH7.5, 7.5% triton x 100, 7.5% Sodium dioxycholate, 0.7 M NaCl, 5mM PMSF, and 0.25% Sodium azide). 2μg of carrier yolk-proteins was added if samples were radioactive. 25μl of anti-YP antibody was added to each sample and incubated at 30°C for one hour. Samples were left at 4°C overnight, then vortexed and left at 20°C for 30 minutes before laying over 200μl 1M sucrose in 1 x APB cushions and centrifuging for 15 minutes at 10k. Pellets were twice resuspended in 0.4 ml 1 x APB and centrifuged for 15 minutes before resuspending in 1 x laemmli buffer (Laemmli, 1970).
Preparation of $^{32}$P-labelled YP-Probes

Genomic DNA sequences containing yolk protein genes cloned into plasmid pBR322 (called pYP1, pYP2 and pYP3) of Barnett et al (1980) were nick-translation. 30µl of $^{32}$P d CTP was dried down in an eppendorf tube. To the tube, on ice, was added 5µl 4 x NT buffer (210mM Tris-Hcl pH 7.5, 21mM MgCl2, 20µg/mlBSA), distilled H2O (to make final volume 20µl), 4µl dATP, dGTP, TTP mix (0.5mM each), 1µg DNA, and 2µl DNase (1 x 10^-5 mg ml^-1). This was mixed and incubated at 37°C for 15 minutes, then 2µl DNA polymerase I (2u/µl) was added, mixed and incubated at 16°C for 1½ hours. 1µl samples were spotted directly onto Whatman glass fibre filters and also precipitated with TCA in order to determine radioactive incorporation into DNA. The remainder was passed through a G50 Sephadex column to separate labelled DNA from unincorporated nucleotides. The first radioactive "peak" of DNA was collected and stored at 4°C. Probe activities were typically $10^7 - 10^8$ cpm/µg DNA.

Preparation of RNA Dot Blots

RNA samples were spotted directly onto nitrocellulose filters (pretreated with H2O, equilibrated with 20 x SSC and dried). 5µg samples (in no more than 5µl of distilled H2O) were spotted onto the nitrocellulose, dried and baked for 2 hours at 80°C in a vacuum oven.

Electrophoretic RNA Separation on Formaldehyde/Formamide Gels

RNA samples were prepared in 50% formamide, 2.2M HCHO, 1 x gel buffer (20mM MOPS, 5mM Na acetate, 1mM EDTA; pH to 7.0 with Acetic acid). They were heated to 60°C for 5 minutes and ficoll dye added to 1 x (2% ficoll, 0.02% bromophenol blue in water). They were then separated on a 1% agarose gel in 1 x gel buffer plus 2.2M HCHO, overnight at 50v. Gels were treated with 2µg/ml ethidium bromide in 0.1M Ammonium acetate at room temperature for a few hours before viewing under UV light. Gels to be transferred to nitrocellulose were not treated with ethidium bromide.
Northern Transfer of RNA onto Nitrocellulose Filters

The untreated gel was placed on blotting paper soaked in 20 x SSC connected to a reservoir of 20 x SSC. Nitrocellulose, presoaked in 20 x SSC, was layed onto the gel and a further piece of blotting paper, also presoaked in 20 x SSC, placed on top. A stack of dry blotting paper was placed on top followed by approximately 500g of weights. The apparatus was covered with clingfilm and transfer allowed to continue for at least 24 hours. The nitrocellulose was then baked at 80°C in a vacuum oven for 2 hours.

Hybridization of RNA dot blots and Northern transfers with $^{[32]}$P-DNA Probes

Filters were prehybridized for 8 - 20 hours at 37°C, shaken in a polythene bag, in an excess of 50% formamide, 5 x SSC, 50mM Sodium phosphate buffer pH 6.5, 250μg/ml sonicated salmon sperm DNA, 1 x Denhardts. The radioactive probe was made up to 10 mls with 4 parts prehybridization buffer to 1 part 25% dextran sulphate. This was heated in a boiling waterbath for 5 - 10 minutes and rapidly cooled on ice before hybridizing with the filters, in a plastic bag. Filters were hybridized for at least 24 hours at 37°C, shaking.

Filters were washed 4 times for 10 minutes each shaking at room temperature in 2 x SSC, 0.1% SDS. They were further washed 2 times for 20 minutes each shaking at 52°C in 0.1 x SSC, 0.1% SDS, then dried and set up against x-ray film for autoradiography. Such stringency gave, for D. melanogaster, no cross-hybridization between pYP1 and pYP3, or pYP2 and pYP3, but pYP1 and pYP2 showed 10% cross hybridization. The YP1 specific probe (YP1 HBM13) appears entirely specific to pYP1 under these conditions. This would be expected from the sequences of these genes (Hovemann and Galler, 1982). For dot blots the area x density on the autoradiographs were calculated. This technique is useful for comparing transcript levels between samples, but does not give absolute quantitation of transcripts, nor does it distinguish hnRNA from mRNA. The weak hybridization seen when male RNA is spotted is due to the DNA isolated along with the
RNA. A similar signal is seen after alkaline hydrolysis of similar quantities of female RNA, and the signal is removed when the samples are treated with LiCl to remove the DNA.

Reprobing of Nitrocellulose Filter

Whenever filters were to be reprobed with a different \(^{32}\)P-labelled probe they were first washed, shaking, at 65°C overnight in wash off probe (WOP) buffer (3.75mM Tris/HCl pH 8.0, 0.15mM EDTA, 0.037% sodium pyrophosphate, and 0.0015% of each of BSA, Ficoll, and polyvinylpyrrolidone. When such washed filters were exposed to x-ray film for several days no signals on the autoradiograph could be detected.
Separation of Polypeptides by Gradient SDS Polyacrylamide Electrophoresis

7 - 20% gradient SDS polyacrylamide gels were poured using a stirring gradient-making apparatus. The 20% gel solutions consisted of 0.37 M Tris/HCl pH 8.8, 0.1% SDS, 20% acrylamide, 0.53% bisacrylamide and 13.6% sucrose. The 7% gel solutions consisted of 0.37 M Tris/HCl pH 8.8, 0.1% SDS, 7% acrylamide and 0.19% bisacrylamide. 35 µl of 15% ammonium persulphate and 5.5 µl of TEMED were used to set each of these solutions. The running buffer was 14.4 g/l glycine, 3 g/l Tris, 0.1% SDS. Samples were boiled in Laemmli buffer for 15 minutes, vortexed and briefly centrifuged, and loaded with a Hamilton syringe. Gels were run for 16 - 20 hours at 140V.

SDS Polyacrylamide Gels Comassie Blue Staining

Gels were fixed, shaking, for 30 minutes in 20% TCA. They were stained, shaking, for 2 - 4 hours in 0.1% Comassie blue, 50% methanol. They were then destained and kept in 50% methanol.

SDS Polyacrylamide Gel Silver Staining

Silver staining has proved a more sensitive method of protein staining compared with use of Comassie Blue. Gels were washed in 45% methanol, 10% acetic acid for 30 minutes, and 5% methanol, 7% acetic acid for 30 minutes. They were then fixed in 10% glutaraldehyde for 30 minutes and washed in water for at least one hour before washing in 5 µg/ml Dithiothreitol for 30 minutes, and 0.1% silver nitrate for 30 minutes. After rinsing in water they were developed in 3% sodium carbonate, 0.037% formaldehyde and stopped with 0.1M Citric acid. They were then washed in water and stored, in the dark, in 0.03% sodium carbonate.

Fluorography of SDS Polyacrylamide Gels

Gels containing samples pulse labelled with [35S] - methionine were enhanced for autoradiography essentially as described by Bonnar and
Laskey (1974). Gels were fixed in 20% TCA for 30 minutes followed by three washes in DMSO for 30 minutes each, then washed in 22% PPO in DMSO for 3 hours, and washed in water for at least 30 minutes, dried on a gel dryer and set up for autoradiography.

Western Transfer of Proteins to Nitrocellulose and anti-YP Antibody Detection of Yolk Polypeptides

Untreated SDS polyacrylamide gels were transferred using a Bio-Rad Transfer Cell for 4 hours at 0.3 Amp (50-60v) in TGM (14.4 g/l glycine, 3 g/l Trisbase, 20% methanol), onto nitrocellulose. The nitrocellulose was washed for 1 hour in OTS (5% ovalbumin, 20mM Tris/Hcl pH 7.4, 0.9% NaCl, 0.5mg/ml Sodium azide) followed by 1½ hours in OTS plus 50μl anti-YP antibody (50 mls volume). It was next washed in TS (20mM Tris/Hcl pH 7.4, 0.9% NaCl), 5 changes over 30 minutes, before washing for 1 hour in OTS plus 60μl Goat anti-rabbit IgG conjugated to horseradish peroxidase (30ml volume). After another 5 changes of TS over 30 minutes the filters were washed in enzyme substrate solution (10mM Imidazole pH 7.5, 0.3% hydrogen peroxide, 25μl/ml -dianisidine) until the polypeptides stained. Filters were washed in water and stored, dry, in the dark.

For densitometer scanning it was necessary to bind iodinated [125I]-Protein A to the filters. Protein A binds specifically to all antibodies. Filters were preincubated in OTS at 37°C for 30 minutes, followed by incubation in 30 mls OTS plus 20μl [125I]-Protein A for 1 hour at 37°C, and finally washed 5 times in TS for at least 30 minutes, dried and set up for autoradiography.

Determination of RNA Concentrations

RNA concentrations were calculated by optical density absorbance measurements by a UV spectrophotometer. OD_{260} = 40μg RNA. In a more accurate control [32p]-labelled probes of ribosomal DNA genes were used to standardize amounts of RNA. Thus nitrocellulose
filters which were probed with radioactive YP-genes were also reprobed with radioactive ribosomal DNA cloned genes (18s and 28s DNA cloned into pBR322).
MEASUREMENT OF DOT BLOT HYBRIDIZATION

The area X density of the dot blots on the autoradiograph was measured using a densitometer. It was shown by using standards of different quantities of RNA, exposed to X-ray film for various times, that there is a linear response between transcript quantities from 2.5 µg to 15 µg and values of hybridization strength determined by densitometer, for the varying times of autoradiograph exposure used.
2.3 RESULTS

Yolk polypeptide synthesis was measured by injecting D. melanogaster male adult flies with \(^{35}\text{S}\)-methionine and collecting the haemolymph two hours later for analysis by SDS polyacrylamide gel electrophoresis and fluorography. Results for D. melanogaster are shown in Fig. 2.1, quantitations shown in Table 2:1, and as a histogram in Fig. 2:2. In this species there is a burst of yolk polypeptide synthesis lasting for at least 24 hours after hormone treatment, maximal synthesis being at about 12 hours, when 76% of the female level was reached.

To investigate the possibility of induction of yolk polypeptide synthesis in males of other species (sibling species of D. melanogaster, plus species, D. funebris and D. virilis, which are phylogenetically outside the Melanogaster Species Group) similar experiments were carried out. After injection of 20-OH ecdysone into male flies the presence of yolk polypeptides was analysed in these species. As in D. melanogaster, 16 hours after hormone treatment, there are high levels of yolk
polypeptides, so the flies were analysed at this time point. To detect the yolk polypeptides, haemolymph samples were collected and the polypeptides separated by SDS polyacrylamide gradient gel electrophoresis. The proteins were stained by the silver staining technique, the results shown in Fig. 2:3, quantitations shown in Table 2:1. Total proteins loaded in each track, and those corresponding to yolk polypeptides were scanned using a densitometer. By calculating the total protein content of each track variable loading could be corrected for. As in D. melanogaster, there are proteins which co-migrate with the yolk-polypeptides which are present in non-induced male tracks (see Fig. 2:3). Quantitation of the yolk polypeptide present, taking into account both of these problems, are shown in Table 2:1. Yolk polypeptides have accumulated in males, 16 hours after induction, in all eight species studied. That the induction is specific to the yolk polypeptides and perhaps a small number of other proteins is supported by the fact that other haemolymph proteins are present at approximately equal amounts in males, females and induced males. Others still (see polypeptide marked PE1 in Fig. 2:3) are present in large amounts in males, and present in small amounts
or not detectable in females, while after induction, the male levels appear to fall towards that of females.

To be sure that the co-migrating polypeptides were indeed yolk polypeptides was determined using their cross-reactivity with the anti-YP antibody prepared against *D. melanogaster* yolk proteins after Western blotting. The results are shown in Fig. 2:4. In order to quantitate the results, $^{125}\text{I}}$-Protein A was incubated with the filters and the resulting autoradiographs scanned by densitometry (results shown in Table 2:1). Most species show a clear induction of yolk polypeptides in hormone treated males. With *D. simulans* only a small accumulation of yolk polypeptides in induced males is detectable, while in *D. mauritiana* there is no significant presence of yolk polypeptides in induced males. This may have been due to the poor ability of the antibodies raised from *D. melanogaster* to cross react with the yolk polypeptides from these two species, but this is unlikely since the female control track in both cases shows strong cross-reactivity. However, such an explanation is likely to account for the apparent lack of induction observed in *D. funebris* after Western blotting.
Gels stained for protein (Fig. 2:3) suggest a strong induction of yolk polypeptides in induced males. Therefore, the apparently conflicting results shown in Table 2:1 for *D. funebris* are because even female control tracks show no cross reactivity with antibodies raised against *D. melanogaster* yolk proteins. This is probably due to the high degree of evolutionary divergence between these two species, which is confirmed in Chapter Three. The situation for *D. virilis* is beyond doubt as, by Western blotting, yolk polypeptides are present in induced male haemolymphs.

Accumulation of yolk protein transcripts was analysed by RNA Dot Blots. Fig. 2:5 shows Dot Blots of *D. melanogaster* RNA samples and Fig. 2:6 for the other species, hybridised against pYP1/2/3 probes. Quantitations are shown in Table 2:1 and as histograms in Fig. 2:2 and Fig. 2:7. For *D. melanogaster* the peak of transcript accumulation was at 16 hours (101% of female level) and fell by 24 hours (54% of female level). In this group of flies the peak of yolk polypeptide synthesis preceded the peak of YP-transcript accumulation. The accumulated transcript levels dropped by 50% between 16 and 24 hours after induction, suggesting a half-life for
the male YP-transcripts of not more than 8 hours. In all the sibling species RNA transcripts homologous to the yolk protein transcripts have accumulated in the induced males, at the 16 hour time point (Table 2:1), often being present in quantities in excess of female values. Although not all Dot Blots are from the same filter, they were prehybridized, hybridized and washed together. As might be expected, the female hybridization signal using YP-DNA probes was greater for D. melanogaster than that observed in any of the sibling species female values, this probably being due to divergent evolution, at the nucleotide level, of the yolk protein genes in the sibling species. Therefore, for the same number of yolk protein transcripts present, the D. melanogaster cloned probes would hybridize more strongly to D. melanogaster YP-transcripts than to those from sibling species. D. virilis and D. funebris have diverged further still from D. melanogaster and here the D. melanogaster YP-gene probes are unable to show significant hybridization to female RNA.

It is interesting to compare the results of Dot Blot hybridization to the YP1-specific probe (YPLHBM13) with those to the mixed YP1/2/3 gene probe. The
induced male:female ratio of hybridization signals tend to differ, often markedly, as shown in Fig. 2:7. In particular, the induced male:female ratios for YP1-like transcript accumulation are high for four species *D. erecta*, *D. teissieri*, *D. simulans* and *D. mauritiana*; but the ratios for mixed YP1/2/3-like transcript accumulations are lower. This could be the result of induction of YP-transcripts occurring in a non-co-ordinated fashion. In these instances, the YP1-like genes appear to be transcribed preferentially in induced males, compared with the other yolk protein genes. However, data of the yolk polypeptides present in the same flies (see Fig. 2:3 and Fig. 2:4) does not support this theory. There are no high levels of YP1 yolk polypeptides in any of these species, relative to the other yolk polypeptides, although this could be the result of post-transcriptional controls.

To be sure that the increase in YP-transcripts after hormone treatment was not a result of 20-OH ecdysone generally stimulating transcription, all RNA Dot Blots were reprobed with ribosomal DNA (cloned into pBR322). These genes are expressed in all cells at all developmental stages. In all cases the RNA Dot
Blots showed almost identical hybridization signals when probed with $^{32}\text{P}$-labelled ribosomal DNA cloned genes (see Fig. 2:5 for an example). Firstly, this confirmed that equal amounts of RNA were loaded on the Dot Blots. Secondly, the results are consistent with the view that 20-OH ecdysone specifically stimulates transcription of the YP-genes, or at least just a small set of genes. However, as the ribosomal DNA genes are transcribed by RNA polymerase I, the results do not eliminate the possibility that 20-OH ecdysone stimulates transcription of all genes transcribed by RNA polymerase II.

At the 16 hour time point, after induction, both D. simulans and D. mauritiana show high levels of YP1-like transcripts present (113% and 77% of female values). However, the same flies contain non-detectable or very low levels of yolk polypeptides present in their haemolymph (2.5% and 3.4% of female values from Western analysis).

