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Tucker, Mark Allen, Ph.D.

Rice University, 1988
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Isolation of Oogenesis-Specific Genes Transcribed in the Germ-Line of Calliphora erythrocephala and Drosophila melanogaster

by

Mark A. Tucker

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

DOCTOR OF PHILOSOPHY

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Houston, Texas
December, 1987
Isolation of Oogenesis-Specific Genes Transcribed in the Germ-Line of Calliphora erythrocephala and Drosophila melanogaster

Mark A. Tucker

ABSTRACT

Clones containing DNA uniquely transcribed during oogenesis were isolated from genomic DNA phage libraries for the two related Dipteran flies C. erythrocephala and D. melanogaster. Poly(A)^+ RNA from early or mid-stage ovarian follicles of C. erythrocephala was used to generate radiolabelled oogenesis-specific cDNA probes for screening the phage libraries. A cDNA probe made from mid-stage embryo poly(A)^+ RNA was used as the differential screening probe. Thus plaques hybridizing to the two oogenesis-specific probes but not the mid-stage embryo probe were selected as potentially containing oogenesis-specific genes. Two further rounds of screening were used to eliminate false positives and, after plaque purification, restriction digests of the remaining clones were screened by Southern blot hybridization to identify DNA fragments transcribed in an oogenesis-specific manner. To date, 22 oogenesis-specific clones have been isolated from C. erythrocephala and two from D. melanogaster by this method.

In situ hybridization to sections of ovarian follicles has been used to determine the cell types within the follicles in which the various genes are expressed. Radiolabelled RNA probes for four of the C. erythrocephala oogenesis-specific clones and the two D. melanogaster clones have been hybridized to ovarian follicles. Two of the C. erythrocephala clones and both of the D. melanogaster clones have thus been shown to be transcribed in the germ-line cells of the follicles as opposed to the somatic follicle cells.

Further studies have been concentrated on the two germ-line transcribed, oogenesis-specific clones isolated from the D. melanogaster clone library. In situ hybridization of the two D. melanogaster clones to the salivary gland polytene chromosomes has established that one clone is derived from chromosome region 31B/D (clone DA) and the second clone from region 98E/F (clone DM). Region 31B/D is rich in female sterile mutations and detailed genetic mapping of the DA clone and of these mutations was performed (using deficiency chromosomes) to determine which mutations might represent the DA gene. cDNA clones have been isolated for the transcribed region of clone DA and have been used to further define the transcription unit from this region of the D. melanogaster genome.
Acknowledgements

I would like to thank Esther Belikoff for her help in performing the C. erythrocephala library screening and D. melanogaster library screening. I would like to thank Tamsen Valoir for her help in performing and analyzing the whole tissue in situ and for the artwork she helped prepare for this thesis. I am also grateful to Laurie Camp for her work performing restriction enzyme site mapping for DA and DM. I am especially grateful to Kate Beckingham for the time, help and advice she has given me throughout my time at Rice and in writing this thesis.
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CHAPTER ONE

INTRODUCTION

The development of a single cell into a differentiated, multicellular organism is one of the most fundamental and complex problems facing developmental biologists today. The details of blastoderm formation, cell differentiation and gastrulation have been described by classical biologists many years ago but little progress has been made in understanding the mechanisms controlling these processes. Recently in the organism *Drosophila melanogaster* a combination of genetic studies and recombinant DNA technology has led to breakthrough achievements in this area. Genes involved in regulating early embryogenesis have been identified and isolated offering the potential for real insights into the underlying processes in development.

**OOGENESIS AND EMBRYOGENESIS IN Drosophila melanogaster**

The cells of the *D. melanogaster* ovary are derived from separate cell lineages: the somatic cells are derived from the mesoderm, and the germ cells are derived from the pole cells (see below). The somatic tissues of the ovary include the tracheoles, ovariole sheath and follicle cells while the germ line components are the oocyte proper and nurse cells. The follicle cells surround each developing follicle and are mainly responsible for secreting the egg coverings including the vitelline membrane and chorion. The roles of the germ-line components of the follicle are less well defined. Their products include ubiquitous "housekeeping" components such as RNA polymerases and ribosomes and other products required later in embryogenesis. Both the somatic and germ-line components of the follicle are responsible for the correct polarity of the egg (1). Each ovary consists of a cluster of ovarioles with a germarium at the anterior end of each ovariole (Figure 1). Stem cells in each germarium undergo mitotic divisions each yielding one stem cell and one presumptive oocyte. The presumptive oocyte then undergoes four rounds of incomplete mitotic division to give the oocyte and fifteen nurse cells connected by cytoplasmic bridges. During these mitotic divisions the somatically derived follicle cells envelope the oocyte forming the ovarian follicle.
Figure 1. A dorsal view of the internal reproductive system of an adult *D. melanogaster* female. Two ovarioles have been exposed from the right ovary with follicles at different stages of development shown as ovals of varying size. The uterus is shown expanded as it would be when it contains a mature egg (modified from King (2)).
The follicle then begins its migration down the ovariole into the vitellarium. During stages 1 to 7 the nurse cells and oocyte grow at equal rates. Most if not all of the transcription of RNA and translation of protein of the germ-line tissue occurs in the nurse cells. Endomitotic replication of nurse cell nuclear DNA begins during stage 1. Vitellogenesis, or the uptake of yolk, takes place between stages 8 and 12. During these stages, the oocyte grows four to five times faster than the nurse cells. One reason for the faster growth of the oocyte is that during this period the nurse cell cytoplasm migrates by a little understood process into the oocyte. The follicle cells become very active during stage 9 producing and secreting the egg coverings. By stage 10, the endomitotic replication of the nurse cell nuclear DNA is complete and the nurse cell nuclei achieve a DNA content of approximately 512 - 1024 X C. The nurse cells begin degenerating during the later stages of vitellogenesis. The last two stages of oogenesis are marked by ooplasmic changes and little or no growth. The nurse cells and follicle cells have degenerated and are absent. The external structures (chorion, micropyle and dorsal appendages) are completed and the mature oocyte is ready for laying (2).

Fertilization of the egg by a single sperm occurs internally in the uterus, however, meiotic activation of the egg can occur without fertilization. After fusion of the pronuclei, the first eight divisions occur synchronously at approximately nine minute intervals without cytokinesis (Figure 2). Nuclei surrounded by cytoplasmic islands gradually segregate towards the periphery of the embryo and, by the ninth division, have reached the periphery of the embryo forming the syncytial blastoderm. Nuclei left in the interior region of the embryo cease division and become highly polyploid. These are often referred to as vitellophages. Six to seven nuclei arrive at the posterior pole of the embryo and cellularize to form the pole cells, or germ line precursor cells. These are the first cells committed to their final fate in the embryo. It has been demonstrated that the fate of these cells is determined by the inclusion of the polar granules (RNA granules in the posterior egg cytoplasm) into their cytoplasm. The peripheral nuclei undergo four more synchronous divisions with gradually lengthening interphases. Non-ribosomal RNA synthesis begins before the thirteenth division. Nuclear division ceases after the thirteenth cleavage until later in gastrulation although the DNA content of each nuclei is 4C. Ribosomal RNA (rRNA) synthesis begins along with the appearance of the
Figure 2. Embryonic development of *Drosophila melanogaster*. T1-3, thoracic segments; A1-8, abdominal segments. (a) Zygote, (b) cleavage, (c) cellular blastoderm, (d) gastrulation, (e) maximum germ band extension, (f) after dorsal closure (from Slack (3)).
nucleoli, chromocenters and heterochromatic regions in the DNA. Cellularization begins with the formation of the plasma membrane moving interiorly from the egg periphery between nuclei. Blastoderm formation is complete ventrally first, although midbody-like regions continue to connect the cells with the internal yolky region of the embryo.

Gastrulation begins with a series of almost simultaneous cell movements in various regions of the blastoderm. Ventral furrow formation begins with the inward migration of 1/6 of the ventral blastoderm cells to form the mesoderm. At the posterior tip of the embryo, the initiation of endoderm begins with the formation of the posterior midgut invagination. The pole cells are included in this inward migration and will later pass through the midgut wall into the gonads to form germ cells. Simultaneously with these invaginations, the anterior midgut invaginates ventrally in front of the mesoderm followed one hour later by the stomodeal invagination. Germ band extension begins when the ventral cell mass extends around the posterior of the egg and then anteriorly along the dorsal side of the embryo. Neuroblasts separate from the ectoderm thirty minutes after gastrulation begins and continue to form for the next two hours. After germ band extension primary organogenesis occurs followed by germ band shortening, head involution, and arrangement of organs into their final positions. Twenty two hours after fertilization (at 25°C) the larva hatches from the egg case (4).

Determination, at least in a very global sense, has been shown to occur at or just before blastoderm formation. Nuclei isolated during the preblastoderm cleavage stages from the anterior portion of the egg, when transplanted to the posterior region of a donor egg, will form posterior structures (5,6). Similarly, cleavage stage nuclei and syncytial blastoderm nuclei were shown to support development through embryogenesis when transplanted into unfertilized eggs indicating the totipotency of these nuclei (7). However, as demonstrated by Simcox and Sang (8), cells at the blastoderm stage are fixed in terms of their segmental determination. Blastoderm cells from the first thoracic segment primordia were transplanted into a region corresponding to the third thoracic segment of a donor blastoderm stage embryo. Donor cells integrated into the third thoracic leg but formed structures (sex combs) found only on first thoracic legs. Genetic mosaics constructed using the formation of gyandromorphs (9) and X-ray induced somatic crossing over (10) also indicate that blastoderm
cells are fixed in terms of compartmental (possibly segmental) boundaries. Clones produced by blastoderm cells were found only in specific compartments of imaginal discs with little or no mixing of cells across these boundaries. The results of these three types of experiments indicate that at the time of removal from the donor (or at the time of clone formation) these cells were committed to a fixed compartmental (segmental) identity.

**MATERNAL CONTRIBUTIONS TO EMBRYOGENESIS IN *D. melanogaster***

Most of the information contributing to our understanding of early determinative events comes from the study of mutations that disrupt normal ovarian and/or embryonic development. While the actual gene and gene products that these mutations affect are unknown, the period when the developing fly dies or show defects indicates the time frame when the activity of the gene is required. Temperature sensitive alleles of mutations, if available, also help to define the active period of the gene products. These alleles show phenotypically normal development at the permissive temperature but mutant phenotypes are observed at restrictive temperatures. By doing temperature shift experiments throughout the life cycle of the fly, insight can be gained into the active period of the gene product. Mutations affecting ovarian development will produce phenotypes such as reduction in fecundity or female sterility. Mutations affecting the pattern formation of the embryo will be characterized by embryonic lethality as a result of disruption of the normal body axes or abnormalities in segmental differentiation. Female sterile mutants can be divided into two broad classes: those that do not lay eggs and those that produce non-viable eggs. Studies of these genes underline the role of maternal information in controlling early embryonic events. Large scale mutagenesis screens have been carried out to identify female sterile mutations, that is mutations have no effect on the viability of either sex but which render females sterile (11,12,13,14). These mutants define genes whose expression is required during oogenesis for formation of a normal oocyte or for the subsequent development of the embryo. Table 1 lists many of the mutations known to affect oogenesis.

**MUTANTS THAT DO NOT LAY EGGS.**
Table 1. Several mutations known to affect oogenesis in *D. melanogaster* (from King (2)).

<table>
<thead>
<tr>
<th>Genetic locus</th>
<th>Mutation</th>
<th>Alleles studied</th>
<th>Comments$^b$</th>
</tr>
</thead>
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<tr>
<td>X- 0.3</td>
<td>stubarista</td>
<td>sta</td>
<td>9,12</td>
</tr>
<tr>
<td>X- 0.3</td>
<td>deep orange</td>
<td>- dor</td>
<td>8,12</td>
</tr>
<tr>
<td>X- 4.6</td>
<td>diminutive</td>
<td>dm</td>
<td>5,15,16</td>
</tr>
<tr>
<td>X- 21.0</td>
<td>singed</td>
<td>sn$^{s}$, sn$^{s}$, sn$^{s6a}$</td>
<td>4,13,15</td>
</tr>
<tr>
<td>X- 23.1</td>
<td>ocelliless</td>
<td>oc</td>
<td>7,15</td>
</tr>
<tr>
<td>X- 27.7</td>
<td>almondex</td>
<td>amx</td>
<td>8</td>
</tr>
<tr>
<td>X- 27.7</td>
<td>lozenge</td>
<td>Lz$^{s}$, Lz$^{s1}$</td>
<td>1,7,13</td>
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<td>X- 32.8</td>
<td>raspberry</td>
<td>ras$^{t}$</td>
<td>4,13</td>
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<tr>
<td>X- 44.5</td>
<td>tiny</td>
<td>ty$^{o}$</td>
<td>5,15,16</td>
</tr>
<tr>
<td>X- 54.5</td>
<td>rudimentary</td>
<td>r</td>
<td>8,13</td>
</tr>
<tr>
<td>X- 59.5</td>
<td>fused</td>
<td>fu$^{o}$, fu$^{o}$, fu$^{s7o}$, fu$^{s8o}$, fu$^{p2-t}$, fu$^{p2-2}$, fu$^{p2-3}$</td>
<td>2,8,16</td>
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<td>2- 0</td>
<td>lethal (2) giant larva</td>
<td>l(2)gl, l(2)gl$^{1}$</td>
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<td>female sterile (2) B</td>
<td>fs(2)B* = fes</td>
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<td>2- 55.4</td>
<td>aperous</td>
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<td>fs(2)E$_{1}^{*} = fs$ 2.1</td>
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<td>2- 63.3</td>
<td>spermatheca</td>
<td>spt</td>
<td>7</td>
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<tr>
<td>2- 83</td>
<td>narrow</td>
<td>nuo$^{s}$, nuo$^{d}$</td>
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<td>adp$^{s}$</td>
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<td>2-100</td>
<td>meiotic S332a</td>
<td>mei S332a</td>
<td>3</td>
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<tr>
<td>2-104.7</td>
<td>minus</td>
<td>mi</td>
<td>15</td>
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<td>2-106.7</td>
<td>morula</td>
<td>mr, mr$^{t}$</td>
<td>4</td>
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<td>Meiotic S322b</td>
<td>Mei S322b</td>
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<td>transformer</td>
<td>tra$^{s}$</td>
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<td>round</td>
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<td>double sex</td>
<td>dsx</td>
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<td>3- 54.8</td>
<td>suppressor of Hairywing</td>
<td>su(Hw)$^{*}$</td>
<td>4,15</td>
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<td>crossover suppressor (3) of Gouen</td>
<td>c(3)C</td>
<td>3,16</td>
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<td>3- 58.2</td>
<td>stublaid</td>
<td>sbd$^{t}$</td>
<td>3</td>
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<td>3-100.7</td>
<td>claret-nondisjunctional</td>
<td>can$^{d}$</td>
<td>3</td>
</tr>
<tr>
<td>?</td>
<td>meiotic S282</td>
<td>mei S282</td>
<td>3</td>
</tr>
<tr>
<td>2 + 3</td>
<td>meiotic S51</td>
<td>mei S51</td>
<td>3</td>
</tr>
</tbody>
</table>

$^b$ Key to numbers: (1) "Sterile" ovary cured by transplantation; (2) supernumerary cystocyte divisions; (3) meiotic mutants; (4) nurse cell nuclei abnormal; (5) follicle cells abnormal; (6) endocrinology abnormal; (7) duct system abnormal; (8) female fertile, if outcrossed (progeny are daughters); (9) eggs stored; (10) sexual differentiation of gonad disturbed; (11) ovarian abnormality generates abnormalities in other organs; (12) cytological locations determined by Rayle and Hoar (1969); (13) female fertile alleles known; (14) males sterile also; (15) vitellogenesis retarded; (16) ovarian cytology of hemizygote studied.
Mutants that do not lay eggs (or produce so few eggs that they are classified as not laying eggs) are usually blocked during ovarian development and can be subdivided into five subgroups: mutants affecting i) sexual differentiation of the gonads, ii) the nurse cells, iii) the follicle cells iv) ovarian tumor mutants or v) mutants affecting hormone production/activity. Mutants affecting the follicle cells are beyond the scope of this project and will not be discussed here. Few mutations affecting hormone production/activity have been isolated and very little is known about this class of mutations. Therefore I will not discuss these mutants here.

