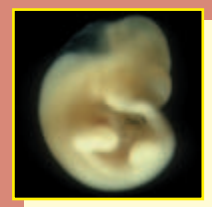
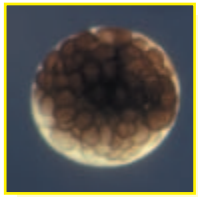
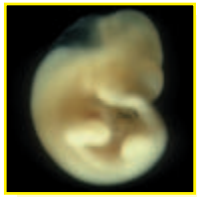
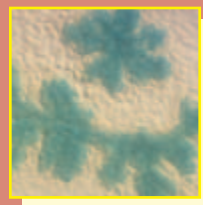
Section 2

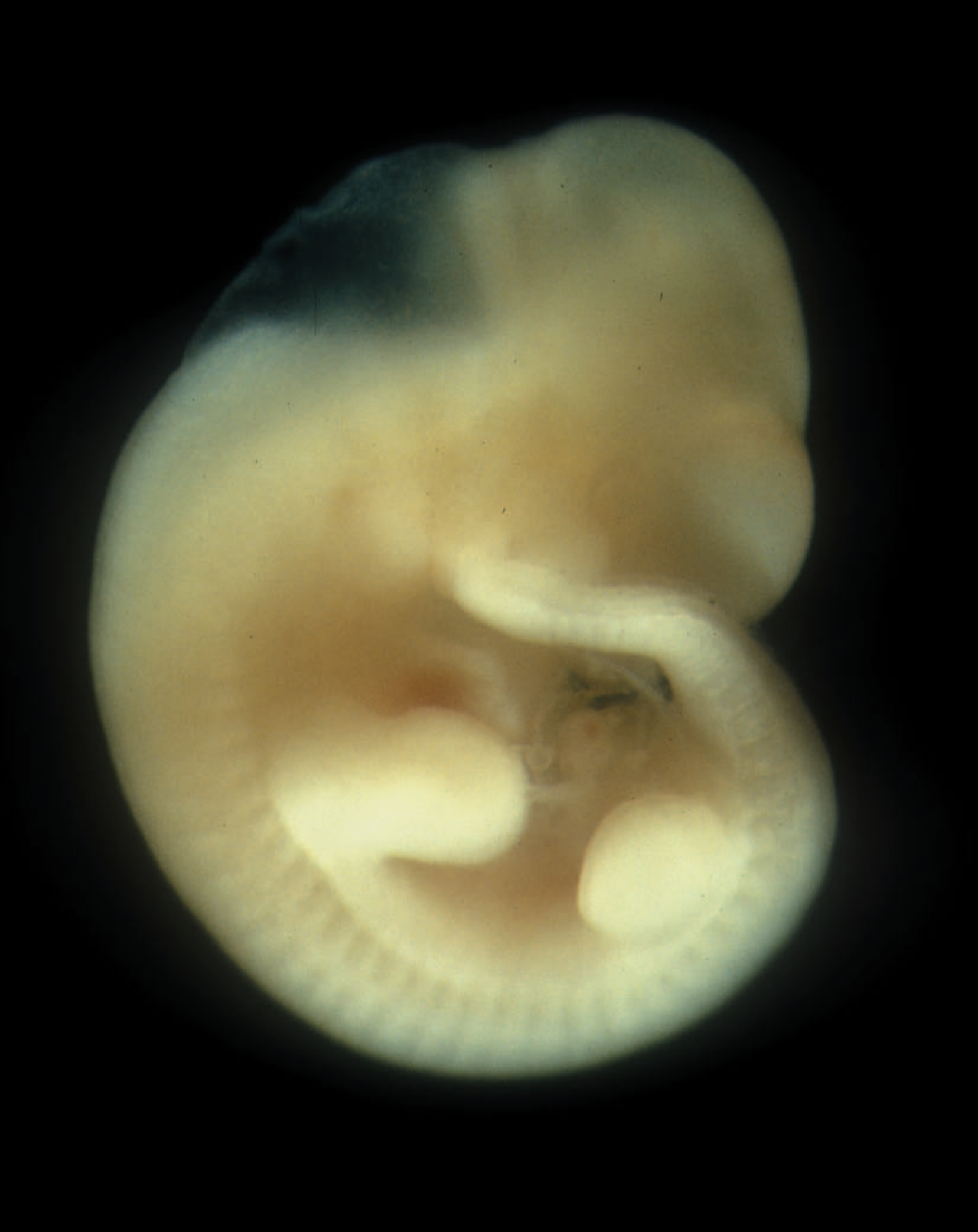


Major model organisms

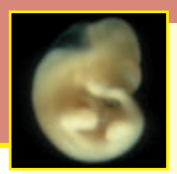








Chapter 6



Model organisms

Out of over 1 million animal species, modern developmental biology has focused on a very small number which are often described as "model organisms." This is because the motivation for their study is not simply to understand how that particular animal develops, but to use it as an example of how all animals develop. Much developmental biology research is supported by medical research funding bodies and their ultimate goal is to understand how the human body develops, even if this is not the immediate goal of the investigators themselves. For this reason there is often an attraction to studying a process that also occurs in humans. In this chapter it will be explained why research activity has focused down onto a small number of species, with some comparative indication of their strengths and weaknesses. In a short book a line has to be drawn somewhere and so just six of the most important models organisms are covered in detail: the mouse, the chick, the frog *Xenopus*, the zebrafish, the fruit fly *Drosophila*, and the nematode *Caenorhabditis elegans*. Other species have also been extensively studied, the next most popular being various species of sea urchin, but worldwide the largest number of active researchers are working with the big six.

The big six

