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Germ-line Development and Fertilization

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I. INTRODUCTION
Classically, nematode germ cells have provided an excellent source of material for studies of fundamental questions of cell biology. In 1883, Van
Beneden used the germ cells of the parasitic nematode, Parascaris equorum to show that germ cells contain only a haploid number of
chromosomes, compared with the diploid number found in somatic cells. This study led Boveri and others to propose that chromosomes are the cellular component that provides genetic continuity from one generation to the next (Wilson 1896). Subsequently, several workers exploited the simplicity and clarity of the cellular architecture of nematode germ cells to explore chromosome structure and the role of the centrosome in cell division.

Today, nematode germ cells continue to provide unique advantages for the study of basic questions of cell biology and development. How is a germ cell signaled to leave the mitotic cell cycle and to enter meiosis? How is a germ cell specified to differentiate as a sperm or as an oocyte? And how is this differentiation carried out? What is the molecular mechanism of ameboid motion? The answers to these questions are beginning to emerge from studies of Caenorhabditis elegans germ cells, using a combination of morphological, genetic, and biochemical approaches.

In this chapter, we cover those aspects of C. elegans germ-line development that are unique to germ-line tissue. These include the processes of spermatogenesis, oogenesis, and fertilization, as well as a discussion of the control of germ-line proliferation and the onset of meiosis. In addition, we summarize our current knowledge of the cell biology of C. elegans germ cells. Here, we know much more about sperm than we know about oocytes—in part, because sperm and spermatocytes can be isolated in quantity and in part because spermatogenesis can be studied in vitro. Embryonic determination of the germ lineage is covered in Chapters 5 and 8, and the decision between spermatogenesis and oogenesis is discussed in Chapter 9.

II. OVERVIEW OF GONADAL ANATOMY AND DEVELOPMENT

The main features of the anatomy and development of the C. elegans reproductive system are summarized below and diagramed in Figure 1. More thorough discussions of these subjects can be found in Chapters 4, 5, and 9 and in the original papers (Hirsh et al. 1976; Klass et al. 1976; Kimble and Hirsh 1979; Kimble and White 1981).

A. The Gonadal Primordium

In both sexes, an L1 worm hatches with a gonadal primordium consisting of four cells: two precursors of the somatic gonad, Z1 and Z4, and two precursors of the germ-line tissue, Z2 and Z3. Z1 and Z4 descend from the embryonic precursor cell, EMS; Z2 and Z3 arise as daughters of the embryonic stem cell, P. The gonadal primordia of the two sexes are morphologically identical; however, the adult gonads of the two sexes differ dramatically. The adult hermaphrodite gonad retains the twofold rotational symmetry of the four-cell primordium, whereas the adult male gonad is asymmetrical. This sexual dimorphism results from differences in development of the somatic gonad in the two sexes.

B. Anatomy of the Adult Gonad

The hermaphrodite adult reproductive system consists of two tubular ovosteses, one anterior and one posterior. The ovosteses are joined centrally by two spermathecae and a uterus; the uterus opens mid-ventrally to the exterior via the vulva. The male reproductive system consists of a single
tubular testis that is connected to the cloaca via the seminal vesicle and vas deferens. Each hermaphrodite ovotestis and the male testis is actually a U-shaped tube possessing distal and proximal arms that are joined by a loop. (The distal–proximal axis is used to describe relative position along the length of the tubular gonad; a distal structure is defined as being further from the gonadal opening to the exterior than a proximal structure. In hermaphrodites, this opening is the vulva; in males, it is the cloaca.)

In both sexes, the distal arm is composed primarily of immature germ-line tissue. Somatic cells in the distal arm include one (hermaphrodites) or two (males) distal tip cells, located at the apex, and two somatic epithelial cells in the hermaphrodite. The germ-line nuclei of the distal arm include mitotic nuclei most distally and meiotic nuclei more proximally. The nuclei in meiosis progress from leptotene distally through diplotene of meiotic prophase I proximally. The distal arm contains a central, anucleate core of cytoplasm, surrounded by a peripherally disposed layer of germ-line nuclei. An incomplete plasma membrane demarcates an alveolus of cytoplasm for each nucleus, such that the cytoplasm of each germ “cell” is continuous with the central core of cytoplasm that extends the length of the distal arm. The germ-line tissue is therefore a syncytium from the distal tip to the loop region where differentiation of germ-line cells into sperm or oocytes begins. By Nomarski microscopy, one can see that the anucleate core of the distal arm is enlarged and granular in a gonad that is making oocytes, whereas it is barely detectable in a gonad making sperm.

In both sexes, the proximal arm is the site of gametogenesis. In hermaphrodites, the germ line of this arm is encapsulated by a somatic contractile epithelial sheath, or oviduct. In males, the germ line is only partially ensheathed by the distal cells of the seminal vesicle.

Sperm are produced continuously in males, whereas they are made only transiently in hermaphrodites. Sperm maturation culminates at the proximal edge of the proximal arm in the two meiotic divisions that generate four haploid sperm from each tetraploid primary spermatocyte. After about 150 sperm are made in each ovotestis, germ cell differentiation switches to oogenesis, and thereafter only oocytes are produced. Developing oocytes are arranged in single file along the hermaphroditic proximal arm. Oocytes become arrested at diakinesis in meiotic prophase I. When an oocyte is fertilized, the zygote moves through the spermatheca to the uterus where meiosis of the oocyte nucleus is completed. Two polar bodies are extruded, and the two pronuclei become apposed after a complex series of movements. The pronuclear membranes then break down, and embryonic divisions begin (Albertson 1984a). No zygote nucleus is formed.