**Mutants Affecting Sexual Differentiation of the Gonads.**

Most of the genes identified which affect sexual differentiation of the gonads are zygotically acting. That is they are genes which are active during embryogenesis rather than oogenesis. *Sex lethal (Sxl), tra, tra-2, double sex (dsx) and intersex (ix)* cause either the transformation of females into pseudomales or produce intersex flies, all appear to be active after blastoderm formation and all affect the somatic sex determination of the fly. These genes respond through *daughtercless* (see below) to the X chromosome to autosome ratio (15).

**Mutations Affecting the Nurse Cells.**

The mutants so far isolated affecting the nurse cells in *D. melanogaster* mostly disrupt some aspect of DNA replication and/or distribution (polyploidy or polyteny of the chromosomes). Oogenesis in these mutants appears normal in the early stages followed by degeneration of the egg chamber later in oogenesis. In *morula, fs(2)E1, singed, raspberry and rotund*, vitellogenesis is also delayed. *suppressor of Hairy wing* appears to affect rRNA synthesis and half-life. rRNAs are not retained in the nucleolus for a normal time and much of it is degraded prematurely. Although little is known about these mutations, they are found throughout the *D. melanogaster* genome and have phenotypic affects on other organs as diverse as wings, bristles and eyes (2).

**Ovarian Tumor Mutants.**

The ovarian tumor mutant *fused (fu)*, located on the X chromosome, was discovered and received its name from its phenotype of abnormal wings in adult flies. Females homozygous for *fused* have reduced fecundity due to a reduction
in the number of ovarioles per ovary, the fusion of many adjacent developing ovarian follicles and the production of tumorous egg chambers (2). However, some normal appearing eggs are produced in young ful/ful females. Eggs from homozygous ful/ful females fertilized with fused or Y bearing sperm die during embryogenesis at the beginning of gastrulation showing deletion/duplication of partial segments without reduction in total segment number (16). These eggs develop normally if fertilized with wild type X bearing sperm, however, indicating that some substance is absent in eggs from ful/ful females that can be supplied after fertilization to produce phenotypically wild type embryos. Heterozygous fu female eggs develop normally regardless of sperm genotype indicating a maternal supply of fu is adequate to support embryogenesis. Experiments with fu homozygotes and hemizygotes show that hemizygotes produce tumors at a higher rate than homozygotes. Therefore, the fused phenotype is not the result of complete loss of gene function. If that were the case, the homo- and hemizygote flies would produce tumors at the same rate.

Adult flies homozygous for the female sterile (fes) ovarian tumor mutation, unlike fu, are normal in appearance. Although there is no effect on male fertility, females are sterile. The fes gene is not required for embryogenesis and fes/fes female embryos have normal viability. Cross sections through whole fes/fes female ovaries show several sausage shaped cell masses surrounded by ill defined follicular epithelium (2). The follicles are filled with thousands of undifferentiated tumor cells. fes tumor cells appear to be modified nurse cells in that many of them are connected by ring canals similar to those seen between nurse cells during normal follicle development.

Flies with the double mutation fes/fes; ful/ful develop fused wings and female sterile type tumors (the tumors appearing at eclosion as for fes instead of developing as oogenesis proceeds as in ful follicles) indicating that fes acts earlier than fu in the follicle. The ovaries of ful/ful; fes/+ were examined and found to have a higher rate of tumor incidence than ful/ful; +/+ females. Because fes type tumors were found in the follicle of the double mutant and fes/+ flies caused an increase in the number of fu tumors, these experiments led Smith and King to speculate that in the ovary, fes acts earlier than fu and that the fes and fu products interact (2).

MUTANTS THAT PRODUCE AND DEPOSIT NON-VIABLE EGGS.
Among female sterile mutants that produce and deposit non-viable eggs, two classes can be distinguished: i) mutants with abnormal egg structure and ii) mutants affecting pattern formation during embryogenesis. This latter class are the so-called maternal effect mutations. Maternal effect mutants affect embryogenesis independently of the genetic make up of the embryo. These could be further subdivided into a) mutations affecting the "housekeeping" functions of the embryo and b) mutants affecting the pattern formation of the embryo. The most widely studied of these mutations and those of greatest interest in our laboratory are those that affect pattern formation of the embryo and therefore I will concentrate my discussion around these mutations. Maternal effect mutations have been shown to be involved in the formation of the anterior/posterior body axis, the dorsal/ventral body axis and many other aspects of embryogenesis. In general, it appears that maternal information specifies the overall sequence and polarity of embryonic pattern formation, which leads to regional responses of the zygotic genome.

**Mutations Affecting Egg Structure.**

Two maternal effect mutations that affect the structure of the egg also affect embryogenesis. *dicephalic (dic)* is a semi dominant mutation that affects the anterior/posterior axis of both the egg and the embryo (17,18,19). During normal oogenesis, the 15 nurse cells are found at the anterior end of the developing follicle. At stage 9, a cluster of anterior follicle cells begin migrating through the nurse cells to occupy a position at the nurse cell/oocyte border. These border cells are responsible for the formation of the micropyle through which the egg will be fertilized during oviposition. Instead of the normal configuration of the 15 nurse cells at the anterior end of the egg, in *dic* mutants the nurse cells split into two groups, one at the anterior end of the egg and one at the posterior end with the oocyte occupying a central position. Two sets of border cells develop forming micropylar structures at both poles of the egg. In the embryo, *dic* eggs produce a mirror image duplication of the anterior half of the embryo.

*fs(1)K10* is a gene involved in dorsal/ventral axis formation. Some eggs laid by females homozygous for this mutation show dorsal type structures around the circumference of the egg (20). The oocyte nucleus is found in an abnormal position, more ventral and lateral than the wild type position.
Embryos developing from \textit{K10} females fail to form ventral structures during gastrulation. They develop a cuticular pattern characteristic of dorsal regions of the embryo and lack structures derived from both the mesoderm and endoderm.

In both these mutations, parallel changes are produced in the pattern of the egg and the embryo. This suggests that both the anterior/posterior and dorsal/ventral polarity of the embryo is dependent on the same polarities being established during oogenesis. The results also suggest that \textit{dic} and \textit{K10} may be among the earliest acting genes involved in establishing embryonic pattern formation.

\textit{claret-non-disjunctional} is an allele of a mutation originally isolated as affecting \textit{D. melanogaster} eye color. This mutation affects the formation of the meiotic spindle during the first meiotic maturation division. These defects include more than one spindle forming, unipolar spindles and abnormally wide spindles. It is unknown what affect this mutation has in the second meiotic maturation division. This mutation also causes the formation of abnormal eggs including defects such as more than one dorsal appendage formed, having the anterior pole of the egg truncated and abnormally small eggs (21).

\textbf{Mutations Producing Morphologically Normal Eggs.}

\textbf{Mutations affecting "housekeeping" functions.} Very few mutations affecting the "housekeeping" functions of the embryo have been characterized. One example of this type of mutation is the maternal effect lethal mutation that affects mitosis recently described in \textit{D. melanogaster}. This mutation, named \textit{gnu}, is defective in nuclear division but not in DNA replication. Embryos from homozygous \textit{gnu} mothers form giant nuclei that are reduced in number relative to wild type embryos. Meiosis does not appear to be affected by this mutation as the normal complement of three polar bodies and one female pronucleus are observed. However, 25 minutes into embryogenesis mutant embryos are distinct from wild type; the nuclei are larger and more diffuse.

In eucaryotes, there are both cyclical nuclear and cytoplasmic mitotic activities associated with cell division. \textit{gnu} embryos do display centrosomes and they appear to be unaffected by the absence of nuclear division. While the mitotic spindle components appear to be unaffected by \textit{gnu}, the distribution of these components is abnormal. Thus, it appears that the nuclear division cycle of these mutants are disrupted but many aspects of the cytoplasmic cycle
remain unaffected indicating that DNA replication and cytoplasmic components of the mitotic division cycle are not coupled (22).

**Mutations affecting sexual differentiation.** *daughterless* is a mutation affecting sexual differentiation in *D. melanogaster*. Studies of a temperature sensitive *da* mutant indicate that both maternal and zygotic activity of the *da* gene are important (15). Homozygous *da* females produce no female offspring when grown at 25°C regardless of zygotic genotype. However, at semi-permissive temperatures, some *da* females do survive. The survival rate is increased if the mothers are mated with wild type males, but increasing the number of *da* alleles in the zygote does not appreciably affect survival indicating that the amount of *da* activity is not the limiting factor of rescue. The *da* females that survive are sexually normal but have morphological defects. Gynandromorph analysis of *da* function suggests that its morphological functions are cell autonomous and that *da* function is required in all diplo-X tissue. At 29°C, *da* is a zygotic lethal; nearly all homozygous *da* offspring die indicating a requirement for *da* function in all embryos. Homozygous *da* females, when shifted to 29°C become sterile (due to fragile eggs) indicating that at least one *da* function during oogenesis may be distinct from the embryonic function. However, in a more recent report, the *da* locus activity was found to be required in all somatic cells for the normal development of both sexes but was not required for growth and differentiation of the germ cells. The *da* gene does function in female germ cells, but only to provide a product required specifically for female development in the subsequent generation (23).

**Mutations affecting pattern formation during embryogenesis.** Most mutants identified affecting the anterior/posterior body axis of the embryo show defects at the posterior end of the embryo. This is probably a result of the fact that anterior structures are rudimentary at all stages of early development making identification of abnormal phenotypes in the anterior region extremely difficult. However, gross abnormalities can easily be identified. Females homozygous for *bicoid* (*bcd*) lack both head and thorax. The more severe phenotypes of *bcd* also show abdominal segmentation abnormalities and the formation of posterior structures (telson and reversed polarity abdominal segments) at the anterior pole of the embryo. Cytoplasmic transplant experiments indicate that *bcd* dependent activity is localized in the anterior pole of wild type embryos. This activity can induce anterior development in mutant
embryos at any position along the antero/posterior axis and suppress posterior development (24). *bicaudal* (*bic*) mutations cause mirror image duplication of the posterior pole of the embryo (25), the exact opposite of *dicephalic*. During gastrulation, posterior midgut invaginations form at both ends of the embryo. Although no affects are seen in the oocyte, temperature shift experiments indicate a temperature sensitive period during vitellogenesis (26).

Five mutants affecting the posterior pole of the embryo are called *torso*-like, named after the first mutant of this class discovered. The development of *torso*-like embryos appears normal up to blastoderm formation with the exception of a small group of cells just anterior to the pole cells that fail to cellularize (27). During gastrulation, no posterior midgut invagination forms although germ band extension proceeds normally. No structures derived from the posterior 20% of the embryo (except pole cells) are seen. There is incomplete formation of the seventh abdominal segment and no terminal structures. In some mutants, defects at the anterior end are also seen. The cephalopharyngeal apparatus is abnormal and head involution sometimes does not occur. The *torso*-like mutation *fs(1)N211* has been shown to alter the expression of the zygotically active pair rule gene *fushi tarazu* (see below) in the regions that will be absent from *torso*-like embryos as early as blastoderm formation (28). These results were interpreted as indicating that the blastoderm fate map was being altered in *torso*-like embryos.

*tudor*-like mutations have a double phenotype. The absence of polar granules precludes the formation of pole cells in all embryos. A second maternal effect phenotype is characterized by a failure of abdominal segments 1 through 7 to form (29,30). Abnormalities in the anterior region of the embryo are seen in some members of this class. *tudor* is a true maternal effect gene in that the presence of a wild type gene during oogenesis is sufficient to insure the formation of fertile offspring and *tudor* activity is not required at any other stage of the life cycle.

*tudor* and *torso*-like maternal effect mutations affect reciprocal areas of the embryo. *torso*-like mutations affect areas of the embryo between the pole cells and seventh abdominal segments while *tudor*-like mutations affect the pole cells and regions between abdominal segments A1 through A8. The events controlled by these loci are as yet ill defined and further analysis must await the cloning of these genes.
Ten loci have been identified that affect the dorsal/ventral polarity of the embryo (Table 2). These mutants (the dorsal group mutants) fail to form characteristic ventral structures and at gastrulation are a hollow tube of epidermis without internal structures (31). Seven of the ten mutants are at least partially rescuable by wild type cytoplasm or RNA. Cytoplasm from embryos carrying a mutation which is rescuable by wild-type cytoplasm will rescue at least one other mutant. However, the pattern of interactions shown by these experiments indicate that these mutants are not part of a simple biochemical pathway. Because rescue can occur with either RNA or cytoplasm from wild type embryos, at least some of the maternal information is stored as RNA. A protein store of maternal product is also suggested by the fact that rescue with cytoplasm is usually more complete than with RNA alone. Injection of cytoplasm has also led to the conclusion that it is the amount of morphogen, not its presence or absence that determines dorsal/ventral fate. Temperature sensitive mutant studies for six of the ten loci have allowed the grouping of mutants into two categories based on their active periods: gastrulation defective and spatzle are active during oogenesis while pelle, Toll, easter and dorsal are active during embryogenesis. Toll is unique among dorsalizing mutants in that different alleles can cause three phenotypes: i) dorsalization, ii) lateralization and iii) ventralization of cuticular structures of the embryo (32). Data from injection experiments also show that contrary to what is found for mutants of the other six rescuable dorsal group genes, the site of injection of wild type cytoplasm establishes the dorsal/ventral axis of the embryo of Toll mutants. These results indicate that in the absence of the Toll gene product, the embryo has no inherent dorsal/ventral polarity. Thus, Toll appears to be unique in that its gene product not only establishes pattern elements but also helps in establishing the polarity of a body axis.