For D. melanogaster, RNA isolated from males after hormone treatment was analysed by Northernns and, as can be seen in Fig. 2:8, YP-transcripts were always of similar size to those observed in females. There
is no signal in male samples before hormone treatment, and the lack of unusual-sized transcripts suggests that there are no major differences in the transcripts induced in males compared to those normally present in females. Small differences in processing or transcript size would not be detected by this method.

*D. melanogaster* total RNA from induced males and control females was translated in the rabbit reticulocyte lysate cell-free translation system (Fig. 2:9). As shown in Fig. 2:2, the synthesis of yolk polypeptides follows the general trend of total transcripts present, but the male YP-transcripts were, in all cases, translated less well in vitro than were the female YP-transcripts. This is not true of all other male messages since the general stimulation of protein synthesis in the cell free system was from 19000 - 52000 cpm/µl of lysate stimulated with male RNA and 27000 cpm/µl of lysate stimulated with female RNA. These in vitro translation studies, indicating that a peak of yolk polypeptide synthesis occurs at least 16 hours after 20-OH ecdysone treatment in male flies, and that total RNA from female flies translates more efficiently into yolk polypeptides than does RNA
from male flies, are reproducible. Data from one such experiment is shown in Table 2:2.
Fig 2:1

Induction of yolk polypeptide synthesis in D. melanogaster males by 20-OH ecdysone. Autoradiograph of haemolymph polypeptides, \[^{35}S\] methionine-labelled for 2 hours, separated on a gradient SDS polyacrylamide gel. The three yolk polypeptides are shown (YPs).

\[ \varphi = \text{control female haemolymph yolk polypeptides} \]
\[ \circ = \text{control male comigrating polypeptides} \]
\[ 4-24 = \text{hours after 20-OH ecdysone treatment of male flies.} \]

Fig 2:9

Autoradiograph of in vitro translation of yolk protein transcripts by the rabbit reticulocyte lysate cell-free system, followed by precipitation with anti YP-antibody. The precipitated proteins were separated on a gradient SDS polyacrylamide gel.

\[ E = \text{endogenous rabbit reticulocyte translation track} \]
\[ \varphi T = \text{control female haemolymph yolk polypeptides} \]
\[ \varphi = \text{control female haemolymph yolk polypeptides after antibody precipitation} \]
\[ \circ = \text{control male haemolymph polypeptides} \]
\[ 4-24 = \text{hours after 20-OH ecdysone treatment of male flies} \]
FIG 2:1

FIG 2:9
YP-synthesis in vivo, YP-transcript accumulation, and YP-translation in cell-free translation system. A group of males was injected with 20-hydroxyecdysone and the haemolymph proteins labelled with [35S]methionine at various times. RNA was then extracted and YP-transcripts measured by dot blotting and the RNA was translated in the rabbit reticulocyte lysate cell-free translation system and precipitated with anti-yolk-protein antibody. Each graph plots the relative YP synthesis or YP-transcript accumulation against time after hormone treatment. In the in vitro and in vivo YP-synthesis the units are the area under the YPs on the autoradiograph after the polypeptides had been separated by gel electrophoresis. In the transcript accumulation experiments they are the area x density of the dot. The female control from the same population is shown in each case as the hatched area on the histograms.
Fig 2:3

Gradient SDS polyacrylamide gel electrophoresis of haemolymph polypeptides showing induction of yolk polypeptide synthesis in *Drosophila* species males by 20-OH ecdysone.

(e) male flies injected with 20-OH ecdysone 16 hours before haemolymph samples were collected.

(AB) polypeptides precipitated with anti-YP antibodies

(Pei) PEI polypeptides
Western transfers of gradient SDS polyacrylamide gels of haemolymph polypeptides from *Drosophila* species, showing induction of yolk polypeptide synthesis after treatment of males with 20-OH ecdysone.

\( e = \) male flies injected with 20-OH ecdysone 16 hours before haemolymph samples were collected

\( Ab = \) polypeptides precipitated with anti-YP antibodies
Fig 2:5

Induction of YP-transcript accumulation in *D. melanogaster* males following 20-OH ecdysone treatment.

(A) RNA dot blots hybridized against a mixed YP1/2/3 probe.

(B) The same dot blots hybridized against a ribosomal DNA probe.

♀ = female control
♂ = male 0 hour control
4-24 = hours after 20-OH ecdysone injection of males
Fig 2:6

Dot blots showing the induction of YP-homologous transcripts in males of several Drosophila species, after 20-OH ecdysone treatment. The dot blots were hybridized against a mixed YP1/2/3 probe. The MEL CONT track shows male and female control dot blots.

♂ = 5µg male RNA
♀ = 5µg female RNA
♂E = 5µg male RNA 16 hours after 20-OH ecdysone injection
RELATIVE AMOUNTS OF YP1-HOMOLOGOUS (OPEN BARS) AND YP1/2/3-HOMOLOGOUS (SHADED BARS) TRANSCRIPTS IN MALE 20-OH ECIDYSONE TREATED ADULTS COMPARED WITH FEMALE ADULTS.
Northern analysis of RNA extracted from whole male flies induced to produce YP-transcripts with 20-hydroxyecdysone. RNA was separated on formaldehyde gels, transferred to nitrocellulose and hybridized to pYP1, pYP2, and pYP3. The resulting autoradiograph is shown. (a) ♀ control; (b) 0 h after hormone treatment; (c) 4 h after hormone treatment; (d) 8 h after hormone treatment; (e) 12 h after hormone treatment; (f) 16 h after hormone treatment; (g) 20 h after hormone treatment; (h) 24 h after hormone treatment.

**FIG 2:8**
\[ Z = \text{corrected for background in untreated male samples} \]

<table>
<thead>
<tr>
<th>Species of Males Injected</th>
<th>Time After Treatment in Hours</th>
<th>Haemolymph YPs</th>
<th>Haemolymph YPs After Western Blotting</th>
<th>YP1/2/3-Homologous Transcripts</th>
<th>YP1-Homologous Transcripts</th>
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**TABLE 2:1**  
YP-Synthesis and YP-Transcript Accumulation in Males Treated with 20-OH ecdysone compared to wild-type Females
<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>COUNTS PER MINUTE</th>
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<tr>
<td>Endogenous Lysate</td>
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<tr>
<td>Male flies treated with 20-OH ecdysone after 0 hrs</td>
<td>760</td>
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<tr>
<td>&quot; &quot; &quot; &quot; &quot; &quot; &quot; &quot; &quot; 4 hrs</td>
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<td>2590</td>
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<tr>
<td>&quot; &quot; &quot; &quot; &quot; &quot; &quot; &quot; 24 hrs</td>
<td>2010</td>
</tr>
<tr>
<td>Female Control</td>
<td>5340</td>
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</table>

**TABLE 2:2** In vitro translation of RNA from male *D. melanogaster* flies treated with 20-OH ecdysone. 5 μg of total RNA was *in vitro* translated in each case, the yolk polypeptides synthesized were precipitated using anti-YP antibodies, precipitated with TCA and counted on a scintillation counter.
2:4 Discussion

2:4.1 Transcriptional and Post-transcriptional Regulations

The results presented for D. melanogaster suggest that when the YP-genes are expressed in males, following treatment with 20-OH ecdysone, the actual level of gene expression may be modulated by post-transcriptional as well as transcriptional controls. After hormone treatment yolk polypeptide synthesis begins in males as YP-transcripts appear. At first they are translated efficiently into yolk polypeptides, compared to translation of transcripts present in females. Shirk et al (1983), measured YP-transcript accumulation and yolk polypeptide synthesis at just one time point (8 hours) after hormone treatment. Their data also show that at this time the transcripts present are translated efficiently into yolk polypeptides. Following this, translation into protein becomes inefficient in males, in relation to the transcripts accumulated. The peak YP-transcript accumulation was at 16 hours and fell by 24 hours. The peak of yolk polypeptide synthesis (12 hours) preceded the peak of YP-transcript accumulation, which suggests that
post-transcriptional control mechanisms may regulate expression of the YP-genes as well as factors at the transcriptional level. The half-life of male YP-transcripts is no more than 8 hours.

Post transcriptional control in this context means any type of control being exerted on the production of yolk polypeptides from the time when primary transcripts have been synthesized to the time when the yolk polypeptides are present in the haemolymph. This includes processing and export of mRNA into the cytoplasm, translation, processing of primary yolk polypeptides and cellular export mechanisms. Control could be the result of an increasing or decreasing action of any controlling factor(s). However, it is also possible that the results are due to a secondary or indirect effect of the steroid hormone on the system, for instance, by altering amino acid pools, which would not necessarily be acting specifically on the yolk protein transcripts, translation or processing of yolk polypeptide products.

The yolk proteins produced in a cell-free translation system using RNA, isolated from the same animals used to analyse the proteins directly, are
present in relative quantities which are similar to the YP-transcript - accumulation histograms rather than the histograms for yolk polypeptide synthesis in vivo. This suggests that the factor regulating in vivo rates of translation does not lie within the structure of the mRNA. Northern analysis suggests that there are no major size differences in the transcripts induced in males compared to those normally present in females. Male YP-transcripts translate very poorly in a cell-free translation system compared to female YP-transcripts (also observed at the 8 hour time point by shirk et al., 1980). The reason for this is unclear, but it does not help to explain the post-transcriptional control observed in vivo in males, because this poor translation in vitro occurs at all points after hormone treatment, not just at the time where there is evidence for post-transcriptional control.

It should be emphasised that total RNA and not poly A⁺ or other selected RNA was used in these in vitro translation studies. Therefore, it is possible that other RNA types, such as nuclear RNA, or the large amounts of ribosomal RNAs, could interfere with the translational ability of any messenger RNAs, possibly by forming RNA-RNA duplexes. Furthermore,
nuclear precursors of yolk protein mRNA molecules would be present in total RNA samples, yet would not be translated into complete yolk polypeptides. However, as the mRNAs coding for yolk proteins in *D. melanogaster* have variable length poly A tails, (Hung *et al.*, 1982) it is also possible that any affinity purification for poly A⁺RNA will result in a bias of selection. Therefore, it was decided not to use poly A⁺RNA.

The arguments for there being post-transcriptional control of the YP-genes rely upon measured rates of secretion of yolk polypeptides into the haemolymph being an accurate reflection of the rates of yolk polypeptide synthesis *in vivo*. This was confirmed by investigation of the incorporation of [³⁵S]-methionine into protein in fat bodies, which showed that yolk polypeptides are not accumulated there (Bownes *et al.*, 1983A). Presumably, YP-gene expression results from a combination of transcriptional control of the YP-genes, mRNA turnover and the rate of translation of the YP-mRNAs. Regulation at each of these levels is likely to operate at some point during hormonal induction, although it is possible that the inefficient translation into yolk polypeptides
several hours after hormone treatment could result from altered amino acid uptake, and hence, availability, and be a non-specific effect of the hormone rather than a specific mechanism to inhibit YP-translation in males.

It cannot be excluded that 20-OH ecdysone does not generally stimulate transcription and as a result, we see more YP-transcripts, but there are reasons why this is unlikely. Firstly, looking at the profiles of protein synthesis in the presence and absence of 20-OH ecdysone, only a few bands are affected, suggesting a differential response to the hormone although some of this specificity could reside at the post-transcriptional level. Secondly, there is no increase in YP-transcripts in ovarian cells, only in the body walls when females are treated with hormone (Bownes et al, 1983A). Thirdly, the hormone does not induce transcription of the YP-genes in all cells, although it is possible that transcription is generally increased in just those cells with appropriate receptors.

2:4-ii Yolk Protein Induction in Other Species

The experiments on yolk protein induction in other
Drosophila species indicate that this phenomenon is neither confined to *D. melanogaster* nor to sibling species of the *Melanogaster* Species Group. These results were not necessarily expected as, although the males of a variety of organisms can be hormonally induced to synthesize yolk proteins (including *Xenopus* (Pearlman *et al.*, 1983; Knowland, 1983), chickens (Wang, 1981; Protter, 1983) and *Sarcophaga* (DeLoof and Huybrechts, 1982), there are also species where such induction has not been observed, including locusts (Applebaum *et al.*, 1984) and cockroaches (Kunkel *et al.*, 1984). Results for two of the species, *D. simulans* and *D. mauritiana*, give supportive evidence that regulation of yolk-protein gene expression in males occurs at post-transcriptional as well as transcriptional levels of control. In these two species, results suggest that YP- transcripts are present which are not being efficiently translated into yolk-polypeptides, in males treated with 20-hydroxyecdysone. It is possible that in these instances the proteins were being rapidly degraded. There is no evidence to support this notion while there is evidence for post-transcriptional regulation in male *D. melanogaster* (Bownes *et al.*, 1983A) and for dsx^D-intersexual flies) (Bownes *et
Various lines of research are now being, or should be, undertaken. Further work is required on the species *Drosophila* other than *D. melanogaster*. For instance, Northern analysis may distinguish YP1, YP2 and YP3-like transcripts in some of these species. Also, *in vivo* pulse-labelling of newly synthesized polypeptides and RNA transcripts should be carried out to accurately determine efficiency of transcription and translation as well as stability of these yolk polypeptides and their transcripts. *In vivo* RNA labelling has proved difficult due to low specific activity of radioactive labelling.

There are several likely candidates for the mechanism or mechanisms for post-transcriptional controls on the induced male transcripts. Northern analysis has ruled out severe degradation of transcripts. If the transcripts lack 5' capping pyrophosphate bonds, which could interfere with translation, this could be rectified *in vitro* by addition of pyrophosphatase plus *s*-adenosylmethionine. Instability of YP-transcripts
could be due to reduced, or complete absence of, 3' polyadenosine tails. In vitro addition of poly(A) polymerase could rectify such a problem. However, both male and female YP-transcripts appear to have similar half-lives (Bownes et al, 1983; A. Bownes et al unpublished). As the three YP-genes of D. melanogaster have only small introns and variable length poly A tails (Hung et al, 1982), we cannot readily distinguish hnRNA from mRNA. It could be that much of the male YP-transcripts are primary hnRNAs and not processed, mature mRNAs. Such hnRNA transcripts would probably not be translatable. Sl nuclease mapping experiments, or total RNA fractionation into cytoplasmic and nuclear RNA types, could detect such a situation. More than one mechanism is likely to be occurring in male flies. Lack of 5' caps or poly(A) tails could account for male YP-transcripts at all time points translating less well, in the in vitro rabbit reticulocyte-lysate system, compare with female transcripts. Perhaps at later time points a negative feedback mechanism in males prevents hnRNA processing. This would account for why male transcripts are less efficiently translated into yolk polypeptides, at later time points compared with earlier time points, in vivo.
It would be useful to have a culture system to investigate yolk protein synthesis in a more controlled fashion. Our group has been unable to define such a system where in vitro cultured male fat body cells will synthesize yolk polypeptides after hormone induction (Bownes, 1982).

Using gene-specific cloned YP-gene probes, (such as YP1 HBM13) it is now possible to study the control of yolk protein synthesis in D. melanogaster for individual genes. In this way we can determine whether the three genes are regulated in a co-ordinated fashion. Melanogaster sibling species induction experiments suggest that the YP-genes are not all induced to the same degree. A similar situation of non-co-ordinated induction of chicken vitellogenin genes, following oestrogen treatment, was observed by Wang (1981). Non co-ordinated induction also occurs in a primary oestrogen induction of vitellogenin gene transcription in livers of male Xenopus (Pearlman et al, 1983).

We are still ignorant of the mechanism used to achieve the normal sex-specific synthesis of yolk-proteins. Male and female adults have almost
identical levels of 20-OH ecdysone (Handler, 1982; Bownes, Dubendorfer and Smith, 1984) and they both rapidly metabolize injected 20-OH ecdysone into more polar products (Bownes, Dubendofer and Smith, 1984). This suggests that the control of expression of these genes resides in the fat body cells themselves, probably some process ultimately under the control of the sex genes dsx, tra, tra-3, and ix (Baker and Ridge, 1980; Postlethwait et al, 1980; Bownes and Nothiger, 1981; Bownes et al, 1983B). However, with high doses of hormone these sex differences can be transiently overcome. The 20-OH ecdysone could act upon other genes in the fat body which indirectly activate the YP-genes, or it may act on another tissue in the adult which then stimulates the fat body to transcribe the YP-genes. Possibly, the tissue specificity of the response lies in the availability of the appropriate hormone receptors.

We want to compare 20-OH ecdysone receptor levels between males and females. It it also possible that male and female fat body cells contain different affinities for the hormone. Either, or both, of these situations could explain the sex specific nature of yolk protein synthesis in Drosophila. In
contrast, in *Xenopus*, it appears that the differences between male and female normal oestrogen levels could, alone, account for the sex-specificity of vitellogenin synthesis (Knowland, 1983).

Finally, of interest is the likelihood that the *Drosophila* sex-determining genes have a primary role to play in controlling not only yolk protein synthesis, but other sex-limited genes also. Cloning of sex genes *dsx*, *tra*, *tra-3* and *ix* would give us tools for investigation of the molecular roles they play. Some male-specific transcript coding regions have already been cloned (Schafer, 1984). We know that the synthesis of yolk proteins can be uncoupled from the normal XX/XY chromosomal determination of sex, as shown in pseudomales and *dsx*<sup>D</sup> mutants (Bownes and Nothiger, 1981; Bownes et al, 1983B). Intersexual flies which show some female morphology always produce yolk polypeptides, but often in intermediate amounts. Such flies could be mosaic (Having both male and female type cells) or cells could be 'intermediate' in nature somewhere between male and female type.