The organisms discussed in detail in this book are listed in Table 6.1 and their positions in the phylogenetic tree of animals are shown in Fig. 6.1. It will be noted that they do not provide a very good coverage of the animal kingdom, as there are just two invertebrates and four vertebrates. However, the evolutionary distance between them is sufficiently large to make it likely that any developmental features possessed by all six are shared by the entire animal kingdom.

The six model species have each been selected because they have some particular experimental advantages for developmental biology research. It is true that sometimes individual scientists choose their organisms just because they like looking at them and working with them. But there are also some more objective practical considerations that govern the selection of organisms for particular purposes which are summarized in Table 6.2.

Availability and cost

All the six species are available all year round. Without this facility they would not have been selected as model organisms at all. It is worth remembering that it can be very difficult to "domesticate" an organism for laboratory life, and to attempt this for a "new" species is a major undertaking. Although some marine invertebrates have been used in developmental research (see below), none of the big six is a marine organism, probably because of the extra difficulties involved in keeping them in the lab. For example it is extremely difficult to breed and rear sea urchins in the lab.

Table 6.1 Organisms discussed in detail in this book.

Species	Common name	Phylum, subphylum, class
Caenorhabditis elegans	Worm	Nematode, phasmida
Drosophila melanogaster	Fruit fly	Arthropod, uniramian, insect
Brachydanio rerio	Zebrafish	Chordate, vertebrate, fish
Xenopus laevis	African clawed frog	Chordate, vertebrate, amphibian
Gallus domestica	Chicken	Chordate, vertebrate, bird
Mus musculus	Mouse	Chordate, vertebrate, mammal