**ZYGOTIC LOCI AFFECTING PATTERN FORMATION SHOWING A MATERNAL EFFECT.**

True maternal effect loci, those that are expressed during oogenesis and no other time of the life cycle of the fly, appear rare. However, several loci identified as zygotic lethals have proved to show a maternal effect. These loci are expressed during oogenesis but can be rescued, at least partially, by the
Table 2. The ten "dorsal" group mutations of *D. melanogaster* (from Nusslein-Volhard (29)).

<table>
<thead>
<tr>
<th>Weak alleles isolated</th>
<th>Probable time of action*</th>
<th>Dominant alleles</th>
<th>Additional features</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>gastrulation</em></td>
<td>+</td>
<td>Oogenesis</td>
<td>-</td>
</tr>
<tr>
<td><em>defective (gd)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>nudel (ndl)</em></td>
<td>-</td>
<td>Oogenesis</td>
<td>-</td>
</tr>
<tr>
<td><em>pipe (pip)</em></td>
<td>-</td>
<td>Oogenesis?</td>
<td>-</td>
</tr>
<tr>
<td><em>spätzle (spz)</em></td>
<td>+</td>
<td>Oogenesis</td>
<td>-</td>
</tr>
<tr>
<td><em>dorsal (dl)</em></td>
<td>+</td>
<td>Blastoderm?</td>
<td>+</td>
</tr>
<tr>
<td><em>tube (tub)</em></td>
<td>-</td>
<td>Blastoderm</td>
<td>-</td>
</tr>
<tr>
<td><em>snake (snk)</em></td>
<td>-</td>
<td>Blastoderm</td>
<td>-</td>
</tr>
<tr>
<td><em>easter (ea)</em></td>
<td>+</td>
<td>Blastoderm</td>
<td>+</td>
</tr>
<tr>
<td><em>pelle (pll)</em></td>
<td>+</td>
<td>Blastoderm</td>
<td>-</td>
</tr>
<tr>
<td><em>Toll (T1)</em></td>
<td>+</td>
<td>Blastoderm</td>
<td>+</td>
</tr>
</tbody>
</table>

*Additional features: Fragile eggs, Conditionally haploinsufficient, Rescue activity stable, Complete rescue by injection, Rescue activity primarily RNA, Cold-sensitive alleles, All dorsal-ventral polarity lost.

*The probable time of action was defined in some cases from temperature-sensitive periods, in others by if and when the mutant phenotype is rescuable by injection. Blastoderm here means syncytial blastoderm.
product derived from the paternal gene at fertilization. Many more of these zygotic loci affecting pattern formation during embryogenesis may show a maternal effect but this aspect has not yet been examined (33). Zygotic genes responsible for determination in *D. melanogaster* can be separated into two broad classes: the segmentation genes, which are responsible for the formation of the correct number and polarity of embryonic segments, and the homeotic selector genes, which give each segment its identity. The latter group of genes act after the segmentation genes but still as early as cellular blastoderm. Experiments involving pole cell transplantation and mitotic recombination have shown that many zygotically expressed genes show a maternal effect. The progeny from homozygous mutant germ-line clone females show a stronger mutant phenotype than the progeny resulting from normal heterozygous mutant adults indicating the importance of the maternally expressed product. As more zygotically acting genes involved in pattern formation are isolated and their pattern of expression studied, the question of possible expression during oogenesis can be addressed.

**Segmentation Genes Showing a Maternal Effect.**

The genetic analysis of the segmentation genes has suggested that there is a temporal succession of gene activities for increasing specification of the segmentation pattern of the embryo. In general, maternal effect genes act first, followed by the three categories of segmentation genes: gap genes, pair rule genes and segment polarity genes. Gap genes, when mutant, cause deletion of large contiguous areas of the embryonic body pattern. Mutant pair rule genes have an approximate two segment periodicity in their deletion patterns while segment polarity mutants cause deletion/duplication of regions within each segment (Figure 3). Of all the segmentation genes studied, only *hunchback* (*hb*) and *caudal* show a maternal effect.

*hunchback* belongs to the gap class of segmentation genes. Homozygous mutants show defects in two regions of the embryo (35). At the anterior end, the labium and all three thoracic segments are deleted while posteriorly the 8th abdominal segment and adjacent areas of the 7th abdominal segment are absent. In homozygous *hb* embryos derived from a homozygous mutant germ-line the anterior deletion is larger and includes the first three abdominal segments. In addition, three abdominal segments with reversed
Figure 3. Schematic representation of the phenotypes of the four gap and nine pair-rule mutations. The diagram at the top depicts the normal array of denticle bands and cuticular elements on the ventral side of a developed embryo. For each mutant a set of darkened bars indicates the positions of the pattern deletions characteristic for that locus. The thoracic and abdominal patterns are shown in detail. In the telson, only the anal plates, tuft and posterior spiracles are indicated and, with the exception of the labial segment, head structures are not shown (modified from Weischaus (32)).
polarity are formed between the remaining head structures and posterior abdomen. However, a wild type allele of *hunchback* from the father completely restores normal development in embryos derived from homozygous mutant germ-line females.

*Kruppel* is the only gap gene that has been cloned and characterized at the molecular level (36). The similarity in phenotype between the gap genes may reflect a similar function of these genes in different areas of the embryo. *Kruppel* is active before blastoderm formation and is expressed in specific regions of the embryo until the stage of germ band extension. *Kruppel* activity is a requirement for normal segmentation in the areas expressed and adjacent regions during development (37).

The *caudal* locus has been cloned and subsequently mutations at this locus were shown to produce phenotypes of the pair rule class of mutations. A maternal transcript of 2.4 kb was found to accumulate in the nurse cells and oocyte during oogenesis. These maternal transcripts establish a concentration gradient along the anterior/posterior axis of the embryo at the syncytial blastoderm stage. At the cellular blastoderm stage, these transcripts are confined to a single band from 13%-19% egg length. A 2.6 kb zygotic transcript is detected at the blastoderm stage and persists until the onset of metamorphosis. In caudal mutants all even-numbered abdominal sections are absent while the head and thorax are normal. This is the first maternally expressed gene known to affect embryonic development which has proved to contain a homeo box, a short DNA element found in the 3' coding region of many homeotic selector genes (38,39).

**Loci Affecting Homeosis in *D. melanogaster* Displaying a Maternal Effect.**

The identities of most if not all the body segments in *D. melanogaster* are under the direct control of the zygotically active genes located in the bithorax complex (BX-C) and the Antennapedia complex (ANT-C) (40,41,42,43). BX-C genes specify the normal identities of the segments in the posterior of the embryo from thoracic segment three (T3) through abdominal segment 8 (A8) while ANT-C genes specify gnathocephalic and anterior thoracic segment identity. Each gene complex contains at least three homeotic lethal complementation groups. These complementation groups are thought to control the developmental fate of a cell by the *trans* regulation of target genes.
Molecular clones of these six "selector" loci (44,45,46,47,48) have been used to
determine the expression pattern of mRNAs by in situ hybridization to embryo
tissue sections. On the basis of these experiments, it has been found that the
regions affected by mutants in these genes are the same regions that contain
transcripts from these 6 ANT-C/BX-C loci (47,49,50,51,52,53). Thus, the
regions where a homeotic gene functions corresponds to the region where its
transcripts accumulate.

There are at least two mechanisms involved in regulating the expression
of a homeotic gene to the correct region of the embryo. A number of genetic loci
unlinked to the ANT-C or BX-C and maternally expressed influence the
expression of these gene complexes (54,55,56,57,58,59,60). There is also
evidence that at least some ANT-C/BX-C genes interact with each other to
maintain normal spatial expression (53,60,51,62). Polycomb (Pc) is one of the
most extensively analyzed examples of a regulatory gene outside the ANT-
C/BX-C that is required for proper expression of these complexes. Embryos that
lack Pc + function display homeotic transformations of anterior segments to
more posterior segments. The phenotypes observed for Pc mutants result from
altered patterns of expression for certain genes of the ANT-C and BX-C
complexes (63). Both Ubx and Antp expression were found to be altered in Pc −
embryos and the alterations were consistent with the Pc − phenotype.

Most of these regulatory loci appear to be required continuously during
development and show a maternal effect (64). Whether the zygotic and
maternal activity are the same remains to be determined. In Pc, the maternal
effect is almost completely rescued by zygotic activity indicating these activities
are the same. The extra sex combs (esc) locus differs from other homeotic
regulatory genes in that its activity is required only during early embryonic
development (65). A maternal supply of esc is sufficient for normal embryonic
development. esc appears to be involved in the initial programming of the
correct patterns of homeotic gene expression while Pc and the others act to
maintain previously established patterns (61). fs(1)h (66) is a homeotic mutant
that appears to interact strongly with regulator of bithorax, another gene
unlinked to ANT-C AND BX-C involved in homeosis. Based on genetic and
molecular analysis of this locus, Digan et al (66) suggested that the maternal
and zygotic activities may be different, with one transcript necessary for normal
embryonic development and the other functioning in homeosis.
The overall relationship of the maternal effect genes and zygotic genes in establishing the metameric pattern of the adult fly remains obscure. The wealth of genetic information available illustrates the need for molecular probes to better understand the interactions between these genes. Molecular probes for the zygotically acting homeotic genes and several of the segmentation genes have led to an increasingly precise analysis of individual genes and their interactions with each other. The ability to identify both transcripts and proteins directly in vivo and in vitro has helped us begin the arduous task of understanding the developmental process.

**ADVANTAGES OF Calliphora erythrocephala FOR THE ISOLATION OF OOGENESIS SPECIFIC GENES**

*Drosophila melanogaster* is well suited for studies on embryonic pattern formation. The intense genetic characterization of *D. melanogaster* and its well defined embryonic fate map make it the organism of choice for genetic studies of embryonic pattern formation. Advanced molecular techniques such as P-element mediated gene insertion and the relatively small genome size aid in the study of developmental genes once molecular probes are available. However, cloning of maternally expressed genes important during both oogenesis and embryogenesis has proved to be difficult. Genes identified genetically as playing a role in embryogenesis may be isolated by transposon tagging (67) or chromosomal walking (68), but these techniques must be applied on a gene by gene basis and genetic analysis must have identified these loci. In addition, genes affecting the internal patterning of the embryo may not be detected by genetic screens. Differential screening of clone libraries provides an alternative procedure for isolating simultaneously many genes expressed specifically during oogenesis and embryogenesis for which no genetic markers are available. Differential gene screens to isolate blastoderm-specific and sex-specific genes have proved the value of this approach (69, 70, 71). However, differential screens to isolate genes specifically expressed in oogenesis is difficult in *D. melanogaster* and no large scale biochemical screen has been attempted such as the genetic saturation screens performed (11,12,13,14) to isolate female sterile mutations. Obtaining large quantities of staged *D. melanogaster* follicles is difficult due to the small size of the organism and ovary. In addition, the *D. melanogaster* ovary contains follicles at all stages
of oogenesis. Thus, generating large quantities of staged *D. melanogaster* follicles involves labor intensive separation of individual follicles and the use of very large fly populations.

We have therefore attempted to isolate oogenesis-specific genes by differential library screening in the dipteran fly *Calliphora erythrocephala*, an evolutionarily close relative of *D. melanogaster*. The physiology of oogenesis in *C. erythrocephala*, along with its much larger size, permits the isolation of biochemical quantities of RNA from any stage of oogenesis. The ovary of *C. erythrocephala* contains 5X as many ovarioles per ovary (72) and 14X more RNA per mature oocyte (73) as *D. melanogaster*. However, the most advantageous feature of *C. erythrocephala* oogenesis is that, unlike *D. melanogaster*, the ovaries are immature at eclosion and development is arrested until triggered by meat feeding. Individual follicles begin developing synchronously in each ovariole only after the female has ingested a meat meal. Collection of follicle populations at any desired stage of oogenesis can be achieved by following a simple meat feeding regime (74). The synchronously matured oocytes are laid as a single clutch of approximately 200 eggs. However, *C. erythrocephala* females retain their eggs until a suitable substrate for oviposition is available. Thus, large quantities of embryos of a given stage can also be easily obtained in the following way. After the flies have been "primed" for several days, all meat is removed from the cage. Oogenesis continues until all follicles reach maturity. Meat is again added to the cage and a large number of females synchronously lay their egg clutches.

**Strategy for the Differential Screening.**

The strategy for the isolation of oogenesis-specific genes is presented in greater detail in Chapter 3. Radiolabelled probes prepared from poly(A)+ RNA from two stages of oogenesis and from mid-stage embryos have been used to identify clones showing transcription specifically during oogenesis. In addition to screening a *C. erythrocephala* genomic library, a *D. melanogaster* genomic library has also been screened with *C. erythrocephala* probes to identify conserved oogenesis-specific genes. The identification of oogenesis-specific genes from both organisms and their characterization as somatic cell or germ-line cell expressed genes is described here. In addition, the further
characterization of two germ-line expressed, oogenesis-specific *D. melanogaster* genes is presented.
CHAPTER TWO

MATERIALS AND METHODS

Preparation of RNA from *Calliphora erythrocephala* Ovaries and Embryos.

Our laboratory fly stock, which is a mixed stock containing flies originally from the Karlson, Levenbook and Cambridge laboratories was used for all experiments. Ovaries of the appropriate stage were obtained from flies which had been primed with a meal of liver homogenate. To obtain previtellogenic stage follicles (PV, stage 1, Figure 4) flies were primed for 24-36 hours. For maximal nurse cell stage follicles (MN, stage 3-4, Figure 4) flies were primed for 72-84 hours. Ovaries were hand dissected into insect saline (0.9% NaCl, 0.025% CaCl₂, 0.04% KCl, 0.02% NaHCO₃, 0.25% glucose) and staged using a dissecting microscope. PV stage follicles were rinsed three times with HSSR (20 mM Tris/HCl pH 8.0, 0.3 M sucrose, 0.1 mM EDTA, 0.5 mM spermine, 0.15 mM spermidine, 0.1% Triton X-100) and homogenized in 10 volumes HSSR using a Dounce homogenizer. After disruption of the tissue, mitochondria and nuclei were removed by two twenty minute centrifugations at 18,000 x g at 4°C. The supernatant was recovered, extracted once with PC (phenol:chloroform:isoamyl alcohol, 50:48:2), once with CA (chloroform:isoamyl alcohol, 24:1) and precipitated with ethanol overnight at -20°C. The RNA pellet was recovered by centrifugation at 10,000 x g, resuspended in 10 mM Tris/HCl pH 8.0, 2% SDS, 0.5 mM EDTA, 200 μg/ml Proteinase K and incubated 60 minutes at 37°C. This solution was then extracted three times with PC and precipitated with ethanol, centrifuged and resuspended in TE (10 mM Tris/HCl pH 8.0, 1 mM EDTA). RNA was quantitated by absorption at 260 nm. For MN follicles, nurse cell nuclei were removed by gently disrupting the tissue using silane-treated Pasteur pipettes in HSSR instead of homogenization. The nuclei were allowed to settle at 1 X g and the supernatant centrifuged to remove mitochondria as above.