It remains to be elucidated what significance the ecdysteroid induced synthesis of yolk polypeptides
has with respect to the normal functioning of the yolk protein genes in *Drosophila* species.
CHAPTER THREE

Identification and Comparison of the Yolk Proteins and their Genes in Sibling Species of the D. melanogaster Group Species

3 : 1 INTRODUCTION

One of the advantages of using Drosophila species as an experimental system is the ability to detect the presence and location of any genomic nucleotide sequence on the chromosome or chromosomes by in situ hybridization (Pardue and Gall, 1972; Gall and Pardue, 1971; Pardue et al, 1970). Drosophila genes which have been cloned have been assigned chromosomal locations by this method. Most of the work has been done on D. melanogaster for which the chromosomal banding pattern of polytene cells (such as the salivary gland cells) is well known (Lefevre Jr. 1976 based on work of Bridges, 1935 and 1938). However the chromosome banding patterns of sibling species of D. melanogaster are less well known despite work by Lemeunier and Ashburner (1976). They showed that the x chromosomes of D. melanogaster, D. simulans, and D. mauritiana are very similar, while those of D. erecta, D. teissieri and D. yakuba differ by a series of inversions. The D. melanogaster x chromosome banding pattern is shown in Fig 4:1. D. simulans
differs from *D. melanogaster* by two small inversions (1E1.2; 1E3 and 3A1.2, 3A5), while the x chromosomes of *D. simulans* and *D. mauritiana* are identical except for the chromosomal tip. The other species have been difficult to analyse but the proposed x chromosome sequences are as follows (Lemeunier and Ashburner, 1976)

**D. erecta:**

1A - 5C/11B - 7D/14DF -

19A/14DF - 11B/5C - 7D/19A - 20

**D. teissieri:**

1A - 2B/6D - 2B/11A -

12D/18D.19A - 12D/11A -

9B/6D - 9B/18D.19A - 20

**D. yakuba:**

1A - 2B/6D - 2B/11A -

12A/8A - 6d/9B - 11A/12D -

18D.19A/12D - 12A/8A -

9B/18D.19A - 20

It is known for *D. melanogaster* that all three yolk protein genes are located on the x chromosome. The YP1 and YP2 genes are closely linked and so both appear to be present in the region 8F/9A (Barnett et al, 1980) or 9A (Bownes and Redfern, unpublished), while the YP3 gene is located at position 12 b/c also on the x chromosome.

The aim was to assign chromosomal locations for all the yolk protein genes of the sibling species of *D.*
*melanogaster*. Any duplication or translocation events which have resulted in yolk protein genes being present at new chromosomal locations should be evident from these experiments.

The yolk proteins and their genes in the sibling species of *D. melanogaster* were identified. In order to do this the assumption was made that these genes in the sibling species would not have evolved too far from *D. melanogaster* and that the proteins would be recognised by a polyspecific anti-yolk protein antibody prepared against *D. melanogaster* yolk protein (Isaac and Bownes, 1982) and the genes could be identified by hybridization to cloned, genomic DNA sequences coding for the three *D. melanogaster* yolk proteins (pYP1, pYP2 and pYP3, Barnett et al 1980). This assumption proved valid and the yolk proteins and the genes coding for them have been identified for all the sibling species. It was also hoped that through using varying stringency condition of post-hybridization washing that the cloned yolk protein gene probes of *D. melanogaster* could be used to distinguish YP1-like, YP2-like and YP3-like genes present in the sibling species. The fact that the coding regions of YP1 and YP2 are 63% homologous (Hung and Wensink, 1984) in *D. melanogaster* may well cause problems in distinguishing YP1 from YP2-like sequences.
Furthermore the YP1 and YP2 genes are closely linked in *D. melanogaster* (Barnett *et al.*, 1980) so if this is also the case in the sibling species it is possible that some restriction enzymes when used for genomic DNA digestion would not separate the two genes. If this was found it would be necessary to search for a restriction endonuclease which did give diagnostic restriction fragment lengths for the two genes.

Initially two restriction endonuclease enzymes (Hind III and EcoRI) were used to fully restrict genomic DNA prepared from the sibling species as these were most likely to enable identification of any YP1-like, YP2-like and YP3-like genes after Southern blotting, and probing with $^{32}$P-labeled cloned YP1, YP2 and YP3 genes from *D. melanogaster*. To avoid problems associated with plasmid contamination of either equipment or *Drosophila* stocks, the probes used were "insert" DNA which had been separated from the plasmid (pBR322) portion of the cloned DNA.

Evolution between the species was compared by determining the number of yolk polypeptides present, by cross-reactivity to *D. melanogaster* anti-YP antibodies, and apparent differences in
molecular weight, observed after SDS gradient polyacrylamide gel electrophoresis. Care in interpretation is required as modification of the yolk polypeptides, such as phosphorylation and glycosylation, will give apparent changes in molecular weights which are not due to alteration in the number or type of amino acids present. Samples were taken from haemolymphs as it was assumed that the polypeptides have had leader sequences cleaved and other modifications which occur in the fat body cells (Warren et al, 1979) before export.

As shown by Upholt (1977) and Nei and Li (1979) it is possible to compare the existence of nucleotide sequences recognised by particular restriction endonucleases, in a quantitative manner. As shown in fig 3:1, for D. melanogaster, Hind III cuts genomic DNA outside the coding region for all three yolk protein genes. EcoRI cuts outside the coding region for YP2 and YP3 but restricts twice within the coding region for YP1. The conservation of these nucleotide sequences (through nucleotide mutation) was investigated for the sibling species.
3:2 MATERIALS AND METHODS

SDS Polyacrylamide Gradient Gels and Cross Reactivity of Yolk Polypeptides to Antibodies Raised Against Yolk Proteins from D. Melanogaster

Described in Chapter Two.

Extraction of Genomic DNA

Several methods were tried but the one found to be most satisfactory is described here. Adult flies (approximately 400) were etherised and either used immediately or stored at -70°C. They were homogenized in 4 mls 0.025M Tris/HCl pH 7.4, 0.05 M EDTA for 10-15 minutes on ice. 400 μg pre-digested non-specific protease (type XI) was added and incubated for 1 hour at 37°C followed by SDS to 1% and further indudcation for 1 hour at 60°C. A second 400μg amount of protease was added and a further 37°C incubation for 4 hours, followed by two phenol extractions, 2 hours each, at 4°C, overnight dialysis against TE buffer, precipitation with ethanol and resuspension in TE. RNase (preincubated at 100°C) was added to 200 μg/ml followed by incubation at 37°C for 1 hour. This was phenol extracted twice, phenol/chloroform extracted once, ether extracted twice, dialysed against TE buffer overnight, precipitated with ethanol twice, and finally resuspended in 1 ml TE buffer.

Determination of DNA Concentrations

DNA concentrations were calculated by observing the optical absorbance at 260nm, where OD_{260}^{-1} = 50μg/ml. To check for protein contamination the optical absorbance at 280 nm was also calculated. As a further check of DNA concentrations, particularly for small quantities, DNA was spotted onto 1% agarose containing 2 μg/ml ethidium bromide, and viewed under a UV lamp. Before Southern blotting, all agarose DNA gels were photographed to enable accurate quantitations of DNA loading by densitometer scanning.
Restriction of DNA by Restriction Endonucleases

For Hind III restrictions DNA was added to 60mM NaCl, 10mM Tris/Hcl pH 7.4, 7mM MgCl₂ and 100 μl/ml gelatin. 10μg of genomic DNA would be incubated with 20 units of Hind III enzyme in a volume between 20μl and 50μl for at least 5 hours at 37°C (sometimes overnight) to ensure complete digestion. Conditions for EcoRI were similar except 100mM Tris/hcl pH 7.4 was used. For molecular weight markers phage lambda C1875 restricted with Hind III was used. This DNA was prepared by heat induction of a thermosensitive C1875 lysogen, followed by PEG precipitation and cesium chloride gradient centrifugation, dialysis, phenol extraction and precipitation with ethanol. C1875 restricted with Hind III gives molecular weight markers of 23.6kb, 9.64kb, 6.64kb, 4.34kb, 2.26kb, 1.98kb, 0.56kb, and 0.14kb. The two smallest size fragments were only observed in overloaded tracks.

Agarose Gel Electrophoresis

Horizontal 1% agarose gels were poured. The running buffer was 40mM Tris, 33mM sodium acetate, 1mM EDTA, adjusted to pH 8.3 with acetic acid (TAE buffer). DNA samples were loaded in 50% glycerol, 0.1% bromophenol blue, 125mM EDTA and 0.5 x TAE buffer. Gels were usually run at 60v overnight. They were shaken in 2μg/ml ethidium bromide for 30 minutes followed by washing in water. They were then photographed under UV light.

Southern Transfer of DNA Agarose Gels onto Nitrocellulose

The method is essentially that of Southern (1975). Gels were soaked in 0.24M Hcl for 20 minutes to promote transfer of large DNA fragments, and washed in water. After denaturing by soaking in 0.5 M NaOH, 1.5 M NaCl for one hour they were washed in water and neutralized by soaking in 1M Tris/Hcl pH 5.5, 3 M NaCl for 90 minutes and then washed in 2 x SSC for 10 minutes before allowing to transfer onto nitrocellulose for at least 24 hours, using 20 x SSC as transfer buffer. Filters were washed in 2 x SSC for one minute before drying and baking in a vacuum oven at 80°C for 2 hours.
Preparation of Large Amounts of Plasmid DNA

For pYP1, pYP2 and pYP3 plasmids 20 mls of L broth with ampicillin (100 μg/ml) was inoculated with the bacteria strain (HB101) containing the plasmid, and left standing overnight at 37°C. The 'overnight' was diluted 1/50 into L broth + 0.2% glucose and grown, shaking, at 37°C to OD_590 = 1. Chloramphenicol was added to 150 μg/ml (to amplify plasmid) and shaking continued overnight. Cells were harvested and resuspended in 6 mls 25% sucrose, 50 mM Tris/HCl pH8.1, 40mM EDTA. One ml 10mg/ml lysozyme and one ml 0.5M EDTA pH8.1 were added, mixed and left on ice for 5 minutes. 13 mls of 0.1% triton x-100, 62mM EDTA pH8.1, and 50mM Tris/HCl pH 8.1 was added, mixed and left on ice for 10 minutes. After centrifugation at 12 k for 45 minutes the supernatant was mixed with caesium chloride (0.95g/ml) and ethidium bromide (0.1 ml 5mg/ml per ml supernatant) and centrifuged for 48 - 60 hours at 38 k. The lower band containing supercoiled plasmid DNA was collected under UV by syringe; ethidium bromide removed by passage over Dowex; caesium removed by dialysis against TE buffer; and the DNA precipitated with ethanol twice before resuspending in TE buffer.

Separation of Insert Yolk Protein Gene DNA from Plasmid DNA

Approximately 20 micrograms of plasmid DNA (either pYP1, pYP2 or pYP3) would be restricted with appropriate restriction enzymes. Thus pYP2 would be restricted with Hind III. Plasmid pYP1 cut with Hind III would give two fragments of approximately equal size so in order to separate plasmid DNA completely a double digestion, Hind III followed by XhoI, was carried out. For pYP3 a triple restriction was necessary to separate insert DNA from plasmid - Hind III followed by SalI, followed by Bgl II. The results of such digestions are shown in fig 3:2. In each case the pBR322 linear plasmid forms a band at 4.3 kb. For pYP2 the insert fragment is at 2.0 kb; for pYP1 the insert gives two fragments at approximately 1.9 kb each; for pYP3 the insert YP3 also forms two fragments at approximately 2.1 kb and 1.8 kb. Two methods proved successful in recovering "clean" DNA in an efficient manner. The first method is
essentially modified from Maniatis et al (1982). The required band was cut out from the agarose gel and placed in a dialysis bag filled with TBE buffer (89mM Tris-boric acid pH 8.0, 5mM EDTA). The bag was laid, at right angles to direction of current, in an electrophoresis tank with TBE buffer. 100v for 2½ hours was applied followed by a two minute reverse of polarity. The buffer was recovered (the gel slice was treated again with ethidium bromide to ensure all the DNA had been eluted) and passed over a DEAE 50 ion-exchange column prepared in a 1ml sterile, plastic syringe. The column was washed with PE buffer (50mM Tris/Hcl pH8.0, 100mM Nacl, 10mM EDTA) before and after passing over the DNA solution (2 times) and the DNA was eluted by passing PEN buffer (PE buffer plus 1M Nacl). Aliquots were collected and those with DNA pooled, phenol/chloroform extracted, butanol extracted twice, ether extracted twice, precipitated with ethanol three times (at -70°C using 0.3M sodium acetate and 10mM magnesium chloride), before resuspending in TE buffer.

The second method involved using a gel consisting of 0.7 – 1.0% low melting point agarose, melted at 70°C and cooled to 37°C before pouring in a 4°C cold room. The gel was prerun for 10 minutes before loading DNA samples and run overnight at approximately 50v. After ethidium bromide treatment the band was cut out under UV, an equal volume of TE buffer added, and the agarose melted by heating at 70°C for 15 minutes. An equal volume of phenol (preheated to 70°C) was added and the mixture shaken at 37°C for 30 minutes before separation by centrifugation, phenol/chloroform extracting, butanol extracting twice, ether extracting twice, precipitating with ethanol twice (using 0.3 M sodium acetate and 10mM MgCl₂), and washing in cold 70% ethanol before resuspending in TE buffer.

Preparation of [32p]-Labelled Nick-translated DNA Probes

These were prepared as described in Chapter Two except that insert DNA was used (0.2 – 0.5 μg per probe). With specific activities of \(10^7 - 10^8\) cpm single copy sequences in Drosophila genomic DNA digests could be detected.
Hybridization of Radioactive DNA Probes with Nitrocellulose Filters Containing Bound DNA

Nitrocellulose filters were prehybridized in hybridization solution (4 x SSC, 50% formamide, 1 x Denhardt's solution, and 0.1% SDS) for at least one hour at 37°C, shaking. The radioactive DNA probe was added to 10 ml of hybridization solution containing 100 µl sonicated salmon sperm DNA (5mg/ml). This was boiled for 5 to 10 minutes and rapidly cooled on ice before adding to the prehybridized filter/s in a sealed polythene bag. Filters were hybridized for at least 24 hours, shaking, at 37°C before washing 4 x 30 minutes in 2 x SSC, 0.1% SDS at 37°C shaking, and 4 x 30 minutes in 2 x SSC at 37°C, also shaking. Filters were dried and set up against X-ray film for autoradiography. Such washes were of fairly low stringency but were employed initially because homologies between probes from D. melanogaster and genomic DNA from sibling species were unknown.

It was usually found that such stringency resulted in the detection of just one yolk protein gene in a sibling species' genome using a particular D. melanogaster YP-gene probe. Cross-hybridization only occurred between DNA fragments containing putative yolk protein genes.

Measurement of DNA Fragment Sizes Separated by Electrophoresis

The method employed is from Southern (1979). Size standards were obtained from phage lambda CI857 DNA restricted with Hind III and separated on the same gel as the DNA to be measured. The mobility (mm) of the fragments could be read directly from the gel photograph using a ruler. The standard graph would consist of one axis indicating 1/mobility, the other axis indicating size of fragment (Kb). The standard curve would result in a straight, or very nearly straight, line. Using a 1% agarose gel fragment size estimates were particularly accurate between about 1 kb and 12 kb. Sizes of unknown fragments were estimated by directly reading from the standard line. However, accuracy cannot be guaranteed to greater than about 100 base pairs by any such method.
Preparation of Slides and Coverslips

Slides and coverslips were washed in 1M HCl for about 30 minutes, rinsed in distilled water, and individually washed in Decon detergent, before rinsing well in water and air drying. Slides, but not coverslips, were then dipped individually into a subbing solution (0.1% gelatin, 0.025% chrome alum) and allowed to dry in a dust free atmosphere. Some coverslips were also siliconized.

Squashes of Drosophila Larval Salivary Glands

Late third instar larvae were used in all cases, which had been reared in uncrowded conditions and the food supplemented with extra yeast. The salivary glands were dissected out into Ringers solution, using a binocular microscope, and adjoining fat cells removed. The glands were fixed in 3:1 ethanol: acetic acid for 5 minutes and transferred to a drop of 50% propionic acid on a siliconized coverslip. A subbed slide was placed over the coverslip and squashed by thumb pressure plus tapped with a metal rod. The preparation was then viewed under high power magnification of a phase contrast microscope to check for good chromosome separation and spreading. Good preparations were immersed in liquid nitrogen for one minute, the coverslips flicked off with a razor blade, and the slides placed into 95% ethanol for 5 minutes. Slides were then air dried and stored dry (using silica gel dessicant) at 4°C.

Squashes which were to be photographed, but not used for in situ hybridization, were photographed immediately after squashing under phase contrast, through high power magnification (x1250).