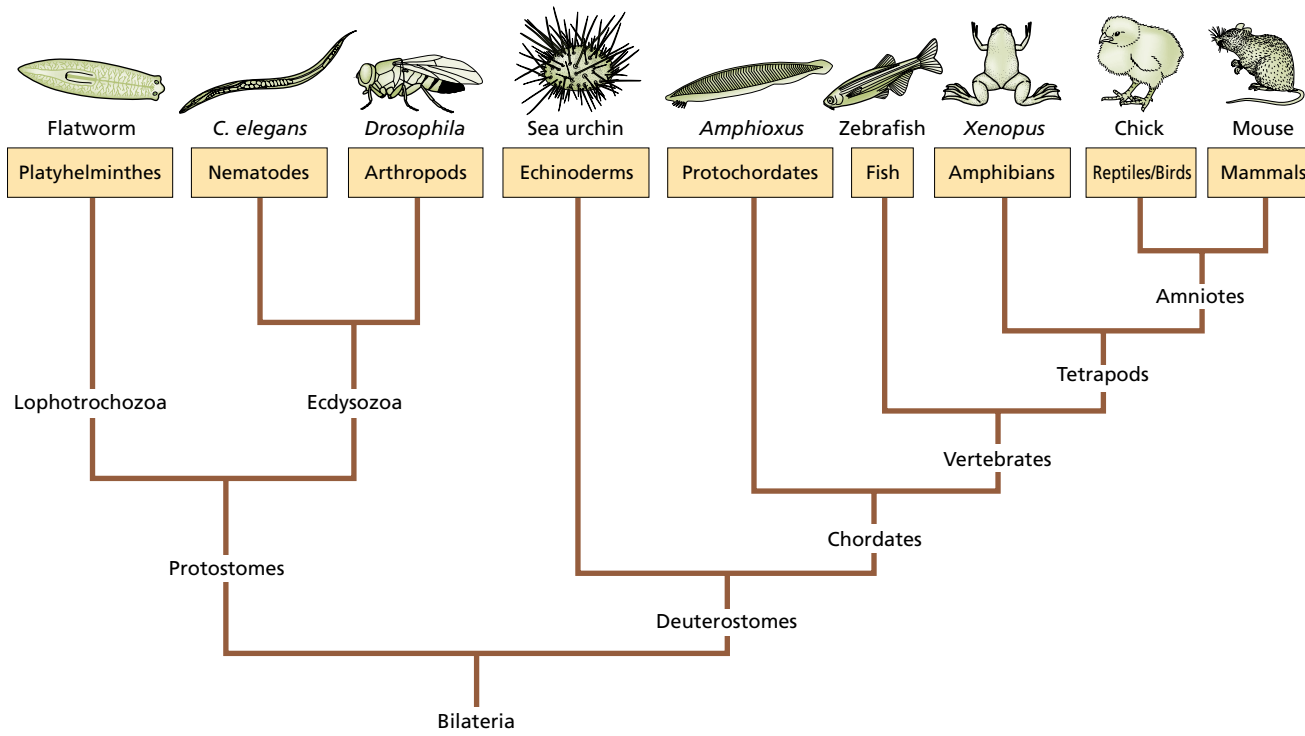


Fig. 6.1 Phylogenetic tree showing the positions of the big six model organisms used in developmental biology.

Table 6.2 Experimental advantages and disadvantages of six model organisms.

	C. elegans	*Drosophila*	Zebrafish	*Xenopus*	Chick	Mouse
Numbers of embryos	high	high	high	high	low	low
Cost	low	medium	medium	medium	low	high
Access	good	good	good	good	good	poor
Micromanipulation	limited	limited	fair	good	good	limited
Genetics	good	good	fair	none	none	good
Gene inventory	known	known	known	known*	known	known

**X. tropicalis*

In terms of numbers, it is relatively easy to obtain thousands of eggs from the first four species. Chick eggs are normally not bred in the lab but are purchased from commercial hatcheries. A large incubator will accommodate a few hundred eggs. Mice are less prolific than the other species but a mated pair are likely to produce a litter of, say, 12 embryos, so it is not too difficult to produce moderate numbers.

Cost is an important consideration because the pressure of modern research demands a weekly or even daily supply of embryos. *Caenorhabditis elegans* are very cheap, as they can be grown on agar plates coated with bacteria, and genetic stocks can be stored frozen. *Drosophila* are potentially cheap to keep but may need temperature- and humidity-controlled fly rooms and significant technician time to maintain all the different genetic stocks. They cannot presently be frozen. Zebrafish need an expensive aquarium facility and large-scale stock maintenance. The capital cost of setting up a lab is quite high and the running cost is probably similar to *Drosophila*. *Xenopus* also need an aquarium, although these have traditionally been of a less sophisticated type than those used for zebrafish. The care is relatively cheap since they have "no genetics" and therefore there are few stocks to maintain. Chicks are very cheap to keep as they are purchased from chick hatcheries and just require an egg incubator in the lab. However, if a lab needs to do genetic crosses or have access to very early stages, it will need a chicken-breeding facility, and since the animals are quite large this will be costly. Mice may seem small and cheap but are actually by far the most expensive organisms on the list. The logistics of mammalian breeding and the regulations about space and standards in laboratory animal facilities mean that the technician time and space required for a significant mouse operation is very high. Mouse sperm and embryos can, however, be frozen, reducing the long-term cost of stock maintenance.