For embryo collections, flies were primed by meat feeding three days and then maintained on sugar and water only for four more days. Embryos were collected on liver pieces wrapped in 100 μ Nitex for two hours. Embryos were allowed to develop for nine hours at 25°C giving 10 ± 1 hour embryos (ME) and then were dechorionated by treatment for two minutes with 50% bleach and
Figure 4. The six stages of *C. erythrocephala* oogenesis. Six micron sections of staged *C. erythrocephala* follicles stained with methylene blue and basic fuchsin are shown. 1 = previtellogenic stage. 2-5 = vitellogenic stages. 6 = mature follicle.
then rinsed on a mesh screen with six liters of water. Embryos were immediately homogenized in HSSR and RNA prepared as above.

Poly(A)+ RNA was selected by column chromatography on oligo(dT) cellulose (Type III, Collaborative Research). The column matrix was allowed to swell in elution buffer (EB, 10 mM Tris/HCl pH 7.5, 1 mM EDTA) and poured into Biorad Econocolumns. The column was then equilibrated with binding buffer (BB=EB + 0.5 M NaCl). Total RNA (3 mgs RNA/ml column matrix) made 0.5 M in NaCl, denatured two minutes at 70°C and quick chilled on ice was passed over the column. The flow-through was collected, denatured and reapplied twice. The column was washed with binding buffer and the RNA then eluted with ten column volumes EB. The eluted RNA was then treated exactly as above for two more passes over the column. The last elution was collected in 10 X 1 ml fractions. Fractions containing RNA were pooled, precipitated with ethanol and quantitated either by absorption at 260 nm or ethidium bromide fluorescence (75).

**Preparation of 32P Radiolabelled cDNA.**

Reverse transcriptase was used to generate single stranded 32P-labelled cDNA complementary to poly(A)+ RNA according to Maniatis et. al. (75). Ten μg of poly(A)+ RNA and 0.5 mCi of 32P-dCTP were combined and the RNA denatured by the addition of methyl mercuric hydroxide. β-mercaptoethanol, oligo(dT)12-18, buffer, dATP, dCTP, dGTP, dTTP were added and the reaction incubated two hours at 42°C with one unit of reverse transcriptase. The reaction was stopped by the addition of EDTA and the RNA template destroyed by alkaline hydrolysis. The solution was neutralized and the amount of radioactivity incorporated into acid precipitable counts was measured. Unincorporated label was removed by three ethanol precipitations. The size of the cDNA was measured by alkaline agarose gel electrophoresis.

**Differential Screening of a C. erythrocephala Genomic Library.**

The *C. erythrocephala* genomic library in the lamda phage vector EMBL 3 was provided by David Ivaninski and screened as described previously (76). Briefly, NM538 bacteria were infected with 3,000 plaque forming units (pfu) per plate on large (150 mm) L-plates and incubated at 37°C overnight. Phage DNA was transferred to nitrocellulose filters by placing a filter onto the plate until
wetted. Two more filters were stacked on top of the first filter as above and allowed to absorb for 15-20 minutes. The filters were key marked with India ink to allow the alignment of the filters with the plate. The filters were sequentially removed from the plate and transferred through Whatman 3MM papers soaked in denaturant (0.5 M NaOH, 1.5 M NaCl), neutralizer (3 M NaCl, 0.5 M Tris/HCl pH 7.0) and 2 X SSC (20 X SSC= 3M NaCl, 0.3 M sodium citrate). The filters were baked under vacuum at 80° for 2-4 hours.

The filters were prehybridized at 42° overnight in 50% formamide, 5 X Denhardt's, 6 X SET (20 X SET= 3 M NaCl, 0.6 M Tris/HCl pH 8.0, 20 mM EDTA), 0.5% SDS and 100 µg/ml boiled, sheared salmon sperm DNA in sealed bags. The prehybridization solution was removed and replaced with fresh prehybridization solution containing 1X 10⁷ cpm/ml radiolabelled cDNA probe. One of the three triplicate filters was hybridized to each of the cDNA probes (that is the PV follicle, MN follicle or ME embryo probe). Hybridization was for 24-60 hours at 42° with shaking. The filters were then washed in 2 X SSC five minutes at room temperature followed by two washes at 65° in 2 X SSC and 1% SDS for 60 minutes. A final one hour wash was performed in 0.1 X SSC, 0.1% SDS at room temperature. Filters were blotted dry and autoradiographed using Kodak XAR-5 film. The autoradiographs were marked from the filters for alignment with the plates and replicate filters compared to identify signals present on the PV and/or MN autoradiographs but not on the ME autoradiograph. Signals demonstrating oogenesis-specific hybridization (that is, signals present on the PV and/or MN autoradiographs but not present on the ME autoradiograph) were then related to the original plate and plaques corresponding to the signals were pulled as agar plugs and eluted in SM (0.1 M NaCl, 50 mM Tris/HCl pH 7.5, 1 mM EDTA).

For each original signal selected it was necessary to pull approximately 10 plaques from the plates as a result of stretching and misalignment of the primary screen nitrocellulose filters during processing. We therefore initiated secondary screening to eliminate the plaques that did not correspond to signals. Five µl aliquots of each plaque eluate was dotted onto freshly poured NM538 bacteria lawns in an ordered pattern in duplicate. After incubation at 37° overnight, 5-6 mm lysed dots arising from the phage eluates were present. Replicate filters were prepared and screened as for the primary screening above.
Phage clones surviving the secondary screen were then plaque purified. Individual phage were poured at very low density (200 pfu/plate) and, after incubation overnight at 37°C, single, well isolated plaques were pulled as agar plugs. Twelve plaques for each secondary signal were isolated and rescreened as in the secondary screening. Comparison of the autoradiographs identified oogenesis-specific phage clones.

**Screening of D. melanogaster Genomic Libraries.**

The screening of *D. melanogaster* genomic libraries was carried out essentially as above with the following changes. Two different genomic libraries were screened: the Maniatis random shear library and a subset of this library prepared by Linda Ambrosio (see **Screening of D. melanogaster Genomic Libraries** below). The primary screen of the Maniatis library was done with phage plates at a density of 5,000 pfu/plate. At this density individual plaques were hard to isolate and therefore large agar plugs were pulled (about 6 mm) containing many phage. These large plugs were pooled and rescreened at a lower density (3,000 pfu/plate) and positive signals treated in the same way as primary signals from the *C. erythrocephala* library screen. Washes were performed at reduced stringency since we were probing the *D. melanogaster* clones with *C. erythrocephala* cDNA probes which may have reduced homology. All filters were washed in 2 X SSC, 1% SDS at 65°C for one hour and twice in 1 X SSC, 0.1% SDS at 65°C for one hour.

**Preparation of DNA.**

Bacteriophage DNA was prepared from four ml liquid lysates (75). A four ml culture was infected with 3 X 10^5 pfu bacteriophage and 1.6 X 10^8 bacteria and incubated 12-18 hours at 37°C with shaking. Cells were pelleted at 6,000 x g at 4°C and the supernatant transferred to clean centrifuge tubes. DNase I and RNase A were added to a final concentration of 1 µg/ml and incubated at 37°C for 15 minutes followed by the addition of an equal volume of 1.5 M NaCl, 20% polyethylene glycol and incubation on ice for one hour. Bacteriophage were pelleted at 12,000 x g, 4°C for 20 minutes and taken up in TE. SDS and Proteinase K were added to final concentrations of 1% and 5 µg/ml respectively and incubated at 65°C for 15 minutes. This solution was then extracted three
times with PC, three times with CA and precipitated with ethanol. DNA was quantitated by ethidium bromide fluorescence.

Plasmid DNA was prepared by the method of Birnboim and Doly (77). A 1 ml saturated overnight of appropriate cells were pelleted at 12,000 x g at 40°C for 30 seconds and taken up in 200 μl solution one (0.5 M glucose, 25 mM Tris/HCl pH 8.0, and 10 mM EDTA). 400 μl solution two (0.2 M NaOH, 1% SDS) was added, gently mixed and incubated on ice for ten minutes. 300 μl 5 M Potassium Acetate pH 4.8 was added, mixed gently and again incubated on ice ten minutes. Chromosomal DNA and protein was removed by a 15 minute centrifugation at 12,000 x g and the supernatant transferred to a fresh tube. 0.6 volumes isopropanol was added to the supernatant, mixed and the mixture incubated for 15 minutes at room temperature. Plasmid DNA was recovered by centrifugation at 12,000 x g, 15 minutes. The pelleted DNA was taken up in 200 μl TE, PC extracted three times and CA extracted twice. Plasmid DNA was then precipitated for 15 minutes on ice in ethanol and quantitated by ethidium bromide fluorescence.

Preparation of Radiolabelled Probes.

Plasmid DNA was radiolabelled according to the method of Feinburg and Goldstein (78). DNA was cleaved with the appropriate restriction endonuclease to remove the insert from the vector and subjected to electrophoreses in a 1% Tris acetate (TA=40 mM TrisHCl pH 7.5, 10 mM sodium acetate, 1 mM EDTA) low melt agarose gel. The fragment corresponding to insert DNA was excised from the gel and 3 ml water per gr of gel slice were added. The water/gel solution was boiled seven minutes and 13 μl aliquots stored at -20°C. When ready for use, the DNA was boiled three minutes and placed at 37°C until the addition of 5 μl OLB (90 mM Hepes, pH 6.6, 50 μM each dATP, dGTP and dTTP, 50 mM Tris/HCl pH 8.0, 5 mM MgCl2, 10 mM β-mercaptoethanol), 2 μl 5 mg/ml BSA, 5 μl 32P dCTP (~3,000 Ci/mmol) and five units Klenow fragment of DNA polymerase I. This reaction was then incubated overnight at 37°C. The radiolabelled DNA was separated from free label by the passage over a Sephadex G-50 column poured in a one ml syringe. Incorporated label was measured by scintillation counting.

Radiolabelled single stranded RNA probes (cRNA) were prepared from subclones in the cloning vector Bluescribe M13+ (Stratagene) as
recommended by the suppliers. This vector has the bacteriophage T7 and T3 transcription initiation sites on either side of the multiple cloning site. DNA was linearized using either Eco RI (for T3 transcription) or Hind III (for T7 transcription) and PC extracted a minimum of three times. 0.25 picomoles of either 35S UTP or 32P UTP (~1,300 Ci/mmol) was lyophilized and rehydrated with nine μl of a solution containing 40 mM Tris/HCl pH 8.0, 6 mM MgCl2, 4 mM spermidine, 20 mM NaCl, 500 uM each ATP, CTP, GTP, and 1 μg template DNA. Ten units of either T7 or T3 RNA polymerase were added and this reaction was incubated two hours at 40°. Unincorporated label was removed by ethanol precipitation and radiolabel incorporation measured by scintillation counting.

RNA probes used for in situ hybridizations were further treated with five units RNase free DNase I for thirty minutes at 37° to remove plasmid DNA. The transcribed RNA was then reduced in size to an average of 200 nucleotides by alkaline hydrolysis. An equal volume of 2 X Bicarb (80 mM NaHCO3, 120 mM Na2CO3) was added to the RNA transcription reaction and incubated at 60° for the appropriate time. Hydrolysed RNA was recovered by ethanol precipitation and the amount of radiolabelled RNA recovered measured by scintillation counting.

Southern Transfers and Hybridization.

DNA was transferred to Genescreen nylon membranes (NEN) essentially as described by the suppliers. DNA was digested with the appropriate restriction endonuclease(s) and subjected to electrophoreses through TA agarose gels. After photographing the gel on a medium wavelength (300 nm) U. V. box, the DNA was denatured in 3 M NaCl, 0.5 M NaOH for one hour, neutralized one hour in 3 M NaCl, 0.5 M Tris pH 7.0 and soaked 15 minutes in 20 X SSC. The gel was then placed upside down on a piece of Whatman 3MM paper saturated with 20 X SSC and resting on a sponge. The gel was then covered with Genescreen cut to fit the gel and 3 pieces of Whatman 3MM paper also cut to fit the gel. 20 X SSC was absorbed through the gel/membrane sandwich by a three inch layer of paper towels again cut to fit the gel. The transfer was allowed to proceed until all the paper towels were soaked or at least 12 hours had passed.
After transfer to Genescreen, filters were baked two hours at $80^\circ$ and prehybridized in 50% formamide, 1 X Denhardt's (100 X Denhardt's=0.2% BSA, 0.2% ficoll and 0.2%polyvinyl-pyrrolidone M.W. 40,000), 5 X SSC, 1% SDS and 200 $\mu$g/ml boiled, sheared Salmon testes DNA at $42^\circ$ overnight. The prehybridization solution was then removed, replaced with the same solution plus from 1 X $10^6$ - 1 X $10^7$ cpm radiolabelled probe and hybridized at $42^\circ$ for a minimum of 18 hours. The hybridization solution was removed and the filters washed twice for five minutes at room temperature with 2 X SSC, twice for 30 minutes at $65^\circ$ with 2 X SSC, 1% SDS and finally twice for 30 minutes at room temperature with 0.1 X SSC, 0.1% SDS. Filters were patted dry using Kimwipes and autoradiographed using Kodak XAR-5 film and intensifying screens at $-70^\circ$.

**In Situ Hybridization of $^{35}$S Labelled Probes to Whole Tissues.**

To determine whether the oogenesis-specific clones we had isolated were transcribed in the somatic or germ-line tissue, *in situ* hybridizations to either whole *C. erythrocephala* follicles or *D. melanogaster* ovaries were performed. The hybridization was performed essentially as described by Mahoney et al (79). MN stage follicles or whole ovaries were hand-dissected from the fly and washed once with insect saline and once with 1 X AM buffer (80 mM KCl, 20 mM NaCl, 2.5 mM EDTA, 0.5 mM spermidine, 0.2 mM spermine, 15 mM PIPES pH 7.4, 0.1% β-mercaptoethanol). The tissue was transferred to ten ml's. 0.2% paraformaldehyde, 0.5 X AM, 50% heptane and agitated ten minutes. The above solution was removed and replaced with 4.0% paraformaldehyde, 0.6 X PBS (1 X PBS=0.13 M NaCl, 7 mM Na$_2$HPO$_4$, 30 mM NaH$_2$PO$_4$), 0.1% deoxycholate, 0.1% Triton X-100 and incubated 15 minutes at room temperature. The tissue was then washed five times for five minutes each with 1 X PBS, treated twenty minutes with 0.2 N HCl and again washed with 1 X PBS four times for five minutes each. The tissue was then treated with 0.6 mg/ml Proteinase K for five minutes at room temperature. After removal of the Proteinase K, 25 µl. of a 40 mg/ml glycine stock was added and incubated 30 seconds at room temperature. The tissue was once again washed twice for five minutes each with 1 X PBS and then refixed in 4.0% paraformaldehyde, 1X PBS for twenty minutes. The tissue was washed five times for five minutes each with 1 X PBS and stored at $4^\circ$. 
The fixed tissue was prehybridized overnight in 50% formamide, 0.6 M NaCl, 10 mM Tris/HCl pH 7.5, 1 mM EDTA, 10 mM DTT, 1 X Denhardt's and 1 mg/ml Salmon testes DNA at 50°. The prehybridization solution was then drawn off and replaced with the same solution plus 2 X 10^5 cpm/µl 35S radiolabelled cRNA probe and incubated at least eighteen hours at 50°. The hybridization solution was removed and the tissue washed two times with 1 X PBS. The tissue was then treated with 20 µg/ml RNAse A for 30 minutes at 37°. The RNAse solution was removed and the tissue washed four times ten minutes each with 2 X SSC, 10 mM DTT at 60°, two times ten minutes each in 0.1 X SSC, 10 mM DTT at 60° and one times 60 minutes in 0.1 X SSC, 10 mM DTT at 60°.