Preparation of Nick Translated, Tritiated DNA Probes for In Situ Hybridization

The D. melanogaster cloned yolk protein genes (pYP1, pYP2 and pYP3) were nick translated as described in Chapter Two. However, no cold
nucleotides were added, and all four nucleotides were $^{3}$H-tritiated derivatives. Aliquots were collected from a G50 Sephadex column and 2μl samples counted in a scintillation counter. In this way the unincorporated tritiated nucleotides were separated from nucleotides incorporated into the DNA gene probes. Specific activities of DNA probes were greater than $10^7$ cpm.

DNA gene probes were precipitated with ethanol using 10x excess of carrier sonicated salmon sperm DNA, washed once in 70% ethanol and dried down. The pellet was redissolved in 50μl 50% deionized formamide, 4 x SSC, 1 x Denhardts. For each slide 10$^6$ cpm, or greater, in a volume of 10μl was used.

Preparation of Slides for In Situ Hybridization

Slides, containing salivary gland squashes, were heat treated by incubating in preheated 2 x SSC at 70°C for 30 minutes. They were then dehydrated through ethanol (2 x 10 minutes in 70% ethanol, then 1 x 5 minutes in 95% ethanol) and air dried. Next, slides were RNase treated by placing 200μl of RNase (100μg/ml in 2 x SSC) onto the slide, covered with a large coverslip, and incubated for one hour at 37°C in a moist chamber. [The moist chamber consists of a large plastic petri dish with filter paper containing the required salt solution. Small petri dish lids were used to support the slides]. Slides were rinsed 2 x 10 minutes in 2 x SSC and dehydrated and air dried as described above.

The DNA of the salivary gland chromosomes was denatured by soaking slides in 0.07 N NaOH for 3 minutes, then dehydrating (3 x 10 minutes in 70% ethanol, then 2 x 5 minutes in 95% ethanol) and air drying.

In Situ Hybridization

The prepared $^{3}$H-labelled DNA gene probe was heated to 100°C for 4 minutes and immediately placed on ice. Slides were placed into a moist chamber dampened with hybridization buffer (50% formamide, 4 x
SSC, 1 x Denhardts) and 10μl (approximately 10^6 cpm) of probe added to each slide. The slides were covered carefully with acid-washed coverslips, the chamber's lid placed back, and incubated at 70°C for 5 minutes, followed by incubation at 37°C for at least 24 hours.

Following hybridization the slides were washed, and coverslips carefully removed, by two changes of 50% formamide, 4 x SSC at 37°C for 10 minutes each; then two changes of 50% formamide, 4 x SSC at room temperature for 10 minutes each; then two changes of 2 x SSC at room temperature for 10 minutes each. Finally, slides were dehydrated and air dried, and stored dry at 4°C until coating with emulsion.

**Autoradiography**

Hybridized slides were coated with photographic emulsion (Ilford L4 nuclear emulsion, gel form). The following work was carried out in absolute darkness. Emulsion was melted in a 42°C water bath and 5mls added to 5mls 2% glycerol in a glass slide-dipping chamber and stirred. Slides were dipped, one at a time, and placed in light-proof slide boxes containing silica gel dessicant. Slides were left, for at least three weeks, to expose at 4°C.

Slides were developed by placing in 1D19 Developer (2.2g metol, 72g sodium sulphite, 8.8g hydroquinone, 48g sodium carbonate, and 4g potassium bromide made upto one litre with distilled water, and filtered) for 3 minutes; stopped in 3% acetic acid for 30 seconds; fixed in Hypan for 1½ minutes; washed in running water for 10 minutes and air dried.

**Staining Slides**

Stock Giemsa stain was prepared by adding 0.5g Giemsa to 33mls of glycerol and heating at 60°C for two hours. Then 33mls methanol was added and mixed.
Developed slides were soaked in 10mM sodium phosphate buffer pH 7.0 for a few minutes, and then transferred to diluted Giemsa stain (3 mls stock in 100 mls phosphate buffer). Slides were stained for 10 minutes after which the metallic surface film was removed with running water. They were then rinsed in phosphate buffer for a few minutes, rinsed well in distilled water, and air dried. They were then examined and photographed under the high power magnification lens of the microscope (x1250), under oil immersion.
Restriction Maps and RNA Complementary Regions of the Cloned Drosophila DNA That Contains Yolk Protein Genes

The inserted Drosophila DNAs are grouped into (A and B) according to their overlapping restriction sites. (a) Each horizontal line labeled by a λ DmYP name represents the Drosophila DNA included in the phage of the same name. (b) Composite restriction maps are drawn with Hind III (I), Sal I (●), Xba I (○), Xho I (†) and Eco RI (†) sites shown. The dashed lines indicate ambiguity in our localization of that site. (c) More detailed maps of the subclones that include the gene regions (Hind III or Hind III/Sal I inserts into pBR322) are shown along with the RNA complementary regions (heavy black line). No poly (A)+ RNA complementary regions were found outside these subcloned restriction fragments. pBR322 DNA is represented by thick unfilled lines. Their tagged ends indicate that some of the pBR322 sequence was not drawn.

**Group A**

- λDmYP1B
- λDmYP3C
- λDmYP5A
- λDmYP2A
- λDmYP1A

**Group B**

- λDmYP1D
- λDmYP2B
- λDmYP3D
- λDmYP1C
- λDmYP2C

**YP1 Gene Probe** = The 3.8 kb Hind III Fragment Containing The YP1 Gene.

**YP2 Gene Probe** = The 2.0 kb Hind III Fragment Containing The YP2 Gene.

**YP3 Gene Probe** = The 3.9 kb Hind III- Sal I Fragment Containing The YP3 Gene.

**FIG. 3.1. RESTRICTION MAPS OF THE THREE DROSOPHILA MELANOGASTER YOLK PROTEIN GENES.** From Barnett et al., 1980.
Restriction enzyme digestions of pYP1 and pYP2 plasmids in order to prepare insert DNA. Arrow indicates area of DNA removed from agarose gels.

(A) Preparation of *D. melanogaster* YP1 insert DNA by Hind III and Xho I double restriction of pYP1

(B) Preparation of *D. melanogaster* YP2 insert DNA by Hind III restriction of pYP2
3:3 RESULTS

Examples of hybridization experiments using the three *D. melanogaster* yolk protein gene probes for each of the sibling species are shown in Fig 4:2 to 4:8. A diagramatic representation is given for each of the photographs shown. Although only one example is shown for each hybridization experiment, it should be noted that the chromosomal locations are based upon a number of observations. The results are summarised in Table 4:1.

The x chromosomes of the sibling species were compared with that of *D. melanogaster* which is shown in Fig 4:1. Banding patterns appeared similar for *D. mauritiana* and *D. simulans*. For the other species the chromosome sequences appeared the same as proposed by Lemeunier and Ashburner (1976) and the x chromosome of *D. orena* appeared similar to that of *D. erecta*.

In every case the regions of hybridization were confined to the x chromosomes, which were easily recognised by the distinctive chromosome tips present in all the sibling species (x chromosome region 1-2 shown for *D. melanogaster* in Fig 4:1.)
In most cases one particular gene probe (YP1, YP2, or YP3) hybridized to just one region on the x chromosome. However, occasionally, weak second sites of hybridization were observed, but these could always be explained by a gene probe cross hybridizing to a second yolk protein gene. The YP3 gene probe weakly cross hybridized to the YP1/2 region in D. melanogaster, D. mauritiana, D. yakuba, D. orena and D. erecta indicating that there may be some homology between YP1/2 genes and YP3 genes.

Haemolymph samples were collected from female flies of various species of Drosophila and were separated on gradient SDS polyacrylamide gels. The results are shown in Fig 3:3. The "D. melanogaster control" tracks show D. melanogaster haemolymph polypeptides precipitated with anti-D. melanogaster YP-antibodies. Fig 3:4 shows polypeptides in female haemolymphs which are precipitated by antibodies raised against D. melanogaster yolk proteins. The "D. melanogaster control" tracks here show the total D. melanogaster haemolymph polypeptide profile. The sibling species of D. melanogaster, all have three yolk polypeptides present in the haemolymph of females. There are two points to note from Fig 3:4. Firstly, the anti-yolk protein antibodies do not precipitate YP3 polypeptide as well as YP1 and YP2, even in the
case of D. melanogaster. And secondly, due to evolution of the amino acid sequences, the sibling species yolk polypeptides often react less strongly with the antibodies compared with D. melanogaster itself. The position of the "light" and "heavy" rabbit IgG antibody chains are shown in Fig 3:4.

Fig 3:4 shows that haemolymph proteins from species outside the Melanogaster Group Species, D. virilis and D. funebris, fail to be precipitated using the D. melanogaster anti-YP antibodies, suggesting that there is not adequate homology between the yolk proteins of these two species and those of D. melanogaster. All the sibling species produce three yolk polypeptides, as has been shown in Chapter Two, Fig 2:5. The female control tracks of these Western transfers, incubated with anti-D. melanogaster yolk protein antibodies, clearly show the presence of three yolk polypeptides for all sibling species. D. funebris female haemolymph proteins show, again, no cross reactivity with the yolk protein antibodies but the situation for D. virilis is more interesting. The Western transfer results show two yolk polypeptides in D. virilis female haemolymph samples, while no cross-reactivity can be observed in the results shown here in Fig 3:4. This is likely to be due to the
differences in methods of reacting yolk polypeptides with the anti-YP antibodies. In the Western transfers the polypeptides were bound, denatured, to the nitrocellulose before incubating with the yolk protein antibodies; however in the gels shown in Fig 3:4 the haemolymph proteins were presented to the yolk protein antibodies in solution, and these haemolymph proteins were probably not in a denatured state. Thus it can be speculated that in a bound, denatured state the D. virilis yolk polypeptides have conserved antigenic sites exposed which are not exposed when the yolk polypeptides are in solution.

The D. melanogaster yolk polypeptides (YP1, YP2 and YP3) have apparent molecular weights (when finally processed and present in the haemolymph) of about 47000, 45700, and 44700 (Bownes and Hames, 1977, Warren and Mahowald 1979) or 45700, 46000 and 44500 (Mintzas and Kambysellis, 1982) respectively, as determined by gradient SDS polyacrylamide gel electrophoresis. The molecular weights, calculated from the nucleotide sequences, are 48700 (YP1) and 49682 (YP2) (Hovemann et al, 1981; Hung and Wensink, 1984) but do not take into account processing steps of these polypeptides. The sibling species yolk polypeptides are of approximately the same apparent molecular weights
as those of *D. melanogaster* although there is some variation. Yolk polypeptides of *D. mauritiana*, *D. simulans*, *D. yakuba* and *D. orena* are indistinguishable from those of *D. melanogaster*. *D. teissieri*’s smallest yolk polypeptide has an apparent larger molecular weight compared with the smallest yolk polypeptide (YP3) of *D. melanogaster*. *D. erecta*’s two largest yolk polypeptides both appear to be larger than the two largest of *D. melanogaster* (YP1 and YP2). We cannot assume that the sibling species' largest yolk polypeptides are YP1-like (related to *D. melanogaster*), or the medium sized YP2-like. However, the smallest yolk polypeptide in all cases is likely to be equivalent to *D. melanogaster*’s YP3 yolk polypeptide, as this polypeptide always reacts poorly with the polyspecific antibodies used.

All the tracks shown in Fig 2:5, Fig 3:3 and Fig 3:4 have been scanned by densitometer. The results confirm that the anti-YP antibodies precipitate YP3 yolk polypeptide less well than the other two yolk polypeptides for all species. The smallest yolk polypeptide appears in different relative amounts compared with the two larger yolk polypeptides. The two larger yolk polypeptides always appeared in equal, or almost equal, quantities. Thus, the two
larger yolk polypeptides appear to be co-ordinately regulated, but the same cannot be suggested for the regulation of all three yolk polypeptides. It has been assumed that Comassie Blue stains all three yolk polypeptides equally so that the densitometer scans are proportional to numbers of molecules in each case.

Genomic DNA from the Drosophila species was restricted with endonuclease Hind III or EcoRI, Southern transferred, and hybridized with (32p-) labelled DNA probes of YP1, YP2 or YP3 yolk protein coding genes from D. melanogaster. The particular DNA fragments used as radioactive probes are shown in Fig 3:1.

Southern transfer results using the YP1-DNA probe are shown in Fig 3:5. Fragment sizes showing hybridization have been calculated and are shown in Table 3:1. These results are based on more than one hybridization experiment, using different DNA samples. Nitrocellulose filters were stripped of probe and rehybridized with the D. melanogaster YP2 gene probe, the results shown in Fig 3:6, and Table 3:1. Finally, nitrocellulose filters were again stripped of probe and rehybridized with the YP3 containing DNA probe. Hybridization signals were not identified for a few of the species using EcoRI...
restricted DNA. There could be an absence of EcoRI binding hexanucleotide sequences in and around the YP3-like genes for these species. Or this could be caused by gradual damage to the nitrocellulose filters, although this is unlikely as no hybridization bands were observed in fresh filters prepared. YP3 hybridizations are shown in Fig 3:7 and combined with the other data in Table 3:1.

**In situ** hybridization results for *D. melanogaster* are shown in Fig 4:2, YP1 hybridizes to region 9A, YP2 to 9A, and YP3 to 12 B/C all on the X chromosome. These are consistent with the published locations (Barnett et al., 1980) and YP1/2 has been more precisely localised. Southern blot hybridization results using the YP1 probe are shown in Fig 3:5, those using the YP2 probe shown in Fig 3:6, and those using the YP3 probe shown in Fig 3:7. Together fragments showing hybridization are shown in Table 3:1. Restriction with Hind III results in signals at 3.8 kb, 2.0 kb and 4.8 kb with YP1, YP2 and YP3 probes respectively. Thus the three probes have detected the three yolk protein genes as expected from previous mapping experiments (Barnett et al., 1980). After EcoRI restriction the YP1 probe hybridization picks out 3.7 kb and 9.6 kb fragments, while the YP2 probe
only picks out the larger fragment. The hybridization of a 9.6 kb fragment to both gene probes is expected because a 9.6 kb genomic fragment contains a section of the YP1 gene probe (5' end) as well as the complete YP2 gene probe (see Fig 3:1). So the very close linkage between the D. melanogaster YP1 and YP2 yolk protein genes has been identified. The EcoRI 3.7 kb fragment is diagnostic for the YP1 gene and contains the 3' end of this gene.

Hybridization with the YP3 gene probe identifies the D. melanogaster YP3 gene as a 4.8 kb Hind III and a 15 kb EcoRI genomic fragment.

For D. mauritiana in situ hybridization results are shown in Fig 4:3 which shows that YP1 hybridizes to 9A-C, YP2 to 9A/B and YP3 to 12 B/C. Southern blot results are summarized in Table 3:1. YP1 and YP2 hybridization after Hind III digestion results in two bands. A hybridization signal for the smaller 2.9 kb band is relatively stronger after YP3 probe hybridization so it is likely that the 2.9 kb fragment contains the main part of a YP3-like gene and that the presence of this band after use of the YP1 and YP2 probes is the result of cross hybridization. However, it is also possible that different genomic sequences are being detected by
each probe which by coincidence are all 2.9 kb in size. As EcoRI restriction results in a 6.1 kb band after probing with YP1 or YP2 genes it is possible that both probes are identifying just one gene which would agree with the in situ results. However, another possibility is that, like D melanogaster, D. mauritiana has two very closely linked genes (YP1-like and YP2-like) which have not been positively identified by either in situ hybridization or Hind III or EcoRI digestion. One of another restriction endonuclease may separate the YP1 and YP2-like genes to give diagnostically different hybridization bands. Polypeptide data already described suggest the existence of three discrete yolk protein coding genes. The possibility exists that each hybridization signal detected by Southern blotting indicates the presence of a different genomic sequence, of which some fragments do not contain yolk protein genes. Thus for D. mauritiana five discrete Hind III genomic sequences could be identified by use of the three YP gene probes. However, it would be expected that all such sequences are present within just two x chromosome sites, identified by in situ hybridization, as stringency conditions for both techniques are roughly similar.
In situ hybridization results for *D. simulans* are shown in Fig 4:4 and genomic blot results summarised in Table 3:1. Sites of in situ hybridization appear the same for the three genes as is the case for *D. melanogaster*. Genomic blots of Hind III digested DNA show a similar pattern of bands after probing with the three YP genes as found for *D. mauritiana*, except that a weakly hybridizing 6.3 kb fragment is also observed after YP3 gene probing. The same possibility and problems of gene identification exist for *D. simulans* as for *D. mauritiana*. The genomic Hind III 6.3 kb fragment could contain a proportion of a YP3-like gene, or it could be another sequence which happens to show slight homology to the YP3 gene probe. EcoRI restriction has detected a 11.2 kb sequence after YP1 and YP2 probes hybridization which could indicate a single YP1/YP2 homologous sequence, or two discrete sequences, one YP1 homologous and one YP2 homologous, which by coincidence are both 11.2 kb. It is also possible that the 11.2 kb sequence contains both a YP1-like and a YP2-like yolk protein gene which would be consistent with in situ hybridization data. Relatively strong hybridization suggest that a Hind III 7.0 kb fragment contains at least one YP1/2-like gene and a 2.9 kb fragment a YP3-like gene.
Southern blot experiments for *D. sechellia* are summarised in Table 3:1. Hind III digestion patterns are the same as found for *D. simulans* so by the same reasoning it is likely that at least YP1/YP2-like gene is identified by a 7.0 kb Hind III fragment, and one YP3-like gene identified by the 2.9 kb fragment. However, as before, this is not the only possibility. The 2.9 kb fragments identified by the three gene probes, and the 7.0 kb fragments identified by the YP1 and YP2 probes could all be different genomic sequences. EcoRI fragments suggest the presence of a 15 kb YP3-like gene, and at least one YP1/YP2-like gene as a 6.1 kb strongly hybridizing fragment. The 8.1 kb fragment which also hybridizes strongly to the YP1 probe could indicate the presence of a sequence adjoining the 6.1 kb fragment containing at least a portion of a YP1-like gene; or it could be a YP1-like sequence located elsewhere. The 11.2 weakly hybridizing fragment may be an adjoining sequence containing part of one or both of the YP1-like or YP2-like genes, or it may indicate the presence of one or two homologous sequences located elsewhere.