C. Postembryonic Development of the Gonad

Soon after the first divisions of Z1 and Z4, the hermaphroditic gonad can be easily distinguished from the male gonad. In hermaphrodites, Z1 and Z4 together generate 12 cells during L1. During L2, ten descendants of Z1 and Z4 coalesce toward the center of the gonad. This rearrangement establishes two separate regions of germ-line tissue—one anterior and one posterior to the central cluster of somatic cells. These two regions of germ-line tissue develop into the anterior and posterior ovotestes. As larval development proceeds, the somatic cells in the center of the gonad continue to divide according to an invariant pattern of divisions to generate the uterus, spermathecae, and oviducts of the adult hermaphroditic gonad. The two growing ovotestes elongate, one anteriorly and the other posteriorly, during L3. Then, early in L4, each ovotestis turns 180° and continues to elongate in the opposite direction. This directed elongation forms the U-shaped ovotestis. A somatic distal tip cell is positioned at the tip of each elongating ovotestis. These cells do not divide and remain in place throughout adulthood.

In males, Z1 and Z4 together generate ten cells during L1 and L2. The original twofold rotational symmetry of the gonadal primordium is abandoned during L1, as eight somatic cells move to the anterior end and two somatic cells, the distal tip cells, become located at the posterior end of the developing gonad. The testis elongates first in an anterior direction and then reflexes and elongates posteriorly towards the cloaca to make the U shape of the single testis. During L3 and L4, the somatic cells at the elongating end of the gonad generate the vas deferens and seminal vesicle of the male somatic gonad. As in hermaphrodites, the distal tip cells remain at the distal end with no further division.

D. Postembryonic Development of the Germ Line

The postembryonic divisions of the germ-line precursor cells, Z2, Z3, and their descendants are variable. Germ-line divisions are unpredictable with respect to time and plane of division. The number of germ-line nuclei increases during larval growth from 2 to about 1000 in hermaphrodites and to about 500 in males. This number continues to increase during adulthood, because the most distal germ-line descendants continue mitotic division throughout adulthood.

Although embryonic determination of the germ lineage appears to rely on cell ancestry (Chapter 5), postembryonic determination of the germ-line descendants as mitotic or meiotic nuclei and as sperm or oocytes has no apparent dependence on cell ancestry. As discussed below, determination of germ-line nuclei as either mitotic or meiotic relies on their interactions with a somatic cell—the distal tip cell. Determination of germ cells as sperm or oocytes is discussed in Chapter 9.

Germ-line cells first leave the mitotic cell cycle to enter meiosis at the proximal edge of the developing ovotestis or testis. Because the earliest stages of meiotic prophase are difficult to detect, the appearance of pachytene nuclei has been used to indicate entry into meiosis. In hermaphro-
dites, pachytene nuclei are first detected during L3 lethargus (33–34 hr, 20°C, after hatching). In males, they are first seen in mid-L3 (29–32 hr, 20°C, after hatching). As the gonads elongate, more and more distal nuclei enter meiosis. However, a zone of nuclei at the distal end of the gonad always remains mitotic.

Gamete differentiation first occurs at the proximal edge of the developing ovotestis or testis. Primary spermatocytes are first detected at mid-L4 in males and at late L4 in hermaphrodites. More distal nuclei begin to undergo gametogenesis subsequently, so that a progression of stages of gamete maturation can be observed in the proximal arm, with more immature gametes found near the loop region and more mature ones found near the spermatheca. Oocytes begin to form soon after the hermaphrodite molts to adulthood.

III. CONTROL OF PROLIFERATION AND ENTRY INTO MEIOSIS

A. Description of Distal Tip Cells

Two somatic cells, the distal tip cells, regulate germ-line proliferation and entry into meiosis (Kimble and White 1981). These two regulatory cells arise in homologous positions of the lineages of Z1 and Z4 (see Chapter 5). In hermaphrodites, Z1.aa and Z4.pp occupy the anterior and posterior distal tips, respectively; in males, Z1.a and Z4.p both occupy the single distal tip. In hermaphrodites, Z1.aa and Z4.pp both perform two functions essential to gonadal development. One is regulation of germ-line proliferation; the other is control of morphogenesis of the U-shaped germ-line tube. To accomplish the morphogenetic or "leader" function, the cell with leader activity guides the developing germ-line tube as it elongates in one direction, reflexes, and elongates in the other direction to complete the U shape. In males, the two distal tip cells perform a single function: regulation of germ-line proliferation. Another cell in the male gonad, the linker cell, carries out the leader function.

Because the only known function of the male distal tip cells is regulation of germ-line proliferation, their ultrastructure might provide clues about the cellular basis of that regulation. Both male distal tip cells are flat, small cells with a cytoplasm containing free ribosomes and some internal membranes (Golgi and endoplasmic reticulum) (Kimble 1978). The distal tip cells are located within the basal lamina that borders the entire gonad. The cellular membrane of the distal tip cells is closely apposed to the outer membrane of the germ-line syncytium, but no specialized junctions have been observed between them. The ultrastructure of the hermaphrodite distal tip cells is similar to that of the male linker cell (Kimble 1978). Both cells have extensive rough endoplasmic reticulum and Golgi cisternae. This ultrastructure suggests that leader function may require secretory activity.