The tissue was then dehydrated through 25%, 50%, 75%, 95% and absolute ethanol solutions for 30 minutes each (80). After dehydration, the tissue was cleared twice in xylene for 30 minutes each. The xylene was removed by washing three times 30 minutes each with melted paraplast wax at 56°. After the final wax rinse, the tissue was covered with wax and the wax drawn into the tissue by baking one hour at 56° in a vacuum oven. The tissue was then embedded in wax in metal embedding dishes (approximately 4 follicles/ovaries per dish). The wax blocks were removed from the embedding dishes and examined with a dissecting microscope to determine the orientation of the follicles, mounted and sliced into six micron sections using a microtome. The sliced sections were flattened on droplets of water placed on glass slides on a 40° warming plate. After the water had evaporated, the wax was removed from the sections by rinsing two times with xylene and then dehydrating twice with absolute ethanol. The glass slides with the tissue attached were dipped in 50% NTB-2 emulsion and autoradiographed for one to five days. The slides were developed and tissue examined under the microscope.

*In situ* Hybridization to *D. melanogaster* Polytene Chromosomes.

Salivary glands from *D. melanogaster* third instar larvae were hand dissected in insect saline and transferred to 3:1 ethanol: acetic acid for five minutes. The glands were then transferred to a drop of 45% acetic acid on a silanized cover slip and a subbed slide was lowered onto the coverslip. The salivary gland was then squashed between two layers of paper towels and squashes examined under the microscope to check that nuclei had been
disrupted. The chromosomes were then flattened on a hot plate at 40-450° for
for 3-5 minutes. The cover slip was removed and the chromosomal squash
stored in absolute ethanol overnight and then air-dried and stored at -70°.

When ready to hybridize, the squashes were incubated 30 minutes in 2 X
SSC at 70° and then dehydrated through 70%, 80%, 95% and absolute
ethanol. After drying at 37°, the squashes were treated with 200 μg/ml RNase A
for one hour at 37° and again dehydrated through the above ethanol solutions.
The chromosomes were acetylated with 0.1 M triethanolamine and acetic
anhydride, rinsed in 2 X SSC and dehydrated through ethanol as above. The
chromosomes were denatured in 0.07 N NaOH, 2 X SSC for one minute and
again dehydrated. Each squash was incubated overnight at 37° with 2 μl
hybridization solution (50% formamide, 10 mM PIPES pH 6.8, 0.5 M NaCl, 100
μg/ml tRNA, 25 μM each dATP, dCTP, dGTP and dTTP) containing 2 X 10^5
dpm/μl 35S radiolabelled cRNA probe. The coverslips covering the squashes
were sealed with rubber cement to prevent evaporation.

Slides were washed four times two hours each in 50% formamide, 10
mM PIPES pH 6.8, 0.5 M NaCl and dehydrated in 75% ethanol, 4 mM MgCl2 for
30 seconds followed by 95% ethanol, 4 mM MgCl2 for 30 seconds. Slides were
then dipped in 50% NTB-2 emulsion and autoradiographed for one to three
days.

Screening of D. melanogaster cDNA Libraries.

Five pools of an D. melanogaster embryonic cDNA library in the phage
vector λGT10 were obtained from the Hogness laboratory and screened
essentially as described above. The primary screen consisted of preparing
phage grown on the bacteria C600 at a density of 50,000 pfu per 150 mm plate.
Duplicate filters were prepared from each plate and hybridized with a 32P
oligolabelled transcribed fragment of the D. melanogaster genomic clones
isolated above. Positive signals were pulled as large plugs and re-screened at
a density of ~200 pfu per 75 mm plate. Individual plaques were pulled, eluted
into SM and rescreened as above. If every plaque in the tertiary screen was
detected by the probe, the plaque was considered plaque purified.

Preparation of DNA from Homozygous Deficiency Embryos.
Deficiencies from the 31B/D region of *D. melanogaster* chromosome 2 were kept as stocks over a *Curly* balancer chromosome on standard *D. melanogaster* media. Stock deficiency flies were crossed with wild type Oregon R flies. The progeny of this cross will be of the genotypes deficiency/wild type or *Curly/wildtype*. Male and female virgin progeny with straight wings (genotype deficiency/wild type) were collected and maintained at 17°C until sufficient flies were collected to perform the F₁ cross. These straight wing siblings were then crossed with each other generating offspring with the three genotypes: i) deficiency/wild type, ii) wild type/wild type and iii) deficiency/deficiency homozygotes. Embryos from this mating were collected on grape plates supplemented with yeast and allowed to develop for 32 hours. Embryos carrying a wild type chromosome will be viable and hatch while deficiency homozygous flies will be non-viable and fail to hatch. After 32 hours, unhatched eggs were collected under the dissection microscope, counted, washed with K⁺ free insect saline and stored at -70°C. DNA was prepared by grinding 200 embryos in a 1.5 ml eppendorf tube on dry ice followed by homogenization in 50 mM Tris/HCl pH 8.0, 50 mM EDTA, 0.1 M NaCl, 0.5% SDS and 100 µg/ml Proteinase K. After homogenization, the embryos were incubated four hours at 50°C, PC extracted three times and CA extracted twice. DNA was collected by ethanol precipitation and stored at -20°C. DNA from wild type embryos collected over a 12 hour period was also prepared as above.
CHAPTER THREE

SECTION I. THE ISOLATION OF OOGENESIS-SPECIFIC, GERM-LINE EXPRESSED CLONES FROM *Calliphora erythrocephala*.

The goal of our research is to isolate and characterize genes expressed during oogenesis that play a role in establishing the correct formation of the developing follicle and/or early embryo. We have begun the differential screening of genomic libraries aimed at identifying genes expressed during oogenesis in a stage specific manner. Since many of the interesting developments within the germ-line cells of the follicle occur early in oogenesis, we limited our search to genes expressed during the previtellogenic and early vitellogenic phases of oogenesis.

*Drosophila melanogaster* has many advantages for the study of these genes. First, there is a wealth of genetic information available for *D. melanogaster* including identification of mutations that affect the formation of the follicle and/or embryo. No other higher eucaryote system offers this advantage. Second, both the ovarian follicle and embryo of *D. melanogaster* have easily identified, characteristic features so that identification of mutants affecting these structures and therefore oogenesis and/or embryogenesis, is possible. Last, the characterization and utilization of P-element mediated gene insertion into the *D. melanogaster* genome along with the genetic manipulation possible in this organism, allows functional studies *in vivo* of re-introduced genes.

Although *D. melanogaster* is the organism of choice for these studies, the small size of this organism and its reproductive physiology make it difficult to isolate large quantities of staged early follicles. We have therefore taken the approach of working with the related but much larger fly *Calliphora erythrocephala* to isolate genes specifically expressed during oogenesis that are conserved in *Drosophila melanogaster*. Both *C. erythrocephala* and *D. melanogaster* display meroistic, polytrophic follicle development. However, because *C. erythrocephala* shows no ovarian development until triggered by the stimulus of meat feeding, it is possible by carefully controlling the feeding of these flies to obtain large populations of females all carrying follicles at the same stage of development. By using probes prepared from previtellogenic
and maximal nurse cell stage follicles of *C. erythrocephala* we have differentially screened both *C. erythrocephala* and *D. melanogaster* genomic libraries to isolate genes expressed specifically during early oogenesis. The non oogenesis probe used as the differential screening agent in these screenings were prepared from 10±1 hour embryos. Thus, our definition of oogenesis-specific expression is defined by those genes that are expressed in the previtellogenic and/or maximal nurse cell stage follicles but not in the 10 hour embryo. Once these *C. erythrocephala* genomic clones have been isolated, they could then be used to obtain the *D. melanogaster* homologs so that the advantages of both flies can be utilized.

**Screening of Calliphora erythrocephala Genomic Libraries.**

Two different *Calliphora erythrocephala* genomic libraries have been used in our differential screening. Both libraries were constructed in the bacteriophage vector EMBL 3 (Figure 5). High molecular weight genomic DNA partially digested with Sau 3A1 was inserted into the Bam H I restriction sites of this vector. This removes from the vector the middle one third of the phage genome containing the red and gamma genes and replaces them with genomic DNA from 10 to 15 kb in length. Therefore, recombinant phage can be selected on cells containing a P2 lysogen which inhibits the growth of of bacteriophage containing the red and gamma genes. Alice Rubacha prepared one of the libraries with a complexity of 290,000 phage and, along with Esther Belikoff, differentially screened 39,000 phage from this library (76). Four clones from these 39,000 were found to show oogenesis-specific expression. I have performed further characterization of two of these cloned genes (see Localization of Gene Transcripts in the Follicle below.). Esther Belikoff and myself screened 25,000 phage from the second *Calliphora erythrocephala* genomic library (complexity 5,000,000 phage) prepared by David Ivanitsky. These two screenings represent a total of 6.4 X 10^5 kilobases of DNA, or 92% of one *Calliphora erythrocephala* genome, the equivalent of one quarter the number of phage required to be 99% sure of screening all single copy DNA of the *Calliphora erythrocephala* genome (81).

Initially, phage clones were differentially screened for oogenesis-specific genes using poly(A)^+ RNA isolated from the nurse cell nuclei of the developing follicle ^32^P-radiolabelled by T4 polynucleotide kinase. Nurse cell nuclear RNA
Figure 5. Restriction enzyme site map for EMBL3. A. The restriction enzyme sites within the phage vector EMBL3 for Sal I (S), Bam HI (B) and Eco RI (E) are indicated. B. Digestion of EMBL3 with Bam HI and Eco RI results in i) two large phage arms which terminate at a Bam HI site ans a cohesive phage end (cos), ii) a central fragment with Eco RI termini and iii) two small (< 15 bp) linker fragments. The central fragment carries the genes for red and gamma which confer Spi phenotype (sensitivity to P2 interference) (from Rubacha (75)).
was chosen since we had developed a procedure for complete purification of
these germ-line derived nuclei. Probably as a result of transcripts derived from
repetitious DNA in this nuclear RNA, it proved impossible to obtain differential
signals with this probe. The second method attempted was to use post
mitochondrial supernatant (PMS) poly(A)+ RNA from whole follicles again
radiolabelled using T4 polynucleotide kinase. Although many strong signals
were obtained, most of these signals corresponded to rRNA genes and these
signals made it difficult to detect differential signals. We therefore prepared
radiolabelled cDNA probes to eliminate rRNA contamination of our probes.
Oligo(dT) primers, which anneal to the poly(A) tail of mRNA were used to initiate
the synthesis of cDNA using reverse transcriptase. 32P-radiolabelled cDNAs
made from PMS poly(A)+ RNA from previtellogenic and maximal nurse cell
stage follicles were used as the stage specific oogenesis probes while
radiolabelled cDNA made from 10±1 hour embryos PMS poly(A)+ RNA was
used as the non oogenesis probe. cDNAs were routinely checked on alkaline
agarose gels to compare the quality and sizes of the three probes to insure that
the differential screening results would be meaningful (Figure 6). The average
size of the cDNA probes used were approximately 0.5-1 kb in length. Using
probes prepared in this way, we were able to detect differentially expressed
signals. All the screening presented here was performed using probes
prepared in this way. Since we had to use whole follicle PMS poly(A)+ RNA for
preparation of these probes, additional experiments are required to identify
germ-line expressed as opposed to somatically expressed genes (see below).
Also, cDNA probes may not faithfully represent the expression pattern of genes
in vivo. Some transcripts may have secondary structures making the reverse
transcription of these transcripts impossible.

For the primary screening of the 25,000 phage performed by E. Belikoff
and myself, phage plates were prepared at a density of 3,000 pfu/plate.
Triplicate filters were prepared and each filter was hybridized with one of the
three radiolabelled cDNA probes. After autoradiography, the autoradiographs
were compared and 265 potentially oogenesis-specific signals were detected.
The autoradiographs were related back to the primary plates and, due to the
inability to exactly match the autoradiographs with the phage plates, 1,583
individual phage plaques were isolated (about six plaques per primary signal).
792 of these plaques have been rescreened as lysed dots in ordered arrays
Figure 6. Analysis of cDNA quality by alkaline agarose electrophoresis. Radiolabelled cDNAs prepared from PMS poly(A)\(+\) RNA from PV and MN follicles and ME stage embryos were electrophoresed through 1.2% alkaline agarose gels. After drying, the gel was subjected to autoradiography. 32P end labelled λ/Hind III DNA was used as a size marker (M). Relevant fragment sizes are indicated.
with the three cDNA probes with 51 phage still showing oogenesis-specific signals. After plaque purification, these phage were rescreened a third time in ordered arrays. 19 phage survived this round of screening as potentially oogenesis-specific clones (Figure 7). The other 791 primary phage isolated are currently being screened by other members of our laboratory.

**Definitive Identification of Oogenesis-Specific Clones by Southern Blot Analysis.**

During the genomic library plaque screens described above, it was noticed that individual phage clones did not necessarily give the same signal throughout the several rounds of screening. Differences in the intensity of the signal seen for each phage would vary from screen to screen. Technical problems including the level of phage growth and unevenness of DNA transfer to the nitrocellulose filters were probably factors contributing to this problem. In order to obtain more definitive results, the 19 *C. erythrocephala* putative oogenesis-specific clones were therefore subjected to differential screening by using Southern blot hybridization. Southern blot analysis also accomplishes one other objective: this analysis tells us which restriction fragments within the 10-15 kb *C. erythrocephala* insert are being transcribed in an oogenesis-specific manner.