In situ hybridization results for *D. orena* are shown in Fig 4:6 and Southern blot results in Table 3:1. A series of fragments are observed after Hind
III digestion. By comparing relative hybridization signals it is possible that YP1, YP2 and YP3-like genes are identified by a 4.8 kb, 2.4 kb and 6.3 kb fragments respectively. Both YP1 and YP3 probes also hybridize strongly to a 2.7 kb fragment, while other hybridizing fragments give less strong signals. In situ hybridization data suggest at least two yolk protein genes exist. YP1 hybridizes to x chromosome region 9, YP2 to 9A-D and YP3 to region 12 A-E shown in Fig 4:6. Polypeptide data suggest that three yolk protein genes are likely to exist. The strongly YP1/YP3-homologous 2.7 kb fragment or fragments may indicate one or two new YP-like genes, possibly a pseudogene or genes, such as have been described in Xenopus, rabbits, mice and other eukaryotes organisms (reviewed by Hsuing Li, 1983). It is also possible that D. orena's YP1 and YP3-like gene regions each contain similar Hind III fragments of 2.7 kb. Yet another possibility is that the YP1 and YP3-like genes are very closely linked and the 2.7 kb fragment contains portions from both YP1 and YP3-like genes. The EcoRI digestions suggest that at least one 5.0 kb fragment contains a YP1 or YP2-like gene or both, while the 8.0 kb and 15.0 kb bands hybridize relatively strongly to the YP3 gene probe. Thus one or two YP3-like genes are suggested. The in situ and polypeptide evidence considered together
suggest that YP1 and YP2-like genes will be present and possibly closely linked, which may be indicated by the relatively strongly hybridizing 5.0 kb EcoRI fragment to both YP1 and YP2 gene probes. A YP3-like gene is also suggested, but it will not be closely linked to the YP1-like gene.

The in situ hybridization and genomic blot results for D. erecta are shown in Fig 4.5 and Table 3.1. The YP1-like gene appears to be located as a 3.8 kb Hind III fragment. The weak signals of 3.1 kb and 2.0 kb are likely to be a result of the probe cross hybridizing to YP3-like and YP2-like genes respectively. Similarly the weak signals of 3.1 kb and 3.8 kb seen after YP2 probe hybridization are likely to be due to cross hybridization with YP3-like and YP1-like genes. Thus a YP2-like gene is possibly indicated by the strong signal at 2.0 kb, and a YP3-like gene indicated by the strong signal at 3.1 kb after YP3 probe hybridization. The weak signal at 6.3 kb after hybridization to the YP3 probe could indicate a different slightly homologous genomic sequence or it could be that it is a fragment adjoining a YP3-like gene which has only a small or slightly homologous sequence compared with the YP3 probe. In situ hybridization shows YP1 hybridizes to 9 A/B, YP2 to 9 A/B and YP3
to 12 A/C. As there are three yolk polypeptides it is likely that the YP1 and YP2-like genes are closely linked. This is supported by the presence of a strongly hybridizing 4.5 kb EcoRI fragment after both YP1 and YP2 gene probing of Southern blots, suggesting that YP1 and YP2-like genes are located mainly on this fragment. It is however, possible that only the YP2-like gene is located on this EcoRI fragment and that its detection by the YP1 gene probe is a result of cross hybridization. If a YP1-like gene is less homologous to the YP1 gene probe than is a YP2-like gene, then a YP1-like gene or genes could be identified by the 8.3 kb and 9.2 kb EcoRI fragments seen after YP1 gene hybridization.

It should be noted that for D. erecta and D. orena the X chromosomal regions 7D-11B and 11B-14DF appear to have inverted compared to the D. melanogaster standard.

In situ hybridization results for D. teissieri are shown in Fig 4:8. YP1 hybridizes to 11D-12D. YP2 to 12 A-D and YP3 to 12 B/C. Thus it is possible that each probe is identifying only one gene, although polypeptide data suggest that it is most likely that there are three YP-genes. Southern transfer results are shown in Table 3:1. Following
similar arguments to those already put forward it is possible that a YP1-like gene is identified by a 3.8 kb Hind III fragment, a YP2-like gene by a 2.0 kb Hind III fragment, and a YP3-like gene by a 3.3 kb Hind III fragment. Other weakly hybridizing bands of 2.0 kb, 3.3 kb and 3.8 kb are most easily explained by cross-hybridization. The weak 6.9 kb band identified by YP1 and YP2 gene probes, and the 6.3 kb band identified by YP3 gene probe are homologous sequences which may or may not contain yolk protein genes. It is possible that these fragments contain parts of yolk protein genes but probably not major parts. The 6.1 kb and 7.5 kb EcoRI fragment which hybridize strongly to both YP1 and YP2 gene probes could indicate that these genes are closely linked as results of Hind III digestion suggest that we would not otherwise expect the YP1 and YP2 gene probes to strongly cross-hybridize to YP1 and YP2-like sequences. A 9.6 kb EcoRI fragment is also identified using the YP1 gene probe. It could indicate a sequence containing part of the same YP1-like gene or a different YP1 homologous sequence.

For D. yakuba in situ hybridization experiments described in Fig 4:7 show that the YP1 gene probe hybridizes to x chromosome region 9 A/B, YP2 to 9
A/B and YP3 to region 11A-12A on 8A-6D. Thus at least two yolk protein genes may be expected, and polypeptide evidence suggests three genes are most likely. Southern blot results are shown in Table 3:1. These are single EcoRI fragments of 1.5 kb, 2.7 kb and 11.5 kb which hybridize to YP1, YP2 and YP3 gene probes respectively. So it is likely that the three D. yakuba yolk protein genes have been identified. Hind III digestion identifies the YP1 and YP3-like sequences as 13.2 kb and 9.2 kb fragments.

Two species outside the D. melanogaster sibling group were also investigated by genomic blotting. Results for D. funebris, shown in Table 3:1, show that after Hind III digestion a 6.9 kb fragment hybridizes to both YP1 and YP2 gene probes. Two other weak signals of 1.8 kb and 5.5 kb can be identified after YP2 hybridization. After YP3 gene probe hybridization a weak hybridization at 6.3 kb is seen. The number of yolk polypeptides in this species is uncertain but these results suggest that more than one YP-gene may be present.

For D. virilis a strong signal at 3.2 kb after Hind III digestion and YP1 gene probe hybridization suggests the presence of a YP1-like gene. After YP2 gene probe hybridization three signals are
present which may indicate up to three YP2-like genes. The signal at 3.2 kb is likely to be a result of cross hybridization to the YP1-like gene. It is also possible that any gene could be cut by Hind III so one gene could result in more than one hybridization signal. Protein data suggest that there are two yolk polypeptides, and genomic blot data suggests these may be a YP1-like gene and at least one YP2-like gene. No genomic sequence homologous to the YP3 gene probe could be identified in this species.

The strengths of hybridization signals were investigated, being compared with those for D. melanogaster for which we know that the three yolk protein genes are present in single copies. There are three problems associated with such an attempt. Firstly, the amounts of DNA loaded on different tracks will vary to some degree, although densitometer scanning was able to correct for this. The photographs shown in Fig 3:5 and Fig 3:7 indeed show a range of hybridization signal strengths. However, in these and repeated examples, these variations can be explained mainly by correcting for DNA loading. For instance the signals in tracks of D. mauritiana DNA appear relatively weak, and this is due, partly, to a relatively low amount
of DNA being loaded. Secondly, as the yolk protein genes will have evolved different nucleotide sequences the *D. melanogaster* yolk protein gene DNA probes will not be expected to hybridize equally efficiently to all the sibling species' yolk protein genes. Thus the low signal strength seen in *D. mauritiana* tracks, is likely to be also partly due to nucleotide divergence of its yolk protein genes compared with those of *D. melanogaster*. Thirdly, it cannot be assumed that all the DNA loaded on an agarose gel is transferred, by Southern blotting, to the nitrocellulose. This applies in particular to small or large DNA fragments. Thus, I was unable to observe the EcoRI 0.4 kb fragment which is part of the *D. melanogaster* YP1 gene, after hybridization to the YP1 gene probe. There is no evidence to suggest the presence of multiple copies of any of the *Melanogaster* Group Species' yolk protein genes. However, duplication resulting in a tandem repeat of perhaps two or three copies of a particular gene cannot be ruled out.

Yolk-protein genes were hard to detect in the two other species *D. funebris* and *D. virilis*. This is presumably due to the extent of nucleotide evolution of the yolk protein genes in these species. However, sequences homologous to the *D.*
melanogaster yolk protein genes have been identified in *D. funebris* and *D. virilis*, which could indicate the presence of more than one yolk protein gene in each of these species.
The total female haemolymph polypeptide profile for some Drosophila species. The gradient SDS polyacrylamide gel was stained with Comassie Blue. "D. melanogaster control" tracks show the D. melanogaster proteins precipitated with anti YP-antibodies.
Polypeptides in female haemolymph samples which are precipitated by antibodies raised against *D. melanogaster* yolk proteins. The gradient SDS polyacrylamide gel was stained with comassie Blue. "*D. melanogaster control*" track shows the total *D. melanogaster* haemolymph polypeptide profile.

h = Ig G heavy chains
l = Ig G light chains
Fig 3:5

(A) Southern blots of Genomic DNA restricted with Hind III and separated on agarose gels. The filters were hybridized with the \[^{32}P\]-labelled cloned YP1 gene of D melanogaster.

B) Southern blots of Genomic DNA restricted with EcoRI and separated on agarose gels. The filters were hybridized with the \[^{32}P\]-labelled cloned YP1 gene of D melanogaster.

Fragment sizes are in Kilobases

L = lambda DNA restricted with Hind III
(A) Southern blots of Genomic DNA restricted with Hind III and separated on agarose gels. The filters were hybridized with the $^{32}$P-labelled cloned YP2 gene of D. melanogaster.

(B) Southern blots of genomic DNA restricted with EcoRI and separated on agarose gels. The filters were hybridized with the $^{32}$P-labelled YP2 gene of D. melanogaster.

Fragment sizes are in Kilobases.

$L = \text{lambda DNA restricted with Hind III.}$
Fig 3:7

(A) Southern blots of Genomic DNA restricted with Hind III and separated on agarose gels. The filters were hybridized with the $^{32}$P-labelled cloned YP3 gene of D melanogaster.

(B) Southern blots of Genomic DNA restricted with EcoRI and separated on agarose gels. The filters were hybridized with the $^{32}$P-labelled cloned YP3 gene of D melanogaster.

Fragment sizes are in Kilobases

L = lambda DNA restricted with Hind III
Fragment sizes underlined, hybridize relatively stronger when probed with one of the other yolk protein gene probes

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<td>3.7, 9.6</td>
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<td></td>
<td>YP2</td>
<td>2.0</td>
<td>9.6</td>
</tr>
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<td></td>
<td>YP3</td>
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<td>D. sechellia</td>
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<td></td>
<td>YP2</td>
<td>2.9</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td>YP3</td>
<td>6.3</td>
<td>15.0</td>
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<tr>
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<td>6.1</td>
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<tr>
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<td>2.9</td>
<td>6.1</td>
</tr>
<tr>
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**TABLE 3:1** GENOMIC DNA FRAGMENTS HYBRIDIZING TO YP1, YP2 and YP3 GENE PROBES FROM D. MELANOGASTER (SIZES ARE IN KILLOBASES)
FIG 4:2. IN SITU HYBRIDIZATION OF D.MELANOCASTER YOLK PROTEIN GENE PROBES TO POLYTENE CHROMOSOME SQUASHES FROM D.MELANOCASTER. (A), USING YP1 GENE PROBE, (B), USING YP2 GENE PROBE, (C), USING YP3 GENE PROBE. ARROWS INDICATE SITES OF HYBRIDIZATION.
FIG 4:3. IN SITU HYBRIDIZATION OF D. MELANOGASTER YOLK PROTEIN GENE PROBES TO POLYTENE CHROMOSOME SQUASHES FROM D. MAURITIANA. (A), USING YP1 GENE PROBE, (B), USING YP2 GENE PROBE, (C), USING YP3 GENE PROBE. ARROWS INDICATE SITES OF HYBRIDIZATION.
FIG 4:4. IN SITU HYBRIDIZATION OF D. MELANOGASTER YOLK PROTEIN GENE PROBES TO POLYTWNE CHROMOSOME SQUASHES FROM D. SIMULANS. (A), USING YP1 GENE PROBE, (B), USING YP2 GENE PROBE, (C), USING YP3 GENE PROBE. ARROWS INDICATE SITES OF HYBRIDIZATION.
FIG 4:5. IN SITU HYBRIDIZATION OF D. MELANOGASTER YOLK PROTEIN GENE PROBES TO POLYTWNE CHROMOSOME SQUASHES FROM D. ERECTA. (A), USING YP1 GENE PROBE, (B), USING YP2 GENE PROBE, (C), USING YP3 GENE PROBE. ARROWS INDICATE SITES OF HYBRIDIZATION.
FIG 4:6. IN SITU HYBRIDIZATION OF D. MELANOGASTER YOLK PROTEIN GENE PROBES TO POLYTENE CHROMOSOME SQUASHES FROM D. ORENA. (A), USING YP1 GENE PROBE, (B), USING YP2 GENE PROBE, (C), USING YP3 GENE PROBE. ARROWS INDICATE SITES OF HYBRIDIZATION.
FIG 4:7. IN SITU HYBRIDIZATION OF D. MELANOGASTER YOLK PROTEIN GENE PROBES TO POLYTENE CHROMOSOME SQUASHES FROM D. YAKUBA. (A), USING YP1 GENE PROBE, (B), USING YP2 GENE PROBE, (C), USING YP3 GENE PROBE. ARROWS INDICATE SITES OF HYBRIDIZATION.
FIG 4:8. **IN SITU HYBRIDIZATION OF D. MELANOGASTER YOLK PROTEIN GENE PROBES TO POLYTENE CHROMOSOME SQUASHES FROM D. TEISSIERI.** (A), USING YP1 GENE PROBE, (B), USING YP2 GENE PROBE, (C), USING YP3 GENE PROBE. ARROWS INDICATE SITES OF HYBRIDIZATION.
<table>
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<th>YP2</th>
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<td>9A</td>
<td>9A</td>
<td>12B/C</td>
</tr>
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<td>9A/C</td>
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<td>D. simulans</td>
<td>9A</td>
<td>9A</td>
<td>12B/C</td>
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<td>D. erecta</td>
<td>9A/B</td>
<td>9A/B</td>
<td>12A/C</td>
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<td>D. orena</td>
<td>9</td>
<td>9A/D</td>
<td>12A/E</td>
</tr>
<tr>
<td>D. yakuba</td>
<td>9A/B</td>
<td>9A/B</td>
<td>11A-12A or 8A-6D</td>
</tr>
<tr>
<td>D. teissieri</td>
<td>11D/12D</td>
<td>12A/D</td>
<td>12B/C</td>
</tr>
</tbody>
</table>

Table 4:1 Proposed locations of Yolk Protein Genes in the D. melanogaster Sibling Species as determined by in situ hybridization
3:4 DISCUSSION

It has been shown, on the basis of cross reactivity to anti-YP antibodies raised against *D. melanogaster* yolk proteins, that all the *Melanogaster* Group Species have three yolk polypeptides. These were identified in female haemolymph samples and it is assumed that they are there as a result of export from the fat body cells, as has been found to be the case for *D. melanogaster* (Kambysellis, 1977; Hames and Bownes, 1978; Postlethwait and Kashnitz, 1978; Bownes and Hames 1978; Brennan et al, 1982).

