The Genetics of Axis Specification in *Drosophila*

6

THANKS LARGELY TO STUDIES spearheaded by Thomas Hunt Morgan's laboratory during the first two decades of the twentieth century, we know more about the genetics of *Drosophila* than that of any other multicellular organism. The reasons have to do with both the flies themselves and with the people who first studied them. *Drosophila* is easy to breed, hardy, prolific, tolerant of diverse conditions, and the polytene chromosomes of its larvae (see Figure 15.17) are readily identified. The progress of *Drosophila* genetics was aided by the relatively free access of every scientist to the mutants and fly breeding techniques of every other researcher. Mutants were considered the property of the entire scientific community, and Morgan's laboratory established a database and exchange network whereby anyone could obtain them.

Undergraduates (starting with Calvin Bridges and Alfred Sturtevant) played important roles in *Drosophila* research, which achieved its original popularity as a source of undergraduate research projects. As historian Robert Kohler noted (1994), "Departments of biology were cash poor but rich in one resource: cheap, eager, renewable student labor." The *Drosophila* genetics program was "designed by young persons to be a young person's game," and the students set the rules for *Drosophila* research: "No trade secrets, no monopolies, no poaching, no ambushes."

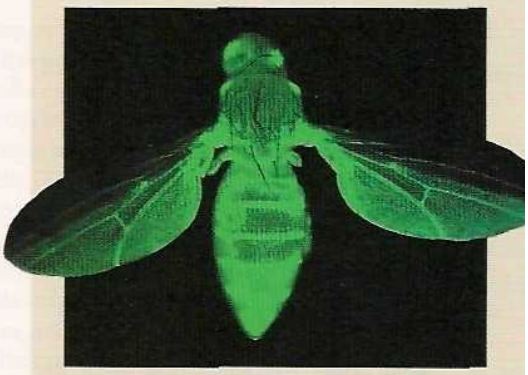
But *Drosophila* was a difficult organism on which to study embryology. Although Jack Schultz (originally in Morgan's laboratory) and others following him attempted to relate the genetics of *Drosophila* to its development, the fly embryos proved too complex and intractable to study, being neither large enough to manipulate experimentally nor transparent enough to observe microscopically. It was not until the techniques of molecular biology allowed researchers to identify and manipulate the genes and RNAs of the insect that its genetics could be related to its development. And when that happened, a revolution occurred in the field of biology. This revolution is continuing, in large part because of the ability to generate transgenic flies at high frequency (Pfeiffer et al. 2009). This enables researchers to identify developmental interactions taking place in very small regions of the embryo, and it identifies enhancers that control developmental processes taking place rapidly and in small areas. The merging of our knowledge of the molecular aspects of *Drosophila* genetics with our knowledge of the fly's development built the foundations on which the current sciences of developmental genetics and evolutionary developmental biology are based.

Those of us who are at work on Drosophila *find a particular point to the question. For the genetic material available is all that could be desired, and even embryological experiments can be done.... It is for us to make use of these opportunities. We have a complete story to unravel, because we can work things from both ends at once.*

JACK SCHULTZ (1935)

The chief advantage of Drosophila *initially was one that historians have overlooked: it was an excellent organism for student projects.*

ROBERT E. KOHLER (1994)



EARLY DROSOPHILA DEVELOPMENT

In Chapter 5 we discussed the specification of early embryonic cells by cytoplasmic determinants stored in the oocyte. The cell membranes that form during cleavage establish the region of cytoplasm incorporated into each new blastomere, and the incorporated morphogenetic determinants then direct differential gene expression in each cell. During *Drosophila* development, however, cellular membranes do not form until after the thirteenth nuclear division. Prior to this time, all the dividing nuclei share a common cytoplasm, and material can diffuse throughout the whole embryo. In these embryos, the specification of cell types along the anterior-posterior and dorsal-ventral axes is accomplished by the interactions of cytoplasmic materials *within* the single multinucleated cell. Moreover, the initiation of the anterior-posterior and dorsal-ventral differences is controlled by the position of the egg within the mother's ovary. Whereas the sperm entry site may fix the axes in ascidians and nematodes, the fly's anterior-posterior and dorsal-ventral axes are specified by interactions between the egg and its surrounding follicle cells.

Fertilization

Drosophila fertilization is not your standard sperm-meets-egg story. First, the sperm enters an egg that is already activated. Egg activation in *Drosophila* is accomplished at ovulation, a few minutes *before* fertilization begins. As the *Drosophila* oocyte squeezes through a narrow orifice, ion channels open, allowing calcium ions to flow into it. The oocyte nucleus then resumes its meiotic divisions and the cytoplasmic mRNAs become translated, even without fertilization (Mahowald et al. 1983; Fitch and Wakimoto 1998; Heifetz et al. 2001; Horner and Wolfner 2008). Second, there is only one site—the **micropyle**, at the future dorsal anterior region of the embryo—where the sperm can enter the egg. The micropyle is a tunnel in the chorion (eggshell) that allows sperm to pass through it one at a time. The micropyle probably prevents polyspermy in *Drosophila*. There are no cortical granules to block polyspermy, although cortical changes are seen. Third, by the time the sperm enters the egg, the egg already has begun to specify its axes; thus the sperm enters an egg that is already organizing itself as an embryo. Fourth, there is competition between sperm. A sperm tail can be many times longer than the adult fly, and this huge tail is thought to block other sperm from entering the egg. In *Drosophilia melanogaster*, the sperm tail is 1.8 mm—about as long as the adult fly, and some 300 times longer than a human sperm. The entire sperm (huge tail and all) gets incorporated into the oocyte cytoplasm, and the sperm cell membrane does not break down until after it is fully inside the oocyte (Snook and Karr 1998; Clark et al. 1999).

See WEBSITE 6.1
Drosophila fertilization

Cleavage

Most insect eggs undergo **superficial cleavage**, wherein a large mass of centrally located yolk confines cleavage to the cytoplasmic rim of the egg. One of the fascinating features of this cleavage pattern is that cells do not form until after the nuclei have divided several times. Karyokinesis (nuclear division) occurs without cytokinesis (cell division), and the rapid rate of division is accomplished (as it is in sea urchin embryos) by eliminating the gap (G) stages of the cell cycle. Cleavage in the *Drosophila* egg creates a **syncytium**, a single cell with many nuclei residing in a common cytoplasm (Figure 6.1). The zygote nucleus under-

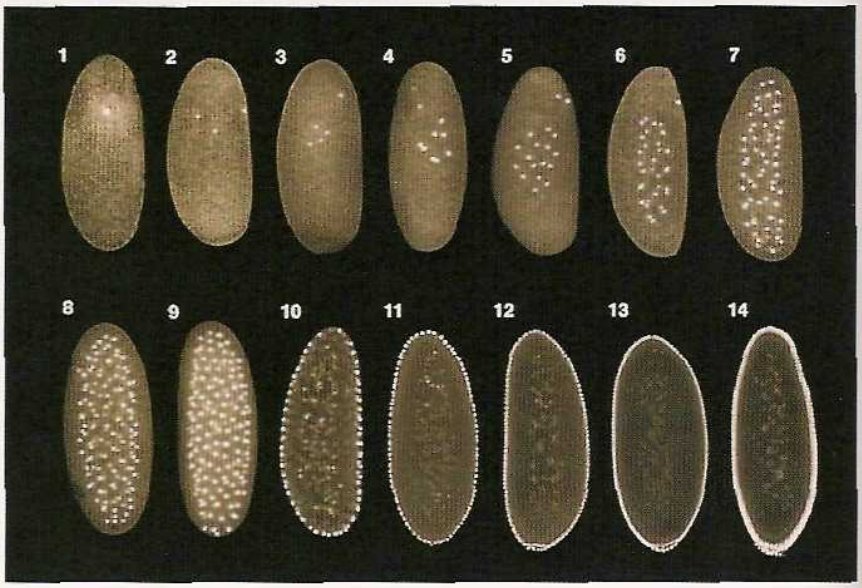


FIGURE 6.1 Laser confocal micrographs of stained chromatin showing superficial cleavage in a *Drosophila* embryo. The future anterior is positioned upward, and the numbers refer to the cell division cycle. The early nuclear divisions occur centrally. Later, the nuclei and their cytoplasmic islands (energids) migrate to the periphery of the cell. This creates the syncytial blastoderm. After cycle 13, the oocyte membranes ingress between the nuclei to form the cellular blastoderm. The pole cells (germ cell precursors) form in the posterior. (Courtesy of D. Daily and W. Sullivan.)

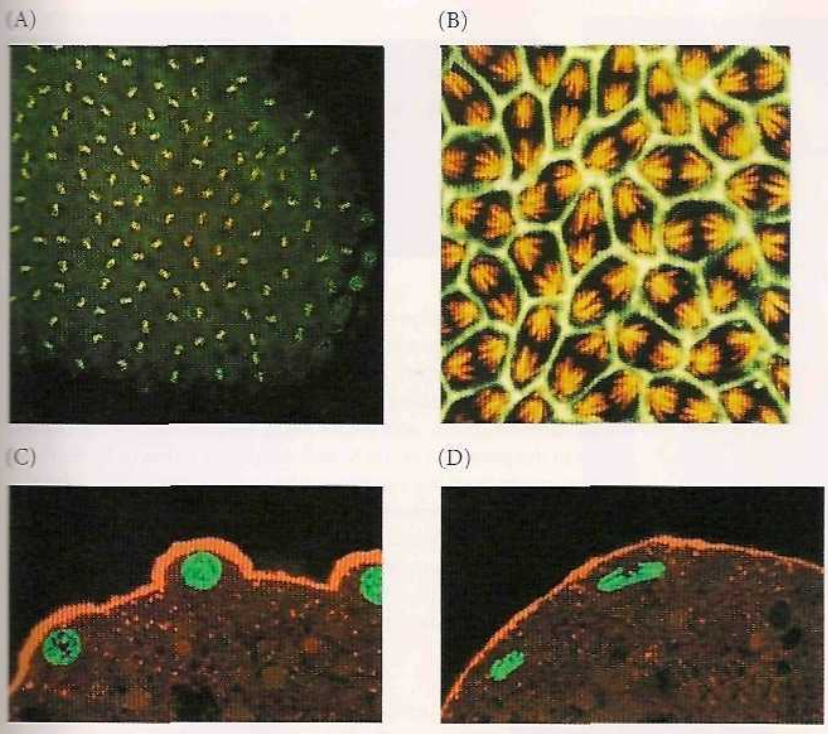


FIGURE 6.2 Nuclear and cell division in *Drosophila*. (A) Nuclear division (but not cell division) can be seen in the single cell of the *Drosophila* embryo using a dye that stains DNA. The first region to cellularize, the pole region, can be seen forming the cells that will eventually become the germ cells (sperm or eggs) of the fly. (B) Confocal fluorescence photomicrographs of nuclei dividing during cellularization of the blastoderm. While there are no cell boundaries, actin (green) can be seen forming regions within which each nucleus divides. The microtubules of the mitotic apparatus are stained red with antibodies to tubulin. (C,D) Cross section of a part of the stage 10 *Drosophila* embryo showing nuclei (green) in the cortex of the syncytial cell, near a layer of actin microfilaments (red). (C) Interphase nuclei. (D) Nuclei in anaphase, dividing parallel to the cortex and enabling the nuclei to stay in the cell periphery. (A from Bonnefoy et al. 2007; B from Sullivan et al. 1993, courtesy of W. Theurkauf and W. Sullivan; C,D from Foe 2000, courtesy of V. Foe.)

goes several mitotic divisions within the central portion of the egg; 256 nuclei are produced by a series of eight nuclear divisions averaging 8 minutes each. During the ninth division cycle, about five nuclei reach the surface of the posterior pole of the embryo. These nuclei become enclosed by cell membranes and generate the **pole cells** that give rise to the gametes of the adult. At cycle 10, the other nuclei migrate to the cortex (periphery) of the egg, where the mitoses continue, albeit at a progressively slower rate (Figure 6.2; Foe et al. 2000). During these stages of nuclear division, the embryo is called a **syncytial blastoderm**, since no cell membranes exist other than that of the egg itself.

The nuclei divide within a common cytoplasm, but this does not mean the cytoplasm is itself uniform. Karr and Alberts (1986) have shown that each nucleus within the syncytial blastoderm is contained within its own little territory of cytoskeletal proteins. When the nuclei reach the periphery of the egg during the tenth cleavage cycle, each nucleus becomes surrounded by microtubules and microfilaments. The nuclei and their associated cytoplasmic islands are called **energids**. Following division cycle 13, the oocyte plasma membrane folds inward between the nuclei, eventually partitioning off each somatic nucleus into a single cell. This process creates the **cellular blastoderm**, in which all the cells are arranged in a single-layered jacket around the yolky core of the egg (Turner and Mahowald 1977; Foe and Alberts 1983).

Like any other cell formation, the formation of the cellular blastoderm involves a delicate interplay between microtubules and microfilaments. The membrane movements, the nuclear elongation, and the actin polymerization each appear to be coordinated by the microtubules (Riparbelli et al. 2007). The first phase of blastoderm cellularization is characterized by the invagination of cell membranes between the nuclei to form furrow canals (Figure 6.3). This process can be inhibited by drugs that block microtubules. After the furrow canals have passed the level of the nuclei, the second phase of cellularization occurs. The rate of invagination increases, and the actin-membrane complex begins to constrict at what will be the basal end of the cell (Foe et al. 1993; Schejter and Wieschaus 1993; Mazumdar and Mazumdar 2002). In *Drosophila*, the cellular blastoderm consists of approximately 6000 cells and is formed within 4 hours of fertilization.

The mid-blastula transition

After the nuclei reach the periphery, the time required to complete each of the next four divisions becomes progressively longer. While cycles 1–10 average 8 minutes each, cycle 13—the last cycle in the syncytial blastoderm—takes 25 minutes to complete. Cycle 14, in which the *Drosophila* embryo forms cells (i.e., after 13 divisions), is asynchronous. Some groups of cells complete this cycle in 75 minutes, other groups take 175 minutes (Foe 1989). Zygotic gene transcription (which begins around cycle 11) is greatly enhanced at this stage. This slowdown of nuclear division, cellularization, and concomitant increase in new RNA transcription is often referred to as the **mid-blastula transition** (see Chapter 5). It is at this stage that the maternally provided mRNAs are degraded and hand over control of development to the zygotic genome (Brandt et al. 2006;

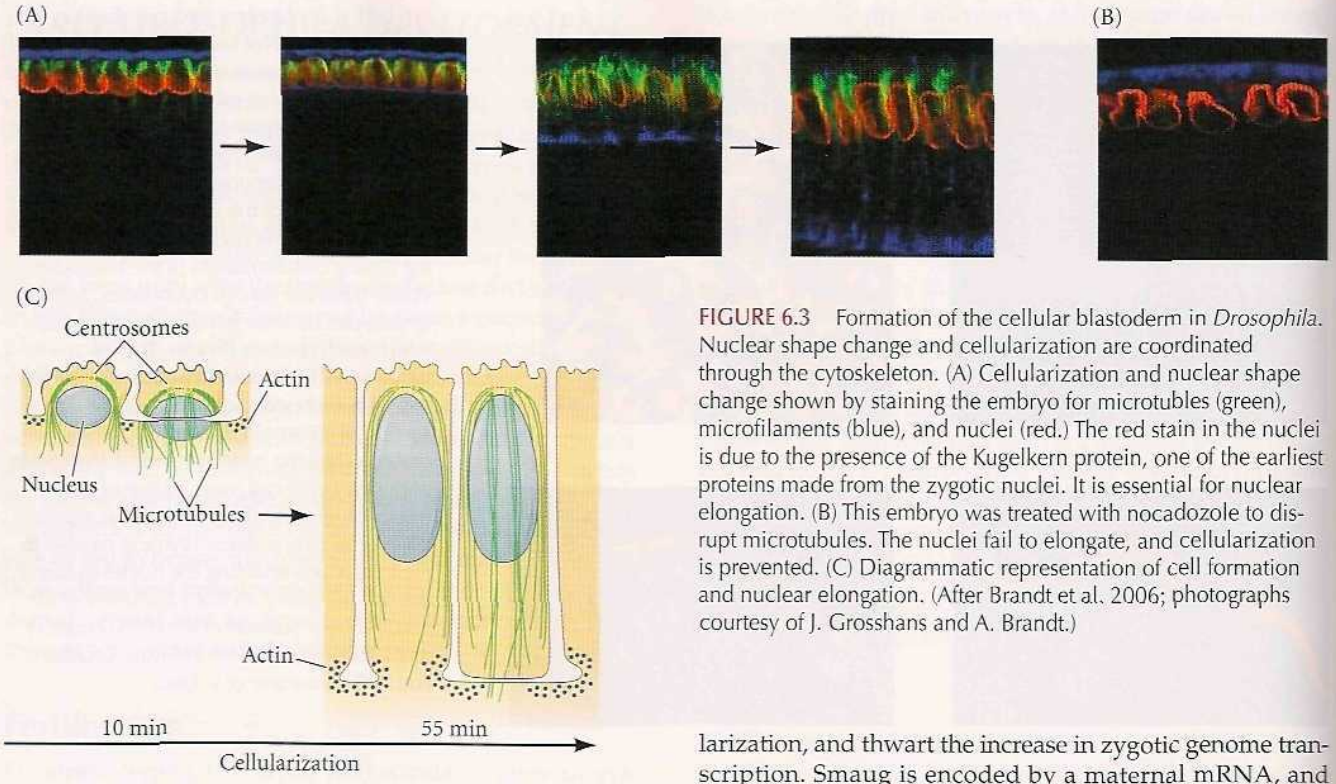


FIGURE 6.3 Formation of the cellular blastoderm in *Drosophila*. Nuclear shape change and cellularization are coordinated through the cytoskeleton. (A) Cellularization and nuclear shape change shown by staining the embryo for microtubles (green), microfilaments (blue), and nuclei (red.) The red stain in the nuclei is due to the presence of the Kugelkern protein, one of the earliest proteins made from the zygotic nuclei. It is essential for nuclear elongation. (B) This embryo was treated with nocadozole to disrupt microtubules. The nuclei fail to elongate, and cellularization is prevented. (C) Diagrammatic representation of cell formation and nuclear elongation. (After Brandt et al. 2006; photographs courtesy of J. Grosshans and A. Brandt.)

De Renzis et al. 2007; Benoit et al. 2009). Such a maternal-to-zygotic transition is seen in the embryos of numerous vertebrate and invertebrate phyla.

In *Drosophila*, the coordination of the mid-blastula transition and the maternal-to-zygotic transition is controlled by several factors, including (1) the ratio of chromatin to cytoplasm; (2) Smaug protein; and (3) cell cycle regulators. The ratio of chromatin to cytoplasm is a consequence of the increasing amount of DNA while the cytoplasm remains constant (Newport and Kirschner 1982; Edgar et al. 1986a). Edgar and his colleagues compared the early development of wild-type *Drosophila* embryos with that of haploid mutants. The haploid *Drosophila* embryos had half the wild-type quantity of chromatin at each cell division. Hence, a haploid embryo at cell division cycle 8 had the same amount of chromatin that a wild-type embryo had at cycle 7. The investigators found that, whereas wild-type embryos formed a cellular blastoderm immediately after the thirteenth division, haploid embryos underwent an extra, fourteenth, division before cellularization. Moreover, the lengths of cycles 11–14 in wild-type embryos corresponded to those of cycles 12–15 in the haploid embryos. Thus, the haploid embryos followed a pattern similar to that of the wild-type embryos—but they lagged by one cell division.

Smaug (yes, it's named after the dragon in *Lord of the Rings*) is an RNA-binding protein often involved in repressing translation. During the mid-blastula transition, however, it targets the maternal mRNAs for destruction (Tadros et al. 2007; Benoit et al. 2009). Maternal Smaug mutants disrupt the slowing down of nuclear division, prevent cellularization, and thwart the increase in zygotic genome transcription. Smaug is encoded by a maternal mRNA, and Smaug protein levels increase during the early cleavage divisions. These levels peak when the zygotic genome begins efficient transcription. Moreover, if Smaug is artificially added to the anterior of an early *Drosophila* embryo, there results a concomitant gradient in the timing of maternal transcript destruction, cleavage cell cycle delays, zygotic gene transcription, cellularization, and gastrulation. Thus, Smaug accumulation appears to regulate the progression from maternal to nuclear control of development and coordinates this progression with the mid-blastula transition.

Cell cycle regulators are critical for introducing the gap stages into the cell cycle and slowing it down. As the maternal replication factors are depleted, the zygotically encoded replication factors take over and regulate the accumulation of cyclins in the cell (Sibon et al. 1997; Royou et al. 2008).

See VADE MECUM *Drosophila* development

See WEBSITE 6.2
The early development of other insects

Gastrulation

Gastrulation begins shortly after the mid-blastula transition. The first movements of *Drosophila* gastrulation segregate the presumptive mesoderm, endoderm, and ectoderm. The prospective mesoderm—about 1000 cells constituting the ventral midline of the embryo—folds inward to produce the ventral furrow (Figure 6.4A). This furrow eventually pinches off from the surface to become a ventral tube within the embryo. The prospective endoderm invaginates

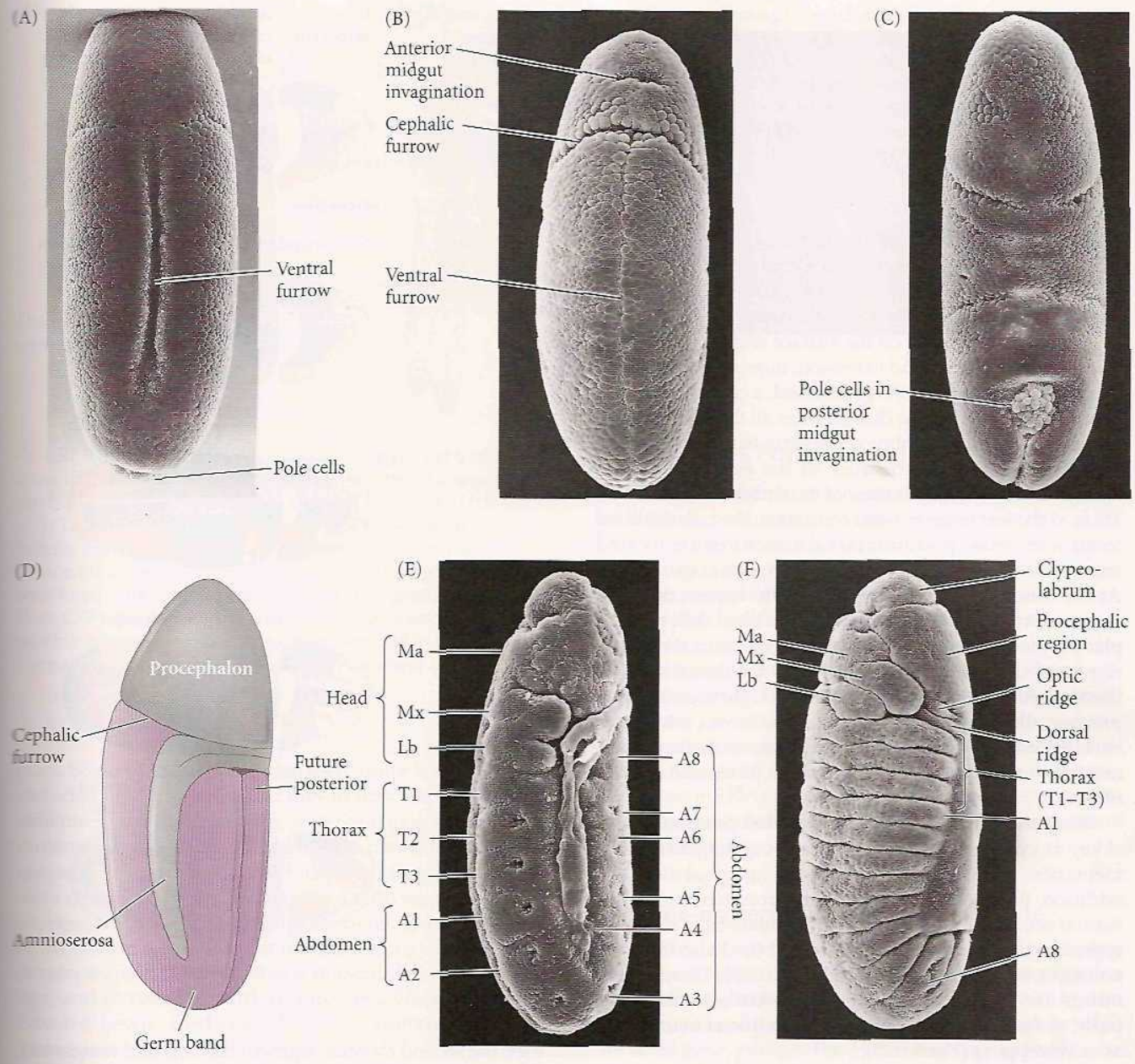
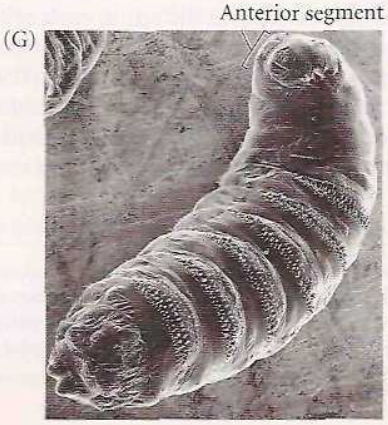


FIGURE 6.4 Gastrulation in *Drosophila*. The anterior points upward in each figure. (A) Ventral furrow beginning to form as cells flanking the ventral midline invaginate. (B) Closing of ventral furrow, with mesodermal cells placed internally and surface ectoderm flanking the ventral midline. (C) Dorsal view of a slightly older embryo, showing the pole cells and posterior endoderm sinking into the embryo. (D) Dorsolateral view of an embryo at fullest germ band extension, just prior to segmentation. The cephalic furrow separates the future head region (procephalon) from the germ band, which will form the thorax and abdomen. (E) Lateral view, showing fullest extension of the germ band and the beginnings of segmentation. Subtle indentations mark the incipient segments along the germ band. Ma, Mx, and Lb correspond to the mandibular, maxillary, and labial head segments; T1–T3 are the thoracic segments; and A1–A8 are the abdominal segments. (F) Germ band reversing direction. The true segments are now visible, as well as the other territories of the dorsal head, such as the clypeolabrum, procephalic region, optic ridge, and dorsal ridge. (G) Newly hatched first-instar larva. (Photographs courtesy of F. R. Turner. D after Campos-Ortega and Hartenstein 1985.)