Phage DNA from the 19 *C. erythrocephala* phage isolated above and six phage isolated previously by Alice Rubacha was prepared and digested with various restriction enzymes to give a wide range of insert fragment sizes. Southern blots were prepared from three identical agarose gels of the digested DNAs and hybridized with ³²P radiolabelled ovarian and embryonic cDNAs as in the above library screening. Two phage clones isolated previously by Alice Rubacha were used as controls. Phage H5C has restriction fragments that hybridize identically to all three cDNAs and therefore provides a control for the quality of all three cDNA probes, while phage A10B has restriction fragments that hybridize only to previtellogenic and maximal nurse cell stage cDNA and not to mid-embryo cDNA, therefore providing a control for the detection of oogenesis-specific hybridization. 18 phage were selected from the autoradiographs of the Southern blots of these 24 phage as being either oogenesis-specific or having a strong differential signal (ovarian signal(s) much stronger than embryonic signal(s)). These phage, shown in Table 3 and Figure
25,000 *C. erythrocephala* recombinant phage

\[ \rightarrow \]

265 oogenesis-specific/differential signals, 1,583 individual plaques

\[ \rightarrow \]

792 phage re-screened to eliminate false positives

\[ \rightarrow \]

51 oogenesis-specific/differential phage, plaque purified and rescreened

\[ \rightarrow \]

791 phage being pursued by other members of the laboratory

\[ \rightarrow \]

19 potential oogenesis-specific/differential phage clones

*Figure 7. Flowchart representation of the *C. erythrocephala* genomic library differential screening protocol.*
8, were selected for further analysis. From the 64,000 phage screened by Alice Rubacha, Esther Belikoff and myself, 22 oogenesis-specific or strong differential clones (.03% of the phage screened) have been isolated, ten by Alice Rubacha and 12 by Esther Belikoff and myself. Of these clones, nine contain fragments which appear to be completely oogenesis-specific in expression while 13 contain fragments which show strong differential hybridization. Eight of these 22 phage also contain fragments which show expression during the timepoints examined in addition to fragments showing oogenesis-specific/differential expression.

**Southern Blot Analysis using cDNA from PMS Poly(A)+ RNA of Mature Oocytes.**

As an indication of whether these clones were being expressed in the germ-line or somatic tissue of the follicle, 13 of the 18 phage isolated in the screening performed by Alice Rubacha, Esther Belikoff and myself were tested for expression in the mature oocyte (Figure 8 and Table 3). The follicle cells have degenerated and are absent at this stage of oogenesis so that transcripts present at this stage of oogenesis are expressed in the germ-line tissue of the follicle. Nine of the clones isolated showed expression in the mature follicle and therefore are being transcribed in the germ-line tissue of the follicle.

**Isolation of D. melanogaster Homologs for the 18 Oogenesis-Specific/Differential Phage.**

* D. melanogaster* homologs for the 18 oogenesis-specific/differential phage are being pursued by Tamsen Valoir and Kathleen Gajewski in our laboratory. A *D. melanogaster* genomic library is being screened using transcribed fragments from each of the 18 *C. erythrocephala* phage isolated as oogenesis-specific/differential in our previous screenings. To date they have isolated *D. melanogaster* homologs for 11 of the 18 *C. erythrocephala* phage.

**Localization of Gene Transcripts in the Follicle--Identification of Somatically and Germ-Line Expressed Genes.**

Because the somatically derived follicle cells are in close association with the germ-line components of the follicle, it is impossible to remove these cells from the follicle before the preparation of RNA. Thus, the ovarian cDNAs used to screen for oogenesis-specific clones contain both germ-line and
Table 3. Summary of *C. erythrocephala* phage clones identified as showing oogenesis-specific/differential expression. PV, MN, MO and ME = previtellogenic, maximal nurse cell, mature oocyte and mid embryo, respectively.

<table>
<thead>
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<th>FRAGMENT</th>
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*"+" signs signal hybridization, the intensity of hybridization is indicated by the number of signs with the weakest hybridization intensity equal to the lowest number of signs , "-" signs indicate no hybridization and "U" indicates these phage have not been checked by that cDNA. Clones originally isolated by A. Rubacha. Partial digestion fragments for clone D2B are not listed. Restriction enzyme fragment sizes from the digests shown in Figure 8. Transcribed fragments used for *in situ* hybridizations to whole follicles.
Figure 8. Definitive Identification of Oogenesis-Specific Clones by Southern Blot Analysis. Suitable digests of clones that survived three rounds of differential screening (see text) were used to prepare identical Southern blots for hybridization to the three cDNA probes. The hybridization pattern of the 19 clones are shown. PV, MN, MO and ME = hybridization patterns to cDNA probes from PV follicles, MN follicles, mature oocytes and ME embryos respectively. Only those clones showing oogenesis-specific/differential hybridization are labelled. The clones identified as showing oogenesis-specific/differential expression are summarized in Table 3.
somatically expressed sequences. Since our goal was to isolate genes that are both oogenesis-specific and expressed in the germ-line tissue of the follicle, it is therefore necessary to perform in situ hybridizations to follicles to determine which cell type within the follicle expresses each of the genes.

Fragments showing oogenesis-specific/differential expression from four C. erythrocephala clones were subcloned into the vector Bluescribe (Figure 9). The Bluescribe vector has the phage T7 and T3 RNA polymerase initiation sequences in opposite orientation on each side of the multiple cloning site allowing the preparation of strand specific, single stranded cRNA probes from inserted DNA. Using the combined procedures of Mahoney et. al (79) and Ingham et. al. (80), 35S radiolabelled cRNA was prepared from both strands of the inserted DNA and hybridized to whole MN stage follicles. The follicles were embedded in wax and sliced into six micron sections. After autoradiography, the sections were examined under the microscope and the hybridization pattern of the cRNAs examined.

I have examined the cell type expression pattern for four clones. Two clones, A10B and GG7K originally isolated by Alice Rubacha as absolutely oogenesis-specific, and two clones, AA2 and 2G3 (see Table 3), isolated as oogenesis-specific/differentials from my screening were further analyzed for their expression pattern within maximal nurse cell stage follicles. MN stage follicles were chosen for this analysis because all four clones were strongly expressed during the MN stage of oogenesis.

The oogenesis-specific restriction fragments used as probes for the in situ hybridizations for clones A10B and GG7K are shown in Figure 10. These subclones of A10B and GG7K were found to be expressed in the peri-oocyte subpopulation of the follicle cells in the developing oocyte (Figure 11, A and B). A10B also shows expression in a further subset of the follicle cells, the border cells. The border cells are a specialized cluster of 7-10 follicle cells that migrate during the early stages of oogenesis from the anterior tip of the follicle through the nurse cell syncytium to the oocyte/nurse cell interface. The border cells are known to be responsible for the formation of the micropyle, or sperm entry point, of the egg. Further experiments carried out by other members of our laboratory have shown that the oogenesis-specific gene within clone A10B is homologous to yolk protein 1 (YP1) of D. melanogaster (82). Although in situ hybridizations of YP1 to D. melanogaster ovaries have been performed, these studies did not
Figure 9. Schematic drawing of the cloning vector Bluescribe showing the T7 and T3 RNA polymerase initiation sites on each side of the multiple cloning site. Arrowheads indicate the direction of transcription of genes contained in this plasmid.
Figure 10. Restriction enzyme site maps for A10B and GG7K. Sites for the restriction enzymes Eco RI, Hind III, Sal I, Bam HI and Pst I were mapped within both clones. Hpa II sites were only mapped in clone GG7K. Ordering of two small Eco RI fragments in A10B was not possible. Two possible positions for one of these Eco RI sites of this clone are therefore shown (symbols with dotted lines). Fragments containing oogenesis-specific transcripts are indicated by (-----), with the direction of the arrow indicating the direction of transcription. The restriction fragments used for in situ hybridizations are shown below the map by (----) and "probe". Fragments within which reside homologies to known D. melanogaster clones are indicated.
Figure 11. Localization of transcripts from the oogenesis-specific genes in ovarian follicles. Hybridization patterns of $^{35}$S-labelled single-stranded RNA probes corresponding to the transcribed fragments of the two oogenesis-specific clones are shown. A. T7 strand hybridization of A10B to a stage 4 follicle. Intense hybridization to the columnar, peri-oocyte follicle cells (FC) and the border cells (BC) is seen. Nurse cell compartment is uppermost, oocyte (O) below. B. T3 strand hybridization of GG7K to a stage 3.5 follicle. This unstained section shows the strong expression of GG7K in the peri-oocyte follicle cells (FC). NCN - nurse cell nucleus; ON - oocyte nucleus. Size bar equals 100 μ.
include sections visualizing the border cells. Our result is thus the first demonstration that the yolk protein genes are expressed in these specialized cells (83). This finding indicates that the border cells perform several roles in oogenesis as do the peri-oocyte follicle cells.

GG7K is also expressed in the peri-oocyte follicle cells but not expressed in the border cells of the follicle (Figure 11 B). Southern blot analysis using three *D. melanogaster* vitelline membrane genes as a "combined" probe carried out by other members of our laboratory indicate that the gene in clone GG7K is homologous to a vitelline membrane protein gene of *D. melanogaster* (84). As was true for the YP1 gene, *in situ* hybridization of the *D. melanogaster* equivalents of the GG7K gene did not establish whether these genes are expressed in the border cells. Thus again, our result is the first demonstration that these genes are not expressed in the border cells.

AA2 and 2G3 show expression in the germ-line tissue of the follicle (Figure 12, A and B). While restriction enzyme site maps of these clones are not available at this time, the transcribed fragments subcloned into Bluescribe for use in *in situ* hybridizations are indicated in Table 3. The transcripts for AA2 are localized in the nurse cell cytoplasm with very few grains seen over the nurse cell nuclei, oocyte or follicle cells. Transcripts for 2G3 are found throughout the nurse cell cytoplasm and oocyte. While the transcripts hybridizing to this clone are transcribed in the germ line tissue, it has not yet been determined if the higher grain density observed in the nurse cell cytoplasm relative to the oocyte is significant. Further analysis of these clones at later stages of oogenesis and early embryogenesis may be pursued at a later date by other members of the laboratory.
Figure 12. Localization of transcripts from the oogenesis-specific/differential genes in ovarian follicles. Hybridization patterns of $^{35}$S-labelled single-stranded RNA probes corresponding to the transcribed fragments (see text) of the two oogenesis-differential clones are shown. A. T3 strand hybridization of AA2 to a stage 4 follicle showing hybridization to the nurse cell cytoplasm. Nurse cell compartment above, oocyte below.
Figure 12B. Phase contrast photograph of T3 strand hybridization of 2G3 to a stage 4 follicle showing hybridization to the nurse cell cytoplasm and oocyte. Nurse cell compartments are uppermost, oocytes below.
SECTION II. ISOLATION AND CHARACTERIZATION OF OOGENESIS-
SPECIFIC, GERM-LINE EXPRESSED CLONES FROM Drosophila
melanogaster.

Screening of D. melanogaster Genomic Libraries.

As indicated in Section I above, Drosophila melanogaster genomic libraries were also differentially screened with our ovarian and embryonic Calliphora erythrocephala cDNA probes. Our rational was to directly identify D. melanogaster genes homologous between the two species that are oogenesis-specific and therefore eliminate one screening (see Isolation of D. melanogaster Homologs for the 18 Oogenesis-Specific/Differential Phage, Section 1) necessary if we first isolate C. erythrocephala oogenesis-specific clones and then isolate homologs. The first library screened was the Maniatis random shear library (83). 10,000 recombinant phage were screened differentially using cDNA probes prepared from PMS poly(A)⁺ RNA from C. erythrocephala PV and MN stage follicles, with cDNA made from PMS poly(A)⁺ RNA from C. erythrocephala 10±1 hr embryos as the non oogenesis probe. This number of phage represents 1.0 X 10⁵ kb of DNA or one half of one entire D. melanogaster genome and is one fifth the number of phage required to be 99% certain of screening all single copy DNA of the D. melanogaster genome.

By altering the wash conditions after hybridization of our cDNA probes, the stringency of hybridization was adjusted (75). The optimum hybridization conditions for DNA duplex formation are at the melting temperature (Tₘ) of the DNA minus 12⁰. 1% mismatched base pairs in the hybrid DNA decreases the Tₘ of the DNA duplex one degree. Many factors can influence the Tₘ of DNA hybrids. A 1% increase in formamide concentration of a DNA solution decreases the Tₘ of the DNA 0.7⁰C. The ionic strength (µ) of the solution also affects the Tₘ of DNA and can be related to the hybridization conditions by the equation:

\[(T_m)_{\mu_2} - (T_m)_{\mu_1} = 18.5\log_{10}(\mu_2/\mu_1)\]

were µ₁ and µ₂ are the ionic strengths of two hybridization solutions. Therefore, by adjusting the ionic strength of our washes, we could allow any degree of mismatched base pair hybridization between our cDNA probes and genomic DNA phage clones. However, increasing the amount of mismatch allowed in
DNA hybridization also increases the background hybridization signal. Because we were using *C. erythrocephala* probes to screen *D. melanogaster* genomic DNA, these screens were performed using conditions appropriate for hybridization of 10% mismatched hybrids. We chose these conditions as a compromise between detecting the highest possible number of *D. melanogaster* homologs and trying to keep the background hybridization down to workable levels.

The screening of this library was done at higher density than the *C. erythrocephala* library screening (approximately 5,000 plaques per plate). Therefore, 6 mm plugs each containing 10-12 plaques were pulled for each of the 46 oogenesis-specific signals obtained. These phage plugs were pooled into 15 groups of three plugs per group and rescreened at a density of 3,000 pfu/150 mm plate. 300 individual plaques were identified and isolated as putative oogenesis-specific clones. This large number of signals presumably resulted from duplications of the 46 original signals as a result of pooling the primary plugs. Eluates of these 300 plaques were rescreened as ordered arrays of lysed dots with 25 plaques being identified by this screen as potentially oogenesis-specific. These phage were rescreened as plaque purified phage in ordered arrays as above. Eight phage were selected as potentially oogenesis-specific and subjected to further analysis (Figure 13).

The second library that was differentially screened was a subset of approximately 600 phage selected from the Maniatis random shear library in P. Schedl's laboratory (personnal communication). These clones were selected from this library as hybridizing to *D. melanogaster* ovarian poly(A)+ RNA of *D. melanogaster*. They have also been hybridized to *D. virilis* ovarian poly(A)+ RNA to identify conserved sequences between these two species. These phage were sent to us by L. Ambrosio as phage lysates in micro-titer wells. Again, three rounds of differential screening were performed. 28 phage were selected from the primary screen as oogenesis-specific, rescreened to eliminate false positives and plaque purified. One phage was selected for further analysis as a potentially oogenesis-specific clone.

**Definitive Identification of Oogenesis-Specific clones by Southern Analysis.**

As a final test for oogenesis-specificity, the nine *D. melanogaster* putative oogenesis-specific phage isolated from these two screenings were analyzed
10,000 D. melanogaster recombinant phage

46 oogenesis-specific/differential signals pulled as large plugs, pooled 3 plugs/pool, rescreened

300 signals identified as oogenesis-specific/differential, re-screened

25 phage identified as oogenesis-specific/differential, plaque purified and re-screened

8 potential oogenesis-specific/differential phage clones

Figure 13. Flowchart representation of the D. melanogaster genomic library differential screening protocol.
by Southern blot analysis. Southern blots were prepared from three identical agarose gels of the phage DNAs double digested with the restriction enzymes Eco RI and Hind III. These filters were hybridized with $^{32}P$ radiolabelled ovarian and embryonic cDNA probes as described for the C. erythrocephala phage (see Definitive Identification of Oogenesis-Specific Clones from C. erythrocephala). H5C and A10B were again included as controls for hybridization to the cDNA probes. Two phage were selected from comparing the three autoradiographs as oogenesis-specific and selected for further analysis (Figure 14).