Species outside the *Melanogaster* Group do not necessarily synthesise three yolk polypeptides. *D. virilis* appear to have only two yolk polypeptides and only one has been observed in *D. funebris* (Bownes, 1980B). Other insects have varying numbers of yolk polypeptides. For instance *Hyalophora cecropia* and *locusta migratoria* have two yolk polypeptides based on polyacrylamide gel electrophoresis (Kunkel and Pan, 1976; Gellissen et al, 1976). All the species investigated here have yolk polypeptides of similar apparent molecular weights, around 44000 to 47000. Evidence is given to suggest that the smallest yolk
polypeptide in each of the *Melanogaster* Group Species is a YP3-like yolk polypeptide on comparison with *D melanogaster*. Some variation in migration rates, and therefore apparent molecular weights, has been observed for two species. The smallest yolk polypeptide of *D. teissieri* and the two largest yolk polypeptides of *D. erecta* appear larger than for the other sibling species. As samples were collected from populations of flies, and not from individuals, such variations are not simply a result of intra-specific polymorphism. It is not known which of the two larger yolk polypeptides are YP1-like or YP2-like for the sibling species. In order to investigate these two areas it will be necessary to either determine the amino-acid sequences for the individual yolk polypeptides, to study specific protease digestion patterns of the individual yolk polypeptides, or to indirectly determine the amino-acid sequences from nucleotide sequences of the genes coding for the yolk polypeptides. Investigations of V8-Protease digestion patterns of sibling species' yolk polypeptides are being carried out in our group. By use of a specific protease, such as V8, and Western transfer experiments, it should be possible to investigate the conservation of particular amino-acid sequences within the sibling species' yolk polypeptides. Such work should be of use in
investigating the evolution of the Melanogaster Group species, as well as detecting polypeptides which are conserved as a result of functional requirements.

The in situ hybridization results are consistent with there being three yolk protein genes in each of the sibling species. However, there are other possibilities. In all the species the YP1 and YP2 gene probes hybridize to similar regions, thus it cannot be ruled out from this data that these probes are simply identifying the same gene, as a result of cross hybridization. It is also possible that tandem repeats of any of the yolk protein genes are present.

It is known that the YP1 and YP2 genes of D. melanogaster are very closely linked (Barnett et al., 1980). The in situ hybridization results presented here are consistent with there being close linkage between these two genes for all the sibling species.

Results presented for Southern blots suggest that all but two of the sibling species have at least three YP genes, although there could be just two in D. mauritiana and D. simulans. Data on the yolk
polypeptides, also presented, suggest that each of the sibling species has three. For these reasons it is probable that each of the YP-gene probes is identifying a different yolk-protein gene, in each of the species, following in situ hybridization.

It is interesting that the three putative YP genes appear at similar, and possibly identical chromosome band positions in D. melanogaster, D. mauritania, D. simulans, D. erecta and D. orena. In D. yakuba the YP1 and YP2 genes appear in similar banding positions as in these species. Finally, in D. teissieri all three YP genes appear to be present within the region 11D to 12D.

The three YP-genes of D. orena and D. erecta are present on regions which have undergone chromosomal inversion to the D. melanogaster standard. In D. yakuba the YP1 and YP2-like genes are closer to the centromere than the YP3-like gene. This can be explained by the chromosomal rearrangements described which have resulted in the region 8A - 9B being located close to the centromere.

Although the YP1 and YP2-like genes of D. teissieri and the YP3-like gene of D. yakuba do not appear to be located in the same banding regions as found for the other sibling species, it is possible that
rearrangement events have occurred involving small chromosome areas, which would not be resolved under the light microscope. Thus, for instance, the YP1 and YP2-like genes of D. teissieri could still be located on a fragment of DNA from region 9A which has ended up within region 11D - 12D.

At least three possible yolk protein coding genes have been identified for all the Melanogaster Group species except two species. Two possible yolk protein genes have been indentified for D. mauritiana and D. simulans, although it is likely that in both cases a third yolk protein gene exists. It is probable that the YP1-like and YP2-like genes of these two species have not been separated by Hind III or EcoRI digestion due to their very close linkage. It is expected that genomic DNA digestion by a different restriction endonuclease will separate these two genes in these two species. Such experiments are being carried out.

Determinations of numbers of putative yolk protein genes are all minimum values. Very divergent genes, which nevertheless code for yolk proteins, would not be detected in these studies based on looking for nucleotide sequences fairly homologous
to the *D. melanogaster* yolk protein genes. Nevertheless the data is consistent with each yolk protein being coded for by a single copy gene in all the sibling species.

Yolk protein genes in species outside the *Melanogaster* Group Species can be detected using gene probes from *D. melanogaster*. Thus, in *D. virilis* two putative yolk protein genes have been identified while at least one yolk protein gene has been identified in *D. funebris*. This is consistent with the polypeptide data presented for these two species.

However, once again genomic blot data do not exclude the possibility of further genomic sequences containing yolk protein coding genes occurring. In order to more confidently assign particular yolk protein genes to particular nucleotide fragments it would be useful to carry out hybridization studies using conditions of increasing stringency. Thus, by increasing the temperature of post-hybridization washes, fragments which contain areas of nucleotide mismatch compared with the probe used will increasingly fail to hybridize. In this way an autoradiograph signal will be lost, while remaining signals are likely to indicate yolk protein genes.
with less ambiguity. Such analysis will also allow quantitative estimates to be made of the nucleotide homologies between gene probe and genomic sequences.

Evidence has been presented from genomic blots and in situ hybridizations to suggest the very close linkage of two of the putative yolk protein genes, the YP1-like and the YP2-like, in all the Melanogaster Group species with the exception of D. yakuba. This does not mean that the YP1-like and YP2-like genes of D. yakuba are not also very closely linked. Different restriction endonucleases are currently being used, with Southern blotting, to try to obtain evidence for close linkage between these two genes in this species. If the YP1-like and YP2-like yolk protein genes are closely linked in all the Melanogaster Group species this suggests that the duplication event, which has been suggested from data for D. melanogaster resulting in the YP1 and YP2 genes, happened in evolution before the divergence of the Melanogaster Group species.

In order to gain a fuller understanding of the organisation and evolution of the yolk protein genes for the Melanogaster Group species, it will
be necessary to isolate, by cloning, these genes. They will have to be sequenced and compared. In this way conserved coding regions and consensus sequences can be identified which may indicate regions of functional importance. Also, rates of nucleotide mutation can be compared between different species which will add valuable knowledge to the understanding of evolution within this species group. The overall aim is similar to that achieved in the comparison of the alcohol dehydrogenase genes (Adh) in some *D. melanogaster* sibling species carried out by Bodmer and Ashburner (1984). It would be interesting to compare their results for the Adh genes with data for another set of genes, those coding for the yolk proteins.

Towards this aim genomic libraries of four of the sibling species, *D. mauritiana*, *D. simulans*, *D. teissieri* and *D. yakuba*, constructed using the vector Horner I (Chia *et al.*, 1982; a gift from Drs. M. Bodmer and M. Ashburner) were screened for yolk protein coding genes. Although no DNA fragments likely to contain complete yp-genes were isolated, given more time success would seem likely.
CHAPTER FOUR

GENERAL CONCLUSIONS AND DISCUSSION

Experiments have been described which investigate some aspects of yolk protein gene organisation, expression and evolution in species of Drosophila.

Yolk protein induction experiments in D. melanogaster males, following 20-OH ecdysone treatment, suggest that transcriptional and post-transcriptional controls regulate these genes. However, it is still not known what role 20-OH ecdysone plays in the normal functioning of the yolk protein genes. It appears that ecdysteroids have little part to play in the control of ovarian yolk polypeptide synthesis, but some role in fat body yolk polypeptide synthesis is likely (Bownes et al, 1983A). There is no evidence to suggest that ecdysteroids play any part in establishing the sex-specific nature of yolk polypeptide synthesis in D. melanogaster.

Nevertheless, ecdysteroids appear to play a part in a variety of other developmental processes in Drosophila. For instance 20-OH ecdysone causes embryonic cells to develop into a range of larval cell types, and causes third larval instar fat body cells to take up specific haemolymph proteins (Dubendorfer and Eichenberger, 1983). It causes the induction of specific genes, such as larval serum protein-2 (LSP-2), in cultured fat body cells (Nakanishi and Garen, 1983), and induces transcription of heat-shock genes in vivo and in vitro (Ireland et al, 1982) and actin genes in Drosophila Kc cell lines (Couderc et al, 1983). Ecdysteroids also induce morphogenesis and differentiation in imaginal discs in vitro and in vivo (Fristrom et al, 1984), and causes a sequence of changes in polytene chromosome puffing patterns in third instar larval salivary gland cells which correlates with increased transcriptional activity of specific genes (Cherbas et al, 1983).

Hormonal induction of the yolk protein genes, in males, is not limited to D. melanogaster as it is possible to do the same in other
Drosophila species, even outside the Melanogaster Group species. In these species 20-OH ecdysone appears to mediate its effect, at least partly, by causing increased transcription of the yolk protein genes, although not necessarily all these genes to the same extent.

By Southern blotting and in situ hybridization techniques, putative yolk protein genes have been identified, and by anti-D. melanogaster YP antibody cross reactivity yolk polypeptides have been identified, in Drosophila species.

Three yolk polypeptides, of similar sizes to those from D. melanogaster, have been detected in the female haemolymph of all the D. melanogaster sibling species. Three putative yolk protein genes have been detected in all the sibling species except D. simulans and D. mauritiana, while at least two yolk protein genes have been detected in these two species.

The sibling species' yolk protein genes appear to be present on the X chromosome. The YP1 and YP2-like genes occupy similar chromosomal banding locations for all the sibling species except D. teissierii. The YP3-like genes are also present at similar chromosomal locations for all the sibling species except D. yakuba. The YP3-like gene is always located closest to the centromere, except in the case of D. yakuba, and possibly D. teissierii.

Overall, it is probable that the sibling species contain similar yolk protein genes and synthesize similar yolk polypeptides. The expression of the yolk protein genes is likely to be governed by similar cellular and/or hormonal control systems, and the yolk polypeptides are likely to serve similar functions. Despite homologies between nucleotide sequences containing yolk protein genes (detected by Southern blotting) and between yolk polypeptides (detected by anti-YP antibody cross reactions) present in the sibling species, it is apparent that evolution has occurred within this species group. Evolution has been detected by investigating the conservation of restriction endonuclease recognition sequences in and around the yolk protein genes; and by slight variations in
Two species outside the *Melanogaster* Group species were also investigated. Despite showing little homologies to *D. melanogaster* at the DNA and polypeptide levels, two yolk polypeptides and YP-genes have been detected in *D. virilis* and one yolk polypeptide and YP-gene detected in *D. funebris*.

Direct, or indirect, determination of yolk protein amino acid sequences from sibling species could be compared to indicate areas which are conserved, both within and between species. Functional domains of the proteins may be identified, and the evolution within and between such domains could be investigated. It is likely that certain domains have sequences conserved more than other domains, as a result of differing selective forces.

So far, there is little direct data on the *D. melanogaster* yolk proteins, such as determined by specific protease digestion patterns, (Bownes and Hames, 1978; Warren and Mahowald, 1979) and these gave differing conclusions. The putative primary sequences of YP1 and YP2 polypeptides have been indirectly determined from the nucleotide sequences (Hung and Wensink, 1984). Their comparisons predict the secondary structures of YP1 and YP2 polypeptides are very similar, with five distinct regions of homology and presumed function detected, despite the amino acid
sequences being only 53% homologous. As well as having similar N-terminal leader sequences, which are thought to be important for correct processing of the primary polypeptides for export, other domains could have functions, for instance, in achieving inter-molecular binding, uptake into oocytes, and in conferring properties for storage.

In a similar way determination and comparisons of the DNA sequences for the sibling species YP genes would identify conserved nucleotide regions. Outside the coding regions consensus sequences may be expected, indicating important cis-acting control regions such as TATA boxes, enhancer sequences and steroid-receptor binding sequences. A consensus sequence similar to a progesterone-receptor binding site (Mulvihill et al., 1982) has been detected in the YP1 - YP2 intergenic sequence from D. melanogaster (Hung and Wensink, 1984). It will be interesting to see whether this sequence is conserved in other sibling species' YP-genes. As yolk polypeptide synthesis can be induced in these species, by 20-OH ecdysone treatment, it is likely that this sequence will be conserved.

From work of Miyata et al. (1980) it may be expected that most nucleotide substitutions in coding regions will rarely result in coding for a different amino acid, and this has been found to be the case for the Adh gene in Drosophila species (Bodmer and Ashburner, 1984). It will be interesting to determine whether this holds true for the Drosophila yolk protein genes. Determination of nucleotide divergence can be used to construct a phylogenetic tree for these Drosophila species and then compare it with those which have already been constructed. The neutral theory of evolution (see Kimura, 1983 for review), which states that chance events are more important than natural selection in bringing about evolution, predicts that the rate of evolution of a class of molecules will be high for molecules with few selective constraints, and low for those which are tightly constrained. By comparing YP-gene sequences with those for other genes we may gain an idea of how tightly constrained the yolk proteins are.
Comparisons of YP-gene sequences within and between species could be used as evidence for or against concerted evolution. Work by Coen et al (1982) and Dover et al (1984), comparing ribosomal and histone gene families as well as non-genic families, has described concerted evolution within the Melanogaster sibling species. An obvious question to ask is whether YP-gene sequences show a similar situation.

Another approach to study the regulation of the genes coding for the yolk proteins is to use P element-mediated transformation, as developed by Rubin and Spradling (1982). The technique has been used to stably transform D. melanogaster with dopa decarboxylase (Scholnick et al, 1983), alcohol dehydrogenase (Goldberg et al, 1983) and glue protein genes (Richards, 1983) amongst others. Interestingly these genes, when incorporated into host chromosomes, are capable of functioning in a stage and tissue-specific manner as found with normal endogenous genes. Glue protein genes, so incorporated, even appear to be under normal ecdysteroid-mediated control.

Modified yolk protein genes from D. melanogaster are being used to transform D. melanogaster embryos. In this way it is hoped to detect control regions required for the normal functioning of these genes. One problem encountered involves the ability to identify inserted genes and gene products from endogenous ones. Although this can be accomplished in D. melanogaster by using modified YP-genes, it could also be possible by taking advantage of naturally occurring inter-specific variations of the YP-genes and their products. Results in Chapter Three clearly show there are many inter-specific variations at the DNA level, but yolk polypeptide differences are less common or obvious between the sibling species. Two dimensional gel electrophoresis or specific protease digestion comparisons may possibly distinguish yolk polypeptides from different species. Work is in progress to determine whether there are inter-specific variations in YP-transcript sizes. So it is possible that YP-genes from sibling species could be used to transform D. melanogaster or vise versa. In this way we can ask
whether a gene from one species can be correctly expressed in a related species, and use of gene constructs may enable us to detect particular DNA sequences which are necessary for functions such as transcription, transcript processing, and hormonal regulation.

Finally, results presented in Chapter Four show that the YP-genes are always present on the x chromosome in all the D. melanogaster sibling species. It would be interesting to use P element-mediated transformation to ask whether the YP-genes will function when present on the autosomes, and if so, will they function in a sex-specific manner?

It can be seen that the Drosophila yolk protein genes continue to offer an interesting system for studying the molecular biology and evolution of a set of genes which are controlled in a developmental, tissue, and sex-specific manner. It has been pointed out that there are various aspects which require further investigation, and many questions to be answered. What are the factors which determine that the genes are not expressed until after eclosion? Why are the genes only expressed in the fat body and follicle cells? What is the molecular basis for the genes, normally, only being expressed in females and what are the precise controls exercised by hormones such as Juvenile Hormone and ecdysteroids? Despite much research in these areas, the answers remain elusive. However, we now have a variety of molecular "handles" on the system, and techniques available, to enable us to directly investigate the questions posed.
REFERENCES


Cameron-Clarke, A. (1980)

Chan, L.N. and Gehring, W. (1971)


Cohn, R.H. and Brown, E.H. (1968)

Collins, J. and Hohn, B. (1978)


Garcia-Bellido, A. (1982) Patterns in


Giddings, L.V. and Templeton, A.R. (1983)


Giddings, L.V. and Templeton, A.R. (1983)
Giorgi, F. and Jacob, J.  

Giorgi, F. and Jacob, J.  


Jenson, E.V. (1968) P.N.A.S. 59, 632.


Cell 34, 37-45.


Developmental Biology 96, 182-188.

Sharrock, W.J. (1983)

Journal of Molecular Biology 174, 419-431.


P.N.A.S. 80, 186-190.

Southern, E.M. (1975)

Journal of Molecular Biology 98, 503-517.

Southern, E.M. (1979)

Analytical Biochemistry, 100, 319-323.


Experientia 34, 1572-1574.


Wilhelm Roux's Archives of Developmental Biology 187, 255-266.

Sturtevant, A.H. (1942)

University of Texas Publication 4213, 5-51.

Throckmorton, L.H. (1975)


P.N.A.S. 5, 1574.


Upholt, W.B. (1977)

Nucleic Acid Research 4, 1257-1265.

Wahl, W. and Dawid, I.B. (1979)

Cell 16, 535-549.