B. Evidence for a Regulatory Role of the Distal Tip Cell

The regulation of proliferation of the germ-line cells by the somatic distal tip cells was discovered in a series of laser ablation experiments (Kimble and White 1981). As described in Chapter 5, individual cells can be killed with a laser microbeam. Laser ablation of the distal tip cells in either sex and at any time during gonadal development leads to arrest of mitosis and initiation of meiosis in all descendants of Z2 and Z3 (Figs. 2 and 3). Thus, if the distal tip cells are killed during L1, soon after their formation, the few germ cells that have been generated stop dividing after a few more mitoses, enter meiosis, and differentiate as sperm. If the distal tip cells are killed later, again all cells of the germ lineage stop dividing, enter meiosis, and differentiate as sperm or oocytes (depending on the sex and stage of development.) In males, both distal tip cells must be destroyed to obtain this effect. In hermaphrodites, if one distal tip cell is killed, only germ cells occupying the ovotestis of the ablated cell enter meiosis. Ablation of the immediate precursors of the distal tip cells in hermaphrodites (Z1.a and Z4.p) mimics the effect of killing the distal tip cells. However, if the two precursor cells of the somatic gonad, Z1 and Z4, are killed, the germ cells do not divide mitotically and do not enter meiosis. Indeed, if the precursors to Z1 and Z4 are killed in the embryo, the two germ cells die during L1.

Figure 2  Nomarski micrographs of distal tip cells (→). A hermaphrodite posterior distal tip cell is shown in an L3 before (A) and after (B) laser ablation. A male distal tip cell is shown in an L3 before (C) and after (D) laser ablation. (Reprinted, with permission, from Kimble and White 1981.)
An alteration in the distance between the distal tip cell and the most proximal germ-line nuclei shows that the influence of the distal tip cells over neighboring germ cells acts over a distance. If one of the two germ-line precursor cells, Z2 or Z3, is destroyed by laser ablation in a hermaphrodite larva that has just hatched, the remaining precursor cell normally contributes half its descendants to one ovotestis and half to the other ovotestis of the gonad. Therefore, each ovotestis in the operated animal is smaller than normal, containing only about half the normal complement of germ-line nuclei at any given stage of gonadal development. In unoperated hermaphrodites, the most proximal germ cells enter meiosis 33–34 hours after hatching. However, in animals with the gonad reduced in size by ablation of Z2, the most proximal germ-line nuclei do not enter pachytene until 38–44 hours after hatching. Control experiments demonstrate that this delay is not simply an artifact of secondary damage caused by the ablation. For example, if the distal tip cells are destroyed in addition to Z2, nuclei of the shorter gonadal arm display a pachytene morphology as usual at 33–34 hours. The presence of the distal tip cell is therefore essential to the delay in entry to meiosis observed in the smaller gonads.

An alteration in the position of distal tip cells confirms the idea that the position of the distal tip cell activity is responsible for establishing the spatial organization of the germ-line tissue. In males, if the sister cells of the distal tip cells are killed, the position of the distal tip cells is often abnormal. The changed position of the distal tip cell results in a corresponding shift in the axial polarity of the germ-line tissue. For example, if the distal tip cells become located at the anterior end of the gonad, the germ cells at the anterior end remain mitotic and the germ cells at the posterior end enter meiosis and begin spermatogenesis. The polarity of the germ-line tissue is therefore reversed by a change in the position of the distal tip cells.

C. A Gene Central to Control of Germ-line Proliferation, glp-1

One gene has been identified that appears to affect the distal tip cell control over germ-line proliferation. Many mutant alleles of this gene, glp-1 III, have been isolated (Austin and Kimble 1987; Priess et al. 1987). The germ line of animals of either sex, when homozygous for any of the glp-1 alleles, is defective in the decision between mitosis and meiosis. Thus, the germ-line cells that are present at hatching divide mitotically only a few times. The descendant germ cells all enter meiosis and differentiate as sperm earlier than normal. This mutant phenotype is essentially the same as the effect observed after ablating both distal tip cells during L1. In the mutant, however, the distal tip cells are still physically present: Z1.aa and Z4.pp in hermaphrodites and Z1.a and Z4.p in males assume their normal morphology and position. In glp-1 mutants, the leader function of the hermaphrodite distal tip cell is not affected. Therefore, an ovotestis in a glp-1 homozygous animal assumes its normal U shape but is filled throughout with only a small number of sperm. The function of glp-1 is unknown. It is plausible that the gene encodes a signal or a receptor in the distal tip cell–germ-line interaction.

Animals homozygous for weak alleles of glp-1 make sufficient germ-line cells to produce some embryos; however, these embryos die. This embryonic lethal phenotype of glp-1 shows a strict maternal effect, so that a homozygous glp-1 hermaphrodite mated to a wild-type male produces only dead embryos, whether or not they are homozygous or heterozygous for glp-1. A temperature-sensitive allele of glp-1 suggests that activity of the glp-1 gene product is required only early in embryogenesis for embryonic viability but that it is required throughout larval development for growth of the germ line. Thus, glp-1 encodes a product that is required for the proper growth and development of the germ line but also for development of the embryo (also see Chapter 8).