The phage clone DM3HH (DM) contained two fragments of 3.0 kb and 1.5 kb which hybridized weakly but uniquely to the MN stage cDNA probe. A 6.0 kb fragment showed expression in both the MN and ME stage cDNA probes. Further analysis established that the clone contained no Hind III sites and that these fragments were all Eco RI fragments. The rather faint signal detected for phage DM could indicate either a low abundance for this transcript during oogenesis or that the C. erythrocephala and D. melanogaster homologs for this gene are not very similar. Although in initial hybridizations the 1.5 kb Eco RI fragment appeared to hybridize more strongly to the MN stage probe, in later hybridizations both with the whole phage and subcloned fragments, the 3.0 kb fragment gave a more reproducible signal. Therefore, the 3.0 kb Eco RI restriction fragment was used in all further experiments (see below).

The initial hybridization with clone DA005C38 (DA) indicated that three fragments of 5.8, 1.8 and 0.7 kb have regions expressed much more strongly in oogenesis than embryogenesis. Further hybridizations and restriction enzyme site mapping established that the signal detected at 5.8 kb was due to a partial digestion product. In addition, what appeared to be hybridization of the embryonic cDNA probe to a 1.7 kb fragment of this clone proved on subsequent hybridizations to subcloned fragments to be an artifactual background problem. The 1.8 kb fragment, which proved to be an Eco RI/Hind III fragment, was used in all further experiments (see below). The results of this analysis are summarized in Table 4.

Localization of Transcripts for DM and DA in the D. melanogaster Ovary.

To test whether these genes were expressed in the germ-line or somatic tissue of the follicle, in situ hybridization to whole D. melanogaster ovaries was
Figure 14. Definitive Identification of Oogenesis-Specific Clones by Southern Blot Analysis. Suitable digests of clones that survived three rounds of differential screening (see text) were used to prepare identical Southern blots for hybridization to the three cDNA probes. The hybridization pattern of the 19 clones are shown. PV, MN and ME = hybridization patterns to cDNA probes from PV follicles, MN follicles and ME embryos respectively. Only those clones showing oogenesis specific hybridization are labelled. The clones identified as showing oogenesis-specific/differential expression are summarized in Table 4.
Table 4. Summary of the Two *D. melanogaster* Phage Clones Isolated as Oogenesis-Specific by Southern Blot Analysis. PV, MN and ME = previtellogenic, maximal nurse cell and mid embryos, respectively.

<table>
<thead>
<tr>
<th>CLONE</th>
<th>PV</th>
<th>MN</th>
<th>ME</th>
<th>FRAGMENT SIZES (KB)</th>
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<tbody>
<tr>
<td>DA005C38</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>1.8</td>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>0.7</td>
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<tr>
<td>DM3HH</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>6.0</td>
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<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>3.0</td>
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<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>1.5</td>
</tr>
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</table>

\(^a\)"+" signs signal hybridization, the intensity of hybridization is indicated by the number of signs with the weakest hybridization intensity equal to the lowest number of signs ,"-" signs indicate no hybridization. \(^b\)Restriction enzyme fragment sizes from the digests shown in Figure 14.
performed. The oogenesis-specifically transcribed fragments of DM and DA, shown in Figure 16 (see Generation of Restriction Enzyme Site Maps for DA and DM below) were subcloned into Bluescribe and $^{35}$S cRNA probes prepared. Whole ovaries were dissected, fixed and hybridized with the single stranded, strand-specific probes. The tissues were then washed, embedded in wax, and sliced into six micron sections. After autoradiography, the sections were examined under the microscope and the hybridization pattern analyzed.

DM3HH hybridizes very strongly to the previtellogenic and very early vitellogenic follicles (Figure 15 A). The hybridization is seen throughout the nurse cell cytoplasm with background hybridization seen throughout the rest of the follicle.

DA005C38 also hybridizes to both previtellogenic and maximal nurse cell stage follicles in the nurse cell cytoplasm, but its time of maximal expression differs from DM (Figure 15 B). Whereas DM appears to be maximally expressed in early stage follicles, DA shows maximal expression during the middle vitellogenic stages of *D. melanogaster* oogenesis. Transcripts for both DM and DA are also detected in the mature follicle.
Figure 15. Localization of transcripts from the oogenesis-specific genes in *D. melanogaster* ovaries. Hybridization patterns of 35S-labelled single-stranded RNA probes corresponding to the transcribed fragments of the two oogenesis-specific clones are shown. A. T7 strand hybridization of DM3HH to previtellogenic stage follicles. Hybridization throughout the nurse cell cytoplasm (NCC) is seen.
Figure 15B. T3 strand hybridization of DA005C38 to vitellogenic stage follicles. This unstained section shows the strong expression of DA005C38 in the NCC of stage 10 follicles.
CHAPTER FOUR

FURTHER CHARACTERIZATION OF DA005C38 AND DM3HH

**Generation of Restriction Enzyme Site Maps for DA and DM.**

Restriction enzyme site maps were generated for the two phage clones DA and DM by Southern blot analysis using a technique developed in our laboratory by Michael Nazimiec. DNA prepared from the two parent phage was digested individually with each of the restriction enzymes to be examined. One restriction digest was chosen as the "probe" digest and each of the fragments from that digest was isolated and individually oligolabelled using $^{32}$P dCTP. A Southern blot was prepared of each phage digested with each of the restriction enzyme digests to be examined for hybridization to each of the radiolabelled "probe" fragments. After hybridization and autoradiography, the pattern of hybridization of each "probe" fragment to the fragments within each of the other digests was analyzed. These hybridization patterns and the known sizes of fragments within each digest permitted ordering of the restriction enzyme sites. Figure 16 A and B shows the restriction enzyme site maps maps of the two clones.

Since the "probe" digest itself was included on all of these Southern blots, homology between individual fragments of this digest could be detected. No homologies of this kind were detected for clone DM, but several of the DA fragments were found to cross-hybridize. From an analysis of the cross-hybridization patterns the number and location of the internal homologies identified could be determined. Of the Eco RI fragments of the clone, the 5.8 kb fragment cross-hybridized to the 2.1 and 1.7 kb fragments, but these two fragments do not show homology to one another, although both show the expected hybridization to the 5.8 kb fragment. Similarly the 2.1 and 3.5 kb Eco RI fragments cross-hybridize to one another but the 3.5 kb fragment shows no homology to the 5.8 kb or the 1.7 kb fragments. Thus these cross-hybridization patterns indicate that the clone must contain at least three different internal repeats. The internal repeating elements were further analyzed by hybridization of the individual Hind III fragments of the clone to the fragments in Hind III, Eco RI and Hind III/Eco RI double digests of the clone. This allowed better localization of the repeat elements but also revealed the greater
Figure 16. Structure of DM3HH and DA005C38. Restriction enzyme site maps for both clones are shown. Fragments containing oogenesis-specific transcripts are indicated by (■■■■) below the maps. The restriction fragments used for in situ hybridizations are shown by (−) and "probe". A. Restriction enzyme site map of DA005C38. Regions of the clone cross hybridizing with each other are indicated by the patterned boxes. Matching boxes show homology to each other. The exact location of these homologies has not been determined, they have only been localized to the Eco RI, Eco RI/Hind III or Hind III fragments concerned. Areas showing homology to a 1.5 kb cDNA clone isolated using the "probe" fragment (see text) are indicated by (■■■■) beneath the map. The arrow head on the "probe" fragment indicates the direction of transcription within this fragment.
Figure 16 B. The restriction enzyme site map of DM3HH. aThis is the only site for the restriction enzyme Pst I mapped in the clone DM. bThe position of the 0.5 kb Bam HI fragment could not be localized further. The two possible positions of this fragment are indicated by the dashed lines. The position of a 0.2 kb Bam HI restriction fragment could not be identified. cThe two possible locations of this 0.2 kb Eco RI fragment are shown (dashed lines).
complexity of the pattern of internal repeats within the clone. The final analysis provided a consistent pattern of at least five pairs of internal repeats localized within the Eco RI/Hind III fragments as shown in Figure 16 A. Sequence studies will be necessary to identify the extent of homology between these various pairs of elements.

**Localization of DM and DA within the *D. melanogaster* Chromosome Complement.**

To localize the region of the *D. melanogaster* genome represented by the clones DA and DM, *in situ* hybridization to the polytene chromosomes of third instar larvae salivary glands was performed. Salivary glands were dissected from wandering stage third instar larvae and fixed for five minutes in ethanol:acetic acid. The glands were transferred to a glass slide and squashed in 45% acetic acid. After treatment with RNase, acetylation, and denaturation, the chromosomes were hybridized with 35S radiolabelled cRNA from the transcribed fragments shown in Figure 16 overnight at 37°C. The chromosome squashes were then washed and autoradiographed for one to three days.

The results of these experiments are shown in Figure 17. DM hybridizes to the 98 E/F region of the right arm of chromosome 3. DA hybridizes to the 31 B/D region on the left arm of chromosome 2.

**Further Mapping of DA and DM and Correlation of DA and DM with Known *D. melanogaster* Mutations.**

DM is located in a poorly characterized region of the *D. melanogaster* genome. There are no known deficiencies which cover this region of the *D. melanogaster* genome. Although intensive screening for maternal effect and female sterile mutations on the third chromosome has been performed (86, C. Nusslein-Volhard, personal communication), none have yet been mapped to this region. Therefore, it appears that DM is an example of a gene that is expressed during oogenesis but that genetic screenings have not yet been able to identify.

In contrast, the 31 B/D region in which DA is located is rich in genetic information. Several deficiencies with known breakpoints in this region have been isolated (87). Four of these deficiencies with the breakpoints defined by D. Sinclair and J. Tonkiel (personal communication) were provided by D.
Figure 17. Localization of DM3HH and DA005C38 within the *D. melanogaster* genome. The hybridization of $^{35}$S cRNA probes to polytene chromosomes of *D. melanogaster* third instar larvae salivary glands are shown. Arrows point to the region of hybridization. A. Hybridization of DM3HH. Hybridization to the 98 E/F region of the right arm of chromosome 3 is seen.
Figure 17B. Hybridization of DA005C38. Hybridization to the 31 B/D region of the left arm of chromosome 2 is observed.
Sinclair's laboratory (Figure 18). In addition, T. Shupbach has isolated a series of female sterile mutations on the second chromosome, several of which have been mapped to this region (88, personal communication). Using complementation analysis, Kathy Beckingham has genetically mapped these female sterile mutations within the deficiencies. These deficiencies were used to define further the location of the DA gene and thus to identify possible mutations that may correspond to this gene. Southern hybridizations to DNA derived from embryos homozygous for each of the deficiencies were used for these mapping studies. Homozygous deficiencies are usually embryonic lethal and therefore homozygous embryos can be separated from wild type embryos since the wild type embryos in an egg collection will hatch while the embryos homozygous for the deficiency will not. It is important to note that the DNA obtained from "dead" embryos is not degraded. The term dead refers to organismal death and not cell death. DNA prepared from embryos homozygous for each deficiency can then be digested, blotted and hybridized with radiolabelled DNA from clone DA. If the region of the genome corresponding to clone DA is deleted from the DNA from the embryos homozygous for a given deficiency, theoretically there will be no hybridization of clone DA to the Southern blot. However, a certain percentage of unhatched wild type embryos will be collected with the homozygous deficiency embryos. As a result, some hybridization of clone DA will be present as a result of wild type contamination. The known breakpoints of that deficiency will therefore limit the region of the D. melanogaster genome within which the DA clone can be located.

DNA was prepared from embryos homozygous for each deficiency (Figure 19), digested with Eco RI and subjected to electrophoreoses through 0.6% agarose gels. Eco RI digested DNA prepared from wild type Oregon R embryos was also included on each gel. Southern blots were prepared from these gels and hybridized with the 1.8 kb Eco RI/Hind III fragment of clone DA oligolabelled using $^{32}$P dCTP. A hybridization signal which is present in all of the DNA preparations on the Southern blot and therefore serves as a control for differences in the amounts of DNA loaded in the individual lanes of the blot was also desired. Therefore, the 2.0 kb Eco RI/Pst I fragment of clone DM $^{32}$P oligolabelled was also hybridized to the Southern blots. The DA fragment will hybridize with a 5.8 kb Eco RI genomic fragment while the DM probe will
Figure 18. Deficiency mapping of clone DA005C38 and correlation with known maternal effect mutations. The 30-33 region of chromosome 2 is shown with the regions deleted for J-der 2, J-der 77, J-der 27 and J-der 106 indicated by black bars beneath the chromosome. The female sterile mutations isolated by T. Shupbach and mapped within the deficiencies by K. Beckingham are also shown. Autoradiographs of Southern Blots hybridized with the "probe" fragments of DM3HH and DA005C38 (Figure 15) are shown. DA005C38 hybridizes to a 5.8 kb fragment in Eco RI digests of genomic DNA while DM3HH hybridizes to a 3.0 kb Eco RI genomic fragment. The DM3HH fragment serves as a control for the amount of DNA in each lane while wild type Oregon R DNA (+) provides a control for hybridization intensities. Hybridization of DA005C38 is seen in J-der 77 (77), J-der 27 (27) and J-der 106 (106) DNA while it is absent from J-der 2 (2) DNA. Partial digestion products are present in the wild type lane of the J-der 77 Southern blot hybridization.
Figure 19. Crosses performed to isolate homozygous deficiency embryos. Stock deficiency flies maintained over a CyO balancer were mated with wild type Oregon R flies. Straight wing progeny were collected as virgins and maintained at 17°C until ~100 flies of each sex were obtained. These siblings were mated and embryos collected over a 12 hour period. After incubation at room temperature for 32 hours, unhatched embryos were collected and stored at -70°C.
hybridize with a 3.0 kb Eco RI fragment (see Figure 16).

The results of this analysis are shown in Figure 18. As can be seen, the deletions J-der 27, J-der 106 and J-der 77 do not delete the region of the genome corresponding to clone DA, therefore the DA gene can not lie in the 31 C/D region of the chromosome. The J-der 2 deletion does lack the DNA corresponding to clone DA establishing the position of this DNA in the 31 B region of chromosome 2. The only known female sterile mutation mapping in the 31 B region is chalice. Nothing is known about this mutation except that it causes female sterility.

Isolation of cDNA Clones for DA and DM.

An embryonic cDNA library prepared in vector λGT10 obtained from the D. Hogness laboratory was screened using the transcribed fragments of both DA and DM. 50,000 plaques from each of five distinct sub-pools of the library were grown on 150 mm L-plates and duplicate filters were prepared. After processing, the filters were hybridized with $^{32}$P oligolabelled transcribed fragments (Figure 16) of the two clones. After autoradiography, signals were related back to the phage plate and 6 mm plugs removed containing several phage. These phage were rescreened at low density (250 pfu/75 mm plate) as above and individual plaques corresponding to positive signals selected. These plaques were rescreened a third time to ensure they were plaque purified.