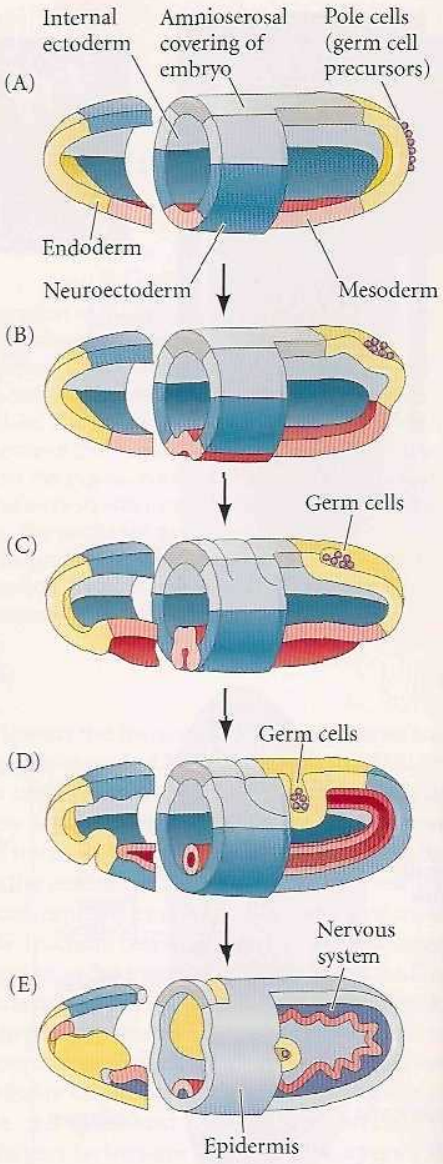


FIGURE 6.5 Schematic representation of gastrulation in *Drosophila*. Anterior is to the left; dorsal is facing upward. (A,B) Surface and cutaway views showing the fates of the tissues immediately prior to gastrulation. (C) The beginning of gastrulation as the ventral mesoderm invaginates into the embryo. (D) This view corresponds to Figure 6.4A, while (E) corresponds to Figure 6.4B,C. In (E), the neuroectoderm is largely differentiated into the nervous system and the epidermis. (After Campos-Ortega and Hartenstein 1985.)

to form two pockets at the anterior and posterior ends of the **ventral furrow**. The pole cells are internalized along with the endoderm (Figure 6.4B,C). At this time, the embryo bends to form the **cephalic furrow**.

The ectodermal cells on the surface and the mesoderm undergo convergence and extension, migrating toward the ventral midline to form the **germ band**, a collection of cells along the ventral midline that includes all the cells that will form the trunk of the embryo. The germ band extends posteriorly and, perhaps because of the egg case, wraps around the top (dorsal) surface of the embryo (Figure 6.4D). Thus, at the end of germ band formation, the cells destined to form the most posterior larval structures are located immediately behind the future head region (Figure 6.4E). At this time, the body segments begin to appear, dividing the ectoderm and mesoderm. The germ band then retracts, placing the presumptive posterior segments at the posterior tip of the embryo (Figure 6.4F). At the dorsal surface, the two sides of the epidermis are brought together in a process called **dorsal closure**. The amnioserosa, which had been the most dorsal structure, interacts with the epidermal cells to encourage their migration (reviewed in Panfilio 2007; Heisenberg 2009).

While the germ band is in its extended position, several key morphogenetic processes occur: organogenesis, segmentation, and the segregation of the imaginal discs.* In addition, the nervous system forms from two regions of ventral ectoderm. Neuroblasts differentiate from this neurogenic ectoderm within each segment (and also from the nonsegmented region of the head ectoderm). Therefore, in insects like *Drosophila*, the nervous system is located ventrally, rather than being derived from a dorsal neural tube as in vertebrates (Figure 6.5).

The general body plan of *Drosophila* is the same in the embryo, the larva, and the adult, each of which has a distinct head end and a distinct tail end, between which are repeating segmental units (Figure 6.6). Three of these segments form the thorax, while another eight segments form the abdomen. Each segment of the adult fly has its own identity. The first thoracic segment, for example, has only legs; the second thoracic segment has legs and wings; and the third thoracic segment has legs and *halteres* (balancing organs). Thoracic and abdominal segments can also be distinguished from each other by differences in the cuticle of the newly hatched first-instar larvae.

*Imaginal discs are those cells set aside to produce the adult structures. The details of imaginal disc differentiation will be discussed in Chapter 15. For more information on *Drosophila* developmental anatomy, see Bate and Martinez-Arias 1993; Tyler and Schetzer 1996; and Schwalm 1997.

GENES THAT PATTERN THE DROSOPHILA BODY PLAN

Most of the genes involved in shaping the larval and adult forms of *Drosophila* were identified in the early 1990s using a powerful "forward genetics" approach. The basic strategy was to randomly mutagenize flies and then screen for mutations that disrupted the normal formation of the body

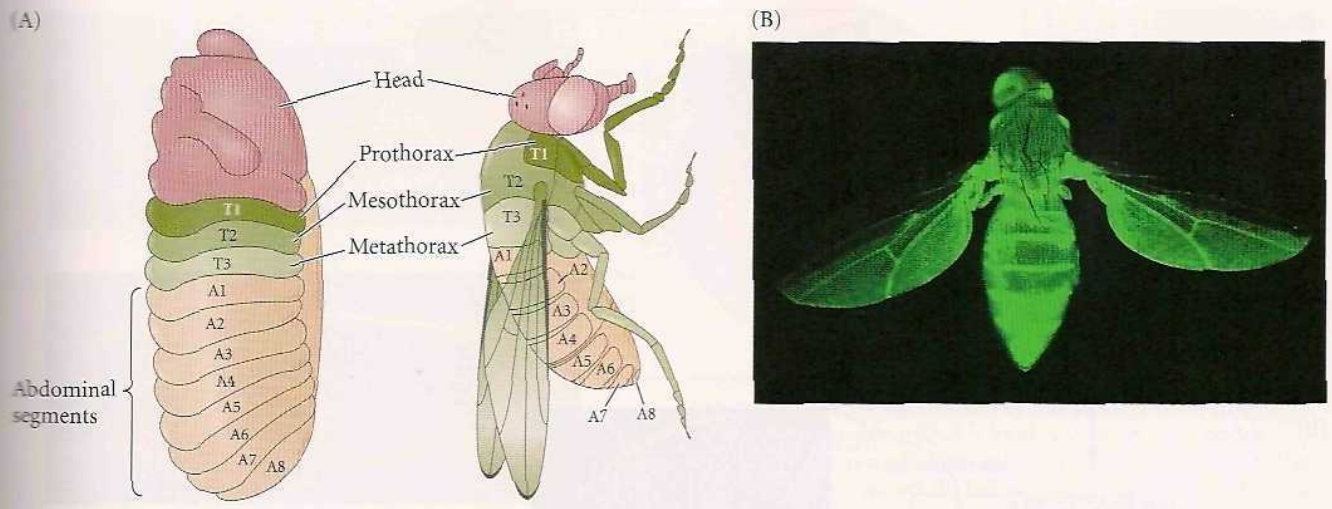


FIGURE 6.6 Comparison of larval (left) and adult (right) segmentation in *Drosophila*. (A) In the adult, the three thoracic segments can be distinguished by their appendages: T1 (prothorax) has legs only; T2 (mesothorax) has wings and legs; T3 (metathorax) has halteres (not visible) and legs. (B) Segments in adult transgenic *Drosophila* in which the gene for green fluorescent protein has been fused to the *cis*-regulatory region of the *engrailed* gene. Thus, GFP is produced in the areas of *engrailed* transcription, which is active at the border of each segment and in the posterior compartment of the wing. (B courtesy of A. Klebes.)

plan. Some of these mutations were quite fantastic, and included embryos and adult flies in which specific body structures were either missing or in the wrong place. These mutant collections were distributed to many different laboratories. The genes involved in the mutant phenotypes were cloned and then characterized with respect to their expression patterns and their functions. This combined effort has led to a molecular understanding of body plan development in *Drosophila* that is unparalleled in all of biology, and in 1995 the work resulted in a Nobel Prize for Edward Lewis, Christiane Nüsslein-Volhard, and Eric Wieschaus.

The rest of this chapter details the genetics of *Drosophila* development as we have come to understand it over the past two decades. First we will examine how the dorsal-ventral and anterior-posterior axes of the embryo are established by interactions between the developing oocyte and its surrounding follicle cells. Next we will see how dorsal-ventral patterning gradients are formed within the embryo, and how these gradients specify different tissue types. The third part of the discussion will examine how segments are formed along the anterior-posterior axis, and how the different segments become specialized. Finally, we will briefly show how the positioning of embryonic tissues along the two primary axes specifies these tissues to become particular organs.

Primary Axis Formation during Oogenesis

The processes of embryogenesis may "officially" begin at fertilization, but many of the molecular events critical for *Drosophila* embryogenesis actually occur during oogenesis. Each oocyte is descended from a single female germ cell—the **oogonium**—which is surrounded by an epithelium of follicle cells. Before oogenesis begins, the oogonium divides four times with incomplete cytokinesis, giving rise to 16 interconnected cells: 15 **nurse cells** and the single oocyte precursor. These 16 cells constitute the **egg chamber** (ovary) in which the oocyte will develop, and the oocyte will be the cell at the posterior end of the egg chamber (see Figure 16.4). As the oocyte precursor develops, numerous mRNAs made in the nurse cells are transported on microtubules through the cellular interconnections into the enlarging oocyte.

Anterior-posterior polarity in the oocyte

The follicular epithelium surrounding the developing oocyte is initially uniform with respect to cell fate, but this uniformity is broken by two signals organized by the oocyte nucleus. Interestingly, both of these signals involve the same gene, ***gurken***. The *gurken* message appears to be synthesized in the nurse cells, but it becomes transported specifically to the oocyte nucleus. Here it is localized between the nucleus and the cell membrane and is translated into Gurken protein (Cáceres and Nilson 2005). At this time the oocyte nucleus is very near the posterior tip of the egg chamber, and the Gurken signal is received by the follicle cells at that position through a receptor protein encoded by the ***torpedo*** gene* (Figure 6.7A). This signal results in the "posteriorization" of these follicle cells (Fig-

*Molecular analysis has established that *gurken* encodes a homologue of the vertebrate epidermal growth factor (EGF), while *torpedo* encodes a homologue of the vertebrate EGF receptor (Price et al. 1989; Neuman-Silberberg and Schüpbach 1993).

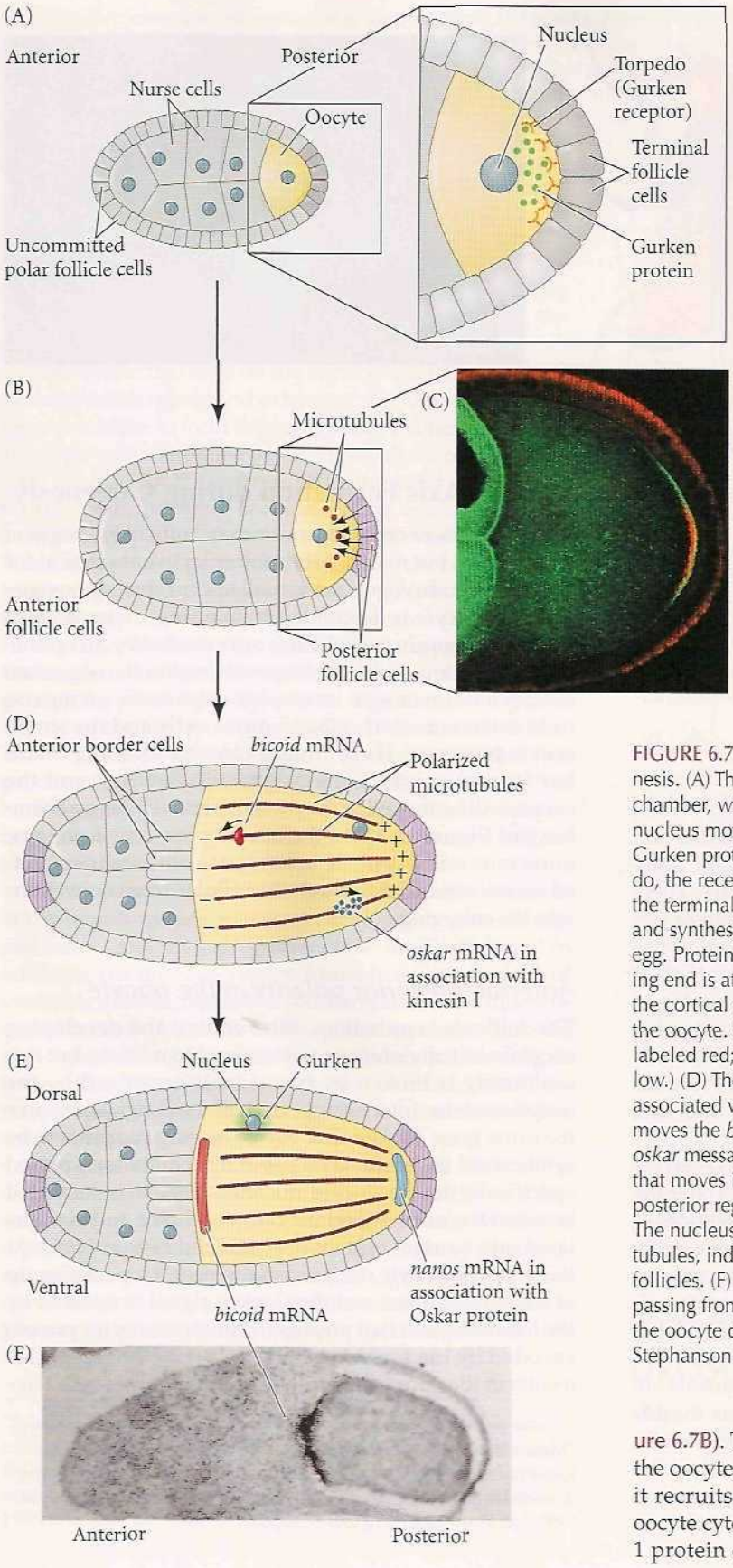


FIGURE 6.7 The anterior-posterior axis is specified during oogenesis. (A) The oocyte moves into the posterior region of the egg chamber, while nurse cells fill the anterior portion. The oocyte nucleus moves toward the terminal follicle cells and synthesizes Gurken protein (green). The terminal follicle cells express Torpedo, the receptor for Gurken. (B) When Gurken binds to Torpedo, the terminal follicle cells differentiate into posterior follicle cells and synthesize a molecule that activates protein kinase A in the egg. Protein kinase A orients the microtubules such that the growing end is at the posterior. (C) The Par-1 protein (green) localizes to the cortical cytoplasm of nurse cells and to the posterior pole of the oocyte. (The Stauffen protein marking the posterior pole is labeled red; the red and green signals combine to fluoresce yellow.) (D) The *bicoid* message binds to dynein, a motor protein associated with the non-growing end of microtubules. Dynein moves the *bicoid* message to the anterior end of the egg. The *oskar* message becomes complexed to kinesin I, a motor protein that moves it toward the growing end of the microtubules at the posterior region, where Oskar can bind the *nanos* message. (E) The nucleus (with its Gurken protein) migrates along the microtubules, inducing the adjacent follicle cells to become the dorsal follicles. (F) Photomicrograph of *bicoid* mRNA (stained black) passing from the nurse cells and localizing to the anterior end of the oocyte during oogenesis. (C courtesy of H. Doerflinger; F from Stephanson et al. 1988, courtesy of the authors.)

ure 6.7B). The posterior follicle cells send a signal back into the oocyte. The identity of this signal is not yet known, but it recruits the par-1 protein to the posterior edge of the oocyte cytoplasm (Figure 6.7C; Doerflinger et al. 2006). Par-1 protein organizes microtubules specifically with their

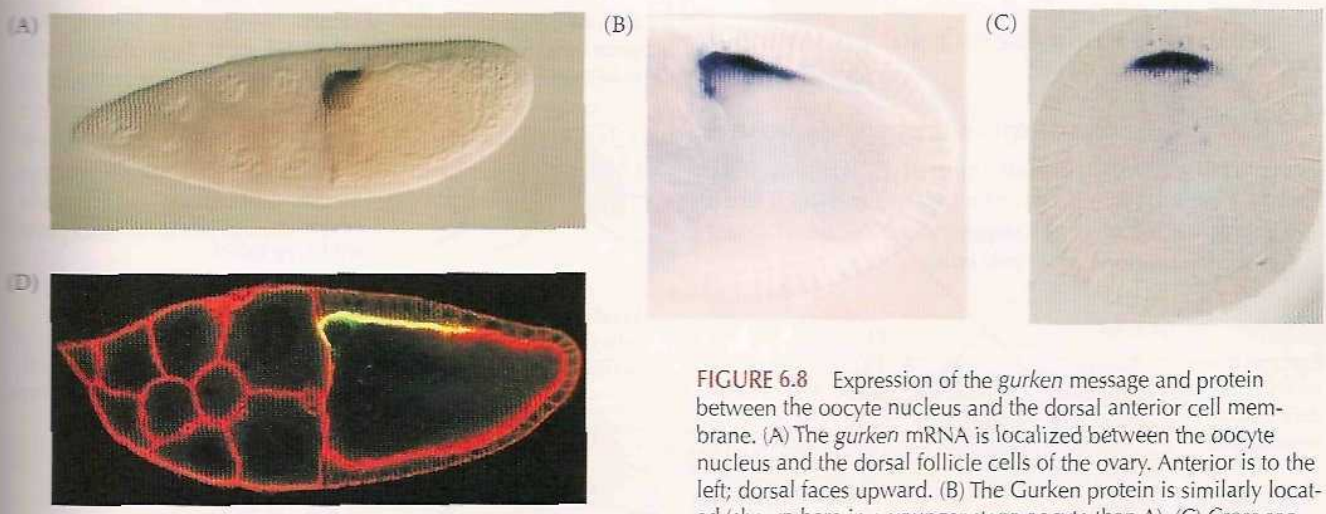


FIGURE 6.8 Expression of the *gurken* message and protein between the oocyte nucleus and the dorsal anterior cell membrane. (A) The *gurken* mRNA is localized between the oocyte nucleus and the dorsal follicle cells of the ovary. Anterior is to the left; dorsal faces upward. (B) The Gurken protein is similarly located (shown here in a younger stage oocyte than A). (C) Cross section of the egg through the region of Gurken protein expression. (D) A more mature oocyte, showing Gurken protein (yellow) across the dorsal region. The actin is stained red, showing cell boundaries. As the oocyte grows, follicle cells migrate across the top of the oocyte, becoming exposed to Gurken. (A from Ray and Schüpbach 1996, courtesy of T. Schüpbach; B,C from Peri et al. 1999, courtesy of S. Roth; D courtesy of C. van Buskirk and T. Schüpbach.)

minus (cap) and plus (growing) ends at the anterior and posterior ends of the oocyte, respectively (Gonzalez-Reyes et al. 1995; Roth et al. 1995; Januschke et al. 2006).

The orientation of the microtubules is critical, because different microtubule motor proteins will transport their mRNA or protein cargoes in different directions. The motor protein kinesin, for instance, is an ATPase that will use the energy of ATP to transport material to the plus end of the microtubule. Dynein, however, is a "minus-directed" motor protein that will transport its cargo the opposite way. One of the messages transported by kinesin along the microtubules to the posterior end of the oocyte is *oskar* mRNA (Zimyanin et al. 2008). The *oskar* mRNA is not able to be translated until it reaches the posterior cortex, at which time it generates the Oskar protein. Oskar protein recruits more par-1 protein, thereby stabilizing the microtubule orientation and allowing more material to be recruited to the posterior pole of the oocyte (Doerflinger et al. 2006; Zimyanin et al. 2007). The posterior pole will thereby have its own distinctive cytoplasm, called **pole plasm**, which contains the determinants for producing the abdomen and the germ cells.

This cytoskeletal rearrangement in the oocyte is accompanied by an increase in oocyte volume, owing to transfer of cytoplasmic components from the nurse cells. These components include maternal messengers such as the ***bicoid*** and ***nanos*** mRNAs. These mRNAs are carried by motor proteins along the microtubules to the anterior and posterior ends of the oocyte, respectively (Figure 6.7D–F). As we shall soon see, the protein products encoded by *bicoid* and *nanos* are critical for establishing the anterior-posterior polarity of the embryo.

Dorsal-ventral patterning in the oocyte

As oocyte volume increases, the oocyte nucleus moves to an anterior dorsal position where a second major signaling event takes place. Here the *gurken* message becomes localized in a crescent between the oocyte nucleus and the oocyte cell membrane, and its protein product forms an anterior-posterior gradient along the dorsal surface of the oocyte (Figure 6.8; Neuman-Silberberg and Schüpbach 1993). Since it can diffuse only a short distance, Gurken protein reaches only those follicle cells closest to the oocyte nucleus, and it signals those cells to become the more columnar **dorsal follicle cells** (Montell et al. 1991; Schüpbach et al. 1991; see Figure 6.7E). This establishes the dorsal-ventral polarity in the follicle cell layer that surrounds the growing oocyte.

Maternal deficiencies of either the *gurken* or the *torpedo* gene cause ventralization of the embryo. However, *gurken* is active only in the oocyte, whereas *torpedo* is active only in the somatic follicle cells. This fact was revealed by experiments with germline/somatic chimeras. In one such experiment, Schüpbach (1987) transplanted germ cell precursors from wild-type embryos into embryos whose mothers carried the *torpedo* mutation. Conversely, she transplanted the germ cell precursors from *torpedo* mutants into wild-type embryos (Figure 6.9). The wild-type eggs produced mutant, ventralized embryos when they developed in a *torpedo* mutant mother's egg chamber. The *torpedo* mutant eggs were able to produce normal embryos if they developed in a wild-type ovary. Thus, unlike Gurken, the Torpedo protein is needed in the follicle cells, not in the egg itself.