IV. SPERMATOOZA AND SPERMATOGENESIS

A. Morphology of Spermatozoa

C. elegans spermatozoa, like those of other nematodes and certain crustaceans, are nonflagellated crawling cells. They are approximately 4.5 × 7 μm, with a single knobby pseudopod protruding from one side of a hemispherical cell body (Fig. 4a) (Wolf et al. 1978; Nelson and Ward 1980; Nelson et al. 1982). When spermatozoa are conventionally fixed, sec-
Figure 4  (a) Scanning electron micrograph of spermatozoa. The cell on the lower right was crawling over the substrate when fixed; the other cells were not properly attached by their pseudopods to crawl, but they were moving their pseudopods. (b) Transmission electron micrograph. The organelles concentrated in the rounded cell body include the nucleus (Nuc), which is surrounded by a less electron-dense halo containing the centrioles (C), mitochondria, and the MOs that have fused with the plasma membrane, forming a stable pore and releasing their fibrous glycoprotein contents. A thin laminar membrane (LM) separates the organelles from the amorphous contents of the pseudopod. No microtubules or microfilaments are found in the spermatozoa, but with other fixation conditions, 2-nm-diameter filaments are abundant in the pseudopod cytoplasm (Roberts 1983). (Reprinted, with permission and slight modification, from Ward et al. 1981 [copyright, permission of the Rockefeller University Press].)

B. Protein Composition of Sperm

Sufficient sperm for biochemical analysis can be obtained by growing mutant strains that produce males at high frequency (from mutants) in liquid cultures. Two glass plates are used to separate the released sperm cells from the culture. Monospecific sera are then added to the sperm suspension and centrifuged through liquid culture. Maleic acid is added to the supernatant and the separated male cells are washed with phosphate-buffered saline (PBS). A 2 μl droplet of sperm supernatant is applied to a glass slide, allowed to dry, and stained with 1% w/v crystal violet. The sperm are then identified by the presence of characteristic structures or by the absence of certain organelles.

A prominent spot on two-dimensional gels of sperm proteins is made up of the actin found in sperm preparations. The absence of actin in an acetic acid wash of the actin found in sperm preparations may also be due to the presence of the actin filament network, which is observed in the absence of microfilaments and microtubules in the sprinter sperm. Some of the tubulin is found in cytoskeletal elements, and this may account for all of the actin found in sperm preparations. The absence of actin in an acetic acid wash of the actin found in sperm preparations may also be due to the presence of the actin filament network, which is observed in the absence of microfilaments and microtubules in the sprinter sperm. Some of the tubulin is found in cytoskeletal elements, and this may account for all of the actin found in sperm preparations. The absence of actin in an acetic acid wash of the actin found in sperm preparations may also be due to the presence of the actin filament network, which is observed in the absence of microfilaments and microtubules in the sprinter sperm. Some of the tubulin is found in cytoskeletal elements, and this may account for all of the actin found in sperm preparations.
of at least three and probably more members of a family of similar proteins called the major sperm proteins (MSPs). All three classes of MSPs are detected among proteins synthesized in vitro from male mRNA, so each must represent one or more independently synthesized polypeptide chains. MSPs are not detected by immunofluorescent staining or immunotransfer labeling in any tissue except spermatocytes and sperm, nor can their mRNA be detected (see Section IV.C); therefore, the MSPs are sperm-specific proteins. In sperm, they are present in the cytoplasm of both the cell body and the pseudopod where they polymerize into 2–3-nm filaments (Ward and Klass 1982; Roberts et al. 1986).

In addition to the MSPs, two-dimensional gels resolve a number of other polypeptides that appear to be sperm-specific because they are not found on gels of other worm tissue. Some of these polypeptides are numbered in Figure 5. Antibodies to the polypeptides p10, p11, and others have been prepared and used to confirm their sperm specificity. The functions of these proteins are unknown, but like the MSPs, p10 and p11 are both found in the cytoplasm of the pseudopod (Ward 1986).

In addition to these cytoplasmic proteins, sperm-specific membrane proteins have been identified by monoclonal antibodies prepared against whole sperm. Three different antibodies have been found to react with a polypeptide antigen shared by at least eight different minor sperm-specific proteins (Ward et al. 1986). A gold conjugate of one of these monoclonal antibodies detects the antigens in the plasma membrane, the membrane of the body of the MO, and the contents of the MO (Roberts et al. 1986). The appearance and localization of this antigen during sperm development will be described below.

C. MSP Genes

Genes encoding members of the MSP family have been cloned independently in two laboratories (Klass et al. 1982, 1984; Burke and Ward 1983). Because the messages for these proteins are the most abundant mRNAs in whole males, the genes were easily selected by differential screening of genomic and cDNA libraries with male versus hermaphrodite RNA. The identification of the genes was confirmed by hybrid-selected in vitro translation and by comparing the protein sequence predicted from DNA sequencing to that obtained by partial microprotein sequencing.

When genomic Southern blots are probed with cDNA clones of MSP genes, more than 30 bands are found to hybridize. Thus, the MSPs are encoded by a large multigene family with many more members than are indicated by the three protein components separable by electrophoresis (Burke and Ward 1983; Klass et al. 1984). Fourteen cDNA clones have been sequenced and 13 different sequences found, showing that many of the different MSP genes are transcribed into RNA (Klass et al. 1988; Ward et al. 1988). The protein-coding regions of these genes are 87–90% similar, with nearly all substitutions occurring in the third position of codons, so that the encoded MSP proteins are 96–100% conserved.

Unlike the coding-region sequences, the 3' untranslated sequences of MSP cDNA clones are not conserved. Synthetic nucleotide probes corresponding to these sequences have been used to probe genomic Southern blots. Each hybridizes to only one or a small subset of the MSP genes.
(Ward et al. 1988). Many of the transcribed MSP genes have been isolated from a genomic library using these synthetic probes.