Screening of this library produced no cDNA clones corresponding to the clone DM. However, three clones containing cDNAs of 1.5, 0.8 and 0.4 kb in length were isolated for clone DA. The 1.5 kb cDNA contains an internal Eco RI restriction enzyme site that cleaves the insert DNA into fragments of 1.3 kb and 0.2 kb. The hybridization of these two fragments to various digests of the genomic DA clone were analyzed by Southern blot analysis. The results of these experiments are represented in Figure 16 A. The hybridization of the 1.3 kb fragment is limited to the 1.8 kb Eco RI/Hind III fragment. Hybridization of this 1.3 kb fragment to digests of clone DA in which the 1.8 kb Eco RI/Hind III fragment was further cleaved at its Bam HI or Kpn I sites revealed that the this fragment showed homology to all three regions of the 1.8 kb fragment defined by these digests. The 0.2 kb Eco RI fragment of the cDNA clone hybridizes to the 1.1 and 0.7 kb Eco RI/Hind III restriction enzyme fragments of clone DA.
Since the hybridization of the 1.3 kb Eco RI fragment of the 1.5 kb cDNA clone is limited to the 1.8 kb Eco RI/Hind III fragment of the genomic DA clone but hybridization of the 0.2 kb Eco RI fragment to the 1.1 kb and 0.7 kb Eco RI/Hind III fragments of the genomic clone is observed, the 1.5 kb cDNA clone contains at least two exons localized in the genomic DA clone. Sequencing of the 0.2 kb Eco RI fragment of this cDNA clone (data not shown) has shown that there are no Hind III sites present within this fragment. Therefore, the hybridization of the 0.2 kb fragment to the 1.1 kb and 0.7 kb Hind III fragments of the genomic DA clone can not be the result of one exon. This result, along with those above, indicate that there are at least three exons present in the genomic DA clone showing homology with this cDNA clone.

At a gross level, the 0.4 and 0.8 kb cDNA clones appear to be shorter versions of the 1.5 kb cDNA. These clones both hybridize to the 1.3 kb Eco RI fragment of the 1.5 kb cDNA clone but do not hybridize to the 0.2 kb Eco RI fragment of this clone. Further analysis of these two cDNAs will be required to establish whether these clones are indeed shorter versions of the largest cDNA clone.
CHAPTER FIVE

DISCUSSION

Limitations of the Screening Procedure.

We have isolated oogenesis-specific, germ-line expressed clones from both *D. melanogaster* and *C. erythrocephala* genomic libraries. To accomplish this task, we used cDNA probes specific for two stages of oogenesis and cDNA prepared from 10±1 hour embryos as the differential screening agent. Only transcripts of medium or high abundancies are likely to be represented in our probe preparations in quantities high enough to detect a signal in our differential screening procedure. All clones isolated as oogenesis-specific that have been examined by Northern analysis (data not shown) and/or in situ hybridization to date have proved abundant enough to give readily detectable signals. These results indicate that we are unlikely to find genes expressed at low levels using our differential screening procedure. We used cDNA probes instead of kinased poly(A)+ RNA to lessen the degree of contamination of our probes with rRNA. While successful on this point, the cDNA probes have problems unique to themselves. Some transcripts are resistant to the formation of cDNA probes. Even though the RNA is denatured before cDNAs are prepared, secondary structure can interfere with the formation of cDNA as evidenced by the aberrant cDNAs found in any cDNA library. Because of this, certain messages may not be represented in the cDNA population and therefore will not be detected.

Further limitations to our screening procedure derive from the problems of reproducibility within the technique itself. In the primary screening performed by Esther Belikoff and myself, 265 primary signals were found of which only 12 proved to be oogenesis-specific after further screening. That means that 95% of the primary signals obtained were false positives, clearly an unattractive number to contemplate. An even more disturbing thought is the possible number of false negative signals obtained due to high background hybridization levels. The high background levels are obtained whenever heterogeneous probes (e.g. cDNA probes) are used in a screening procedure and probably result in many clones not being selected as oogenesis-specific. The cumulative
effects of differential phage growth, uneven transfer of phage DNA to nitrocellulose filters and variation in each cDNA preparation all probably contributed to the variability of signals seen from one screening to the next and hindered the analysis of the clones selected. This lack of reproducibility necessitated the final definitive differential screen using Southern blots of individual phage DNAs. This final screen eliminated the problems of differential growth and also improved detection of hybridization signals since much more phage DNA was available for hybridization. This analysis also indicated to us the restriction fragments within each phage clone containing regions transcribed in an oogenesis-specific manner.

A final limitation inherent in the differential screening technique is the problem of clones containing constitutively transcribed DNA along with oogenesis-specifically transcribed DNA. It is impossible to estimate the number of clones that we were not able to detect as oogenesis-specific because these regions were masked by some other transcription unit within that clone showing expression during mid embryogenesis. Alternate exon usage (88) could also mask an oogenesis-specific transcript if the alternative transcript(s) were not expressed in a differential manner. However, based on the number of clones we have isolated as oogenesis-specific that do contain other regions showing constitutive expression, this problem may not be as severe as might be predicted (see Table 3 above). This problem may not be as severe in the screening of the *C. erythrocephala* genomic libraries as in screening *D. melanogaster* libraries. The existence of overlapping transcription units (TU)s in *D. melanogaster* has been demonstrated (89, 90, R. Schultz, personal communication). Unless both TU}s of an overlapping pair were expressed in an oogenesis-specific manner, such clones would probably not be detected by our screening procedure. Our approach of isolating oogenesis-specific clones from *C. erythrocephala* libraries and then finding their *D. melanogaster* homolog should alleviate the problem of overlapping TU{s. Another approach that we have used to alleviate this problem is to concentrate on isolating clones that are expressed differentially, as opposed to specifically, at relatively low levels during oogenesis. Three of the four clones isolated as being expressed at high levels strictly during oogenesis were found to be transcribed in the follicle cells of the developing egg. Given these results, we are now giving higher priority to
those clones showing differential expression at relatively low levels during oogenesis.

Nevertheless, in screening 1/4 the number of phage required to ensure screening the entire *C. erythrocephala* genome, we have been able to identify 22 oogenesis-specific or differential clones by our differential screening procedure. We have also been able to either directly or indirectly isolate 13 *D. melanogaster* clones with our *C. erythrocephala* probes. The data to date indicate that these clones represent different regions of the genome and therefore are not multiple isolations of a few genes. Tables 5 summarizes the known characteristics of each clone.

**Oogenesis-Specific/Differential Clones have been Isolated from C. erythrocephala.**

The screens performed by A. Rubacha, E. Belikoff and myself have led to the identification of 22 oogenesis-specific/differential clones. We have to date screened 1/4 of the number of clones required to be 99% sure of covering the entire *C. erythrocephala* genome. This is clearly not a complete screen and further screening should reveal many more clones. Eight of the 22 clones appear to be absolutely oogenesis-specific, no hybridization to the embryonic probe is seen even after very long exposures. Nine of the 22 clones hybridize to probes prepared from mature follicles. Since this indicates the genes within these clones are expressed in the germ-line cells of the follicle, these clones are strong candidates for further analysis.

**Very Few Clones Show Maximal Expression in Previtellogenic Stage Follicles.**

Only one of the clones isolated was expressed maximally in the previtellogenic stages of oogenesis, and its expression at this stage was barely detectable. Although it seems highly likely that stage specific transcription of some genes occurs during these stages of development, this finding suggests to us that the transcripts of such genes are of low abundance and not readily detectable by a screening procedure such as we have performed.

**Somatic Clones of Known Function and Germ-Line Expressed Clones of Unknown function have been Isolated.**
Table 5. Summary of *C. erythrocephala* phage oogenesis-specific/differential, germ-line expressed clones. PV, MN, MO and ME = previtellogenic, maximal nurse cell, mature oocyte and mid embryo, respectively.

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<sup>a</sup>"+" signs signal hybridization, the intensity of hybridization is indicated by the number of signs with the weakest hybridization intensity equal to the lowest number of signs , "-" signs indicate no hybridization and "U" indicates these phage have not been checked by that experiment. NCC = nurse cell cytoplasm. <sup>b</sup>Clones originally isolated by A. Rubacha. Partial digestion fragments for clone D2B are not listed. <sup>c</sup>Restriction enzyme fragment sizes from the digests shown in Figure 8.
Clones A10B and GG7K are expressed in the somatic tissue of the developing follicle. The oogenesis-specific gene within A10B has been shown to be homologous to yolk protein 1 in *D. melanogaster*. Synthesis of A10B transcripts was detected in two populations of cells - the columnar follicle cells surrounding the oocyte compartment of the follicle and the small group of migratory border cells. A common feature of these cells is that, in the vitellogenic stages of oogenesis, both share cell-cell contact with the oocyte proper, that is, the final destination of their yolk protein products. It would be interesting to determine whether the border cells synthesize yolk protein transcripts before this contact is made and thus whether this cell-cell interaction might be part of the stimulus for yolk protein synthesis by these cells. The only function known previously for the border cells was the formation of the micropyle. Our findings indicate that, like the follicle cells surrounding the oocyte, these cells are multipurpose, performing different functions at different stages of oogenesis. GG7K is transcribed only in the columnar follicle cells but not in the border cells and is homologous to a *D. melanogaster* vitelline membrane protein. It has not been established whether the equivalent vitelline membrane protein genes of *D. melanogaster* are expressed in the border cells.

Three of the 22 clones isolated by the total screening to date have been found to be expressed in the germ-line cells of the follicle. Grains corresponding to the transcripts of AA2 were found throughout the nurse cell cytoplasm with very few grains seen in the oocyte, nurse cell nuclei or follicle cells. Grains corresponding to the transcripts of 2G3 were found throughout the nurse cell cytoplasm and oocyte. No specific localization patterns for the hybridization of these clones were observed.

*In situ* hybridization performed by T. Valoir on the clone C7K, one of the four oogenesis-specific clones isolated by A. Rubacha also showed grains corresponding to transcripts localized in the nurse cell compartment of the follicle. However, these transcripts often appeared to be more intense over the nurse cell nuclei.

*Oogenesis-Specific Genes in D. melanogaster can be isolated using C. erythrocephala cDNA Probes.*

Tamsen Valoir and Kathleen Gajewski have isolated *D. melanogaster* homologs for 11 of the 18 *C. erythrocephala* phage clones. Our rationale of
using related species to isolate developmentally expressed genes appears to be a functional approach to isolate genes that might not be detected by the genetic screenings. *in situ* hybridization of the *D. melanogaster* homologs of these genes will be performed to determine whether any map near loci known to be active during oogenesis or embryogenesis and showing female sterile or maternal effect mutations.

Using *C. erythrocephala* ovarian probes we have been able to identify 2 oogenesis-specific genes in *D. melanogaster*. To date we have screened 1/5 the total number of phage required to be 99% sure of covering the entire *D. melanogaster* genome. This is clearly not an exhaustive screen and further screening should reveal many more clones. However, because *D. melanogaster* has an unusual genome organization relative to many organisms and the positive results of our earlier screening of *C. erythrocephala* genomic libraries isolating *D. melanogaster* homologs, it is unlikely that further direct screening of *D. melanogaster* libraries will be performed. Both DM and DA are expressed in the previtellogenic and vitellogenic stages of oogenesis. Table 6 summarizes the characteristics of these clones.

**Both DM and DA are Expressed in the Germ-line Tissue of the Follicle.**

Both DA and DM are expressed in the germ-line tissue of the developing follicle as shown by *in situ* hybridization to *D. melanogaster* whole ovaries. DM is expressed very strongly in the previtellogenic stage follicles and remains throughout oogenesis. The transcripts are found in the nurse cell cytoplasm with few grains present in the nurse cell nuclei or follicle cells. The data suggest that the transcript does not accumulate throughout oogenesis, but instead is transcribed only during the previtellogenesis stages of oogenesis. The concentration of transcripts is then diluted as the follicle grows throughout oogenesis.

DA is expressed in the PV and MN stage follicles, although in a different pattern than DM. The transcripts corresponding to this gene appear to be expressed at low levels until stages 8-10 of oogenesis. At this time, very high levels of transcripts are found in the nurse cell cytoplasm but not in the nurse cell nuclei or follicle cells. It is interesting that the high concentration of transcripts seen during stages 8-10 is diluted considerably during further growth of the follicle. Low levels of this transcript are found in the mature oocyte.
Table 6. Summary of the two *D. melanogaster* phage oogenesis-specific germ-line expressed clones. PV, MN and ME = previtellogenic, maximal nurse cell and mid embryos, respectively.

<table>
<thead>
<tr>
<th>CLONE</th>
<th>PV</th>
<th>MN</th>
<th>ME</th>
<th>FRAGMENT SIZES (KB)</th>
<th>HYBRIDIZATION LOCALIZATION</th>
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<td>1.5</td>
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</tr>
</tbody>
</table>

\(^a\)"+" signs signal hybridization, the intensity of hybridization is indicated by the number of signs with the weakest hybridization intensity equal to the lowest number of signs , "-" signs indicate no hybridization. \(^b\)Restriction enzyme fragment sizes from the digests shown in Figure 14. \(^c\)NCC = nurse cell cytoplasm.
Both DM and DA have been localized in the *D. melanogaster* Genome.

DM has been located at 98 E/F on the third arm of chromosome three by *in situ* hybridization to the polytene chromosomes of *D. melanogaster*. Because there are no known deficiencies in this region of the *D. melanogaster* genome, the DM location could not be further defined. Genetically, there are no known female sterile mutations mapping to this region of the genome. However, Ait-Ahmed et al (31) recently reported the location of a cluster of genes differentially expressed at the 98 F3-10 region of chromosome 3. Nine transcripts from this region with varying developmental expression profiles were found. Using the transcribed region of clone DM as probe, at least five transcripts are seen on Northern blots of *D. melanogaster* RNA prepared from early embryos (unpublished data). On comparison of the restriction maps of the two regions and the size of the transcripts seen by Northern blot analysis, it is clear that the genes isolated by Ait-Ahmed et al are not the same gene as DM. It is interesting to note the clustering of genes showing a similar expression pattern in this region of the *D. melanogaster* genome possibly suggesting the existence of a large gene complex controlling an as yet undefined aspect of early development.

DA hybridizes to the 31 B/D region of the left arm of chromosome 2. By using deficiency mapping in this region, we were further able to localize this hybridization to the 31 B region. Several female sterile mutations map in the 31 B/D region of the *D. melanogaster* genome including daughterless and trunk (Figure 18). Kathy Beckingham performed a complementation analysis of these mutations with the J-der series of deficiencies mapping these mutations within the deficiencies. Based on the deficiency mapping and the complementation analysis, DA could possibly be chalice. Nothing is known about this locus except that it causes female sterility. Further analysis of DA will be required to determine if indeed this is the chalice locus.
REFERENCES