Access and micromanipulation

"Access" refers to how easy it is to get at embryos at all stages of development. Just about any embryo can be obtained by dissection, but many experiments require that something should be done to the embryo and it should then be maintained alive until a later stage to observe the consequences. From this point of view the free-living organisms with external fertilization are the most favorable. This means *Xenopus* and zebrafish. *Drosophila* eggs are also laid soon after fertilization. *Caenorhabditis elegans* need to be dissected from the mother during cleavage stages, although they can survive perfectly well on their own. Chicks undergo their cleavages in the reproductive tract of the hen and are already a double-layered structure containing about 60,000 cells when the eggs are laid. After this stage they are easy to get at because it just requires a hole to be cut in the egg shell. They will survive perfectly well if the hole is resealed with adhesive tape and they are kept in a humidified incubator. Mice are the least good from the access point of view. For the first 4 days the embryos can be flushed from the reproductive tract and cultured *in vitro*. After this, they become implanted into the uterus of the mother and depend on the placenta for their nutrition. It is very difficult to grow preimplantation embryos into postimplantation stages *in vitro*, and if an early postimplantation embryo is removed from the mother's uterus it can only be cultured *in vitro* for another 1–2 days. However, individual organ rudiments from mouse and chick embryos can usually be cultured for long periods and show good differentiation *in vitro*. This means that they are often favored for studies of late development.

The other requirement for embryonic experimentation is the ease of microsurgical manipulation. This may mean removing a single cell, or a small piece of tissue, grafting an explant to another position in a second embryo, or injecting individual cells with substances. All this is relatively easy in *Xenopus*. Because of the large size of amphibian eggs, microsurgery can be done freehand under the dissecting microscope, and microinjection requires relatively cheap and simple equipment. A good level of micromanipulation can also be achieved in the chick, particularly at later stages. The other organisms are somewhat less favorable. With zebrafish it is possible to remove or inject cells during the early stages. *Caenorhabditis elegans* and *Drosophila* eggs are small and surrounded by a tough outer coat. Mouse embryos are small at preimplantation stages and hard to culture at postimplantation stages.

The *C. elegans* and fish embryos have the particular advantage of being transparent and so it is easier to follow cell movements *in vivo* than it is for the other species.

Genetics and genome maps

All the organisms under consideration have genomes, but they do not all have "genetics" in the sense of a technology for doing genetic experiments in the lab. *Drosophila* genetics is very sophisticated as it was practiced for many decades before *Drosophila* was adopted as a model for the study of development. The short life cycle of 2 weeks, and the ease of keeping large numbers of animals are both decisive. Also, the existence of balancer chromosomes (see Chapter 11) simplifies the keeping of stocks of mutants that are lethal in the homozygous form. *Caenorhabditis elegans* is also very favorable for genetics, because of a short life cycle and ease of keeping large numbers. Because it is a self-fertilized hermaphrodite, new mutations will segregate to the homozygous state automatically without the need to set up any crosses. Mouse genetics has also been practiced for many decades but it still falls below that of *Drosophila* in sophistication, partly because of the difficulty of handling lethal mutations, and also because of the huge cost of keeping the large numbers of animals required for mutagenesis screens. However, the ability to make "knockouts" of selected genes has become an enormously important technique. The zebrafish has a shorter history as a laboratory organism, and so lacks some of the sophisticated technology that exists for the more longstanding models. In terms of numbers and life-cycle duration (4 months) it is worse than the invertebrates but good for a vertebrate. *Xenopus* has never been taken seriously for genetics because of the long life cycle; it takes at least 9 months to rear an animal to sexual maturity. However, a related species, *Xenopus tropicalis*, will grow to maturity in 4 months.

The large-scale genome-sequencing activity of recent years means that all the model organisms now have more or less complete inventories of genes and high-resolution genome maps. The main importance of this for developmental biology is that it takes most of the labor out of cloning a new gene. In the past, to obtain a homolog of a known gene in your organism you had to clone it yourself and this can take considerable time and effort. Even worse, the positional cloning of a gene known only as a point mutation could take several years. When complete gene inventories and maps are available any particular gene can be obtained, at least to the level of a large piece of genomic DNA, from a central depository. Furthermore the complete gene inventory means that all the members of a gene family are known in advance. This is very helpful because there is frequently considerable redundancy of function between members of a gene family and it is necessary to know all the members to be able to interpret the results of both overexpression and loss of function experiments.

Unfortunately it has turned out that *Xenopus laevis* is **pseudotetraploid**. This means that it doubled the number of chromosomes about 30 million years ago, since when mutations have accumulated that make the gene copies on the duplicated chromosomes somewhat different from each other. But the gene pairs still remain fairly similar with similar functions. They are sometimes called "**pseudoalleles**" because the sequences make them look like alternative alleles at the same genetic locus but they are really not alleles at all. Pseudotetraploidy is unfavorable to the experimentalist because it means more genome to

sequence and more redundancy of function. So it is unlikely that *Xenopus laevis* will be sequenced in the near future and attention has focused on *Xenopus tropicalis* which is a true diploid. Fortunately the level of sequence divergence between the two *Xenopus* species is low enough that probes from one species will normally hybridize with the homologous gene from the other.