To study the location and organization of transcribed MSP genes, cosmid clones containing larger regions of DNA that include some of these sequences have been identified by the DNA fingerprinting method (Coulson et al. 1986). A total of 41 MSP genes have been identified on cosmid clones out of an estimated total of 60 MSP genes in this multigene family. From DNA sequencing and hybridization of DNA with probes from different parts of the MSP coding sequence, it is estimated that about half of the MSP genes are pseudogenes.

Thirty-nine of the MSP genes, including all those known to be transcribed, are organized into 6 clusters composed of 3–13 genes each (Ward et al. 1988). Within each cluster, the genes are usually separated by several kilobase pairs of DNA. Pseudogenes are interspersed among functional genes.

These six clusters of MSP genes have been mapped, by overlap with known genes or by in situ hybridization, to only three chromosomal locations, the left arm of chromosome II and the left and center of chromosome IV. Among the MSP genes on chromosome IV are additional sperm-specific genes, including the gene for p10 (Ward et al. 1988).

### D. Spermatogenesis

The determination of cells to become sperm is discussed in Chapter 9. This section will describe the morphological changes leading from spermatocyte to spermatozoan, based on electron microscopy of the male testis and of spermatocytes and spermatids developing in vitro (Klass et al. 1976; Wolf et al. 1978; Nelson and Ward 1980; Ward et al. 1981, 1983). Development proceeds linearly from the spermatogonial cells in the distal tip of the male testis to the spermatozoa adjacent to the vas deferens, making it possible to follow all stages in longitudinal sections or serial cross sections. In addition, a medium that supports spermatozoan motility also allows some primary spermatocytes to develop in vitro to form spermatids, so these steps can be carefully studied by time-lapse video recording. All the intermediates described below for male spermatogenesis have also been seen by electron microscopy in hermaphrodites, so spermatogenesis in males and hermaphrodites is likely to be identical. Male and hermaphrodite spermatozoa are indistinguishable by electron microscopy and by their motility in vitro, but they can be distinguished functionally (see Section VI).

About 90 minutes are required, either in vivo or in vitro, for the tetraploid primary spermatocyte to complete meiosis and form four haploid spermatids. The morphological changes are summarized in Figure 6.

![Figure 6](image.png) **Figure 6** Summary of sperm development. Spermatocytes enter meiosis and proceed through pachytene, attached to a central core of cytoplasm called the rachis. They bud from this core and complete meiosis, forming four haploid nuclei that collect organelles and form spermatids. These bud off a central region of cytoplasm, which is left behind as a residual body. Spermatids are triggered to mature to spermatozoa by mating, or in vitro by monensin, weak bases, or proteases. This maturation includes fusion of the MOs, extension of the pseudopod, and initiation of motility. (Reprinted, with permission, from Ward et al. 1981 [copyright, permission of the Rockefeller University Press].)

The development of the peculiar sperm-specific MO and the association of MSPs with this developing organelle have been studied in detail using both fluorescent and gold-labeled antibodies to follow the localization of sperm-specific antigens (Ward and Klass 1982; Roberts et al. 1986). The MOs first form from the Golgi apparatus in the primary spermatocyte and are recognized by the characteristic electron-dense collar and head. The contents of the head are labeled by a sperm-specific monoclonal antibody (SP56). Subsequently, the membrane of the body of the organelle extends
from the head, and this membrane is also labeled with the SP56 antibody. The first MSP in the spermatocyte, detected by a gold-labeled monoclonal anti-MSP antibody, is found as an amorphous material associated with the MO body membrane. This material increases in amount and becomes an array of parallel fibers, 4–5 nm in diameter, partially enclosed by a double membrane of the body of the MO. This complex organelle, called the fibrous body–MO complex, enlarges as meiosis proceeds.

After the first meiotic division, cytokinesis is commonly incomplete, so that the two secondary spermatocytes remain joined. The second meiotic division immediately follows the first, with the still condensed chromosomes retaining distinct positions on the spindle. Haploid nuclei resulting from this division are highly condensed, and they segregate to the poles of the secondary spermatocyte, together with all the mitochondria and fibrous body–MO complexes. The residual body begins forming in the center of the cell and collects the ribosomes, Golgi apparatus, and most of the intracellular membranes.

Although the chromatin and centrioles have segregated to the developing spermatid, the spindle fails to disassemble and remains in the residual body (Ward 1986a). All the microfilaments also remain behind in the residual body. Spermatids form by rounding up the plasma membrane around the haploid nuclei, mitochondria, and fibrous body–MO complexes (which occupy >30% of the volume). The spermatids separate from the residual body by a budding process involving fusion of membrane vesicles to form the membrane between the residual body and the spermatid (Ward et al. 1981; Roberts et al. 1986). The residual body is eventually degraded and resorbed in the male and presumably in the hermaphrodite as well.

From the antibody-labeling experiments, the fibrous body–MO complex is a transient organelle used to transport both cytoplasmic and membranous sperm-specific proteins to the spermatid. Conversely, the proteins actin and tubulin are prevented from appearing in the spermatid by remaining assembled into fibers after meiosis so that they are left behind in the residual body.

After the spermatid buds off the residual body, the fibrous body disassembles and distributes the MSPs throughout the cytoplasm (Ward and Klass 1982; Roberts et al. 1986). The MO body becomes more compact and moves to the periphery of the spermatid. In males that do not copulate, development arrests with accumulation of more than 3000 spermatids. A single male, mated with several hermaphrodites, is capable of siring more than 2500 progeny (Hodgkin 1983a).