### APPENDIX 1

**Latin and Common Names of Species**

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<th>Species</th>
<th>Common Name</th>
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<tr>
<td>Drosophila species</td>
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<td>Cecropia moth</td>
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<td>Locusta migratoria</td>
<td>African migratory locust</td>
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<tr>
<td>Sarcophaga bullata</td>
<td>Flesh fly</td>
</tr>
<tr>
<td>Xenopus laevis</td>
<td>African clawed toads</td>
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20-hydroxyecdysone stimulates tissue-specific yolk-protein gene transcription in both male and female *Drosophila*

By MARY BOWNES¹, MAIREARAD BLAIR, ROBERT KOZMA AND MAUREEN DEMPSTER

From the Department of Molecular Biology, Edinburgh University

**SUMMARY**

The yolk polypeptides of *Drosophila* are normally synthesized in the fat body and ovarian follicle cells of adult females. In response to 20-hydroxyecdysone males synthesize yolk polypeptides. The actual level of yolk polypeptides synthesized in males is not always a direct reflection of the YP-transcripts present. Initially YP-transcripts are efficiently translated into polypeptides whereas later they are not and the YP-transcripts can have a half-life of less than 8 h in males. We suggest that the expression of the genes coding for the yolk polypeptides in males may be regulated at transcriptional and translational levels. Treatment of females with 20-hydroxyecdysone leads to a transient increase in YP-transcript accumulation, but the response is difficult to assess in whole flies due to the high variability in transcript levels during normal development.

Analysing the response to 20-hydroxyecdysone at the level of specific tissues shows that transcript accumulation is dramatically increased in body walls (fat-body cells, epidermis and oenocytes) of both males and females. Gut, Malpighian tubules, testis and ovaries are not affected. Treatment of females with 20-hydroxyecdysone followed by measuring YP-transcript accumulation over the next 24 h in ovaries and body walls separately, confirms that only body walls respond to the hormone. There is an increase in yolk-polypeptide synthesis during the period of increased YP-transcript accumulation in females.

We conclude that the response of the YP-genes to 20-hydroxyecdysone is tissue-, but not sex-specific.

**INTRODUCTION**

The three yolk polypeptides (YPs) of *Drosophila melanogaster* are synthesized in the follicle cells of the ovary and in the fat body of adult females (Kambysellis, 1977; Hames & Bownes, 1978; Postlethwait & Kashnitz, 1978; Bownes & Hames, 1978; Brennan, Weiner, Goralski & Mahowald, 1982). Adult males do not usually synthesize these polypeptides, however they can be induced to do so by 20-hydroxyecdysone (Postlethwait, Bownes & Jowett, 1980). The response of the males to a single hormone injection or feeding with hormone for 2 h is a short burst of yolk-polypeptide synthesis lasting approximately 24 h, the maximum

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rate of yolk polypeptide synthesis being between 8h and 14h after hormone treatment (Bownes, 1982).

When females first emerge YP mRNAs are only present in small amounts and gradually accumulate over the first 24 h of adult life (Barnett & Wensink, 1981). The mRNA available for translation into yolk polypeptides mirrors the total YP-mRNA present indicating that initially the expression of the genes in females operates at the level of transcription. In males, treatment with 20-hydroxyecdysone leads to increased accumulation of YP-transcripts (Shirk, Minoo & Postlethwait, 1983; Shirras & Bownes, unpublished). However the expression of the genes in response to 20-hydroxyecdysone as measured by yolk polypeptide synthesis in vivo, is short-lived (Bownes, 1982). This suggests either that the YP-mRNAs produced are relatively unstable in a male environment, or that controls operate in the male to make the YP-transcripts less readily translated into proteins. In preliminary experiments measuring the hybridization of labelled cloned YP-genes to total RNA, the peak accumulation of YP-transcripts in males occurred at 16h, not at 8–12 h when the peak of yolk polypeptide synthesis was found to occur. (Shirras & Bownes, unpublished).

Therefore we have investigated the level at which the expression of these three genes is regulated in males by measuring YP-transcript accumulation during a 24 h response to the hormone. The translation of these transcripts into yolk polypeptides by the rabbit reticulocyte lysate cell-free translation system has also been measured. The results indicate that the expression of the genes is modulated in vivo by transcriptional and possibly also post-transcriptional control mechanisms, and that the transcripts are degraded fairly rapidly following induction of the genes by 20-hydroxyecdysone. We then investigated the tissue specificity of the response to hormone. Transcription of the YP-genes only occurs in male carcasses, not in testes, gut or Malpighian tubules. Furthermore the YP-genes in the female body wall are also more actively transcribed, and/or the RNA is made more stable after treatment with 20-hydroxyecdysone. Ovarian transcript levels, however, remain unaffected. 20-hydroxyecdysone therefore seems to affect the expression of the YP-genes in a tissue-specific but not in a sex-limited fashion.

MATERIALS AND METHODS

Maintenance of stocks

Flies were maintained on standard yeast, cornmeal, sugar and agar medium at 25 °C. The pooled haemolymphs of each set (10–15 flies) were collected into 50 μl Laemmli buffer (Laemmli, 1970). Tissues were dissected in Ringers (Chan & Gehring, 1971), transferred to 50 μl Laemmli buffer, vortexed and the debris separated by centrifugation. 1 μl samples were precipitated with TCA to calculate total incorporation into proteins. The polypeptides were separated by
Expression of yolk polypeptide genes in Drosophila

polyacrylamide gel electrophoresis as in Bownes, Dempster & Blair (1983) and the gels were prepared for fluorography either by the technique of Bonner & Laskey (1974) or by using 'enlightening' (New England Nuclear).

The resulting autoradiographs were scanned with a densitometer and the peak areas corresponding to yolk polypeptides were calculated. Where comparisons are made between samples, all samples were run on the same gel and the same autoradiograph was scanned in each case. To ensure that this method of scanning gels was reasonably quantitative we compared the total area under all the proteins of an autoradiograph with the total TCA precipitable counts loaded onto a gel. In a series of five samples the ratios of TCA precipitable counts loaded was 1: 1.4: 2.2: 1.2: 0.7 and the ratios of the total areas of the same samples after autoradiography was 1: 1.7: 2.4: 1.4: 0.7. Thus agreement is fairly good and the method would appear to be valid for providing approximate comparisons between proteins in various samples.

Isolation of RNA

RNA was isolated from whole flies and dissected tissues as described by Bownes et al. (1983).

Translation of RNA in a cell-free translation system

Samples of 5 µg of total RNA were translated in the rabbit reticulocyte cell-free translation system, as described by Pelham & Jackson (1976).

Precipitation with anti-YP antibody

Yolk polypeptides were precipitated after translation in the rabbit reticulocyte lysate cell-free translation system as described in Isaac & Bownes (1982).

Preparation of ³²P-labelled YP-probes

These were prepared as described by Bownes et al. (1983) using pYP1, pYP2 and pYP3 of Barnett, Pachl, Gergen & Wensink (1980). Probe activities were approximately 10⁷ c.p.m./µg DNA.

Specificity of YP-probes

As described by Barnett et al. (1980), we found that the three cloned YP genes pYP1, pYP2, and pYP3 specifically hybridize to the yolk-protein genes and do not hybridize to any other sequences in the Drosophila genome. The experimental conditions used for subsequent hybridization experiments were such that there was no cross-hybridization between pYP1 and pYP3, or pYP2 and pYP3, but that pYP2 and pYP1 showed some cross-hybridization. For example a pYP2 probe hybridized to both pYP2 and pYP1 on nitrocellulose filters showed 10% as much hybridization to pYP1 as to itself. This would be expected from the sequences of these genes (Hovemann & Galler, 1982).
Measurement of YP-RNA levels

Transcripts coding for the yolk polypeptides were measured by the dot hybridization techniques of Thomas (1980). Details of our protocols and methods of quantitation of the resulting autoradiographs are described in Bownes et al. (1983), the only modifications being that filters were hybridized for 40 h and washed finally at 52°C (two times) rather than 50°C. The most accurate method is to measure the area \times density of the dots on the resulting autoradiographs. This technique is useful for comparing transcript levels between samples, but does not give absolute quantitation of transcripts, nor does it distinguish hnRNA from mRNA. The weak hybridization seen when male RNA is spotted is due to the DNA isolated along with the RNA. A similar signal is seen after alkaline hydrolysis of similar quantities of female RNA, and this signal is removed when the samples are treated with LiCl to remove the DNA. We prefer however to see this weak signal to locate precisely where samples are spotted onto the nitrocellulose.

For Northern blots 10 \mu g samples of total RNA were separated on formaldehyde gels at 50 V overnight and transferred to nitrocellulose. Hybridization conditions were similar to those used for dot blots. The transcript sizes are 1.59 to 1.61 kb for YP1, 1.59 to 1.61 and 1.66 to 1.68 kb for YP2 (these two transcripts differ at the 3' end) and 1.53–1.55 for YP3 (Hung, Barnett, Woolford & Wensink, 1982). The genes have only small introns, identified by S1 mapping, about 70–80 bp in length and variable length polyA tails (Hung et al. 1982). Thus separation by electrophoresis and hybridization to the pYP1, 2, 3 probe still does not distinguish hnRNA from mRNA, and Northern blots give little more information than the blots used in the majority of these experiments to avoid loss of RNA which occurs in transferring from the gel to nitrocellulose.

Treatment with hormone

Adults aged 3 days were lightly etherized and either injected with 0.2 \mu l of 10^{-2} \text{M}-20-hydroxyecdysone in Ringers (Chan & Gehring, 1971), or alternatively they were fed on a sugar solution containing 10^{-5} \text{M}-20-hydroxyecdysone for 2 h at 25°C. Control flies for feeding experiments were fed on the sugar solution for a similar period of time. They were subsequently maintained on normal medium at 25°C.

All experiments were performed at different times, on different fly populations, and the transcript levels were measured using different probes. This was done to avoid biasing results because of the characteristics of particular populations of flies. Nonetheless experimental protocol, rearing conditions, method, site and volume of injection, and feeding methods were always repeated as exactly as possible. In some experiments RNA from the same batch of induced males was analysed by dot hybridization (Thomas, 1980) and in the cell-free translation system. The same groups of induced males were also used to label
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their haemolymph yolk polypeptides with $^{35}$S-methionine before RNA extraction in some experiments.

In experiments looking at individual tissues, these were dissected into Ringers at the end of the experiment and transferred immediately into RNA extraction buffer. They were either extracted immediately or frozen rapidly in liquid nitrogen and stored at $-70^\circ$C until the RNA was extracted.

RESULTS AND DISCUSSION

Protein synthesis in vivo

We measured the synthesis of yolk polypeptides and their secretion into the haemolymph by injecting 3-day-old adults with $^{35}$S-methionine and collecting the haemolymph 2 h later for analysis by SDS polyacrylamide gel electrophoresis and fluorography (some of the results are presented in Table 1). Our previous studies showed that the peak of the yolk-polypeptide synthesis in males which have been stimulated with 20-hydroxyecdysone, occurred at about 12 h after hormone treatment, but that this varied between the populations. Yolk-polypeptide synthesis was subsequently reduced, and sometimes undetected, 24 h after treatment (Bownes, 1982). The degree of induction of the yolk polypeptides varies between experiments. Sometimes the yolk polypeptides represent only 12–14% of the total newly synthesized haemolymph proteins, yet in other experiments they can reach 35–40%. The method of hormone treatment contributes to this, feeding with the hormone (10$^{-3}$ M) generally giving a

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time after treatment in hours</th>
<th>% female control level</th>
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<tr>
<td></td>
<td>haemolymph YPs</td>
<td>YP-transcripts</td>
</tr>
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<tr>
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<td>44</td>
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<td>24</td>
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</table>

*Corrected for background in untreated male samples.
larger induction than injecting it (0.2 μl 10⁻² M per fly). Females also show variation in the rate at which they synthesize yolk polypeptides. In 3-day-old females 43–64 % of the total [³⁵S]methionine-labelled haemolymph proteins in a 2 h period were yolk polypeptides. This variability is probably related to many factors including the nutritional condition of the population, time of day, etc., these factors will vary despite maintaining flies under essentially identical culture conditions.

Expressing YP-induction in males in terms of the level of YP-expression in females of the same age from the same population in the same vial (this comparison should avoid, as much as is technically possible, variabilities due to differences between cultures) we obtain values of from 30 % to 100 %. Males have only half the number of genes coding for yolk polypeptides in each cell due to their X-chromosome location, so the observed yolk polypeptide synthesis represents a substantial induction of yolk polypeptide gene expression in male tissue.

**Yolk polypeptide transcript accumulation in hormone-treated male flies**

During the hybridization of labelled cloned YP-gene probes to RNA isolated from induced males and immobilized on DBM paper, we observed that the peak of yolk polypeptide transcript accumulation was at around 14–16 h after hormone induction (Shirras & Bownes, unpublished). This suggested that the peak of yolk polypeptide synthesis occurred before the peak of yolk polypeptide transcript accumulation. However, because of the possibility of variability between groups of flies, we could not be sure that, had we measured the synthesis in vivo of YP’s in flies whose peak transcript accumulation occurred at 14–16 h, the same population would not have shown a 14–16 h peak in yolk polypeptide synthesis.

We decided, therefore, to perform an experiment using the same group of flies measuring protein synthesis in vivo and YP-transcript levels in the whole flies, to look at the relationship between transcription and translation of the yolk polypeptide genes.

3-day-old males were treated with 10⁻² M-20-hydroxyecdysone. Yolk polypeptides synthesized and secreted into the haemolymph during a 2 h labelling period...
Expression of yolk polypeptide genes in Drosophila

In vivo protein synthesis

Transcript accumulation

In vitro protein synthesis

Fig. 1
were measured. Their rate of synthesis was highest at 12 h (84% of the female level) and, in this experiment, was still relatively high at 24 h (52% of the female level). The degree of protein synthesis is shown in Fig. 1A. Transcript levels were measured using the technique of Thomas (1980); dotting 5 μg samples of RNA onto nitrocellulose, hybridizing with a mixed probe containing equimolar quantities of labelled cloned YP1, 2 and 3 genes, and exposing the filters to X-ray film. The area \( \times \) density of the dot is proportional to the amount of YP-transcript present under the conditions we use (Bownes et al. 1983). The results are recorded in Fig. 1B. The peak of transcript accumulation was at 16 h (100% of female level), and fell by 24 h (54% female level). In this group of flies, the peak of yolk polypeptide synthesis preceeded the peak of YP-transcript accumulation, which suggests that post-transcriptional control mechanisms may regulate expression of the YP-genes as well as factors at the transcriptional level. It is interesting that the accumulated transcript levels dropped by 50% between 16 and 24 h after induction, suggesting a half-life for the male YP-transcripts of not more than 8 h. We do not know if further transcription of the genes occurs during this period. If so, then the half-life may be shorter still.

RNA from induced males and control females was translated in the rabbit reticulocyte lysate cell-free translation system. As shown in Fig. 1C the synthesis of yolk polypeptides follows the general trend of the total transcripts present, but the male YP-transcripts were in all cases translated considerably less well \textit{in vitro} than were the female YP-transcripts. This is not true of all other male messages since general stimulation of protein synthesis in the cell free system was from 19 000–52 000 c.p.m. /µl of lysate stimulated with male RNA, and 27 000 c.p.m. /µl of lysate stimulated with female RNA. The curves for cell-free translation product follow the shape of the transcript-accumulation curves, and do not follow the curve for yolk polypeptide synthesis \textit{in vivo}, at least at the early time points. This suggests that the factor regulating \textit{in vivo} rates of translation does not lie within the structure of the RNA.

RNA isolated from males after hormone treatment was analysed by Northernns and, as can be seen in Fig. 2, YP-transcripts were always of similar size to those observed in females. There is also no signal in male samples before hormone treatment, and the lack of unusual-sized transcripts suggests that there are no major differences in the transcripts induced in males compared to those normally present in females. We would not, however, detect small differences in processing or transcript size by this method.

As this group of flies fell towards one of the extremes of variation between populations, in that it showed a very high peak of yolk polypeptide synthesis and still quite substantial synthesis at 24 h, we repeated the experiment twice, measuring transcript levels and protein synthesis \textit{in vivo}, using fewer time points. In both populations yolk polypeptide synthesis was much reduced by 24 h (Table 1) and at 12 h, male yolk polypeptide synthesis \textit{in vivo} represented a smaller proportion of the control female level than did transcript accumulation.
Expression of yolk polypeptide genes in Drosophila

Fig. 2. Northern analysis of RNA extracted from whole male flies induced to produce YP-transcripts with 20-hydroxyecdysone. RNA was separated on formaldehyde gels, transferred to nitrocellulose and hybridized to pYP1, pYP2, and pYP3. The resulting autoradiograph is shown. (a) ♀ control; (b) 0 h after hormone treatment; (c) 4 h after hormone treatment; (d) 8 h after hormone treatment; (e) 12 h after hormone treatment; (f) 16 h after hormone treatment; (g) 20 h after hormone treatment; (h) 24 h after hormone treatment.
Transcript levels reached 64% and 67% of female levels by 12 h, while yolk polypeptide synthesis reached 44% and 40% respectively. Yolk polypeptide synthesis *in vivo* was therefore 69% and 60% of the predicted levels for the number of transcripts available in males compared to females. In the first experiment at 24 h the relationship between protein synthesis *in vivo* and transcript levels showed no discrepancy, but in the latter two experiments there was only a 35% and 25% comparative efficiency of translation (see Table 1). Furthermore at 4, 8 and 12 h in experiment 1 the male RNA seems to be translated into protein more efficiently than the female RNA. Thus there may well be translational controls in flies operating at various points after hormone induction, since the level of protein synthesis in the males, late in the cycle of induction, is often lower than one would predict on the basis of the number of transcripts present and their translation into yolk polypeptides in females. In the second and third experiments the reduction in transcript accumulation was much slower than in the first experiment but yolk polypeptide synthesis *in vivo* fell more rapidly. Presumably YP-gene expression results from a combination of transcriptional control of the YP-genes, mRNA turnover, and the rate of translation of the YP-mRNAs. Regulation at each of these levels appears to operate at some point during hormonal induction, although it is possible that the inefficient translation into yolk polypeptides several hours after hormone treatment could result from altered amino acid uptake and be a non-specific effect of the hormone rather than a specific mechanism to inhibit YP translation in males.