The Gurken-Torpedo signal that specifies dorsalized follicle cells initiates a cascade of gene activities that create

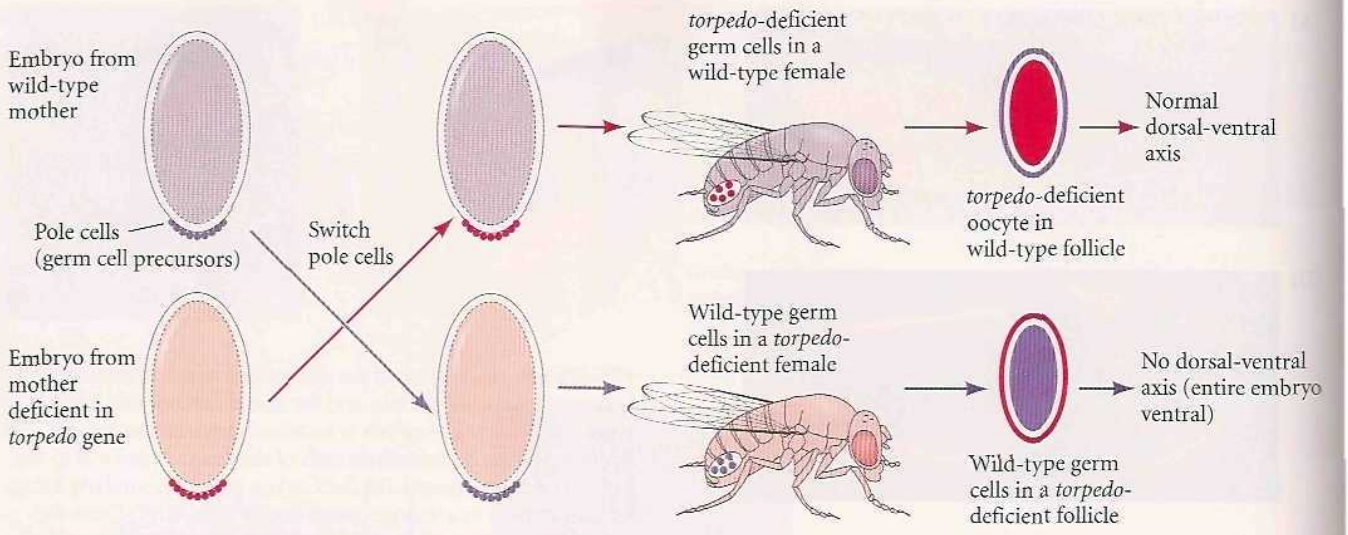


FIGURE 6.9 Germline chimeras made by interchanging pole cells (germ cell precursors) between wild-type embryos and embryos from mothers homozygous for a mutation of the *torpedo* gene. These transplants produced wild-type females whose eggs came from mutant mothers, and *torpedo*-deficient females that laid wild-type eggs. The *torpedo*-deficient eggs produced normal embryos when they developed in the wild-type ovary, whereas the wild-type eggs produced ventralized embryos when they developed in the mutant mother's ovary.

the dorsal-ventral axis of the embryo (Figure 6.10). The activated Torpedo receptor protein inhibits the expression of the ***pipe*** gene. As a result, Pipe protein is made only in the *ventral* follicle cells (Sen et al. 1998; Amiri and Stein 2002). In some as yet unknown way (probably involving sulfation), Pipe activates the Nudel protein, which is secreted to the cell membrane of the neighboring ventral embryonic cells (see Zhang et al. 2009). A few hours later, activated Nudel initiates the activation of three serine proteases that are secreted into the perivitelline fluid (see Figure 6.10B; Hong and Hashimoto 1995). These proteases are the products of the *gastrulation defective* (*gd*), *snake* (*snk*), and *easter* (*ea*) genes. Like most extracellular proteases, these molecules are secreted in an inactive form and are subsequently activated by peptide cleavage. In a complex cascade of events, activated Nudel activates the Gastrulation-defective protease. The Gd protease cleaves the Snake protein, activating the Snake protease, which in turn cleaves the Easter protein. This cleavage activates the Easter protease, which then cleaves the Spätzle protein (Chasan et al. 1992; Hong and Hashimoto 1995; LeMosy et al. 2001).

It is obviously important that the cleavage of these three proteases be limited to the most ventral portion of the embryo. This is accomplished by the secretion of a protease inhibitor from the follicle cells of the ovary (Hashimoto et al. 2003; Ligoxygakis et al. 2003). This inhibitor of Easter and Snake is found throughout the perivitelline space surrounding the embryo. Indeed, this protein is very similar to the mammalian protease inhibitors that limit blood clotting protease cascades to the area of injury. In this way, the proteolytic cleavage of Easter and Spätzle is strictly limited to the area around the most ventral embryonic cells.

The cleaved Spätzle protein is now able to bind to its receptor in the oocyte cell membrane, the product of the *toll* gene. Toll protein is a maternal product that is evenly

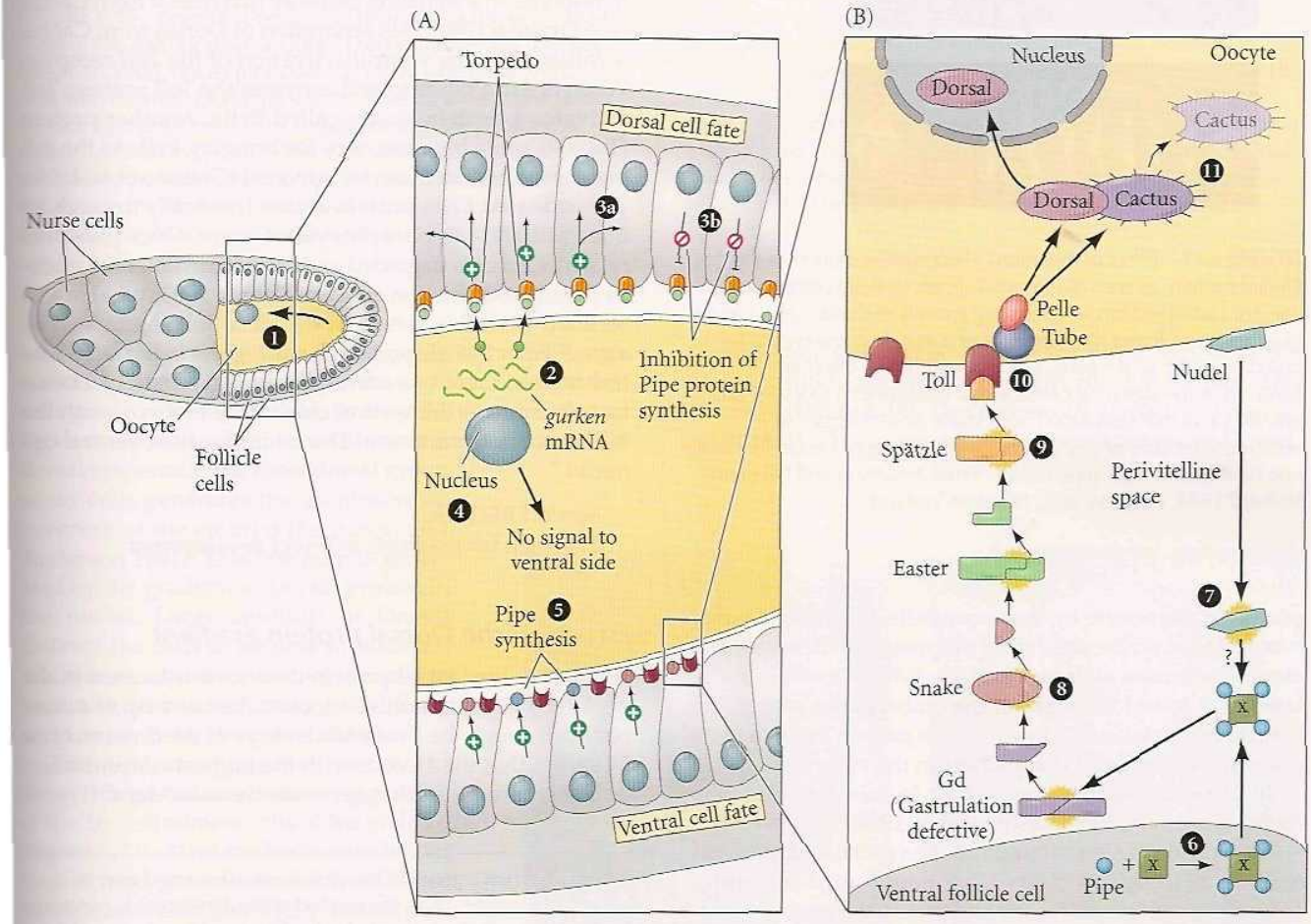
FIGURE 6.10 Generating dorsal-ventral polarity in *Drosophila*. (A) The nucleus of the oocyte travels to what will become the dorsal side of the embryo. The *gurken* genes of the oocyte synthesize mRNA that becomes localized between the oocyte nucleus and the cell membrane, where it is translated into Gurken protein. The Gurken signal is received by the Torpedo receptor protein made by the follicle cells (see Figure 6.7). Given the short diffusibility of the signal, only the follicle cells closest to the oocyte nucleus (i.e., the dorsal follicle cells) receive the Gurken signal, which causes the follicle cells to take on a characteristic dorsal follicle morphology and inhibits the synthesis of Pipe protein. Therefore, Pipe protein is made only by the *ventral* follicle cells. (B) The ventral region at a slightly later stage of development. Pipe modifies an unknown protein (x) and allows it to be secreted from the ventral follicle cells. Nudel protein interacts with this modified factor to split the product of the *gastrulation defective* gene, which then splits the product of the *snake* gene to create an active enzyme that will split the inactive Easter zymogen into an active Easter protease. The Easter protease splits the Spätzle protein into a form that can bind to the Toll receptor (which is found throughout the embryonic cell membrane). This protease activity of Easter is strictly limited by the protease inhibitor found in the perivitelline space. Thus, only the ventral cells receive the Toll signal. This signal separates the Cactus protein from the Dorsal protein, allowing Dorsal to be translocated into the nuclei and ventralize the cells. (After van Eeden and St. Johnston 1999.)

distributed throughout the cell membrane of the egg (Hashimoto et al. 1988, 1991), but it becomes activated only by binding the Spätzle protein, which is produced only on the ventral side of the egg. Therefore, the Toll receptors on the ventral side of the egg are transducing a signal into the egg, while the Toll receptors on the dorsal side of the egg are not. This localized activation establishes the dorsal-ventral polarity of the oocyte.

Generating the Dorsal-Ventral Pattern in the Embryo

Dorsal, the ventral morphogen

The protein that distinguishes dorsum (back) from ventrum (belly) in the fly embryo is the product of the *dorsal* gene. The mRNA transcript of the mother's *dorsal* gene is



1. Oocyte nucleus travels to anterior dorsal side of oocyte where it localizes *gurken* mRNA.
2. *gurken* messages are translated. Gurken is received by Torpedo proteins during mid-oogenesis.
3a. Torpedo signal causes follicle cells to differentiate to a dorsal morphology.
3b. Synthesis of Pipe is inhibited in dorsal follicle cells.
4. Gurken does not diffuse to ventral side.
5. Ventral follicle cells synthesize Pipe.
6. In ventral follicle cells, Pipe completes the modification of an unknown factor (x).
7. Nudel and factor (x) interact to split the Gastrulation-deficient (Gd) protein.
8. Activated Gd splits the Snake protein, and activated Snake cleaves the Easter protein.
9. Activated Easter splits Spätzle; activated Spätzle binds to Toll receptor protein.
10. Toll activation activates Tube and Pelle, which phosphorylate the Cactus protein. Cactus is degraded, releasing it from Dorsal.
11. Dorsal protein enters the nucleus and ventralizes the cell.

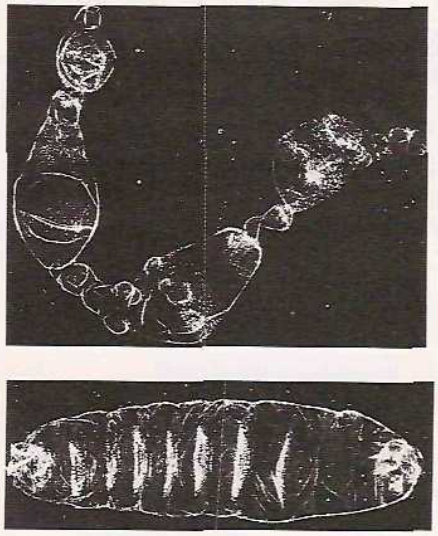


FIGURE 6.11 Effect of mutations affecting distribution of the Dorsal protein, as seen in the exoskeleton (cuticle) patterns of larvae. (A) Deformed larvae consisting entirely of dorsal cells. Larvae like these developed from the eggs of a female homozygous for a mutation of the *snake* gene, one of the maternal effect genes involved in the signaling cascade that establishes a Dorsal gradient. (B) Larva that developed from *snake* mutant eggs that received injections of mRNA from wild-type eggs. Larvae like this one have a wild-type appearance. (From Anderson and Nüsslein-Volhard 1984, courtesy of C. Nüsslein-Volhard.)

placed in the oocyte by the nurse cells. However, Dorsal protein is not synthesized from this maternal message until about 90 minutes after fertilization. When Dorsal is translated, it is found throughout the embryo, not just on the ventral or dorsal side. How can this protein act as a morphogen if it is located everywhere in the embryo?

In 1989, the surprising answer to this question was found (Roth et al. 1989; Rushlow et al. 1989; Steward 1989). While Dorsal is found throughout the syncytial blastoderm of the early *Drosophila* embryo, it is translocated into nuclei only in the ventral part of the embryo. In the nucleus, Dorsal protein acts as a transcription factor, binding to certain genes to activate or repress their transcription. If Dorsal does not enter the nucleus, the genes responsible for specifying ventral cell types are not transcribed, the genes responsible for specifying dorsal cell types are not repressed, and all the cells of the embryo become specified as dorsal cells.

This model of dorsal-ventral axis formation in *Drosophila* is supported by analyses of maternal effect mutations that give rise to an entirely dorsalized or an entirely ventralized phenotype (Figure 6.11; Anderson and Nüsslein-Volhard 1984). In mutants in which all the cells are dorsalized (evident from their dorsal-specific exoskeleton), Dorsal does not enter the nucleus of any cell. Conversely, in mutants in which all cells have a ventral phenotype, Dorsal protein is found in every cell nucleus.*

Establishing a nuclear Dorsal gradient

So how does the Dorsal protein enter into the nuclei only of the ventral cells? When Dorsal is first produced, it is complexed with a protein called Cactus in the cytoplasm of the syncytial blastoderm. As long as Cactus is bound to it, Dorsal remains in the cytoplasm. Dorsal enters ventral nuclei in response to a signaling pathway that frees it from Cactus (see Figure 6.10B). This separation of Dorsal from Cactus is initiated by the ventral activation of the Toll receptor. When Spätzle binds to and activates the Toll protein, Toll activates a protein kinase called Pelle. Another protein (Tube) is probably necessary for bringing Pelle to the cell membrane, where it can be activated (Galindo et al. 1995). The activated Pelle protein kinase (probably through an intermediate) can phosphorylate Cactus. Once phosphorylated, Cactus is degraded and Dorsal can enter the nucleus (Kidd 1992; Shelton and Wasserman 1993; Whalen and Steward 1993; Reach et al. 1996). Since Toll is activated by a gradient of Spätzle protein that is highest in the most ventral region, there is a corresponding gradient of Dorsal translocation in the ventral cells of the embryo, with the highest concentrations of Dorsal in the most ventral cell nuclei.†

See WEBSITE 6.3
Evidence for gradients in insect development

Effects of the Dorsal protein gradient

What does the Dorsal protein do once it is located in the nuclei of the ventral cells? A look at the fate map of a cross section through the *Drosophila* embryo at the division cycle 14 shows that the 16 cells with the highest concentration of Dorsal are those that generate the mesoderm (Figure

*Remember that a gene in *Drosophila* is usually named after its mutant phenotype. Thus, the product of the *dorsal* gene is necessary for the differentiation of ventral cells. That is, in the absence of *dorsal*, the ventral cells become dorsalized.

†Recall that maternal effect mutations (as in the coiling mutant in snails discussed in Chapter 5) involve those genes that are active in the female and provide materials for the oocyte cytoplasm. The process described for the translocation of Dorsal protein into the nucleus is very similar to the process for the translocation of the NF-κB transcription factor into the nucleus of mammalian lymphocytes. In fact, there is substantial homology between NF-κB and Dorsal, between I-B and Cactus, between Toll and the interleukin 1 receptor, between Pelle and an IL-1-associated protein kinase, and between the DNA sequences recognized by Dorsal and by NF-κB (González-Crespo and Levine 1994; Cao et al. 1996). Thus, the biochemical pathway used to specify dorsal-ventral polarity in *Drosophila* appears to be homologous to that used to differentiate lymphocytes in mammals.

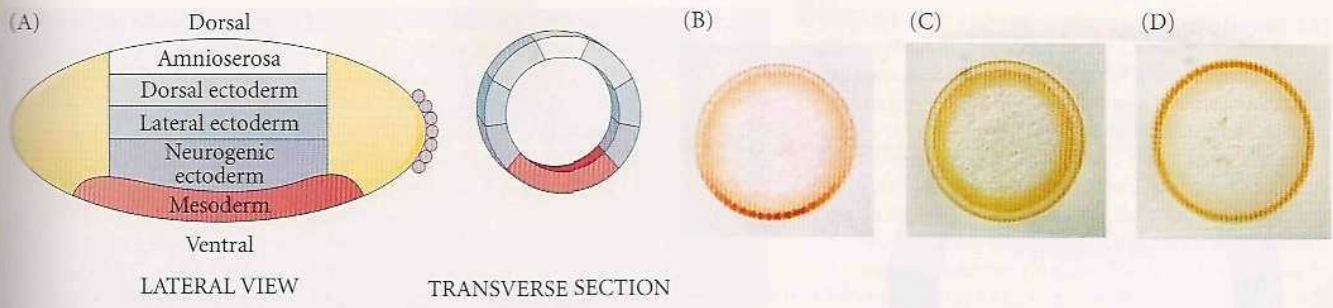


FIGURE 6.12 Specification of cell fate by the gradient of Dorsal protein. The translocation of Dorsal protein into ventral, but not lateral or dorsal, nuclei produces a gradient where the ventral cells with the most Dorsal protein become mesoderm precursors. (A) Fate map of a lateral cross section through the *Drosophila* embryo at division cycle 14. The most ventral part becomes the mesoderm; the next higher portion becomes the neurogenic (ventral) ectoderm. The lateral and dorsal ectoderm can be distinguished in the cuticle, and the dorsalmost region becomes the amnioserosa, the extraembryonic layer that surrounds the embryo. (B–D) Transverse sections of embryos stained with antibody to show the presence of Dorsal protein (dark-stained area). (B) A wild-type embryo, showing Dorsal protein in the ventralmost nuclei. (C) A dorsalized mutant, showing no localization of Dorsal protein in any nucleus. (D) A ventralized mutant, in which Dorsal protein has entered the nucleus of every cell. (A after Rushlow et al. 1989; B–D from Roth et al. 1989, courtesy of the authors.)

6.12). The next cell up from this region generates the specialized glial and neural cells of the midline. The next two cells give rise to the ventrolateral epidermis and ventral nerve cord, while the nine cells above them produce the dorsal epidermis. The most dorsal group of six cells generates the amnioserosal covering of the embryo (Ferguson and Anderson 1991). This fate map is generated by the gradient of Dorsal protein in the nuclei. Large amounts of Dorsal instruct the cells to become mesoderm, while lesser amounts instruct the cells to become glial or ectodermal tissue (Jiang and Levine 1993; Hong et al. 2008).

The first morphogenetic event of *Drosophila* gastrulation is the invagination of the 16 ventralmost cells of the embryo (Figure 6.13). All of the body muscles, fat bodies, and gonads derive from these mesodermal cells (Foe 1989). Dorsal protein specifies these cells to become mesoderm in two ways. First, the protein activates specific genes that create the mesodermal phenotype. Five of the target genes for the Dorsal protein are *twist*, *snail*, *fgf8*, the *fgf8 receptor*, and *rhomboid* (Figure 6.14). These genes are transcribed only in nuclei that have received high concentrations of Dorsal,

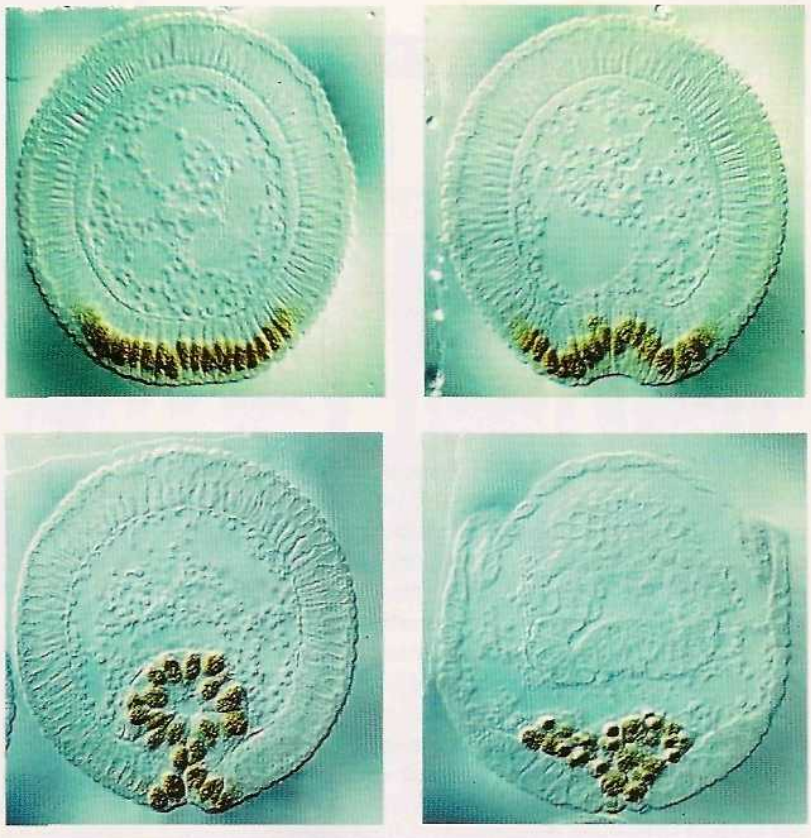


FIGURE 6.13 Gastrulation in *Drosophila*. In this cross section, the mesodermal cells at the ventral portion of the embryo buckle inward, forming the ventral furrow (see Figure 6.4A,B). This furrow becomes a tube that invaginates into the embryo and then flattens and generates the mesodermal organs. The nuclei are stained with antibody to the Twist protein, a marker for the mesoderm. (From Leptin 1991a, courtesy of M. Leptin.)

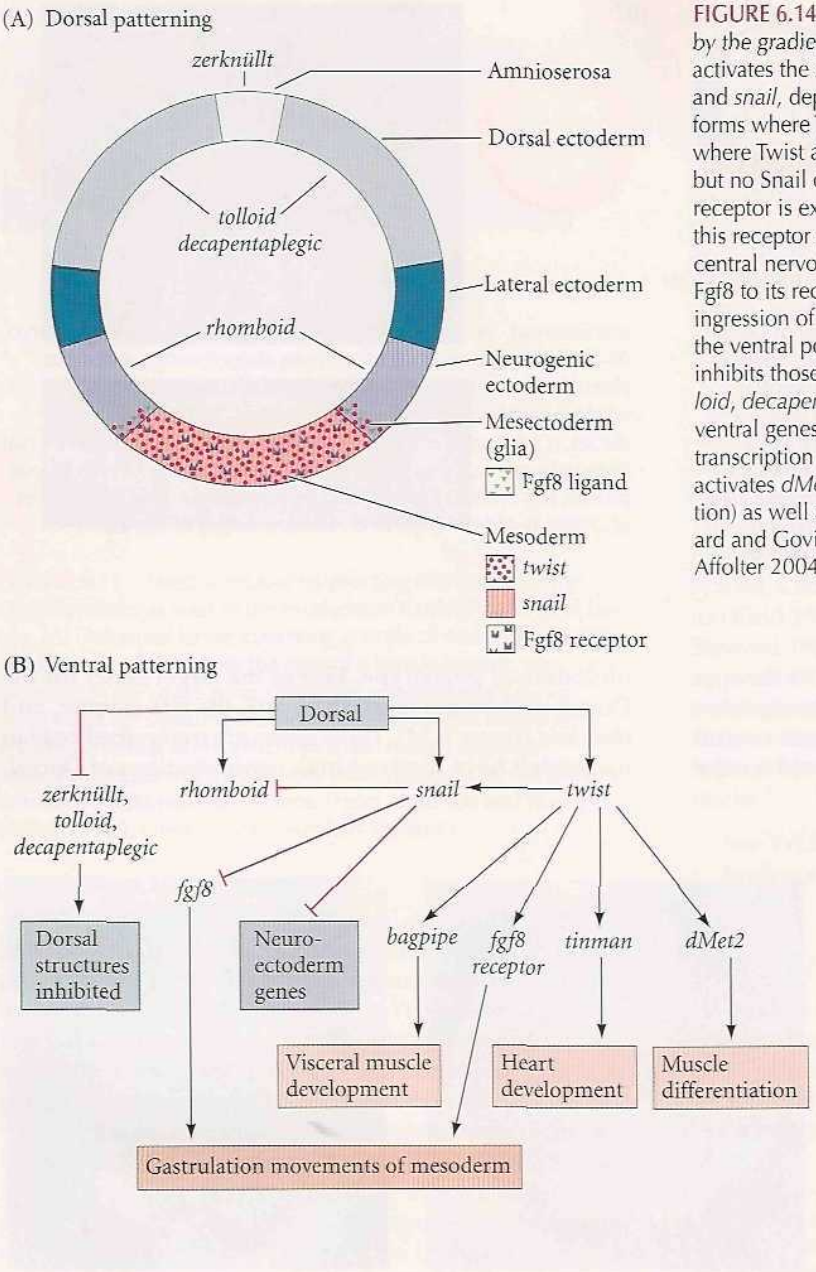


FIGURE 6.14 Subdivision of the *Drosophila* dorsal-ventral axis by the gradient of Dorsal protein in the nuclei. (A) Dorsal protein activates the zygotic genes *rhomboid*, *twist*, *fgf8*, *fgf8 receptor*, and *snail*, depending on its nuclear concentration. The mesoderm forms where Twist and Snail are present, and the glial cells form where Twist and Rhomboid interact. Those cells with Rhomboid, but no Snail or Twist, form the neurogenic ectoderm. The Fgf receptor is expressed in the mesoderm, and the Fgf8 ligands for this receptor are expressed in the mesectoderm (glia and midline central nervous system), adjacent to the mesoderm. The binding of Fgf8 to its receptor triggers the cell movements required for the ingression of the mesoderm. (B) Interactions in the specification of the ventral portion of the *Drosophila* embryo. Dorsal protein inhibits those genes that would give rise to dorsal structures (*tolloid*, *decapentaplegic*, and *zerknüllt*) while activating the three ventral genes. Snail protein, formed most ventrally, inhibits the transcription of *rhomboid* and prevents ectoderm formation. Twist activates *dMet2* and *bagpipe* (which activate muscle differentiation) as well as *tinman* (heart muscle development). (A after Steward and Govind 1993; B after Furlong et al. 2001 and Leptin and Affolter 2004.)

since their enhancers do not bind Dorsal with a very high affinity (Thisse et al. 1988, 1991; Jiang et al. 1991; Pan et al. 1991). Both Snail and Twist are also needed for the complete mesodermal phenotype and proper gastrulation (Leptin et al. 1991b). The Twist protein activates mesodermal genes, while the Snail protein represses particular non-mesodermal genes that might otherwise be active. The *rhomboid* and *fgf8* genes are interesting because they are activated by Dorsal but repressed by Snail. Thus, *rhomboid* and *fgf8* are not expressed in the most ventral cells (i.e., the mesodermal precursors) but are expressed in the cells adjacent to the mesoderm. These *rhomboid*- and *fgf8*-expressing cells will become the mesectoderm. The mesectoderm tissue is fated to become the ventral midline, once the mesoderm invaginates and brings these ventrolateral regions together. This mesectoderm gives rise to glial cells and to the midline structures of the central nervous system. Unlike the neurogenic ectoderm adjacent to it, the mesectoderm cells never form typical neuroblasts, never form epidermis, and are not a stem cell population (see Figure 6.14).