Spermatids are spherically symmetric sessile cells. Those produced in males complete maturation to spermatozoa (spermiogenesis) following copulation. Spermatids transferred to hermaphrodites by copulation form pseudopods and begin movement in the uterus within 5 minutes of transfer. Some spermatids left behind in the male also mature to spermatozoa and can be observed crawling in the vas deferens and seminal vesicle. By analogy to *Ascaris*, where an unidentified substance from the vas deferens triggers spermatid maturation (Burghardt and Foor 1978), *Caenorhabditis* spermiogenesis may also be induced by a secretion from the vas deferens, but this has not been demonstrated. Spermiogenesis can be initiated in vitro with high efficiency and reproducibly by three different treatments: the ionophore monesin, weak bases such as triethanolamine, and proteases (Nelson and Ward 1980; Ward et al. 1983). The first two act by increasing the intracellular pH of the spermatid from its resting value of 7.1 to 7.7 or more. Intracellular pH change triggers many developmental events and have many potential sites of action (reviewed in Nuccitelli and Deamer 1982). Within 2 minutes of this pH increase the spermatid initiates surface movements of its membrane, rearranges its surface projections, fuses its MOs with the posterior end of the cell, and extends its pseudopod anteriorly. This process is completed within 5 minutes, both in vivo and in vitro. Spermiogenesis is an energy-dependent process, which is blocked by metabolic inhibitors. Protease treatment triggers the same maturation but without increasing the intracellular pH.

Motility of the pseudopod is not necessary for its formation, because certain concentrations of the respiratory inhibitor azide block motility but not pseudopod formation, and because some mutants form nonmotile pseudopods (see Section IV.F).

**E. Mutants with Altered Sperm**

One of the reasons for studying spermatogenesis is that mutants affecting sperm development are easily obtained. Sperm-defective mutants are sterile hermaphrodites that can produce progeny if mated to males. If such a homozygous mutant strain produces males that behave normally and copulate with hermaphrodites but yield no outcross progeny, the mutant is most likely sperm defective. The class of sperm-defective mutants most intensively studied are those that make infertile sperm in normal number. Such mutants were originally designated fertilization-defective or *fer* mutants (Ward and Miwa 1978), but all subsequently isolated sperm-defective mutants are named *spe*, irrespective of where in spermatogenesis they are blocked. More than 60 mutations affecting spermatogenesis have been obtained and assigned to more than 40 different genes (Ward and Miwa 1978; Argon and Ward 1980; Ward et al. 1981; S. L’Hernault et al., in prep.). Some of these are summarized in Appendix 4B. Mutations blocking at every stage of sperm development have been obtained. Some mutants accumulate apparently normal intermediates, such as spermatocytes or spermatids that do not mature to spermatozoa. Other mutants accumulate aberrant cells, such as spermatids with crystalline inclusions or spermatozoa that have failed to fuse their MOs with the plasma membrane (Ward et al. 1981). Still other mutants make normal looking motile spermatozoa that fail to fertilize eggs.
F. Sperm Motility

Hermaphrodite spermatozoa must crawl to locate themselves properly in the spermatheca and to avoid being swept away by fertilized eggs passing into the uterus and out of the vulva. Male spermatozoa must be able to crawl from the region in the uterus where they are deposited during copulation to the site of fertilization, the spermatheca, a distance of more than 200 μm. These movements can be observed easily in the transparent hermaphrodite using Nomarski microscopy and time-lapse video recording and can be analyzed in vitro (Nelson et al. 1982). Spermatozoa crawl at about 20 μm/minute, but they can move as fast as 43 μm/minute for short intervals. The pseudopod attaches to the substrate and pulls the cell forward, with the cell body either dragged passively behind or carried atop the rear part of the motile pseudopod. As the cell crawls, new substrate attachments are found continuously under the front of the pseudopod and, in extreme cases, contact under the leading edge of the pseudopod alone provides sufficient traction for locomotion (Roberts and Streitmatter 1984). Cells remain motile for several hours in vitro.

The mechanism of motility cannot be an actin–myosin-based contractile system, because the spermatozoa contain almost no actin and no detectable myosin, and their movements are unaffected by drugs that interfere with microfilaments. Instead, the spermatozoa propel themselves by tip to base flow of membrane over the pseudopod surface (Roberts and Ward 1982a,c). Spermatozoa insert new membrane components at the tips of their pseudopods, and these components flow backwards to be taken up at the base of the pseudopod. This propels the cell forward, because the pseudopod membrane is continually rebuilt at the tip, creating new attachment sites under the leading edge of the advancing cell.

Some of the evidence establishing this membrane flow is the observation that labeled lectins, antibody bodies, and polystyrene beads attached to the pseudopod all move from the tip to the base, as do fluorescently labeled lipids inserted into the membrane. It is not known what provides the driving force for this membrane flow, what establishes its polarity, or how membrane components are inserted and removed.

V. Oogenesis
A. Description of Oogenesis

Oogenesis begins at the loop between the distal and proximal arms of the hermaphrodite ovotestis. Nuclei at the loop are present only at the outer side of the bend, and, compared with the distal arm, there is a marked increase in volume of cytoplasm surrounding each nucleus in this region. Oocytes appear to result from the packaging of a single peripheral nucleus with cytoplasm from the central core of the distal arm. Generally, the first oocytes produced are observed just after the molt to adulthood.