In feeding experiments we had previously observed very high levels of induction, but yolk-polypeptide synthesis was still low by 24 h. We measured YP-transcript levels after feeding 20-hydroxyecdysone to males for 2 h at times 2 h, 6 h, 18 h and 24 h. At 2 and 6 h transcript accumulation was less than 2% female levels, but by 18 h it had reached 60% and by 24 h it had fallen to 3%. Thus virtually all of the transcripts were degraded in a 6 h period.

The arguments for there being post-transcriptional control of these genes rely upon measured rates of secretion of yolk polypeptide into the haemolymph being an accurate reflection of the rates of yolk polypeptide synthesis *in vivo*. We therefore investigated the incorporation of $[^{35}\text{S}]$methionine into protein in male fat bodies. Proteins co-migrating with the yolk polypeptides are abundant but, as in females, we could see no obvious signs of yolk polypeptides. Thus there appears to be no accumulation of proteins in the fat body. To confirm this, we precipitated fat-body-synthesized yolk polypeptides with anti-YP antiserum and found only the very low levels of yolk polypeptides normally present in female fat body. These results show that yolk-polypeptides are not accumulated in the fat body, however the results of $[^{35}\text{S}]$methionine incorporation in yolk polypeptides may still not precisely reflect yolk-protein synthesis in the fat body. There could be changes in the methionine pool available for translation during the course of the experiment. For example, as the hormone treatment induces the synthesis of a new subset of proteins there will be different amino acid
Expression of yolk polypeptide genes in *Drosophila*

Fig. 3. YP-transcript accumulation in hormone-treated females. A) Groups of females were fed with $10^{-3} \text{M}$-20-hydroxyecdysone for 4h. Subsequently RNA was isolated from the whole flies and YP-transcripts measured by dot-hybridization. B) Groups of females were fed continuously on $10^{-3} \text{M}$-20-hydroxyecdysone for 24 h. The graphs show the area $\times$ density of the dot plotted against time after hormone treatment ceased. The points show the mean from three to four separate experiments using flies from two different populations $\pm$ standard deviation.
requirements in the fat body. This may affect the uptake of the additionally injected, labelled methionine and cause artefacts in our results. Thus it is still possible that the apparent inhibition of translation of YP-transcripts could be a non-specific effect.

_Yolk-polypeptide-transcript accumulation in hormone-treated female flies_

Since male YP-genes clearly become actively transcribed in the presence of high doses of 20-hydroxyecdysone we asked whether females also responded in a similar way. The results of feeding 20-hydroxyecdysone to females for 2 h, then measuring YP-transcript accumulation as described earlier for males is shown in Fig. 3A. There does appear to be a slight increase in transcript accumulation, though it is difficult to judge how significant this is against the variability of YP mRNA levels in adult females. Continuous feeding of 20-hydroxyecdysone also shows an increase in transcript levels soon after hormone treatment commences (Fig. 3B).

_Which tissues are induced to transcribe the YP-genes by 20-hydroxyecdysone?_

The above results clearly show that males can transcribe the YP-genes in response to 20-hydroxyecdysone and that perhaps transcription increases in females. However, this requires unphysiological doses of hormone to be injected into the flies, and although it may well be rapidly metabolized and excreted, the high doses do seem to be needed to activate the genes. We therefore asked whether YP-transcripts appear in just the fat body of males, or whether they also appeared in the testes, gut and Malpighian tubules (i.e. non-specifically in all tissues). We have previously shown that in females YP-transcripts are present in body walls and ovaries, but not in the gut and Malpighian tubules (Bownes et al. 1983).

Females and males were injected with 20-hydroxyecdysone and 12 h later RNA was extracted from the carcass, i.e. the head, thorax and abdominal body wall (this contains fat body cells, epidermal cells and oenocytes); the gut and Malpighian tubules, and the gonads. YP-transcript levels were established using the dot blot method. The results (one set of which is shown in Fig. 4) indicate that only the male and female carcass, and the female gonad preparations of RNA contained YP-transcripts. Other tissues in males and females did not respond to the presence of 20-hydroxyecdysone by transcribing the YP-genes. Thus the hormonal induction in males shows the same tissue specificity for YP-gene transcription as is normally observed in untreated females, and high doses of 20-hydroxyecdysone do not induce YP-gene expression in the gut and Malpighian tubules of females. It was also observed that treatment of females with 20-hydroxyecdysone stimulated YP-transcript accumulation in the body wall preparations, but not in the ovaries. This was reproducible in two repeats of the experiments and suggested that in females the YP-genes were responsive to 20-hydroxyecdysone only in the body walls, thus the response is similar in males and females.
Fig. 4. Tissue-specificity of hormone induction. Dot blot of RNA from various tissues hybridized against a YP1, YP2 YP3 probe. (a) untreated whole ♀ (3.7); (b) untreated ♀ carcass (7.9); (c) untreated ♀ ovary (3.4); (d) 20-hydroxyecdysone-treated ♀ ovary (3.2); (e) 20-hydroxyecdysone-treated ♀ carcass (18.1); (f) untreated ♀ gut and Malpighian tubules (0.07); (g) 20-hydroxyecdysone-treated ♀ gut and Malpighian tubules (0.16); (h) 20-hydroxyecdysone-treated ♂ carcass (2.75); (i) 20-hydroxyecdysone-treated ♂ testis and accessory glands (0.00); (j) 20-hydroxyecdysone-treated ♂ gut and Malpighian tubules (0.00); (k) untreated whole ♂ (0.05). Number in brackets is area × density of the dot.
Yolk-polypeptide transcript accumulation in hormone-treated female tissues

It seemed possible that since the response to 20-hydroxyecdysone was limited to the body walls of females that ovarian levels of transcripts had obscured the response to hormone in the female time course shown above. We therefore repeated these experiments feeding females with 20-hydroxyecdysone for 2 h, but separated the ovary and carcass before measuring YP-transcript levels after various times. The results in Fig. 5, show that there is a dramatic increase in YP-transcript accumulation compared to the controls, and that indeed the response is tissue limited.

![Graph showing YP-transcript accumulation in ovaries and body walls of hormone-treated females.](image-url)

Fig. 5. YP-transcript accumulation in ovaries and body walls of hormone-treated females. Flies from the same population were fed either on 20-hydroxyecdysone or sugar for 2 h then transferred to normal medium. At various times the ovaries and body walls were isolated, RNA separated and YP-transcript levels measured. The graph shows the area x density of dot plotted against time after ceasing feeding with 20-hydroxyecdysone. ▲—▲ control body wall; △—△ 20-hydroxyecdysone-treated body wall; ●—● control ovary; ○—○ 20-hydroxyecdysone-treated ovary.
Expression of yolk polypeptide genes in Drosophila

The increased transcript levels are observed as soon as the feeding period ends (i.e. 2 h), thus there is a rapid response to the hormone. We cannot determine from these experiments whether this is due to increased transcription or preventing turnover of mRNAs already present, but since there is a rapid two-fold increase in transcript levels, new transcription seems likely to be involved. YP-transcript levels remained higher than controls for 24 h. The quantity of YP-transcripts present did not fall by a half during the experiment, thus the half-life of the YP-mRNA or the rate of transcription may exceed that in males. However, due to the background of high YP-transcript levels, it is unclear whether the induced transcripts will have the same stability as those present normally. Experiments labelling YP-RNA in vivo directly will be necessary to calculate the half-life of YP-mRNA in females. These experiments are difficult due to the problems of radioactively labelling a specific mRNA with tritium to a sufficient degree to detect it reliably. It is not clear why levels of YP-transcripts fall during this experiment in the control population of flies, but it may be associated with the period spent feeding on sugar rather than on complete medium.

The YP-genes, then, respond to 20-hydroxyecdysone in one tissue, but not in another tissue where they are normally expressed in adult females. One of several possibilities is that the ovarian follicle cells lack the appropriate hormone receptors, but that ecdysone receptors are present in one or more of the cell types found in the body wall preparations. It does suggest that the regulation of expression of the YP-genes may differ in these two tissues. This finding is in agreement with Jowett & Postlethwait (1980) who found that when isolated abdomens were treated with 20-hydroxyecdysone, only the body walls resumed YP-synthesis.

Yolk-polypeptide synthesis in hormone-treated females

We measured yolk polypeptide synthesis by labelling with $[^{35}\text{S}]$methionine in vivo at various points after hormone treatments. The results shown in Fig. 6 show only a slight increase in the hormone-treated flies in the ratio of newly synthesized yolk polypeptides compared to total haemolymph polypeptides at 12 and 24 h, but at 12 h there is a distinct accumulation of yolk-polypeptides in the fat body cells (YP1 and YP2, can be clearly seen accumulating above a protein which comigrates with YP3 in Fig. 6), thus indicating that the new transcripts are translated into proteins, and that they mostly remain in the fat body cells rather than being secreted into the haemolymph.

This shows that in females, rates of YP-gene transcription and translation are not normally maximal. The increased yolk polypeptide synthesis in the females treated with ecdysone did not result simply from increased rates of protein synthesis. Table 2 shows the incorporation of $[^{35}\text{S}]$methionine into TCA-precipitable counts, and it can be seen that although the incorporation is variable as might be expected when haemolymph is collected by capillary action, there is no trend towards increased translation in the ecdysone-treated flies. There are
Expression of yolk polypeptide genes in Drosophila

Table 2. Protein synthesis in hormone-treated females

<table>
<thead>
<tr>
<th>Experiment</th>
<th>T.C.A. precipitated counts/female</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>haemolymph</td>
<td>body wall</td>
<td>ovary</td>
</tr>
<tr>
<td>0 h + 20HE</td>
<td>4450</td>
<td>236810</td>
<td>216220</td>
</tr>
<tr>
<td>control</td>
<td>6270</td>
<td>235570</td>
<td>363770</td>
</tr>
<tr>
<td>12 h + 20HE</td>
<td>9700</td>
<td>208450</td>
<td>180240</td>
</tr>
<tr>
<td>control</td>
<td>5180</td>
<td>194750</td>
<td>354580</td>
</tr>
<tr>
<td>24 h + 20HE</td>
<td>2690</td>
<td>178200</td>
<td>97870</td>
</tr>
<tr>
<td>control</td>
<td>3000</td>
<td>477910</td>
<td>590050</td>
</tr>
</tbody>
</table>

20HE, 20-hydroxyecdysone.

one or two other peptides, indicated in Fig. 6, that also seem to be present in greater quantities in the hormone-treated flies, yet others do not change. The increased YP-synthesis therefore appears to be part of a specific response to the hormone.

GENERAL DISCUSSION

The results presented in this paper suggest that, when the YP-genes are expressed in males following treatment with 20-hydroxyecdysone, the actual level of gene expression, as determined by the synthesis of yolk polypeptides may be modulated by post-transcriptional as well as transcriptional controls. One problem we encounter with this type of experiment is the large variability in YP-transcript accumulation and rates of yolk polypeptide synthesis between groups of flies. This is true of untreated females and hormone-induced males and means that quantitation is difficult and we are only able to discover the general features involved in regulating YP-gene expression. It will prove interesting in the future to look at the expression of the three genes individually to determine if they are co-ordinately regulated.

The general pattern which emerges is that after hormone treatment yolk polypeptide synthesis begins in males as transcripts appear. At first they are one or two other peptides, indicated in Fig. 6, that also seem to be present in greater quantities in the hormone-treated flies, yet others do not change. The increased YP-synthesis therefore appears to be part of a specific response to the hormone.

Fig. 6. Yolk polypeptide synthesis in hormone-treated females. Batches of five females were injected with 10^{-2}mol-hydroxyecdysone and with [35S]-methionine after various times. Tracks a–f shows the autoradiograph of the haemolymphs and g–l the body walls, after separation by SDS polyacrylamide gel electrophoresis. YPs = yolk polypeptides; + = treated with 20-hydroxyecdysone (20HE); – = control. Numbers represent times in hours after hormone injection. Numbers in brackets represent the fraction of total protein synthesis which is represented by proteins comigrating with the YPs. 1. other polypeptides which are present in greater amounts in presence of 20HE. 2. unchanged polypeptides.
translated into yolk polypeptides very efficiently, compared to translation of the transcripts present in females. Shirk et al. (1983) measured transcript accumulation and yolk polypeptide synthesis at just one time point (8h) after 20-hydroxyecdysone treatment. Their data also show that at this time the transcripts present are translated efficiently into yolk polypeptides. Following this we find that translation into protein often becomes inefficient in males in relation to the transcripts accumulated, the time at which this occurs is very variable between groups of flies. Furthermore the total YP-transcripts present begins to drop by 24h, thus the mRNA must be degraded fairly rapidly. From our experiments, measuring transcript levels after hormonal stimulation of females, we cannot deduce the half-life of the YP-mRNA since the natural state is that transcripts are present. We do not know, therefore, whether there are post-transcriptional controls in females, but we have detected them in dxy^D intersexual flies (Bownes et al. 1983). At present we are trying to in vivo label YP-mRNA so we can measure directly when the genes are being transcribed in females. A further point which we observe is that male YP-transcripts translate very poorly in a cell-free translation system compared to female YP-transcripts. Shirk et al. (1983) translated YP-RNA isolated from males 8 h after hormone induction and also observed a poor translation efficiency compared to female YP-RNA. The reason for this is unclear and it does not seem to help to explain the post-transcriptional controls we observe in vivo in males, because this poor translation in vitro occurs at all points after hormone treatment, not just at the times when we have evidence for translational controls. Although the possible translational controls are difficult to interpret conclusively due to the variability between populations of flies, and possible artefacts resulting from changes in amino acid pools, we can be sure that the YP-transcripts induced by 20-hydroxyecdysone in males are relatively unstable.

One of our most interesting findings is that even though we are possibly using unphysiologically high doses of 20-hydroxyecdysone to induce yolk polypeptide synthesis the response is tissue specific. The genes are not transcribed in gut, Malpighian tubules, testes or accessory glands, but only in the carcass which contains the fat body in males. In females, similarly, 20-hydroxyecdysone treatment increases transcript accumulation only in the body walls. It does not even affect transcription in the ovary where the YP-genes are normally expressed (Bownes & Hames, 1978; Brennen et al. 1982; Bownes, 1982). Thus it seems possible that some cell-types in body walls carry the appropriate ecdysteroid receptors for hormone-induction of YP-gene expression but that other cells in the adult do not. Whether induction uses normal ecdysteroid receptors or not is unclear, the fact that males and females have similar concentrations of 20-hydroxyecdysone (Handler, 1982, Smith & Bownes unpublished) suggests that normally the amount of ecdysone/receptor complex formed in male fat bodies is insufficient to induce transcription of the YP-genes. We cannot be sure from our results that 20 hydroxyecdysone does not generally stimulate transcription
and as a result of this we see more YP-transcripts, but there are several reasons why this is unlikely.

Firstly, when we look at the profiles of protein synthesis in the presence and absence of 20-hydroxyecdysone, only a few bands are affected, suggesting a differential response to the hormone although, of course, some of this specificity could reside at a post-transcriptional level. Secondly, we do not see increased YP-transcripts in ovarian cells, only in the body walls when females are treated and thirdly, we do not induce transcription of the YP-genes in all cells. It is still possible though that transcription is generally increased in just those cell types with appropriate receptors, although the patterns of protein synthesis argue against this. Ideally, we need to measure transcription of a gene that is expressed in all cells at all developmental stages and is not hormonally regulated as a control. Experiments to select such a gene are in progress.

Why is it then that male fat body cells do not normally express these genes, but female cells do. Clearly both sexes have the correct machinery to respond to 20-hydroxyecdysone. Male and female adults have almost identical levels of total ecdysteroids (Hodgetts, Sage & O'Connor, 1977; Handler, 1982; Bownes & Dubendorfer in prep.), suggesting that the control of expression of these genes resides in the fat body cells themselves, probably some process ultimately under the control of the sex genes dsx, tra, tra-3, and ix (Baker & Ridge, 1980; Postlethwait et al. 1980; Bownes & Nothiger, 1981; Bownes et al. 1983). Nonetheless with high doses of hormone these sex differences can be transiently overcome. One possible model for the regulation of expression of the YP-genes is that the tissue specificity of the response lies in the availability of the appropriate hormone receptors and that the sex specificity is controlled by the sex genes, perhaps by altering the conformation of the YP-genes in the chromatin or nuclear matrix, and hence their availability to low levels of hormone/receptor complex. The 20-hydroxyecdysone could act upon other genes in the fat body which indirectly activate the YP-genes. Alternatively the added 20-hydroxyecdysone may act on another tissue in the adult which then stimulates the fat body to transcribe the YP-genes.

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REFERENCES


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