The high concentration of Twist protein in the nuclei of the ventralmost cells activates the gene for the Fgf8 receptor (the product of the *heartless* gene) in the presumptive mesoderm (Jiang and Levine 1993; Gryzik and Müller 2004; Strathopoulos et al. 2004). The expression and secretion of Fgf8 by the presumptive neural ectoderm is received by its receptor on the mesoderm cells, causing these mesoderm cells to invaginate into the embryo and flatten against the ectoderm (see Figure 6.13).

Meanwhile, *intermediate* levels of nuclear Dorsal activate transcription of the *Short gastrulation* (*Sog*) gene in two lateral stripes that flank the ventral *twist* expression domain, each 12–14 cells wide (François et al. 1994; Srinivasan et al. 2002). *Sog* encodes a protein that prevents the ectoderm in this region from becoming epidermis and begins the processes of neural differentiation (Figure 6.15).

Dorsal protein also determines the mesoderm indirectly. In addition to activating the mesoderm-stimulating genes (*twist* and *snail*), it directly inhibits the dorsalizing genes *zerknüllt* (*zen*) and *decapentaplegic* (*dpp*). Thus, in the same cells, Dorsal can act as an activator of some genes and a repressor of others. Whether Dorsal activates or represses a given gene depends on the structure of the gene's

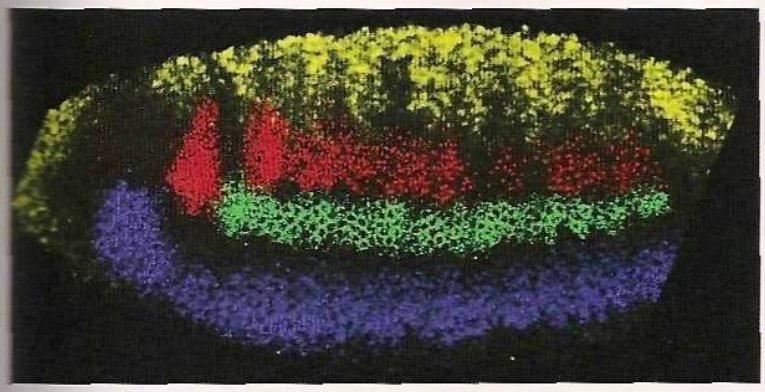


FIGURE 6.15 Dorsal-ventral patterning in *Drosophila*. The readout of the Dorsal gradient can be seen in the anterior region of a whole-mount stained embryo. The expression of the most ventral gene, *ventral nervous system defective* (blue), is from the neurogenic ectoderm. The *intermediate neuroblast defective* gene (green) is expressed in lateral ectoderm. Red represents the *muscle-specific homeobox* gene, expressed in the mesoderm above the intermediate neuroblasts. The dorsalmost tissue expresses *decapentaplegic* (yellow). (From Kosman et al. 2004, courtesy of D. Kosman and E. Bier.)

enhancers. The *zen* enhancer has a silencer region that contains a binding site for Dorsal as well as a second binding site for two other DNA-binding proteins. These two other proteins enable Dorsal to bind a transcriptional repressor protein (Groucho) and bring it to the DNA (Valentine et al. 1998). Mutants of *Dorsal* express *dpp* and *zen* genes throughout the embryo (Rushlow et al. 1987), and embryos deficient in *dpp* and *zen* fail to form dorsal structures (Irish and Gelbart 1987). Thus, in wild-type embryos, the mesodermal precursors express *twist* and *snail* (but not *zen* or *dpp*); precursors of the dorsal epidermis and amnioserosa express *zen* and *dpp* (but not *twist* or *snail*). Glial (mesectoderm) precursors express *twist* and *rhomboid*, while the lateral neural ectodermal precursors do not express any of these five genes (Kosman et al. 1991; Ray and Schüpbach 1996). By the cellular responses to the Dorsal protein gradient, the embryo becomes subdivided from the ventral to dorsal regions into mesoderm, neurogenic ectoderm, epidermis (from the lateral and dorsal ectoderm), and amnioserosa (see Figure 6.12A).

SIDELIGHTS & SPECULATIONS

The Left-Right Axis

Very little is known about the formation of the left-right axis in *Drosophila*. Although the fly may look bilaterally symmetric, there are asymmetries in the embryonic hindgut (which loops to the left) and the adult hindgut and gonads. This asymmetry appears to be regulated by microfilaments (Hozumi et al. 2006; Spéder et al. 2006). The mechanism that produces this asymmetry is different from that known to produce left-right asymmetry in vertebrates, which appears to be regulated by microtubules.

If the actin microfilaments are disrupted in the *Drosophila* embryo, many defects occur, and the left-right pattern is randomized. Loss-of-function mutations of certain genes for myosin-1 proteins (which interact with microfilaments) can reverse the insect's left-right asymmetry (Figure 6.16).

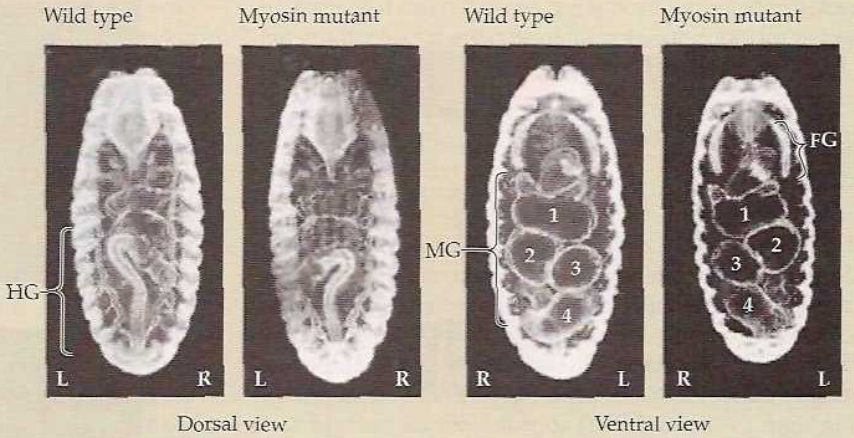


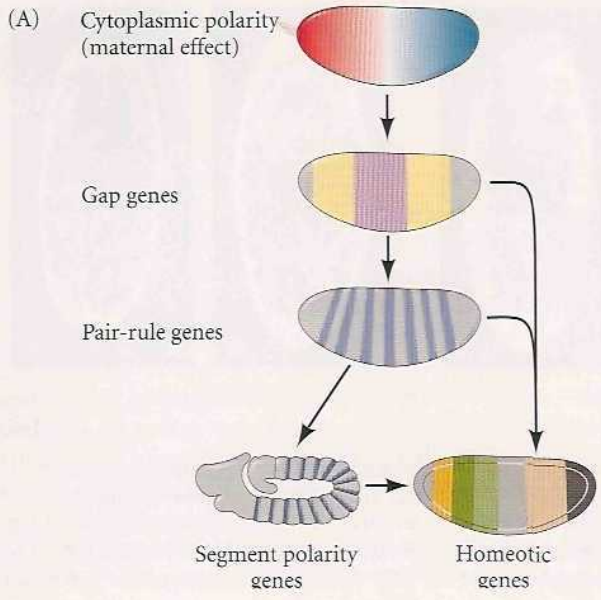
Figure 6.16 Left-right axis formation in *Drosophila* involves the microfilament cytoskeleton. Mutations in the myosin gene *Myo31DF* can reverse the insect's left-right asymmetry. Here the embryonic gut is seen in dorsal and ventral perspectives, showing that in the larva with myosin mutant, the asymmetry of the gut is reversed. HG, hindgut; MG, midgut; FG, foregut. (From Hozumi et al. 2006, courtesy of K. Matsuno.)

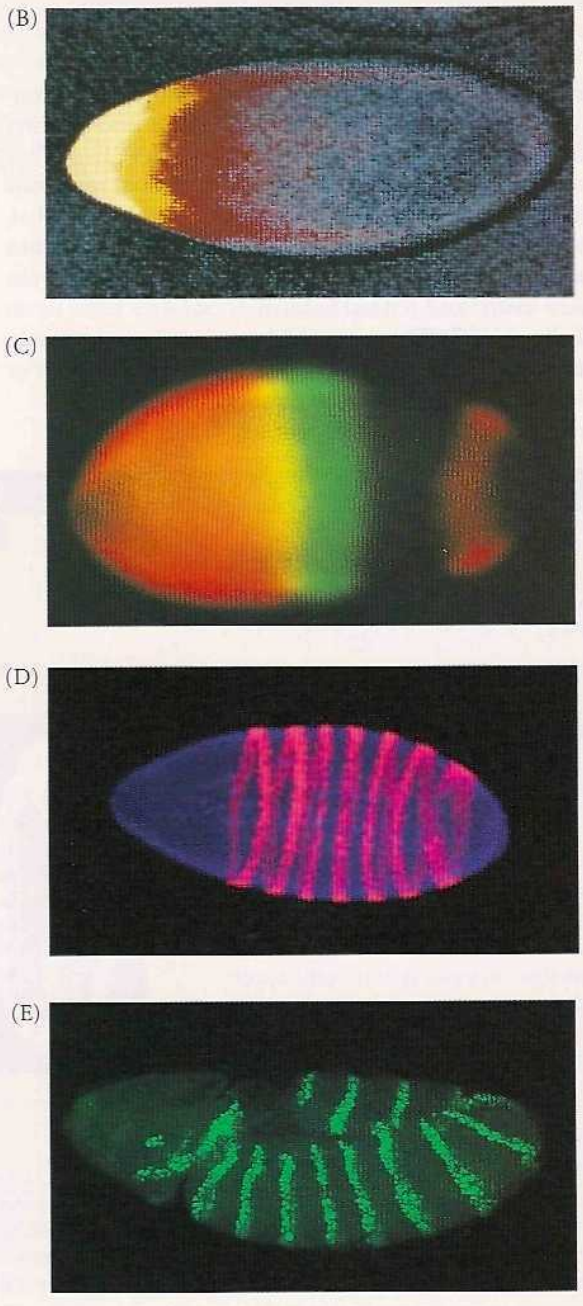
Segmentation and the Anterior-Posterior Body Plan

The genetic screens pioneered by Nüsslein-Volhard and Wieschaus identified a hierarchy of genes that establish anterior-posterior polarity, and divide the embryo into a specific number of segments with different identities (Figure 6.17). This hierarchy is initiated by **maternal effect genes** that produce messenger RNAs that are placed in different regions of the egg. These messages encode transcriptional and translational regulatory proteins that diffuse through the syncytial blastoderm and activate or repress the expression of certain zygotic genes.

The first such zygotic genes to be expressed are called **gap genes** (because mutations in them cause gaps in the segmentation pattern). These genes are expressed in certain broad (about three segments wide), partially overlapping domains. These gap genes encode transcription fac-

FIGURE 6.17 Generalized model of *Drosophila* anterior-posterior pattern formation. Anterior is to the left; the dorsal surface faces upward. (A) The pattern is established by maternal effect genes that form gradients and regions of morphogenetic proteins. These proteins are transcription factors that activate the gap genes, which define broad territories of the embryo. The gap genes enable the expression of the pair-rule genes, each of which divides the embryo into regions about two segments wide. The segment polarity genes then divide the embryo into segment-sized units along the anterior-posterior axis. Together, the actions of these genes define the spatial domains of the homeotic genes that define the identities of each of the segments. In this way, periodicity is generated from nonperiodicity, and each segment is given a unique identity. (B) Maternal effect genes. The anterior axis is specified by the gradient of Bicoid protein (yellow through red). (C) Gap gene protein expression and overlap. The domain of Hunchback protein (orange) and the domain of Krüppel protein (green) overlap to form a region containing both transcription factors (yellow). (D) Products of the *fushi tarazu* pair-rule gene form seven bands across the blastoderm of the embryo. (E) Products of the segment polarity gene *engrailed*, seen here at the extended germ band stage. (B courtesy of C. Nüsslein-Volhard; C courtesy of C. Rushlow and M. Levine; D courtesy of D. W. Knowles; E courtesy of S. Carroll and S. Paddock.)





tors, and differing combinations and concentrations of the gap gene proteins regulate the transcription of **pair-rule genes**, which divide the embryo into periodic units. The transcription of the different pair-rule genes results in a striped pattern of seven transverse bands perpendicular to the anterior-posterior axis. The proteins encoded by the pair-rule genes are transcription factors that activate the **segment polarity genes**, whose mRNA and protein products divide the embryo into 14-segment-wide units, establishing the periodicity of the embryo. At the same time, the protein products of the gap, pair-rule, and segment polarity genes interact to regulate another class of genes, the **homeotic selector genes**, whose transcription determines the developmental fate of each segment.

Maternal gradients: Polarity regulation by oocyte cytoplasm

Classical embryological experiments demonstrated that there are at least two "organizing centers" in the insect egg, one in the anterior of the egg and one in the posterior. For instance, Klaus Sander (1975) found that if he ligated the egg early in development, separating the anterior half from the posterior half, one half developed into an anterior embryo and one half developed into a posterior embryo, but neither contained the middle segments of the embryo. The later in development the ligature was made, the fewer middle segments were missing. Thus it appeared that there were indeed morphogenetic gradients emanating from the two poles during cleavage, and that these gradients interacted to produce the positional information determining the identity of each segment.

Moreover, when the RNA in the anterior of insect eggs was destroyed (by either ultraviolet light or RNase), the resulting embryos lacked a head and thorax. Instead, these embryos developed two abdomens and *telsons* (tails) with mirror-image symmetry: telson-abdomen-abdomen-telson (Figure 6.18; Kalthoff and Sander 1968; Kandler-Singer and Kalthoff 1976). Sander's laboratory postulated the existence of a gradient at both ends of the egg, and hypothesized that the egg sequesters an mRNA that generates a gradient of anterior-forming material.

The molecular model: Protein gradients in the early embryo

In the late 1980s, the gradient hypothesis was united with a genetic approach to the study of *Drosophila* embryogenesis. If there were gradients, what were the morphogens whose concentrations changed over space? What were the genes that shaped these gradients? And did these morphogens act by activating or inhibiting certain genes in the areas where they were concentrated? Christiane Nüsslein-Volhard led a research program that addressed these questions. The researchers found that one set of genes encoded morphogens for the anterior part of the embryo, another

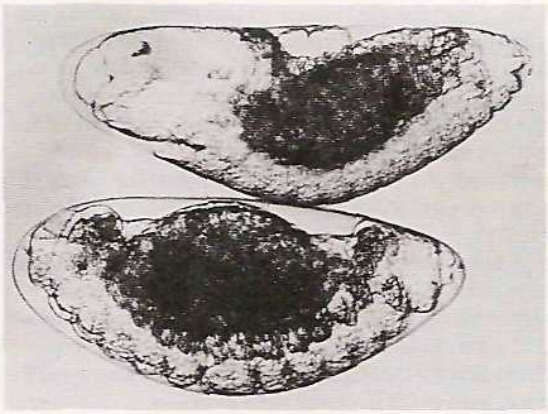


FIGURE 6.18 Normal and irradiated embryos of the midge *Smittia*. The normal embryo (top) shows a head on the left and abdominal segments on the right. The UV-irradiated embryo (bottom) has no head region but has abdominal and tail segments at both ends. (From Kalthoff 1969, courtesy of K. Kalthoff.)

set of genes encoded morphogens responsible for organizing the posterior region of the embryo, and a third set of genes encoded proteins that produced the terminal regions at both ends of the embryo (Table 6.1).

Two maternal messenger RNAs, *bicoid* and *nanos*, are most critical to the formation of the anterior-posterior axis. The *bicoid* mRNAs are located near the anterior tip of the unfertilized egg, and *nanos* messages are located at the posterior tip. These distributions occur as a result of the dramatic polarization of the microtubule networks in the developing oocyte (see Figure 6.7). After ovulation and fertilization, the *bicoid* and *nanos* mRNAs are translated into proteins that can diffuse in the syncytial blastoderm, forming gradients that are critical for anterior-posterior patterning (Figure 6.19; see also Figure 6.17B).

See WEBSITE 6.4
Christiane Nüsslein-Volhard and the molecular approach to development

BICOID AS THE ANTERIOR MORPHOGEN That Bicoid was the head morphogen of *Drosophila* was demonstrated by the "find it, lose it, move it" experimentation scheme. Christiane Nüsslein-Volhard, Wolfgang Driever, and their colleagues (Driever and Nüsslein-Volhard 1988; Driever et al. 1990) showed that (1) Bicoid protein was found in a gradient, highest in the anterior (head-forming) region; (2) embryos lacking Bicoid could not form a head; and (3) when *bicoid* mRNA was added to Bicoid-deficient embryos in different places, the place where *bicoid* mRNA was injected became the head. Moreover, the areas around the site of Bicoid injection became the thorax, as expected from a concentration-dependent signal (Figure 6.20). When inject-

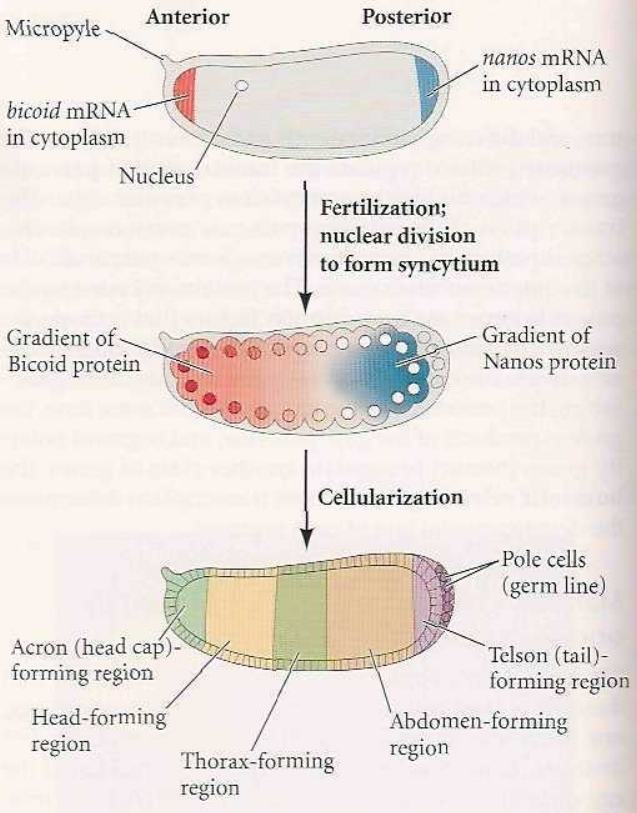


FIGURE 6.19 Syncytial specification in *Drosophila*. Anterior-posterior specification originates from morphogen gradients in the egg cytoplasm. *Bicoid* mRNA is stabilized in the most anterior portion of the egg, while *Nanos* mRNA is tethered to the posterior end. (The anterior can be recognized by the micropyle on the shell; this structure permits sperm to enter.) When the egg is laid and fertilized, these two mRNAs are translated into proteins. The Bicoid protein forms a gradient that is highest at the anterior end, and the Nanos protein forms a gradient that is highest at the posterior end. These two proteins form a coordinate system based on their ratios. Each position along the axis is thus distinguished from any other position. When the nuclei form, each nucleus is given its positional information by the ratio of these proteins. The proteins forming these gradients activate the transcription of the genes specifying the segmental identities of the larva and the adult fly.

ed into the anterior of *bicoid*-deficient embryos (whose mothers lacked *bicoid* genes), the *bicoid* mRNA "rescued" the embryos and they developed normal anterior-posterior polarity. Moreover, any location in an embryo where the *bicoid* message was injected became the head. If *bicoid* mRNA was injected into the center of an embryo, that mid-

TABLE 6.1 Maternal effect genes that establish the anterior-posterior polarity of the *Drosophila* embryo

Gene	Mutant phenotype	Proposed function and structure
ANTERIOR GROUP		
bicoid (bcd)	Head and thorax deleted, replaced by inverted telson	Graded anterior morphogen; contains homeodomain; represses *caudal* mRNA
exuperantia (exu)	Anterior head structures deleted	Anchors *bicoid* mRNA
swallow (swa)	Anterior head structures deleted	Anchors *bicoid* mRNA
POSTERIOR GROUP		
nanos (nos)	No abdomen	Posterior morphogen; represses *hunchback* mRNA
tudor (tud)	No abdomen, no pole cells	Localization of Nanos protein
oskar (osk)	No abdomen, no pole cells	Localization of Nanos protein
vasa (vas)	No abdomen, no pole cells; oogenesis defective	Localization of Nanos protein
valois (val)	No abdomen, no pole cells; cellularization defective	Stabilization of the Nanos localization complex
pumilio (pum)	No abdomen	Helps Nanos protein bind *hunchback* message
caudal (cad)	No abdomen	Activates posterior terminal genes
TERMINAL GROUP		
torso (tor)	No termini	Possible morphogen for termini
trunk (trk)	No termini	Transmits Torso-like signal to Torso
fs(1)Nasrat[fs(1)N]	No termini; collapsed eggs	Transmits Torso-like signal to Torso
fs(1)polehole[fs(1)ph]	No termini; collapsed eggs	Transmits Torso-like signal to Torso

Source: After Anderson 1989.