The proximal arm of adult ovotestis contains a single file of enlarging oocytes. Six bivalents can be distinguished in each nucleus. Mature oocytes are arrested in meiosis at diakinesis of meiotic prophase I. Both meiotic divisions occur after fertilization. The cytoplasm of maturing oocytes contains a large number of osmiophilic granules. Comparison of the ultrastructure of oocytes made in a wild-type animal to that of oocytes made in a mutant that does not contain yolk suggests that these granules contain yolk (Doniach and Hodgkin 1984).

B. Yolk Proteins and Their Synthesis

The yolk proteins of C. elegans were first identified as four abundant proteins specific to adult hermaphrodites (Klass et al. 1979). They include two related proteins (yp170A and yp170B) and two unrelated proteins (yp115 and yp88). All four are glycoproteins (Sharrock 1983). Only three polypeptides specific to adult hermaphrodites are readily observed among the products of RNA translated in vitro (Sharrock 1984). Two of these correspond, with little or no modification, to the yp170A/B doublet. The third is large, with a Mr of about 180,000, and binds antibodies specific for both yp115 and yp88 (Sharrock 1984). These and other observations suggest that the 180,000-Mr protein is a precursor, which is cleaved and modified to yield both yp115 and yp88.

A comparison of proteins synthesized by dissected tissues (intestines, gonads, and body wall) suggests that the intestine is the primary site of vitelloigenin synthesis (Kimble and Sharrock 1983). Although the yp115/yp88 precursor is secreted from the intestine (Sharrock 1984), its cleavage products yp115 and yp88 accumulate in animals possessing no gonadal tissue. Therefore, cleavage of vitelloigenin most likely occurs in the body cavity of the nematode before uptake by the ovary.

The yolk proteins of C. elegans can be visualized in situ by indirect immunofluorescent staining (Sharrock 1983). In the embryo, staining with antibodies against any of the yolk proteins yields a punctate pattern. In the oocyte, yolk is localized in osmiophilic cytoplasmic granules, which accumulate during maturation (Doniach and Hodgkin 1984). Generally, only the two or three oocytes near the spermatheca exhibit the high density of staining characteristic of fertilized eggs.

Yolk protein uptake may be facilitated by the epithelial sheath of the oviduct that surrounds maturing oocytes. Electron micrographs show osmiophilic granules in the cytoplasm of sheath cells and coated pits on both the membranes facing the body cavity and those facing the oocyte. However, oocytes contain these granules even when the epithelial sheath has been eliminated by laser ablation. Therefore, it seems likely that yolk can
be taken up by oocytes either directly from the body cavity or after transport through the sheath.

C. Vitellogenin Genes

The vitellogenins are encoded by six genes. A family of five genes (vit-1, vit-2, vit-3, vit-4, and vit-5) encodes the yp170A and yp170B proteins, and one gene (vit-6) encodes the precursor protein for yp115 and yp88 (Blumenthal et al. 1984; Spieth and Blumenthal 1985). Cloning of these genes was facilitated by the abundance of their mRNAs in a poly(A)-containing fraction of C. elegans high-molecular-weight RNA. Hybrid–arrest translation experiments show that vit-1 and vit-2 correspond to yp170B, whereas vit-3, vit-4, and vit-5 correspond to yp170A. However, vit-1 has a stop codon in frame and is therefore a pseudogene (Spieth et al. 1985a). Moreover, cDNA clones have been obtained only from vit-2 and vit-5, so that vit-3 and vit-4 may also be pseudogenes.

The vitellogenin mRNAs for yp170A and yp170B and for the yp115/yp88 precursor are about 5 kb in length (Blumenthal et al. 1984; Spieth and Blumenthal 1985). They are abundant in adult hermaphrodites but are missing from larvae and males. Furthermore, they are highly enriched in RNA from intestines but are not detectable in RNA isolated from gonads or body walls.

The coding regions of the vit genes are highly conserved (Spieth et al. 1985b). In the region coding for the first 100 amino acids, the vit-1/vit-2 subfamily is 70% homologous to the vit-3/vit-4/vit-5 subfamily, and vit-6 is about 50% homologous to the vit-1/vit-5 family. All six genes begin with a highly conserved sign I sequence, and all have a TATA box at about position –30 from the start site of transcription. The nucleotide sequence of vit-5 has been completed (Spieth et al. 1985a). The vit-5 message is 4869 nucleotides long, including untranslated regions of 9 bases at the 5' end and 51 bases at the 3' end. vit-5 contains four short introns totaling 218 bp. The predicted vitellogenin, yp170A, has a molecular weight of 186,430.

Two highly conserved heptameric sequences are found in the 5'-flanking regions of all six vit genes from C. elegans (Spieth et al. 1985b) and in the same regions of an additional five vit genes from Caenorhabditis briggsae (T. Blumenthal, pers. comm.). These heptameric sequences have not been observed in any other gene cloned from C. elegans. The first heptamer, called box 1 (TGTCAAT), appears in both orientations and repeats between four and six times per promoter region, allowing a 1-bp mismatch. The second heptamer, called box 2 (CTGATAA), is present in only one or two copies per promoter region.