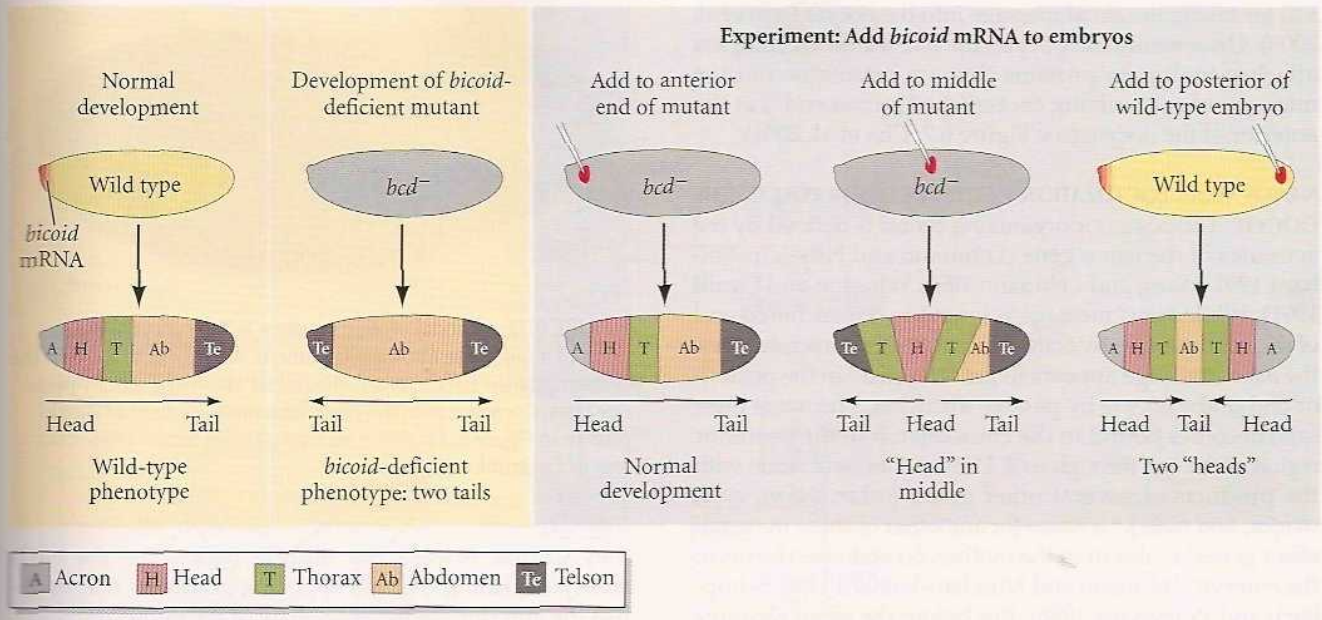


FIGURE 6.20 Schematic representation of experiments demonstrating that the *bicoid* gene encodes the morphogen responsible for head structures in *Drosophila*. The phenotypes of *bicoid*-deficient and wild-type embryos are shown at left. When *bicoid*-deficient embryos are injected with *bicoid* mRNA, the point of injection forms the head structures. When the posterior pole of an early-cleavage wild-type embryo is injected with *bicoid* mRNA, head structures form at both poles. (After Driever et al. 1990.)

dle region became the head, with the regions on either side of it becoming thorax structures. If a large amount of *bicoid* mRNA was injected into the posterior end of a wild-type embryo (with its own endogenous *bicoid* message in its anterior pole), two heads emerged, one at either end (Driever et al. 1990).

***BICOID* MRNA LOCALIZATION IN THE ANTERIOR POLE OF THE OOCYTE** The 3′ untranslated region (UTR) of *bicoid* mRNA contains sequences that are critical for its localization at the anterior pole (Figure 6.21; Ferrandon et al. 1997; Macdonald and Kerr 1998; Spirov et al. 2009). These sequences interact with the **Exuperantia** and **Swallow** proteins while the messages are still in the nurse cells of the egg chamber (Schnorrer et al. 2000). Experiments in which fluorescently labeled *bicoid* mRNA was microinjected into living egg chambers of wild-type or mutant flies indicate that Exuperantia must be present in the nurse cells for anterior localization. But having Exuperantia in the oocyte is not sufficient to bring the *bicoid* message into the oocyte (Cha et al. 2001; Reichmann and Ephrussi 2005). The *bicoid*-protein complex is transported out of the nurse cells and into the oocyte via microtubules. The complex seems to ride on a kinesin ATPase that is crit-

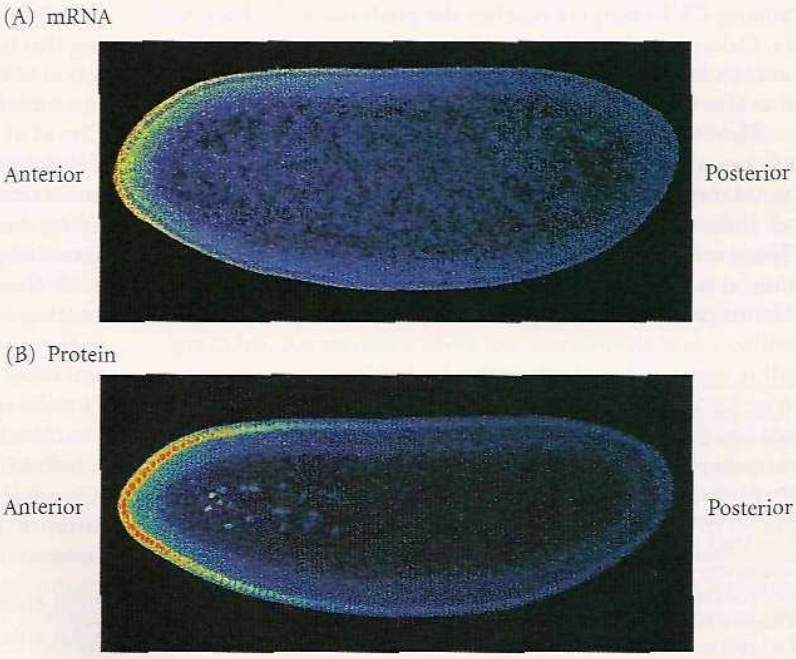


FIGURE 6.21 *Bicoid* mRNA and protein gradients shown by in situ hybridization and confocal microscopy. (A) *Bicoid* mRNA shows a steep gradient across the anterior portion of the oocyte. (B) When the mRNA is translated, the Bicoid protein gradient can be seen in the anterior nuclei. Anterior is to the left; the dorsal surface is upward. (After Spirov et al., courtesy of S. Baumgartner.)

ical for taking the *bicoid* message into the oocyte (Arn et al. 2003). Once inside the oocyte, the *bicoid*-mRNA complex attaches to dynein proteins that are maintained at the microtubule organizing center (the "minus end") at the anterior of the oocyte (see Figure 6.7; Cha et al. 2001).

NANOS MRNA LOCALIZATION IN THE POSTERIOR POLE OF THE OOCYTE The posterior organizing center is defined by the activities of the *nanos* gene (Lehmann and Nüsslein-Volhard 1991; Wang and Lehmann 1991; Wharton and Struhl 1991). While *bicoid* message is bound to the anchored end of the microtubules by active transport along microtubules, the *nanos* message appears to get "trapped" in the posterior end of the oocyte by passive diffusion. The *nanos* message becomes bound to the cytoskeleton in the posterior region of the egg through its 3′ UTR and its association with the products of several other genes (*oskar*, *valois*, *vasa*, *staufen*, and *tudor*).* If *nanos* (or any other of these maternal effect genes) is absent in the mother, no abdomen forms in the embryo (Lehmann and Nüsslein-Volhard 1986; Schüpbach and Wieschaus 1986). But before the *nanos* message can get "trapped" in the posterior cortex, a *nanos* mRNA-specific trap has to be made; this trap is the Oskar protein (Ephrussi et al. 1991). The *oskar* message and the Staufen protein are transported to the posterior end of the oocyte by the kinesin motor protein (see Figure 6.7). There they become bound to the actin microfilaments of the cortex. Staufen allows the translation of the *oskar* message, and the resulting Oskar protein is capable of binding the *nanos* message (Brendza et al. 2000; Hatchet and Ephrussi 2004).

Most *nanos* mRNA, however, is not trapped. Rather, it is bound in the cytoplasm by the translation inhibitors Smaug and CUP. Smaug binds to the 3′ UTR of *nanos* mRNA and recruits the CUP protein that prevents the association of the message with the ribosome. If the *nanos*-Smaug-CUP complex reaches the posterior pole, however, Oskar can dissociate CUP from Smaug, allowing the mRNA to be bound at the posterior and ready for translation (Forrest et al. 2004; Nelson et al. 2004).

Therefore, at the completion of oogenesis, the *bicoid* message is anchored at the anterior end of the oocyte, and the *nanos* message is tethered to the posterior end (Frigerio et al. 1986; Berleth et al. 1988; Gavis and Lehmann 1992). These mRNAs are dormant until ovulation and fertilization, at which time they are translated. Since the Bicoid and Nanos *protein products* are not bound to the cytoskeleton, they diffuse toward the middle regions of the early embryo, creating the two opposing gradients that establish the anterior-posterior polarity of the embryo.

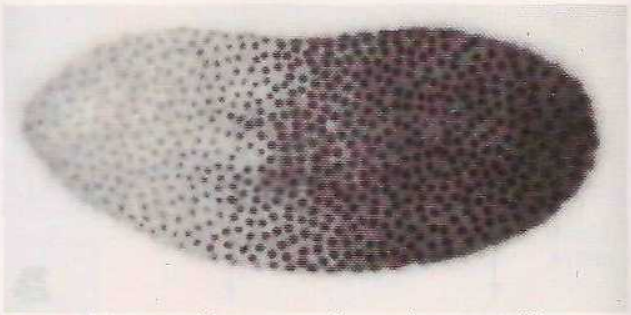


FIGURE 6.22 Caudal protein gradient in the syncytial blastoderm of a wild-type *Drosophila* embryo. Anterior is to the left. The protein (stained darkly) enters the nuclei and helps specify posterior fates. Compare with the complementary gradient of Bicoid protein in Figure 6.21. (From Macdonald and Struhl 1986, courtesy of G. Struhl.)

Two other maternally provided mRNAs (*hunchback*, *hb*; and *caudal*, *cad*) are critical for patterning the anterior and posterior regions of the body plan, respectively (Lehmann et al. 1987; Wu and Lengyel 1998). These two mRNAs are synthesized by the nurse cells of the ovary and transported to the oocyte, where they are distributed ubiquitously throughout the syncytial blastoderm. But if they are not localized, how do they mediate their localized patterning activities? It turns out that translation of the *hb* and *cad* mRNAs is repressed by the diffusion gradients of Nanos and Bicoid proteins, respectively.

In anterior regions, Bicoid binds to a specific region of *caudal*'s 3′ UTR. Here, it binds d4HEP, a protein that inhibits translation by binding to the 5′ cap of the message, thereby preventing its binding to the ribosome. By recruiting this translational inhibitor, Bicoid thus prevents translation of Caudal in the anterior section of the embryo (Figure 6.22; Rivera-Pomar et al. 1996; Chan and Struhl 1997; Cho et al. 2006). This suppression is necessary because if Caudal protein is made in the embryo's anterior, the head and thorax do not form properly. Caudal is critical in specifying the posterior domains of the embryo, activating the genes responsible for the invagination of the hindgut.

At the other end of the embryo, Nanos functions by preventing *hunchback* translation (Tautz 1988). Nanos protein in the posterior of the embryo forms a complex with several other ubiquitous proteins, including Pumilio and Brat. Pumilio appears to direct the complex to the 3′ UTR of the *hunchback* message, and Brat appears to recruit d4HEP, which will inhibit the translation of the *hunchback* message (Cho et al. 2006). The result of these interactions is the creation of four maternal protein gradients in the early embryo (Figure 6.23):

- An anterior-to-posterior gradient of Bicoid protein
- An anterior-to-posterior gradient of Hunchback protein

*Like the placement of the *bicoid* message, the location of the *nanos* message is determined by its 3′ UTR. If the *bicoid* 3′ UTR is experimentally placed on the protein-encoding region of *nanos* mRNA, the *nanos* message gets placed in the anterior of the egg. When the RNA is translated, the Nanos protein inhibits the translation of *hunchback* and *bicoid* mRNAs, and the embryo forms two abdomens—one in the anterior of the embryo and one in the posterior (Gavis and Lehmann 1992). We will see these proteins again in Chapter 16, since they are critical in forming the germ cells of *Drosophila*.

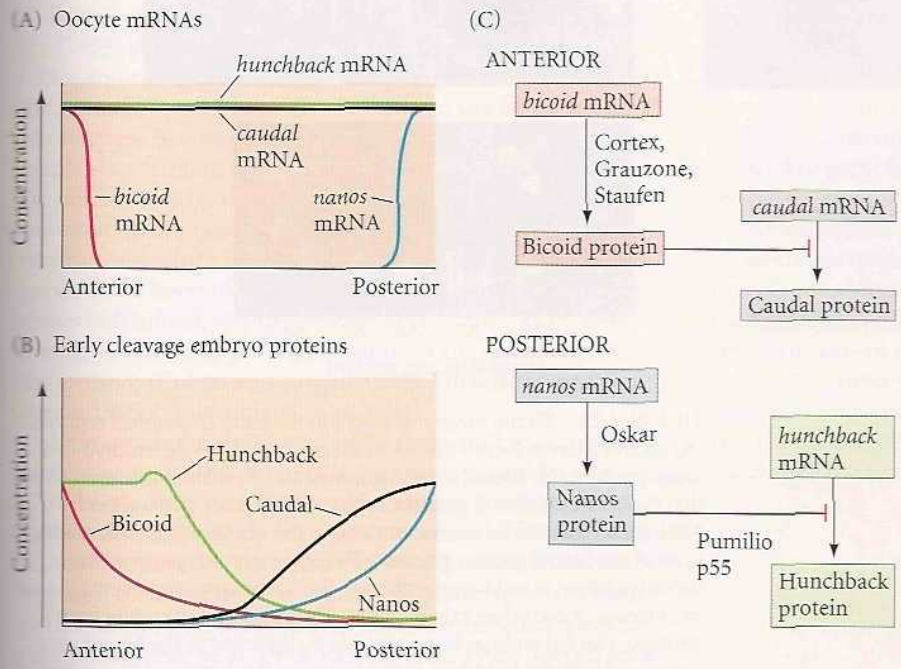


FIGURE 6.23 Model of anterior-posterior pattern generation by *Drosophila* maternal effect genes. (A) The *bicoid*, *nanos*, *hunchback*, and *caudal* mRNAs are placed in the oocyte by the ovarian nurse cells. The *bicoid* message is sequestered anteriorly; the *nanos* message is sent to the posterior pole. (B) Upon translation, the Bicoid protein gradient extends from anterior to posterior, while the Nanos protein gradient extends from posterior to anterior. Nanos inhibits the translation of the *hunchback* message (in the posterior), while Bicoid prevents the translation of the *caudal* message (in the anterior). This inhibition results in opposing Caudal and Hunchback gradients. The Hunchback gradient is secondarily strengthened by transcription of the *hunchback* gene in the anterior nuclei (since Bicoid acts as a transcription factor to activate *hunchback* transcription). (C) Parallel interactions whereby translational gene regulation establishes the anterior-posterior patterning of the *Drosophila* embryo. (C after Macdonald and Smibert 1996.)

- A posterior-to-anterior gradient of Nanos protein
- A posterior-to-anterior gradient of Caudal protein

The Bicoid, Hunchback, and Caudal proteins are transcription factors whose relative concentrations can activate or repress particular zygotic genes. The stage is now set for the activation of zygotic genes in those nuclei that were busy dividing while these four protein gradients were being established.

The anterior organizing center: The Bicoid and Hunchback gradients

In *Drosophila*, the phenotype of the *bicoid* mutant provides valuable information about the function of morphogenetic gradients (Figure 6.24A–C). Instead of having anterior structures (acron, head, and thorax) followed by abdominal structures and a telson, the structure of the *bicoid* mutant is telson-abdomen-abdomen-telson (Figure 6.24D). It would appear that these embryos lack whatever substances are needed for the formation of anterior structures. Moreover, one could hypothesize that the substance these mutants lack is the one postulated by Sander and Kalthoff to turn on genes for the anterior structures and turn off genes for the telson structures (compare Figures 6.18 and 6.24D).

Several studies support the view that the product of the wild-type *bicoid* gene is the morphogen that controls anterior development. The first type of evidence came from experiments that altered the shape of the Bicoid protein gradient. As we have seen, the *exuperantia* and *swallow* genes are responsible for keeping the *bicoid* message at the anterior pole of the egg. In their absence, the *bicoid* message diffuses farther into the posterior of the egg, and the protein gradient is less steep (Driever and Nüsslein-Volhard 1988a). The phenotype produced by *exuperantia* and *swallow* mutants is similar to that of *bicoid*-deficient embryos but is less severe; these embryos lack their most anterior structures and have an extended mouth and thoracic region. Furthermore, by adding extra copies of the *bicoid* gene, the Bicoid protein gradient can be extended

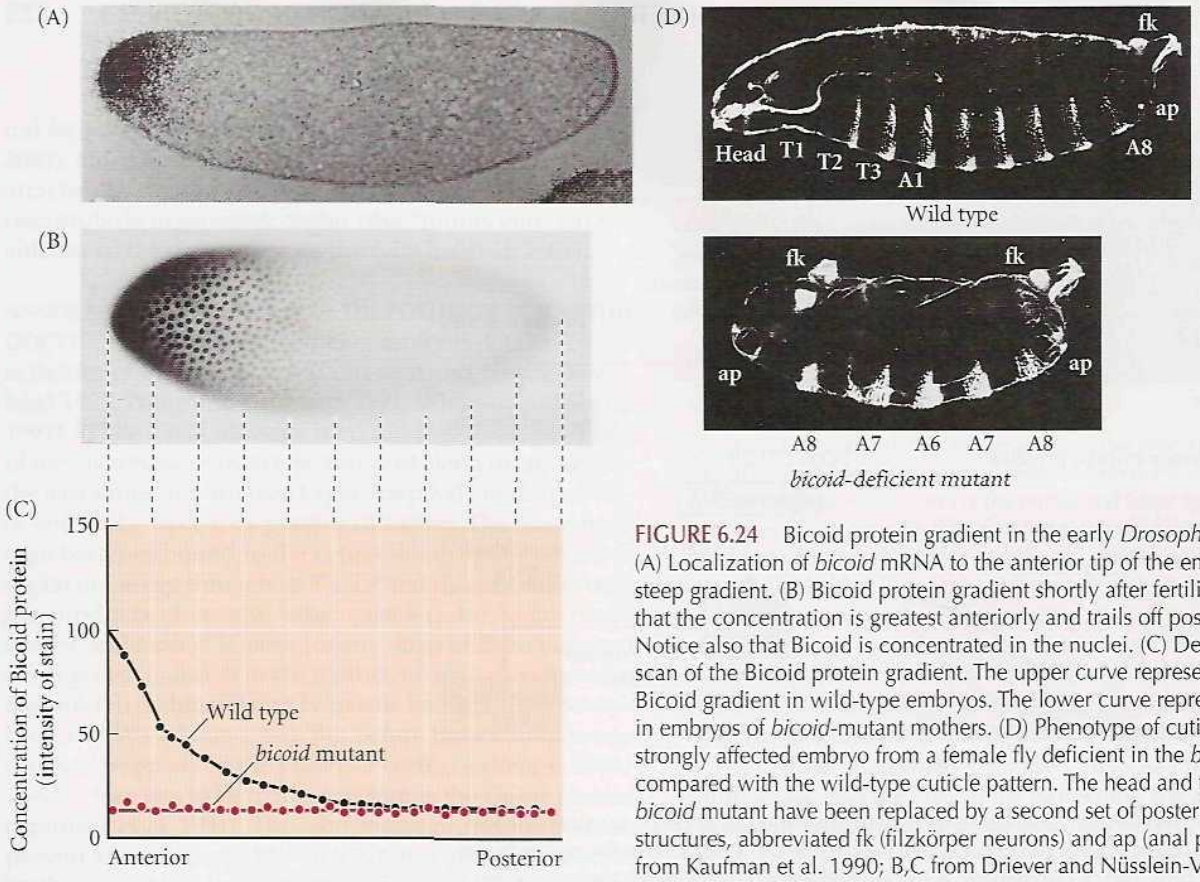


FIGURE 6.24 Bicoid protein gradient in the early *Drosophila* embryo. (A) Localization of *bicoid* mRNA to the anterior tip of the embryo in a steep gradient. (B) Bicoid protein gradient shortly after fertilization. Note that the concentration is greatest anteriorly and trails off posteriorly. Notice also that Bicoid is concentrated in the nuclei. (C) Densitometric scan of the Bicoid protein gradient. The upper curve represents the Bicoid gradient in wild-type embryos. The lower curve represents Bicoid in embryos of *bicoid*-mutant mothers. (D) Phenotype of cuticle from a strongly affected embryo from a female fly deficient in the *bicoid* gene compared with the wild-type cuticle pattern. The head and thorax of the *bicoid* mutant have been replaced by a second set of posterior telson structures, abbreviated fk (filzkörper neurons) and ap (anal plates). (A from Kaufman et al. 1990; B,C from Driever and Nüsslein-Volhard 1988b; D from Driever et al. 1990, courtesy of the authors.)

into more posterior regions, causing anterior structures like the cephalic furrow to be expressed in a more posterior position (Driever and Nüsslein-Volhard 1988a; Struhl et al. 1989). Thus, altering the Bicoid gradient correspondingly alters the fate of specific embryonic regions.

It had been thought that, once the *bicoid* message was translated, the gradient of Bicoid protein would be generated simply by diffusion of the protein; the reality is a bit more complicated. In 2007, Thomas Gregor and his colleagues demonstrated that the speed of diffusion cannot account for the rapid deployment of the Bicoid protein gradient. Shortly thereafter, using highly sensitive confocal microscopy, Weil and colleagues (2008) showed that the anteriorly localized *bicoid* message became dispersed by egg activation (at ovulation), and Spirov and collaborators (2009) showed that the *bicoid* mRNA was transported along microtubules to form a gradient that prefigured the gradient of its protein (see Figures 6.21 and 6.24A,B). The *bicoid* mRNA gradient is established at nuclear cycle 10 (the beginning of the syncytial blastoderm stage), persists through nuclear division 13, and disappears as the mRNA is degraded during the initial stages of cycle 14 (when the blastoderm becomes cellular).

Whether the gradient of Bicoid protein arises from diffusion from a single source or from localized synthesis, it appears to act as a *morphogen*. As described in the Part II opening essay, morphogens are substances that differentially specify the fates of cells by different concentrations. High concentrations of Bicoid produce anterior head structures. Slightly less Bicoid tells the cells to become jaws. A moderate concentration of Bicoid is responsible for instructing cells to become the thorax, while the abdomen is characterized as lacking Bicoid.

How might a gradient of Bicoid protein control the determination of the anterior-posterior axis? As discussed earlier (see Figure II.9), Bicoid protein acts as a translation inhibitor of *caudal*, and *caudal*'s protein product is critical for the specification of the posterior. However, Bicoid's primary function is to act as a transcription factor that activates the expression of target genes in the anterior part of the embryo.*

The first target of Bicoid to be discovered was the *hunchback* (*hb*) gene. In the late 1980s, two laboratories independently demonstrated that Bicoid binds to and activates *hb* (Driever and Nüsslein-Volhard 1989; Struhl et al. 1989). Bicoid-dependent transcription of *hb* is seen only in the anterior half of the embryo—the region where Bicoid is found. This transcription reinforces the gradient of maternal Hunchback protein produced by Nanos-dependent translational repression. Mutants deficient in maternal and zygotic *hb* genes lack mouthparts and thorax structures. Therefore, both maternal and zygotic Hunchback contribute to the anterior patterning of the embryo.

**bicoid* appears to be a relatively "new" gene that evolved in the Dipteran (fly) lineage; it has not been found in other insect lineages. The anterior determinant in other insect groups has not yet been determined but appears to have *bicoid*-like properties (Wolff et al. 1998; Lynch and Desplan 2003).

Based on two pieces of evidence, Driever and co-workers (1989) predicted that Bicoid must activate at least one other anterior gene besides *hb*. First, deletions of *hb* produced only some of the defects seen in the *bicoid* mutant phenotype. Second, the *swallow* and *exuperantia* experiments showed that only moderate levels of Bicoid protein are needed to activate thorax formation (i.e., *hunchback* gene expression), but head formation requires higher Bicoid concentrations. Since then, a large number of Bicoid target genes have been identified. These include the head gap genes *buttonhead*, *empty spiracles*, and *orthodenticle*, which are expressed in specific subregions of the anterior part of the embryo (Cohen and Jürgens 1990; Finkelstein and Perrimon 1990; Grossniklaus et al. 1994).

Driever and co-workers (1989) predicted that the promoters of such a head-specific gap gene would have low-affinity binding sites for Bicoid, causing them to be activated only at extremely high concentrations of Bicoid—that is, near the anterior tip of the embryo. In addition to needing high Bicoid levels for activation, transcription of these genes also requires the presence of Hunchback protein (Simpson-Brose et al. 1994; Reinitz et al. 1995). Bicoid and Hunchback act synergistically at the enhancers of these "head genes" to promote their transcription in a feedforward manner (see Figure 5.13).

In the posterior half of the embryo, the Caudal protein gradient also activates a number of zygotic genes, including the gap genes *knirps* (*kni*) and *giant* (*gt*), which are critical for abdominal development (Rivera-Pomar 1995; Schulz and Tautz 1995).

The terminal gene group

In addition to the anterior and posterior morphogens, there is a third set of maternal genes whose proteins generate the unsegmented extremities of the anterior-posterior axis: the **acron** (the terminal portion of the head that includes the brain) and the **telson** (tail). Mutations in these terminal genes result in the loss of the acron and the most anterior head segments as well as the telson and the most posterior abdominal segments (Degelmann et al. 1986; Klingler et al. 1988). A critical gene here appears to be ***torso***, a gene encoding a receptor tyrosine kinase (RTK; see Chapter 3). The embryos of mothers with mutations of *torso* have neither acron nor telson, suggesting that the two termini of the embryo are formed through the same pathway. The *torso* mRNA is synthesized by the ovarian cells, deposited in the oocyte, and translated after fertilization. The transmembrane Torso protein is not spatially restricted to the ends of the egg but is evenly distributed throughout the plasma membrane (Casanova and Struhl 1989). Indeed, a gain-of-function mutation of *torso*, which imparts constitutive activity to the receptor, converts the entire anterior half of the embryo into an acron and the entire posterior half into a telson. Thus, Torso must normally be activated only at the ends of the egg.

Stevens and her colleagues (1990) have shown that this is the case. Torso protein is activated by the follicle cells only at the two poles of the oocyte. Two pieces of evidence suggest that the activator of Torso is probably the **Torso-like** protein: first, loss-of-function mutations in the *torso-like* gene create a phenotype almost identical to that produced by *torso* mutants; and second, ectopic expression of Torso-like protein activates Torso in the new location. The *torso-like* gene is usually expressed only in the anterior and posterior follicle cells, and secreted Torso-like protein can cross the perivitelline space to activate Torso in the egg membrane (Martin et al. 1994; Furriols et al. 1998). In this manner, Torso-like activates Torso in the anterior and posterior regions of the oocyte membrane.

The end products of the RTK cascade activated by Torso diffuse into the cytoplasm at both ends of the embryo (Figure 6.25; Gabay et al. 1997). These kinases are thought to inactivate the Groucho protein, a transcriptional inhibitor

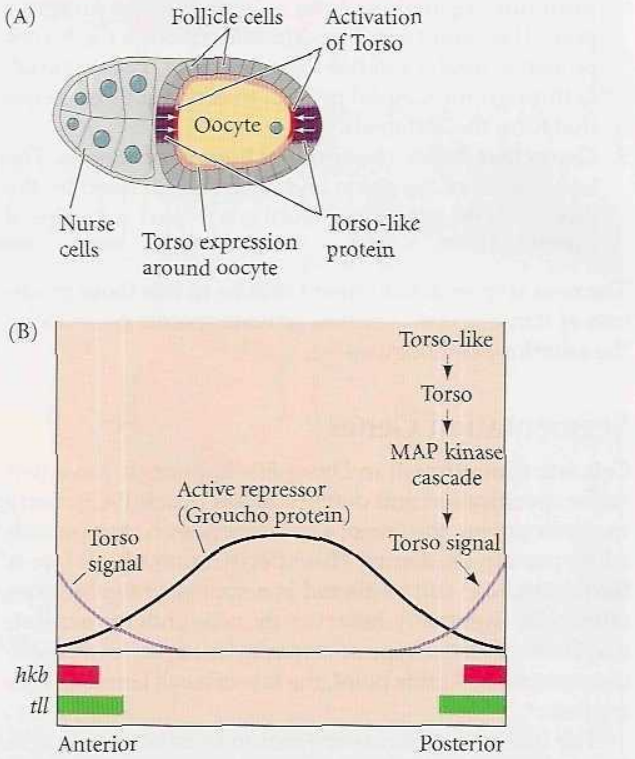


FIGURE 6.25 Formation of the unsegmented extremities by Torso signaling. (A) Torso-like protein is expressed by the follicle cells at the poles of the oocyte. Torso protein is uniformly distributed throughout the plasma membrane of the oocyte. Torso-like activates Torso at the poles (see Casanova et al. 1995). (B) Inactivation of the transcriptional suppression of *huckebein* (*hkb*) and *tailless* (*tll*) genes. The Torso signal antagonizes the Groucho protein. Groucho represses *tailless* and *huckebein* expression. The gradient of Torso is thought to provide the information that allows *tailless* to be expressed farther into the embryo than *huckebein*. (A after Gabay et al. 1997; B after Paroush et al. 1997.)

of the *tailless* and *huckebein* gap genes (Paroush et al. 1997); it is these two gap genes that specify the termini of the embryo. The distinction between the anterior and posterior termini depends on the presence of Bicoid. If *tailless* and *huckebein* act alone, the terminal region differentiates into a telson. However, if Bicoid is also present, the terminal region forms an acron (Pignoni et al. 1992).

Summarizing early anterior-posterior axis specification in Drosophila

The anterior-posterior axis of the *Drosophila* embryo is specified by three sets of genes:

1. **Genes that define the anterior organizing center.** Located at the anterior end of the embryo, the anterior organizing center acts through a gradient of Bicoid protein. Bicoid functions as a *transcription factor* to activate anterior-specific gap genes and as a *translational repressor* to suppress posterior-specific gap genes.
2. **Genes that define the posterior organizing center**. The posterior organizing center is located at the posterior pole. This center acts *translationally* through the Nanos protein to inhibit anterior formation, and *transcriptionally* through the Caudal protein to activate those genes that form the abdomen.
3. **Genes that define the terminal boundary regions**. The boundaries of the acron and telson are defined by the product of the *torso* gene, which is activated at the tips of the embryo.

The next step in development will be to use these gradients of transcription factors to activate specific genes along the anterior-posterior axis.

Segmentation Genes

Cell fate commitment in *Drosophila* appears to have two steps: specification and determination (Slack 1983). Early in development, the fate of a cell depends on cues provided by protein gradients. This specification of cell fate is flexible and can still be altered in response to signals from other cells. Eventually, however, the cells undergo a transition from this loose type of commitment to an irreversible determination. At this point, the fate of a cell becomes cell-intrinsic.*

The transition from specification to determination in *Drosophila* is mediated by **segmentation genes** that divide the early embryo into a repeating series of segmental primordia along the anterior-posterior axis. Segmentation genes were originally defined by zygotic mutations that disrupted the body plan, and these genes were divided into three groups based on their mutant phenotypes (Nüsslein-Volhard and Wieschaus 1980):

1. **Gap** mutants lacked large regions of the body (several contiguous segments; Figure 6.26A).
2. **Pair-rule** mutants lacked portions of every other segment (Figure 6.26B).
3. **Segment polarity** mutants showed defects (deletions, duplications, polarity reversals) in every segment (Figure 6.26C).

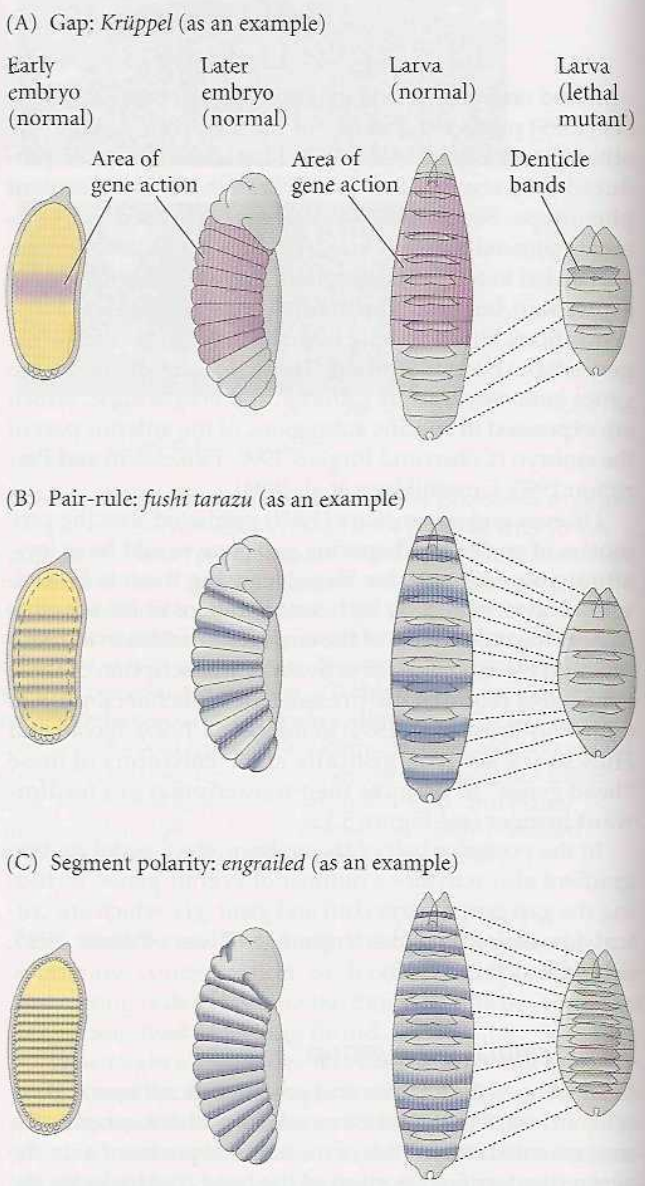


FIGURE 6.26 Three types of segmentation gene mutations. The left panel shows the early-cleavage embryo, with the region where the particular gene is normally transcribed in wild-type embryos shown in color. These areas are deleted as the mutants (right panel) develop.

*Aficionados of information theory will recognize that the process by which the anterior-posterior information in morphogenetic gradients is transferred to discrete and different parasegments represents a transition from analog to digital specification. Specification is analog, determination digital. This process enables the transient information of the gradients in the syncytial blastoderm to be stabilized so that it can be utilized much later in development (Baumgartner and Noll 1990).

SIDELIGHTS & SPECULATIONS

Segments and Parasegments

Mutations in segmentation genes result in *Drosophila* embryos that lack certain segments or parts of segments. However, early researchers found a surprising aspect of these mutations: many of them did not affect actual segments. Rather, they affected the posterior compartment of one segment and the anterior compartment of the immediately posterior segment. These "transegmental" units were named **parasegments** (Figure 6.27A; Martinez-Arias and Lawrence 1985). Moreover, once the means to detect gene expression patterns were available, it was discovered that the expression patterns in the early embryo are delineated by parasegmental boundaries—not by the boundaries of the segments. Thus, the parasegment appears to be the fundamental unit of *embryonic* gene expression.

Although parasegmental organization is also seen in the nerve cord of adult *Drosophila*, it is not seen in the adult epidermis (which is the most obvious manifestation of segmentation), nor is it found in the adult musculature. These adult structures are organized along the segmental pattern. In *Drosophila*, the segmental grooves appear in the epidermis when the germ band is retracted, while the mesoderm becomes segmental later in development.

One can think about the segmental and parasegmental organization schemes as representing different ways of organizing the compartments along the anterior-posterior axis of the embryo. The cells of one compartment do not mix with cells of neighboring compartments, and parasegments and segments are out of phase by one compartment.

Why should there be two modes of metamerism (sequential parts) in flies? Jean Deutsch has proposed that such a twofold way of organizing the body is needed for the coordination of movement. In every group of the Arthropoda—crustaceans, insects, myriapods, and chelicerates (spiders)—the ganglia of the ventral nerve cord are organized by parasegments, but the cuticle grooves and musculature are segmental. Viewing the segmental border as a movable hinge, this shift in frame by one compartment allows the muscles on both sides of any particular epidermal segment to be coordinated by the same ganglion (Figure 6.27B). This in turn allows rapid and coordinated muscle contractions for locomotion.

Therefore, while parts of the body may become secondarily organized according to segments, the parasegment is the basic unit of embryonic construction. A similar situation occurs in vertebrates, where the posterior portion of the anterior somite combines with the anterior portion of the next somite (see Chapter 11).

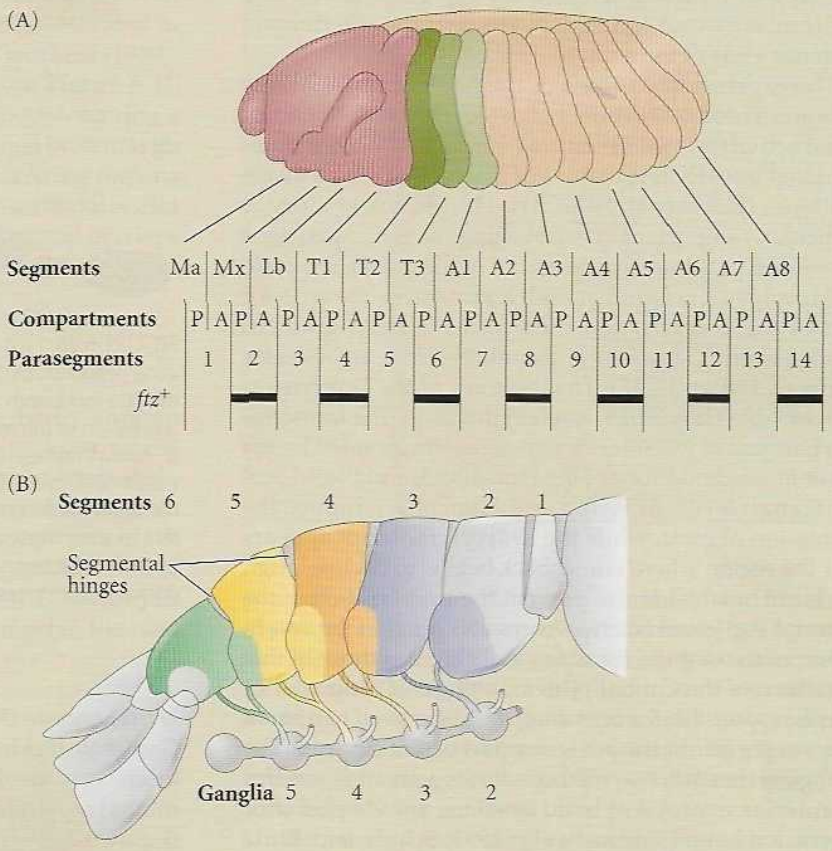


Figure 6.27 Overlap and integration of segments and parasegments. (A) Parasegments in the *Drosophila* embryo are shifted one compartment forward in relation to the segments. Ma, Mx, and Lb are the mandibular, maxillary, and labial head segments; T1–T3 are the thoracic segments; and A1–A8 are abdominal segments. Each segment has an anterior (A) and a posterior (P) compartment. Each parasegment (numbered 1–14) consists of the posterior compartment of one segment and the anterior compartment of the segment in the next posterior position. Black bars indicate the boundaries of gene expression observed in the *fushi tarazu* (*ftz*) mutant (see Figure 6.26B). (B) Segments and parasegments integrated in the body of an adult arthropod (the crustacean *Procambarus*). The ventral nerve cord is divided according to parasegments (color). This allows the neurons of the ganglia to regulate the ectodermal scutes and the mesodermal muscles on either side of a segmental hinge. (A after Martinez-Arias and Lawrence 1985; B after Deutsch 2004.)

The gap genes

The gap genes are activated or repressed by the maternal effect genes, and are expressed in one or two broad domains along the anterior-posterior axis. These expression patterns correlate quite well with the regions of the embryo that are missing in gap mutations. For example, the *Krüppel* gene is expressed primarily in parasegments 4–6, in the center of the *Drosophila* embryo (see Figures 6.26A and 6.16C); in the absence of the Krüppel protein, the embryo lacks segments from these and the immediately adjacent regions.

Deletions caused by mutations in three gap genes—*hunchback*, *Krüppel*, and *knirps*—span the entire segmented region of the *Drosophila* embryo. The gap gene *giant* overlaps with these three, and the gap genes *tailless* and *huckebein* are expressed in domains near the anterior and posterior ends of the embryo.

The expression patterns of the gap genes are highly dynamic. These genes usually show low levels of transcriptional activity across the entire embryo that become consolidated into discrete regions of high activity as cleavage continues (Jäckle et al. 1986). The Hunchback gradient is particularly important in establishing the initial gap gene expression patterns. By the end of nuclear division cycle 12, Hunchback is found at high levels across the anterior part of the embryo, and then forms a steep gradient through about 15 nuclei near the middle of the embryo (see Figures 6.16A and 6.22). The last third of the embryo has undetectable Hunchback levels at this time. The transcription patterns of the anterior gap genes are initiated by the different concentrations of the Hunchback and Bicoid proteins. High levels of Bicoid and Hunchback induce the expression of *giant*, while the *Krüppel* transcript appears over the region where Hunchback begins to decline. High levels of Hunchback also prevent the transcription of the posterior gap genes (such as *knirps* and *giant*) in the anterior part of the embryo (Struhl et al. 1992). It is thought that a gradient of the Caudal protein, highest at the posterior pole, is responsible for activating the abdominal gap genes *knirps* and *giant* in the posterior part of the embryo. The *giant* gene thus has two methods for its activation, one for its anterior expression band and one for its posterior expression band (Rivera-Pomar 1995; Schulz and Tautz 1995).

After the initial gap gene expression patterns have been established by the maternal effect gradients and Hunchback, they are stabilized and maintained by repressive interactions between the different gap gene products themselves.* These boundary-forming inhibitions are thought to be directly mediated by the gap gene products, because all four major gap genes (*hunchback*, *giant*, *Krüppel*, and *knirps*) encode DNA-binding proteins (Knipple et al. 1985; Gaul and Jäckle 1990; Capovilla et al. 1992). The major mechanism involved in this stabilization seems to be strong mutual repression between pairs of *nonadjacent* gap genes (Figure 6.28A). Gene misexpression experiments show that Giant and Krüppel are strong mutual repressors, as are Hunchback and Knirps (Kraut and Levine 1991; Clyde et al. 2003). For example, if *hunchback* activity is lacking, the posterior domain of *knirps* expands toward the anterior. Conversely, if *hunchback* is misexpressed in nuclei that normally express *knirps*, strong repression is detected. This system of strong mutual repression results in the precise placement of gap protein domains but permits overlaps between *adjacent* gap genes.

Jaeger and colleagues (2004) used quantified gene expression data to model how stabilization of the gap gene expression patterns occurs during the thirteenth and fourteenth cleavage cycles (at around 71 minutes). Their data

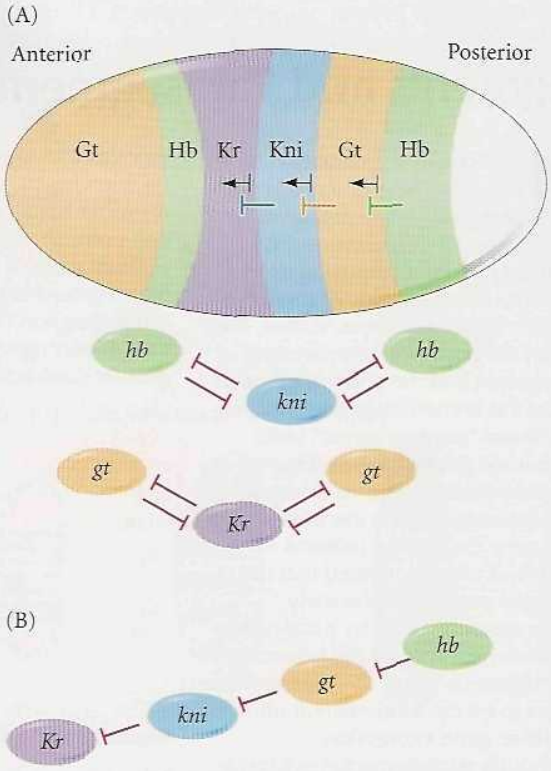


FIGURE 6.28 Expression and regulatory interactions among gap gene products. (A) Schematic expression of the gap genes during the late fourteenth cell cycle. Bars between the domains represent repression of the more anterior domain by the protein posterior to it. Arrows represent the direction in which the domains shift during the cell cycle. (For clarity, overlaps are not shown.) Strong mutual repression (diagrammed below) establishes the basic pattern of gene expression. (B) Asymmetrical repression of gap genes by their posterior neighbors causes an anterior shift in the domains of expression. (After Monk 2004.)

*The interactions between these genes and gene products are facilitated by the fact that these reactions occur within a syncytium, in which the cell membranes have not yet formed.

suggest that the patterns of gap gene expression were stabilized by three major factors. Two of these were strong mutual inhibitions between Hunchback and Knirps and strong mutual inhibitions between Giant and Krüppel. The data also revealed that these inhibitory interactions are unidirectional, with each protein having a strong effect on the anterior border of the repressed genes. This latter part of the model is important because it may explain the anterior "creeping" of the gap gene transcription patterns (Figure 6.28B).

The end result of these repressive interactions is the creation of a precise system of overlapping gap mRNA expression patterns. Each domain serves as a source for diffusion of gap proteins into adjacent embryonic regions. This creates a significant overlap (at least eight nuclei, which accounts for about two segment primordia) between adjacent gap protein domains. This was demonstrated in a striking manner by Stanojevíc and co-workers (1989). They fixed cellularizing blastoderms (see Figure 6.1), stained Hunchback protein with an antibody carrying a red dye, and simultaneously stained Krüppel protein with an antibody carrying a green dye. Cellularizing regions that contained both proteins bound both antibodies and stained bright yellow (see Figure 6.16C). Krüppel overlaps with Knirps in a similar manner in the posterior region of the embryo (Pankratz et al. 1990).

The pair-rule genes

The first indication of segmentation in the fly embryo comes when the pair-rule genes are expressed during cell division cycle 13, as the cells begin to form at the periphery of the embryo. The transcription patterns of these genes divide the embryo into regions that are precursors of the segmental body plan. As can be seen in Figure 6.29 (and in Figure 6.16D), one vertical band of nuclei (the cells are just beginning to form) expresses a pair-rule gene, the next band of nuclei does not express it, and then the next band expresses it again. The result is a "zebra stripe" pattern along the anterior-posterior axis, dividing the embryo into 15 subunits (Hafen et al. 1984). Eight genes are currently known to be capable of dividing the early embryo in this fashion, and they overlap one another so as to give each cell in the parasegment a specific set of transcription factors. These genes are listed in Table 6.2.

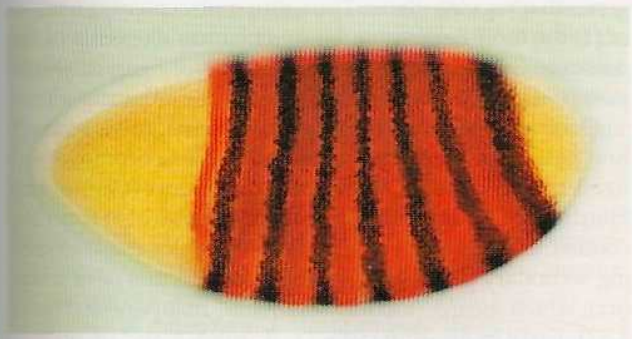


FIGURE 6.29 Messenger RNA expression patterns of two pair-rule genes, *even-skipped* (red) and *fushi tarazu* (black) in the *Drosophila* blastoderm. Each gene is expressed as a series of seven stripes. Anterior is to the left, and dorsal is up. (Courtesy of S. Small.)

TABLE 6.2 Major genes affecting segmentation pattern in *Drosophila*

Category	Gene name
Gap genes	*Krüppel (Kr)* *knirps (kni)* *hunchback (hb)* *giant (gt)* *tailless (tll)* *huckebein (hkb)* *buttonhead (btd)* *empty spiracles (ems)* *orthodenticle (otd)*
Pair-rule genes (primary)	*hairy (h)* *even-skipped (eve)* *runt (run)*
Pair-rule genes (secondary)	*fushi tarazu (ftz)* *odd-paired (opa)* *odd-skipped (odd)* *sloppy-paired (slp)* *paired (prd)*
Segment polarity genes	*engrailed (en)* *wingless (wg)* *cubitus interruptusD (ciD)* *hedgehog (hh)* *fused (fu)* *armadillo (arm)* *patched (ptc)* *gooseberry (gsb)* *pangolin (pan)*

The primary pair-rule genes include *hairy*, *even-skipped*, and *runt*, each of which is expressed in seven stripes. All three build their striped patterns from scratch, using distinct enhancers and regulatory mechanisms for each stripe. These enhancers are often modular: control over expression in each stripe is located in a discrete region of the DNA, and these DNA regions often contain binding sites recognized by gap proteins. Thus it is thought that the different concentrations of gap proteins determine whether or not a pair-rule gene is transcribed.