With the exception of vit-3 and vit-4, whose coding regions are separated by only about 3 kb, the vitellogenin genes of C. elegans do not appear to be clustered. Approximately 50 kb of genomic DNA has been cloned from around each of the four loci of the vit-1–vit-5 family without revealing additional linkage between the genes (Heine and Blumenthal 1986). Mapping of restriction-fragment-length polymorphisms has localized vit-1 and the vit-3/vit-4 pair to the X chromosome. In addition, in situ hybridization experiments indicate that all members of the vit-1–vit-5 family map to the X chromosome (D.G. Albertson, pers. comm. and Appendix 4D).

D. Oogenesis Mutants

No broad search for mutants specifically defective in oogenesis has been carried out to date. A number of embryonic lethal mutants have been obtained (called zyg or emb) in which a gene product contributed by the mother (presumably via the oocyte) is defective (Hirsch and Vanderslice 1976; Schierenberg et al. 1980). These mutants are discussed in more detail in Chapter 8. More recently, recessive mutants, in which hermaphrodites produce sperm continuously and make no oocytes, have been isolated (J. Kimble, unpubl.). In addition, sterile mutants that are defective in oogenesis have been isolated during an intensive screen for mutants of one particular region (Sigurdson et al. 1984). These have been named ooc mutants, but little is known about their effects.

VI. FERTILIZATION

A. Hermaphrodite Self-fertilization

The process of fertilization and the oocyte’s response to sperm penetration were described many years ago (reviewed in Nigon et al. 1960; Nigon 1965). These events have been studied more recently by direct observation with Nomarski microscopy and video recording of fertilization in hermaphrodites (Hirsch et al. 1976; Ward and Carrel 1979). Sperm mature in the hermaphrodite as they pass from the testis into the spermatheca, where they can be seen crawling actively, pushing their pseudopods against the spermathecal walls. Electron micrographs show that the pseudopods protrude into invaginations in the spermathecal cells, presumably to anchor the spermatozoa in place.

Development of the spermatheca is described in Chapter 5. It is a folded tube about 100 μm long, with a constriction at the distal end connecting to the oviduct and a complex valve at the proximal end connecting to the uterus. When an oocyte matures in the oviduct, it is pushed up against the constriction by contractions of the oviduct sheath. The oocyte nucleus moves toward the distal end of the cell, and the nuclear membrane breaks down about 2 minutes before the ripe oocyte is pushed through the constriction by stronger contractions of the oviduct sheath. Multiple sperm contact the oocyte as it enters the spermatheca, but only one sperm
penetrates and fertilizes the egg. The mechanism for preventing polyspermy is not known. From observations by light microscopy, it appears that the sperm is enveloped by the egg, probably pseudopod first as in *Ascaris* (Foor 1970), but electron micrographs of sperm penetration have not been obtained. Fertilization of the egg is recognized by a sudden increase in granule movement in the egg cytoplasm, quickly followed by eggshell formation. The fertilized egg remains in the spermatheca for 3–10 minutes. Contraction of the spermatheca then push the egg against the spermathecal valve, which dilates to allow passage of the egg into the uterus.

The egg usually carries several sperm with it into the uterus. These sperm crawl back through the spermathecal valve to regain their positions on the walls of the spermatheca, so that nearly every sperm eventually fertilizes an oocyte. Oocytes continue to mature after sperm depletion so that in contrast to the situation in almost all other animals, the number of progeny in *C. elegans* is limited by the number of sperm rather than the number of eggs (Ward and Carrel 1979).

The oocytes that are made in old hermaphrodites depleted of sperm or in mutants with defective sperm undergo partial maturation as they pass through the spermatheca. Their nuclear membrane disappears, and they resume meiosis. No eggshell forms, however, and granule motions of the egg cytoplasm are greatly reduced or absent, suggesting that these two events are triggered by sperm contact. After entering the uterus, unfertilized oocytes undergo one nuclear division, and the oocyte nucleus returns to the center of the cell. The DNA and chromosomes replicate, with nuclear membrane breakdown timed roughly as expected for the AB cell lineage but without cytokinesis or karyokinesis. This produces highly polyploid unfertilized oocytes, which are expelled through the vulva. Observation of such oocytes produced by young hermaphrodites is the simplest way to recognize a sperm-defective mutant with the dissecting microscope.

**B. Male Cross-fertilization**

When males copulate with hermaphrodites, their sperm are deposited through the vulva among the fertilized eggs in the uterus. The sperm then crawl among the eggs to reach the spermatheca. Surprisingly, when they arrive at the spermatheca of a young hermaphrodite, which already has hundreds of her own sperm, male sperm outcompete hermaphrodite sperm and preferentially fertilize oocytes (Ward and Carrel 1979). This appears to occur by displacement of the hermaphrodite sperm from their positions on the spermatheca. A hermaphrodite that has been multiply mated will lose her own sperm so that only outcross progeny will be produced after mating. More commonly, predominantly outcross progeny are produced until male sperm are depleted, and then self progeny reappear. This preferential sperm utilization appears to reflect a difference between male and hermaphrodite sperm, because if a spermless hermaphrodite is successively mated by males of two different genotypes, the second male has no advantage at fertilization.

In addition, mating has the effect of stimulating oogenesis by the hermaphrodite. An old hermaphrodite that has ceased oogenesis will reinitiate oocyte maturation after mating, and a multiply mated young hermaphrodite will produce as many as 1400 progeny, four times the normal number. Both preferential male sperm utilization and stimulation of oogenesis by mating are mechanisms to guarantee utilization of the male sperm in a population of predominantly self-fertilizing hermaphrodites, ensuring that if mating occurs, it will result in production of outcross progeny.

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