One of the best-studied primary pair-rule gene is *even-skipped* (Figure 6.30). Its enhancer is composed of modular units arranged such that each unit regulates a separate stripe or a pair of stripes. For instance, *even-skipped* stripe

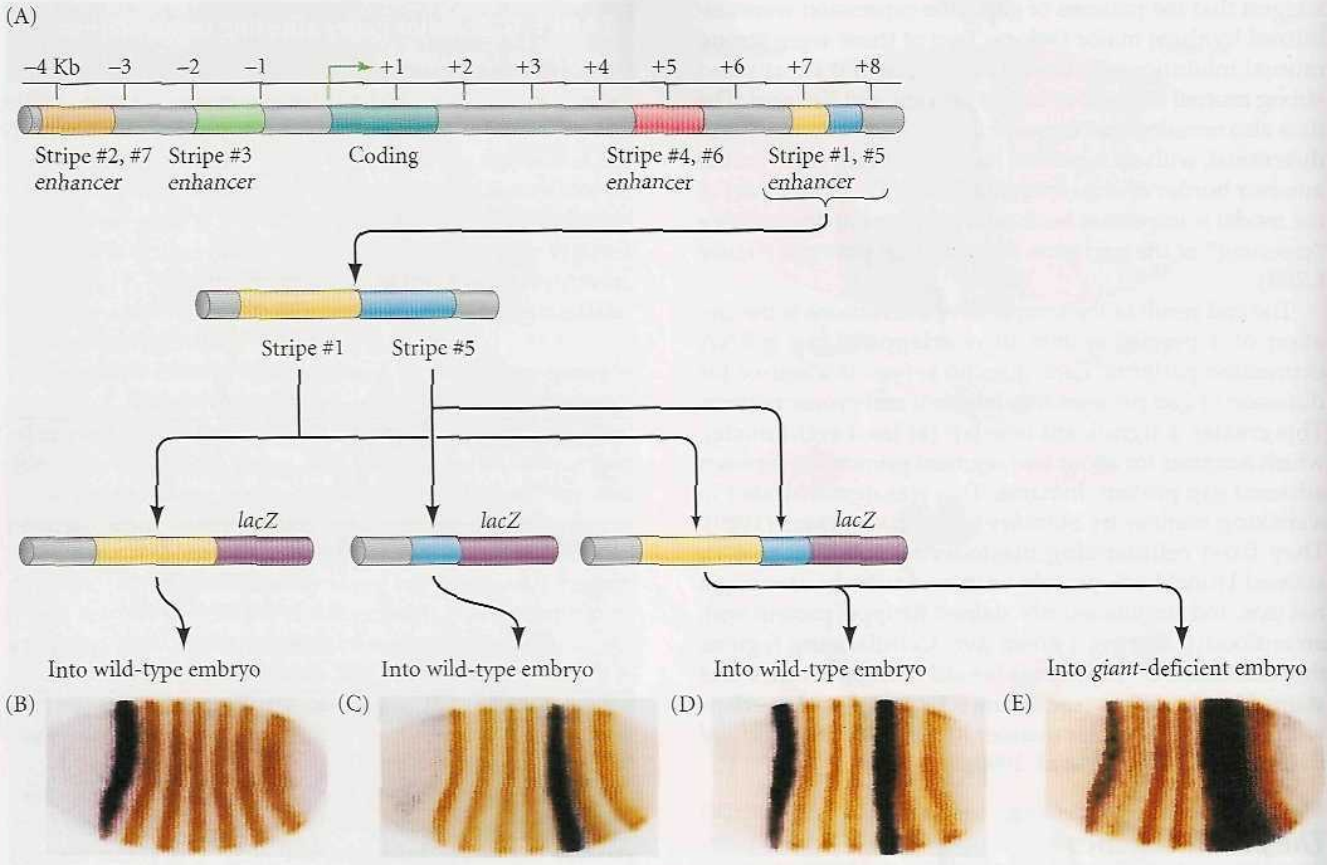


FIGURE 6.30 Specific promoter regions of the *even-skipped* (*eve*) gene control specific transcription bands in the embryo. (A) Partial map of the *eve* promoter, showing the regions responsible for the various stripes. (B–E) A reporter β-galactosidase gene (*lacZ*) was fused to different regions of the *eve* promoter and injected into fly oocytes. The resulting embryos were stained (orange bands) for the presence of Even-skipped protein. (B–D) Wild-type embryos that were injected with *lacZ* transgenes containing the enhancer region specific for stripe 1 (B), stripe 5 (C), or both regions (D). (E) The enhancer region for stripes 1 and 5 was injected into an embryo deficient in *giant*. Here the posterior border of stripe 5 is missing. (After Fujioka et al. 1999 and Sackerson et al. 1999; photographs courtesy of M. Fujioka and J. B. Jaynes.)

2 is controlled by a 500-bp region that is activated by Bicoid and Hunchback and repressed by both Giant and Krüppel proteins (Figure 6.31; Small et al. 1991, 1992; Stanojevíc et al. 1991; Janssens et al. 2006). The anterior border is maintained by repressive influences from Giant, while the posterior border is maintained by Krüppel. DNase I footprinting showed that the minimal enhancer region for this stripe contains five binding sites for Bicoid, one for Hunchback, three for Krüppel, and three for Giant. Thus, this region is thought to act as a switch that can directly sense the concentrations of these proteins and make on/off transcriptional decisions.

The importance of these enhancer elements can be shown by both genetic and biochemical means. First, a mutation in a particular enhancer can delete its particular stripe and no other. Second, if a reporter gene (such as *lacZ*, which encodes β-galactosidase) is fused to one of the enhancers, the reporter gene is expressed only in that particular stripe (see Figure 6.30; Fujioka et al. 1999). Third, placement of the stripes can be altered by deleting the gap genes that regulate them. Thus, stripe placement is a result of (1) the modular *cis*-regulatory enhancer elements of the pair-rule genes, and (2) the *trans*-regulatory gap gene and maternal gene proteins that bind to these enhancer sites.

Once initiated by the gap gene proteins, the transcription pattern of the primary pair-rule genes becomes stabilized by interactions among their products (Levine and Harding 1989). The primary pair-rule genes also form the context that allows or inhibits expression of the later-acting secondary pair-rule genes. One such gene is *fushi tarazu* (*ftz*), which means "too few segments" in Japanese (Figure 6.32). Early in division cycle 14, *ftz* mRNA and its protein are seen throughout the segmented portion of the embryo. However, as the proteins from the primary pair-rule genes begin to interact with the *ftz* enhancer, the *ftz* gene is repressed in certain bands of nuclei to create interstripe regions. Meanwhile, the Ftz protein interacts with its own

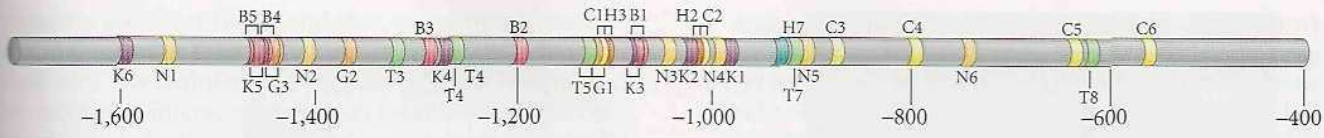


FIGURE 6.31 Hypothesis for formation of the second stripe of transcription from the *even-skipped* gene. The enhancer element for stripe 2 regulation contains binding sequences for several maternal and gap gene proteins. Activators (e.g., Bicoid and Hunchback) are noted above the line; repressors (e.g., Krüppel and Giant) are shown below. Note that nearly every activator site is closely linked to a repressor site, suggesting competitive interactions at these positions. (Moreover, a protein that is a repressor for stripe 2 may be an activator for stripe 5; it depends on which proteins bind next to them.) B, Bicoid; C, Caudal; G, Giant; H, Hunchback; K, Krüppel; N, Knirps; T, Tailless. (After Janssens et al. 2006.)

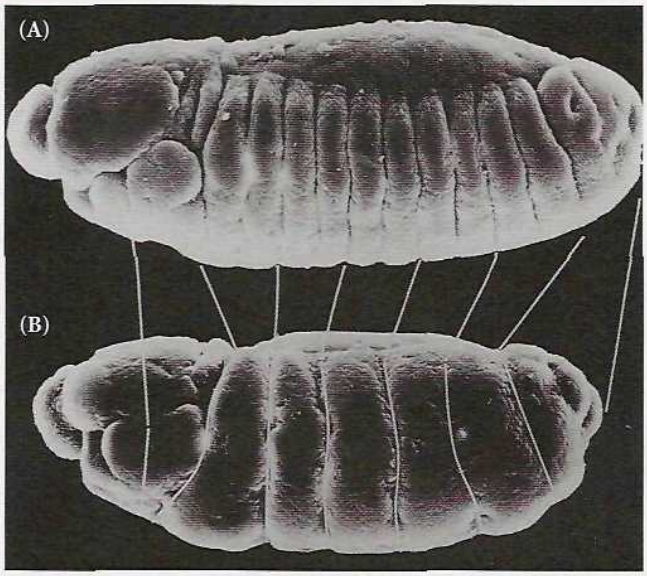
promoter to stimulate more transcription of *ftz* where it is already present (Edgar et al. 1986b; Karr and Kornberg 1989; Schier and Gehring 1992).

The eight known pair-rule genes are all expressed in striped patterns, but the patterns are not coincident with each other. Rather, each row of nuclei within a parasegment has its own array of pair-rule products that distinguishes it from any other row. These products activate the next level of segmentation genes, the segment polarity genes.

The segment polarity genes

So far our discussion has described interactions between molecules within the syncytial embryo. But once cells form, interactions take place between the cells. These interactions are mediated by the segment polarity genes, and they accomplish two important tasks. First, they reinforce the parasegmental periodicity established by the earlier transcription factors. Second, through this cell-to-cell signaling, cell fates are established within each parasegment.

The segment polarity genes encode proteins that are constituents of the Wingless (Wnt) and Hedgehog signal transduction pathways (see Chapter 3). Mutations in these genes lead to defects in segmentation and in gene expression pattern across each parasegment. The development of the normal pattern relies on the fact that only one row of cells in each parasegment is permitted to express the Hedgehog protein, and only one row of cells in each parasegment is permitted to express the Wingless protein. The key to this pattern is the activation of the *engrailed* gene



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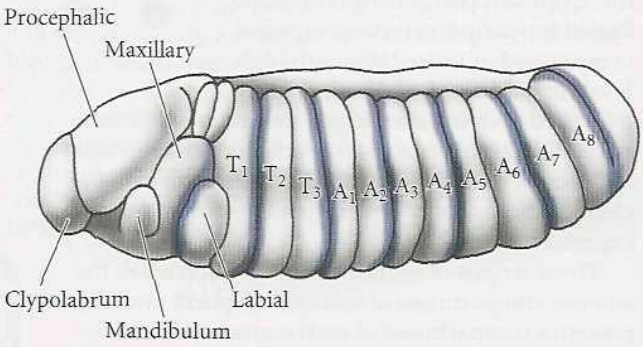

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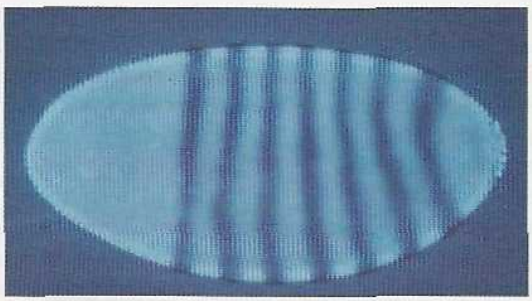

FIGURE 6.32 Defects seen in the *fushi tarazu* mutant. Anterior is to the left; dorsal surface faces upward. (A) Scanning electron micrograph of a wild-type embryo, seen in lateral view. (B) Same stage of a *fushi tarazu* mutant embryo. The white lines connect the homologous portions of the segmented germ band. (C) Diagram of wild-type embryonic segmentation. The shaded areas show the parasegments of the germ band that are missing in the mutant embryo. (D) Transcription pattern of the *fushi tarazu* gene. (After Kaufman et al. 1990; A,B courtesy of T. Kaufman; D courtesy of T. Karr.)

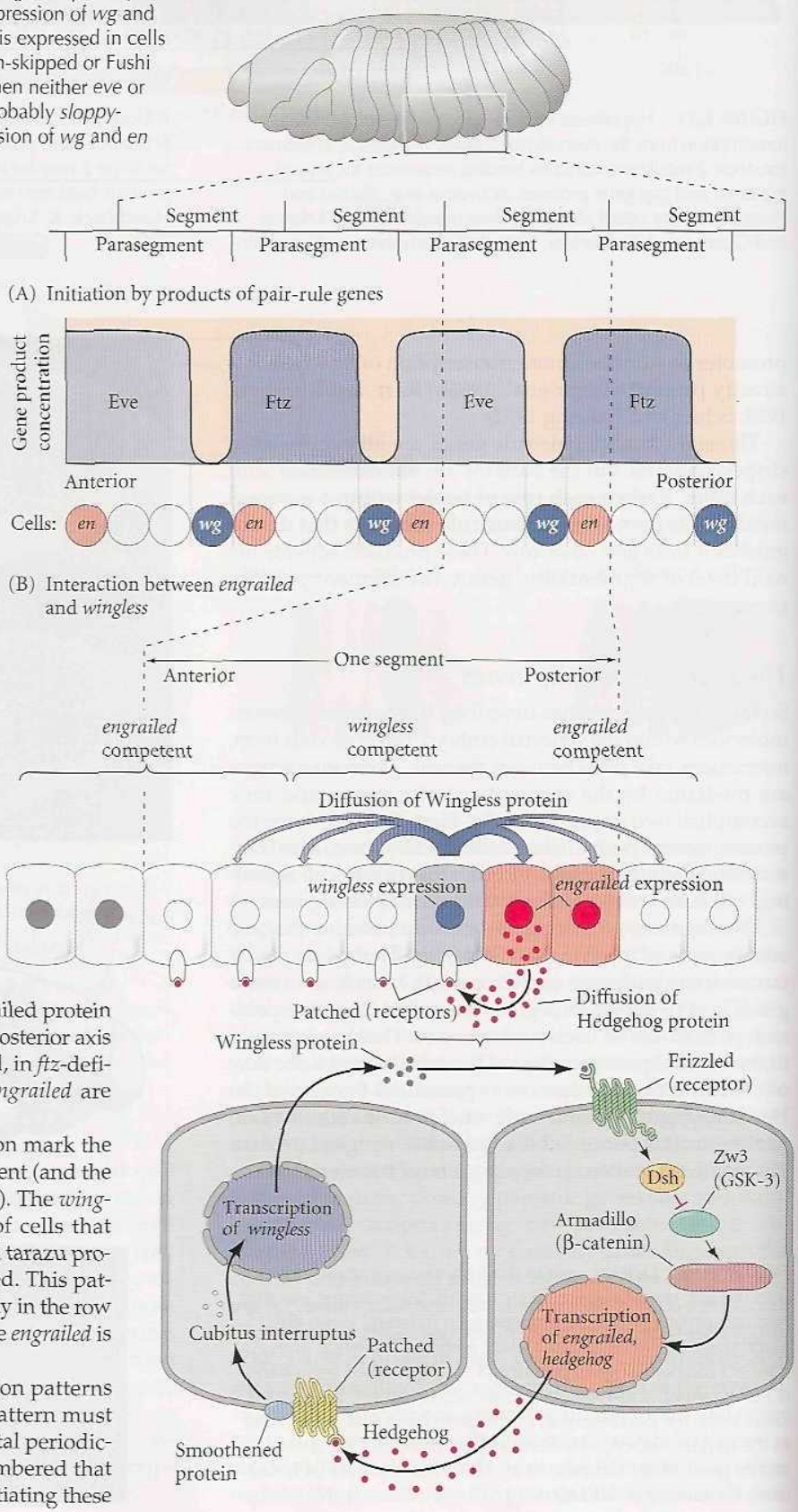

FIGURE 6.33 Model for transcription of the segment polarity genes *engrailed* (*en*) and *wingless* (*wg*). (A) Expression of *wg* and *en* is initiated by pair-rule genes. The *en* gene is expressed in cells that contain high concentrations of either Even-skipped or Fushi tarazu proteins. The *wg* gene is transcribed when neither *eve* or *ftz* genes are active, but when a third gene (probably *sloppy-paired*) is expressed. (B) The continued expression of *wg* and *en* is maintained by interactions between the Engrailed- and Wingless-expressing cells. Wingless protein is secreted and diffuses to the surrounding cells. In those cells competent to express Engrailed (i.e., those having Eve or Ftz proteins), Wingless protein is bound by the Frizzled receptor, which enables the activation of the *en* gene via the Wnt signal transduction pathway. (Armadillo is the *Drosophila* name for β-catenin.) Engrailed protein activates the transcription of the *hedgehog* gene and also activates its own (*en*) gene transcription. Hedgehog protein diffuses from these cells and binds to the Patched receptor protein on neighboring cells. This binding prevents the Patched protein from inhibiting signaling by the Smoothened protein. The Smoothened signal enables the transcription of the *wg* gene and the subsequent secretion of the Wingless protein. For a more complex view, see Sánchez et al. 2008.

in those cells that are going to express Hedgehog. The *engrailed* gene is activated in cells that have high levels of the Even-skipped, Fushi tarazu, or Paired transcription factors; *engrailed* is repressed in those cells with high levels of Odd-skipped, Runt, or Sloppy-paired proteins. As a result, the Engrailed protein is found in 14 stripes across the anterior-posterior axis of the embryo (see Figure 6.16E). (Indeed, in *ftz*-deficient embryos, only seven bands of *engrailed* are expressed.)

These stripes of *engrailed* transcription mark the anterior compartment of each parasegment (and the posterior compartment of each segment). The *wingless* gene is activated in those bands of cells that receive little or no Even-skipped or Fushi tarazu protein, but which do contain Sloppy-paired. This pattern causes *wingless* to be transcribed solely in the row of cells directly anterior to the cells where *engrailed* is transcribed (Figure 6.33A).

Once *wingless* and *engrailed* expression patterns are established in adjacent cells, this pattern must be maintained to retain the parasegmental periodicity of the body plan. It should be remembered that the mRNAs and proteins involved in initiating these

patterns are short-lived, and that the patterns must be maintained after their initiators are no longer being synthesized. The maintenance of these patterns is regulated by reciprocal interaction between neighboring cells: cells secreting Hedgehog activate the expression of *wingless* in their neighbors; the Wingless protein is received by the cells that secreted Hedgehog and maintains *hedgehog* expression (Figure 6.33B). Wingless protein also acts in an autocrine fashion, maintaining its own expression (Sánchez et al. 2008).

In the cells transcribing the *wingless* gene, wingless mRNA is translocated by its 3′ UTR to the apex of the cell (Simmonds et al. 2001; Wilkie and Davis 2001). At the apex, the *wingless* message is translated and secreted from the cell. The cells expressing *engrailed* can bind this protein because they contain Frizzled, which is the *Drosophila* membrane receptor protein for Wingless (Bhanot et al. 1996). Binding of Wingless to Frizzled activates the Wnt signal transduction pathway, resulting in the continued expression of *engrailed* (Siegfried et al. 1994).

This activation starts another portion of this reciprocal pathway. The Engrailed protein activates the transcription of the *hedgehog* gene in the *engrailed*-expressing cells. Hedgehog protein can bind to its receptor protein (Patched) on neighboring cells. When it binds to the adjacent posterior cells, it stimulates the expression of the *wingless* gene. The result is a reciprocal loop wherein the Engrailed-synthesizing cells secrete the Hedgehog protein, which maintains the expression of the *wingless* gene in the neighboring cells, while the Wingless-secreting cells maintain the expression of the *engrailed* and *hedgehog* genes in their neighbors in turn (Heemskerk et al. 1991; Ingham et al. 1991; Mohler and Vani 1992). In this way, the transcription pattern of these two types of cells is stabilized. This interaction creates a stable boundary, as well as a signaling center from which Hedgehog and Wingless proteins diffuse across the parasegment.

The diffusion of these proteins is thought to provide the gradients by which the cells of the parasegment acquire their identities. This process can be seen in the dorsal epidermis, where the rows of larval cells produce different cuticular structures depending on their position in the segment. The 1° row of cells consists of large, pigmented spikes called denticles. Posterior to these cells, the 2° row produces a smooth epidermal cuticle. The next two cell rows have a 3° fate, making small, thick hairs; they are followed by several rows of cells that adopt the 4° fate, producing fine hairs (Figure 6.34).

The fates of the cells can be altered by experimentally increasing or decreasing the levels of Hedgehog or Wingless (Heemskerek and DiNardo 1994; Bokor and DiNardo 1996; Porter et al. 1996). These two proteins thus appear to be necessary for elaborating the entire pattern of cell types across the parasegment. Gradients of Hedgehog and Wingless are interpreted by a second series of protein gradients within the cells. This second set of gradients provides certain cells with the receptors for Hedgehog and (often) with the receptor for Wingless (Casal et al. 2002; Lander et al. 2002). The resulting pattern of cell fates also changes the focus of patterning from parasegment to segment. There are now external markers, as the *engrailed*-expressing cells become the most posterior cells of each segment.

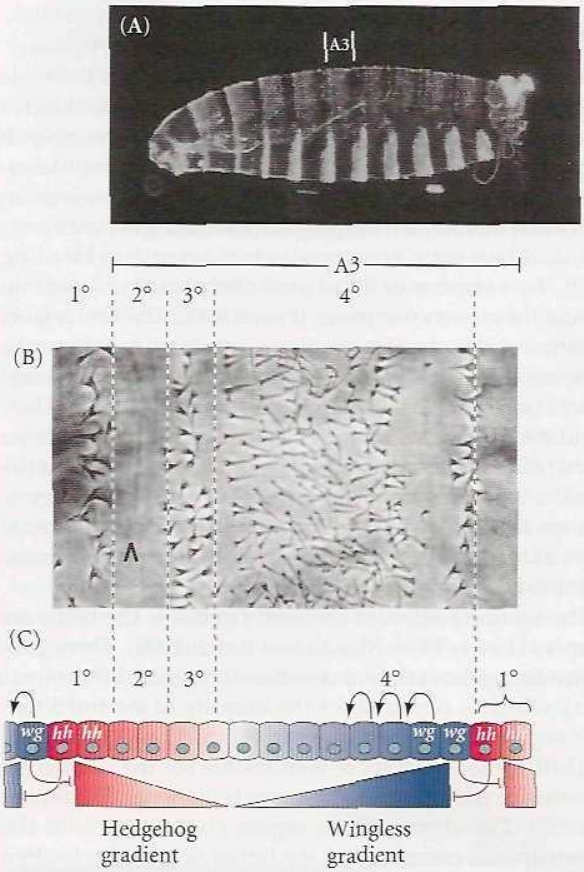

FIGURE 6.34 Cell specification by the Wingless/Hedgehog signaling center. (A) Bright-field photograph of wild-type *Drosophila* embryo, showing the position of the third abdominal segment. Anterior is to the left; the dorsal surface faces upward. (B) Close-up of the dorsal area of the A3 segment, showing the different cuticular structures made by the 1°, 2°, 3°, and 4° rows of cells. (C) Diagram showing a model for the role of Wingless and Hedgehog. Each signal is responsible for about half the pattern. Either each signal acts in a graded manner (shown here as gradients decreasing with distance from their respective sources) to specify the fates of cells at a distance from these sources, or each signal acts locally on the neighboring cells to initiate a cascade of inductions (shown here as sequential arrows). (After Heemskerk and DiNardo 1994; A,B courtesy of the authors.)

See WEBSITE 6.5
Asymmetrical spread of morphogens

See WEBSITE 6.6
Getting a head in the fly

The Homeotic Selector Genes

After the parasegmental boundaries are set, the pair-rule and gap genes interact to regulate the homeotic selector genes, which specify the characteristic structures of each segment (Lewis 1978). By the end of the cellular blastoderm stage, each segment primordium has been given an individual identity by its unique constellation of gap, pair-rule, and homeotic gene products (Levine and Harding 1989). Two regions of *Drosophila* chromosome 3 contain most of these homeotic genes (Figure 6.35). The first region, known as the **Antennapedia complex**, contains the homeotic genes *labial* (*lab*), *Antennapedia* (*Antp*), *sex combs reduced* (*scr*), *deformed* (*dfd*), and *proboscipedia* (*pb*). The *labial* and *deformed* genes specify the head segments, while *sex combs reduced* and *Antennapedia* contribute to giving the thoracic segments their identities. The *proboscipedia* gene appears to act only in adults, but in its absence, the labial palps of the mouth are transformed into legs (Wakimoto et al. 1984; Kaufman et al. 1990).

The second region of homeotic genes is the **bithorax complex** (Lewis 1978; Maeda and Karch 2009). Three protein-coding genes are found in this complex: *Ultrabithorax* (*Ubx*), which is required for the identity of the third thoracic segment; and the *abdominal A* (*abdA*) and *Abdominal B* (*AbdB*) genes, which are responsible for the segmental identities of the abdominal segments (Sánchez-Herrero et al. 1985). The chromosome region containing both the Antennapedia complex and the bithorax complex is often referred to as the **homeotic complex** (**Hom-C**).

Because the homeotic selector genes are responsible for the specification of fly body parts, mutations in them lead to bizarre phenotypes. In 1894, William Bateson called these organisms **homeotic mutants**, and they have fascinated developmental biologists for decades.* For example, the body of the normal adult fly contains three thoracic segments, each of which produces a pair of legs. The first thoracic segment does not produce any other appendages, but the second thoracic segment produces a pair of wings in addition to its legs. The third thoracic segment produces a pair of wings and a pair of balancers known as **halteres**. In homeotic mutants, these specific segmental identities can be changed. When the *Ultrabithorax* gene is deleted, the third thoracic segment (characterized by halteres) is transformed into another second thoracic segment. The result is a fly with four wings (Figure 6.36)—an embarrassing situation for a classic dipteran.†

Similarly, Antennapedia protein usually specifies the second thoracic segment of the fly. But when flies have a

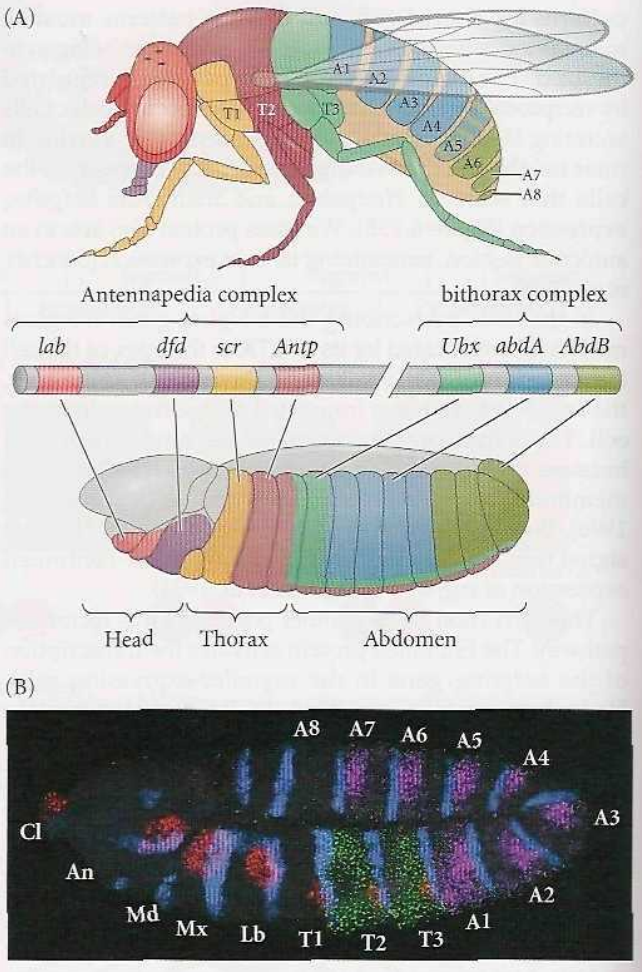

FIGURE 6.35 Homeotic gene expression in *Drosophila*. (A) Expression map of the homeotic genes. In the center are the genes of the Antennapedia and bithorax complexes and their functional domains. Below and above the gene map, the regions of homeotic gene expression (both mRNA and protein) in the blastoderm of the *Drosophila* embryo and the regions that form from them in the adult fly are shown. (B) In situ hybridization for four genes at a slightly later stage (the extended germ band). The *engrailed* (light blue) expression pattern separates the body into segments; *Antennapedia* (green) and *Ultrabithorax* (purple) separate the thoracic and abdominal regions; *Distal-less* (red) shows the placement of jaws and the beginnings of limbs. (A after Kaufman et al. 1990 and Dessain et al. 1992; B courtesy of D. Kosman.)

Homeo, from the Greek, means "similar." *Homeotic mutants* are mutants in which one structure is replaced by another (as where an antenna is replaced by a leg). *Homeotic genes* are those genes whose mutation can cause such transformations; thus, homeotic genes are genes that specify the identity of a particular body segment. The *homeobox* is a conserved DNA sequence of about 180 base pairs that is shared by many homeotic genes. This sequence encodes the 60-amino-acid *homeodomain*, which recognizes specific DNA sequences. The homeodomain is an important region of the transcription factors encoded by homeotic genes. However, not all genes containing homeoboxes are homeotic genes.

†Dipterans (two-winged insects such as flies) are thought to have evolved from four-winged insects; it is possible that this change arose via alterations in the bithorax complex. Chapter 19 includes more speculation on the relationship between the homeotic complex and evolution.

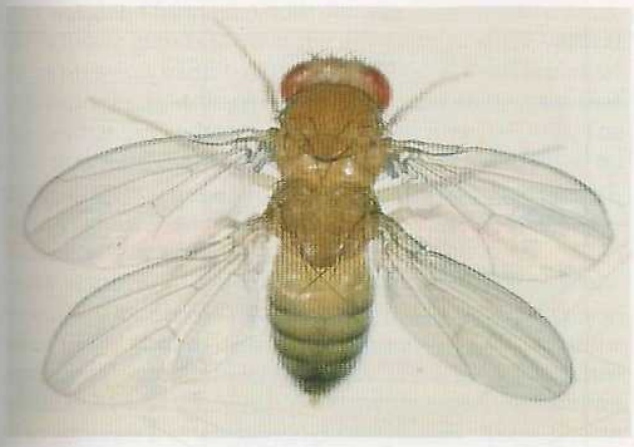

FIGURE 6.36 A four-winged fruit fly constructed by putting together three mutations in *cis*-regulators of the *Ultrabithorax* gene. These mutations effectively transform the third thoracic segment into another second thoracic segment (i.e., halteres into wings). (Courtesy of E. B. Lewis.)

mutation wherein the *Antennapedia* gene is expressed in the head (as well as in the thorax), legs rather than antennae grow out of the head sockets (Figure 6.37). This is partly because, in addition to promoting the formation of thoracic structures, the Antennapedia protein binds to and represses the enhancers of at least two genes, *homothorax* and *eyeless*, which encode transcription factors that are critical for antenna and eye formation, respectively (Casares and Mann 1998; Plaza et al. 2001). Therefore, one of Antennapedia's functions is to suppress the genes that would trigger antenna and eye development. In the recessive mutant of *Antennapedia*, the gene fails to be expressed in the second thoracic segment, and antennae sprout in the leg positions (Struhl 1981; Frischer et al. 1986; Schneuwly et al. 1987).

The major homeotic selector genes have been cloned and their expression analyzed by in situ hybridization (Harding et al. 1985; Akam 1987). Transcripts from each gene can be detected in specific regions of the embryo (see Figure 6.35B) and are especially prominent in the central nervous system.

Initiating and maintaining the patterns of homeotic gene expression

The initial domains of homeotic gene expression are influenced by the gap genes and pair-rule genes. For instance, expression of the *abdA* and *AbdB* genes is repressed by the gap gene proteins Hunchback and Krüppel. This inhibition prevents these abdomen-specifying genes from being expressed in the head and thorax (Casares and Sánchez-Herrero 1995). Conversely, the *Antennapedia* gene is activated by particular levels of Hunchback (needing both the maternal and the zygotically transcribed messages), so *Antennapedia* is originally transcribed in parasegment 4, specifying the mesothoracic (T2) segment (Wu et al. 2001).

The expression of homeotic genes is a dynamic process. The *Antennapedia* gene, for instance, although initially expressed in presumptive parasegment 4, soon appears in parasegment 5. As the germ band expands, *Antp* expression is seen in the presumptive neural tube as far posterior as parasegment 12. During further development, the domain of *Antp* expression contracts again, and *Antp* transcripts are localized strongly to parasegments 4 and 5. Like that of other homeotic genes, *Antp* expression is negatively regulated by all the homeotic gene products expressed posterior to it (Levine and Harding 1989; González-Reyes and Morata 1990). In other words, each of the bithorax complex genes represses the expression of *Antp*. If the *Ultrabithorax* gene is deleted, *Antp* activity extends through the region that would normally have expressed *Ubx* and stops where the *Abd* region begins. (This allows the third thoracic segment to form wings like the second thoracic segment, as in Figure 6.36.) If the entire bithorax complex is deleted, *Antp* expression extends throughout the abdomen. (Such a larva does not survive, but the cuticle pattern throughout the abdomen is that of the second thoracic segment.)

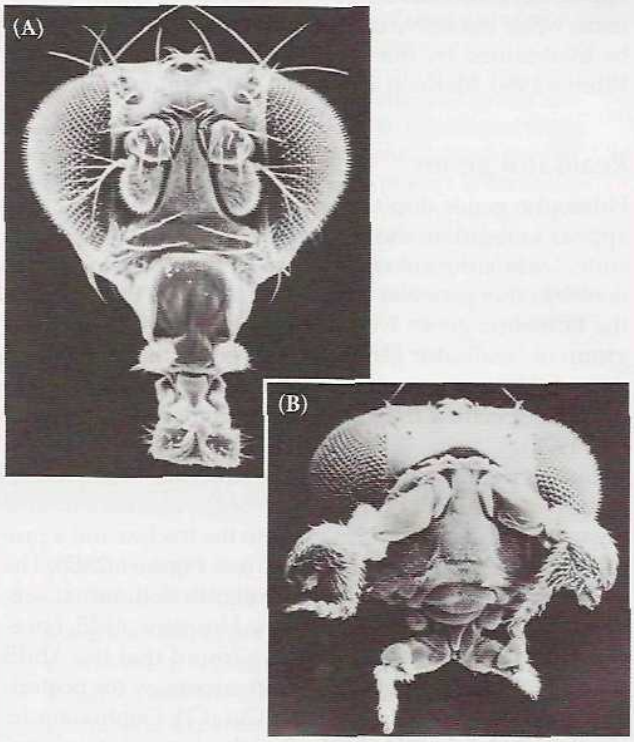

FIGURE 6.37 (A) Head of a wild-type fruit fly. (B) Head of a fly containing the *Antennapedia* mutation that converts antennae into legs. (From Kaufman et al. 1990, courtesy of T. C. Kaufman.)

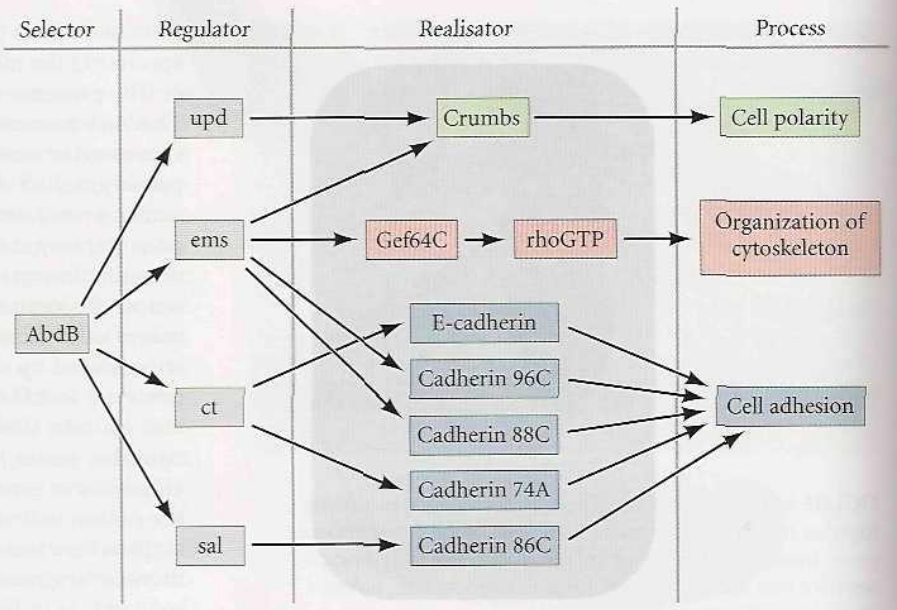

FIGURE 6.38 Developmental control of posterior spiracle formation through AbdB. The homeotic selector protein AbdB (with the interaction of cofactors) activates the transcription of four genes encoding "intermediate" regulators. The proteins encoded by these genes—Spalt (Sal), Cut (Ct), Empty spiracles (Ems), and Unpaired (Upd)—are necessary and sufficient for specifying posterior spiracle development. They control (directly or indirectly) the local expression of a battery of realisator genes that influence *morphogenetic processes such as cell* adhesion (cadherins), cell polarity (crumbs), and cytoskeletal organization (G proteins). (After Lohmann 2006; Lovegrove et al. 2006.)

As we have seen, the proteins encoded by the gap and pair-rule genes are transient; however, in order for differentiation to occur, the identities of the segments must be stabilized. So, once the transcription patterns of the homeotic genes have become stabilized, they are "locked" into place by alteration of the chromatin conformation in these genes. The repression of homeotic genes appears to be maintained by the **Polycomb** family of proteins, while the active chromatin conformation appears to be maintained by the **Trithorax** proteins (Ingham and Whittle 1980; McKeon and Brock 1991; Simon et al. 1992).

Realisator genes

Homeotic genes don't do the work alone. In fact, they appear to regulate the action from up in the "executive suite," while the actual business of making an organ is done by other genes on the "factory floor." In this scenario, the homeotic genes work by activating or repressing a group of "realisator genes"—those genes that are the targets of the homeotic gene proteins and that function to form the specified tissue or organ primordia (Garcia-Bellido 1975).

Such a pathway for one simple structure—the posterior spiracle—is well on its way to being elucidated. This organ is a simple tube connecting to the trachea and a protuberance called the "Filzkörper" (see Figure 6.23D). The posterior spiracle is made in the eighth abdominal segment and is under the control of the Hox gene *AbdB*. Lovegrove and colleagues (2006) have found that the AbdB protein controls four genes that are necessary for posterior spiracle formation: *Spalt* (*Sal*), *Cut* (*Ct*), *Empty spiracles* (*Ems*), and *Unpaired* (*Upd*). The first three encode transcription factors; the fourth encodes a paracrine factor. None of them are transcribed without AbdB. Moreover, if these genes are independently activated in the absence of AbdB, a posterior spiracle will form.

Controlled by AbdB, these four regulator genes in turn control the expression of the realisator genes that control cell structure and function. *Spalt* and *Cut* encode proteins that activate the cadherin genes necessary for cell adhesion and the invagination of the spiracle. *Empty spiracles* and *Unpaired* encode proteins that control the small G proteins (such as Gef64C) that organize the actin cytoskeleton and the cell polarizing proteins that control the elongation of the spiracle (Figure 6.38).

Axes and Organ Primordia: The Cartesian Coordinate Model

The anterior-posterior and dorsal-ventral axes of *Drosophila* embryos form a coordinate system that can be used to specify positions within the embryo (Figure 6.39A). Theoretically, cells that are initially equivalent in developmental potential can respond to their position by expressing different sets of genes. This type of specification has been demonstrated in the formation of the salivary gland rudiments (Panzer et al. 1992; Bradley et al. 2001; Zhou et al. 2001).

Drosophila salivary glands form only in the strip of cells defined by the activity of the *sex combs reduced* (*scr*) gene along the anterior-posterior axis (parasegment 2). No salivary glands form in *scr*-deficient mutants. Moreover, if *scr* is experimentally expressed throughout the embryo, salivary gland primordia form in a ventrolateral stripe along most of the length of the embryo. The formation of salivary glands along the dorsal-ventral axis is repressed by both Decapentaplegic and Dorsal proteins, which inhibit

salivary gland formation both dorsally and ventrally. Thus, the salivary glands form at the intersection of the vertical *scr* expression band (parasegment 2) and the horizontal region in the middle of the embryo's circumference that has neither Decapentaplegic nor Dorsal (Figure 6.39B). The cells that form the salivary glands are directed to do so by the intersecting gene activities along the anterior-posterior and dorsal-ventral axes.

A similar situation is seen with tissues that are found in every segment of the fly. Neuroblasts arise from 10 clusters of 4 to 6 cells each that form on each side in every segment in the strip of neural ectoderm at the midline of the embryo (Skeath and Carroll 1992). The cells in each cluster interact (via the Notch pathway discussed in Chapter 3) to generate a single neural cell from each cluster. Skeath and colleagues (1993) have shown that the pattern of neural gene transcription is imposed by a coordinate system. Their expression is repressed by the Decapentaplegic and Snail proteins along the dorsal-ventral axis, while positive enhancement by pair-rule genes along the anterior-posterior axis causes their repetition in each half-segment. It is very likely, then, that the positions of organ primordia in the fly are specified via a two-dimensional coordinate system based on the intersection of the anterior-posterior and dorsal-ventral axes.

Coda

Genetic studies on the *Drosophila* embryo have uncovered numerous genes that are responsible for specification of the anterior-posterior and dorsal-ventral axes. We are far from a complete understanding of *Drosophila* pattern formation, but we are much more aware of its complexity than we were a decade ago. Mutations of *Drosophila* genes have given us our first glimpses of the multiple levels of pattern regulation in a complex organism and have enabled us to isolate these genes and their products. Most importantly, as we will see in forthcoming chapters, the *Drosophila* genes provide clues to a general mechanism of pattern formation that is used throughout the animal kingdom.

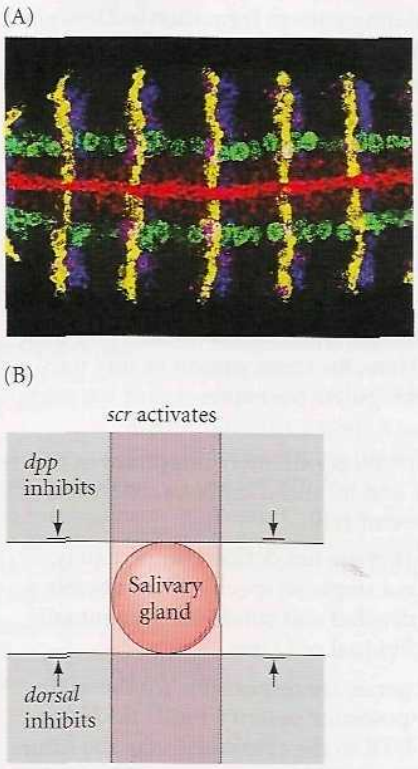

FIGURE 6.39 Cartesian coordinate system mapped out by gene expression patterns. (A) A grid (ventral view, looking "up" at the embryo) formed by the expression of *short-gastrulation* (red), *intermediate neuroblast defective* (green), and *muscle segment homeobox* (magenta) along the dorsal-ventral axis, and by the expression of *wingless* (yellow), and *engrailed* (green) transcripts along the anterior-posterior axis. (B) Coordinates for the expression of genes giving rise to *Drosophila* salivary glands. These genes are activated by the protein product of the *sex combs reduced* (*scr*) homeotic gene in a narrow band along the anterior-posterior axis, and they are inhibited in the regions marked by *decapentaplegic* (*dpp*) and *dorsal* gene products along the dorsal-ventral axis. This pattern allows salivary glands to form in the midline of the embryo in the second parasegment. (A courtesy of D. Kosman; B after Panzer et al. 1992.)

Snapshot Summary: *Drosophila* Development and Axis Specification

1. *Drosophila* cleavage is superficial. The nuclei divide 13 times before forming cells. Before cell formation, the nuclei reside in a syncytial blastoderm. Each nucleus is surrounded by actin-filled cytoplasm.
2. When the cells form, the *Drosophila* embryo undergoes a mid-blastula transition, wherein the cleavages become asynchronous and new mRNA is made. At this time, there is a transfer from maternal to zygotic control of development.
3. Gastrulation begins with the invagination of the most ventral region (the presumptive mesoderm), which causes the formation of a ventral furrow. The germ band expands such that the future posterior segments curl just behind the presumptive head.

4. The genes regulating pattern formation in *Drosophila* operate according to certain principles:
   - There are *morphogens*—such as Bicoid and Dorsal—whose gradients determine the specification of different cell types. These morphogens can be transcription factors.
   - There is a *temporal order* wherein different classes of genes are transcribed, and the products of one gene often regulate the expression of another gene.
   - *Boundaries* of gene expression can be created by the interaction between transcription factors and their gene targets. Here, the transcription factors transcribed earlier regulate the expression of the next set of genes.
   - *Translational control* is extremely important in the early embryo, and localized mRNAs are critical in patterning the embryo.
   - *Individual cell fates* are not defined immediately. Rather, there is a stepwise specification wherein a given field is divided and subdivided, eventually regulating individual cell fates.
5. Maternal effect genes are responsible for the initiation of anterior-posterior polarity. *Bicoid* mRNA is bound by its 3′ UTR to the cytoskeleton in the future anterior pole; *nanos* mRNA is sequestered by its 3′ UTR in the future posterior pole. *Hunchback* and *caudal* messages are seen throughout the embryo.
6. Dorsal-ventral polarity is regulated by the entry of Dorsal protein into the nucleus. Dorsal-ventral polarity is initiated when the nucleus moves to the dorsal-anterior of the oocyte and transcribes the *gurken* message, which is then transported to the region above the nucleus and adjacent to the follicle cells.
7. Gurken protein is secreted from the oocyte and binds to its receptor (Torpedo) on the follicle cells. This binding dorsalizes the follicle cells, preventing them from synthesizing Pipe.
8. Pipe protein in the ventral follicle cells modifies an as yet unknown factor that modifies the Nudel protein. This modification allows Nudel to activate a cascade of proteolysis in the space between the ventral follicle cells and the ventral cells of the embryo. As a result of this cascade, the Spätzle protein is activated and binds to the Toll protein on the ventral embryonic cells.
9. The activated Toll protein initiates a cascade that phosphorylates the Cactus protein, which has been bound to Dorsal. Phosphorylated Cactus is degraded, allowing Dorsal to enter the nucleus. Once in the nucleus, Dorsal activates the genes responsible for the ventral cell fates and represses those genes whose proteins would specify dorsal cell fates.
10. Dorsal protein forms a gradient as it enters the various nuclei. Those nuclei at the most ventral surface incorporate the most Dorsal protein and become mesoderm; those more lateral become neurogenic ectoderm.
11. The Bicoid and Hunchback proteins activate the genes responsible for the anterior portion of the fly; Caudal activates genes responsible for posterior development.
12. The unsegmented anterior and posterior extremities are regulated by the activation of the Torso protein at the anterior and posterior poles of the egg.
13. The gap genes respond to concentrations of the maternal effect gene proteins. Their protein products interact with each other such that each gap gene protein defines specific regions of the embryo.
14. The gap gene proteins activate and repress the pair-rule genes. The pair-rule genes have modular promoters such that they become activated in seven "stripes." Their boundaries of transcription are defined by the gap genes. The pair-rule genes form seven bands of transcription along the anterior-posterior axis, each one comprising two parasegments.
15. The pair-rule gene products activate *engrailed* and *wingless* expression in adjacent cells. The *engrailed*-expressing cells form the anterior boundary of each parasegment. These cells form a signaling center that organizes the cuticle formation and segmental structure of the embryo.
16. The homeotic selector genes are found in two complexes on chromosome 3 of *Drosophila*. Together, these regions are called Hom-C, the homeotic gene complex. The genes are arranged in the same order as their transcriptional expression. The Hom-C genes specify the individual segments, and mutations in these genes are capable of transforming one segment into another.
17. The expression of each homeotic selector gene is regulated by the gap and pair-rule genes. Their expression is refined and maintained by interactions whereby their protein products prevent the transcription of neighboring Hom-C genes.
18. The targets of the Hom-C proteins are the realisator genes. These realisator genes are responsible for constructing the specific structure.
19. Organs form at the intersection of dorsal-ventral and anterior-posterior regions of gene expression.

For Further Reading

Complete bibliographical citations for all literature cited in this chapter can be found at the free-access website **www.devbio.com**

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Go Online

WEBSITE 6.1 *Drosophila* fertilization. Fertilization of *Drosophila* can only occur in the region of the oocyte that will become the anterior of the embryo. Moreover, the sperm tail appears to stay in this region.

WEBSITE 6.2 The early development of other insects. *Drosophila* is a highly derived species. There are other insect species that develop in ways very different from the "standard" fruit fly.

WEBSITE 6.3 Evidence for gradients in insect development. The original evidence for gradients in insect development came from studies providing evidence for two "organization centers" in the egg, one located anteriorly and one located posteriorly.

WEBSITE 6.4 Christiane Nüsslein-Volhard and the molecular approach to development. The research that revolutionized developmental biology had to wait for someone to synthesize molecular biology, embryology, and *Drosophila* genetics.

WEBSITE 6.5 Asymmetrical spread of morphogens. It is unlikely that morphogens such as Wingless spread by free diffusion. The asymmetry of Wingless diffusion suggests that neighboring cells play a crucial role in moving this protein.

WEBSITE 6.6 Getting a head in the fly. The segment polarity genes may act differently in the head than in the trunk. Indeed, the formation of the *Drosophila* head may differ significantly from the way the rest of the body is formed.

Vade Mecum

***Drosophila* development.** The Vade Mecum sites have remarkable time-lapse sequences of *Drosophila* development, including cleavage and gastrulation. This segment also provides access to the fly life cycle. The color coding superimposed on the germ layers allows you to readily understand tissue movements.

Outside Sites

"The Interactive Fly," compiled by Thomas Brody, provides an index to the major *Drosophila* websites worldwide. It is hosted by the Society for Developmental Biology (SDB) at **http://www.sdbonline.org/fly/aimain/1aahome.htm**. Two notable entries accessible through the site are "Atlas of Fly Development" by Voker Hartenstein (**http://www.sdbonline.org/fly/atlas/00atlas.htm**) and "Stages in Fly Development: The Movies" (**http://www.sdbonline.org/fly/aimain/2stages.htm**).