Bony fish, including the zebrafish, also underwent an extra genome duplication, but this is much more ancient and so the gene pairs have diverged considerably. They have acquired rather different functions, and many have also been lost. Because of this the zebrafish is effectively diploid although does tend to have extra copies of many genes important in development.

Relevance and tempo

Table 6.2 summarizes the advantages and disadvantages of the six model organisms. A quick assessment of this table will show that they all have their strengths and weaknesses and that none of them is ideal in all respects. On balance the mouse and chick score lower than the others, and indeed some of their basic processes have been elucidated more recently because they are technically a little less favorable for experimental work. However, they are among the six favorites because of another important consideration, perceived relevance. Both mouse and chick are **amniotes**, and the mouse is a mammal. This means that they appear much more similar to humans than the other models. This consideration has guaranteed the mouse and chick a good share of medical research funding over the years. It has actually turned out that, at the molecular level, the other organisms are much more similar than previously thought to the human, but since we ourselves are mammals we shall always have a special interest in mammalian development.

In today's competitive world it is not possible to work on a system where each experiment will take a very long time. In this context the rates of development of the big six are shown in Fig. 6.2. This shows that the difference of time span to maturity is enormous, ranging from *C. elegans* at 3 days to *Xenopus laevis* at 9 months. Of course only the fast models are used for genetic research, but the very short generation time of *C. elegans* and *Drosophila* is a distinct advantage compared to the mouse or zebrafish in terms of getting experiments done at moderate cost in time and personnel. The top part of the diagram covers the embryonic rather than postembryonic period and shows that all the models do enable experiments to be conducted in a few days. This is because the "endpoint" is not usually the end of development but rather it is the stage at which the developmental process under study has been completed. So for example most *Xenopus* experiments have concerned early development and are completed in 2–3 days from fertilization, while many mouse experiments are scored in mid-gestation.

Other organisms

A very wide variety of organisms has been used at one time or another for developmental research but have not ended up among the big six. A striking feature of the less popular models is that particular research communities tend to work on many different similar species rather than just one species. This is in fact an important reason why they are not major model organisms. In the molecular age research moves faster when probes can be readily exchanged and a total genomic sequence is a very valuable resource enabling the full set of genes in a particular gene family to be known and enabling the rapid identification of mutants. For different species the primary sequences of genes and their genomic organization is inevitably somewhat different, and without worldwide agreement on a single species none of this can be done. For example the various planarian and urodele amphibian species used for regeneration research (see Chapter 19) are too far apart for cross-hybridization of probes to be possible and this is a significant handicap to progress.

Probably the most important of the other models is the sea urchin. This is a "senior citizen" of embryological research and did have a much more prominent position in the pre-molecular era. It was used for the first experiments in the late nineteenth century that demonstrated embryonic regulation (formation of a whole larva from one blastomere), and in the 1930s for experiments demonstrating the existence of a vegetal to animal gradient controlling the body plan. More recently it has been used to build models of developmental genetic networks. It is easy to obtain large quantities of eggs and to fertilize them *in vitro*. Sea urchin embryos are usually transparent and so morphogenetic processes can be observed *in vivo*. But the life cycle is very long, it is hard to rear the animals through metamorphosis, and they are not suitable for experimental genetics. Although microsurgical experiments have been conducted, the embryos are small (<100 μm diameter) so this type of work is very demanding. Sea urchins had their heyday in the 1960–80 period when it was advantageous to be able to obtain gram quantities of embryos for biochemical studies. This culminated in the discovery of the cyclins, proteins controlling the cell cycle. Also sea urchins are very convenient for studying fertilization, but the successful outcome of this work has shown that the molecular mechanisms have rather little in common with mammalian fertilization.

Ascidians are lower chordates that have had a modest following. Again they have a distinguished history having been used for some of the classic cell lineage studies around the beginning of the twentieth century. Isolation and transplantation experiments demonstrated the existence of cytoplasmic determinants, some of which have now been identified. Various gastropods such as *Patella* and *Ilyanassa* have also appealed for studies of cell lineage and cytoplasmic determinants. But in the end the concentration of attention on *C. elegans* has achieved most of what might have been expected to come from these marine invertebrates in terms of understanding the molecular nature and function of cytoplasmic determinants.

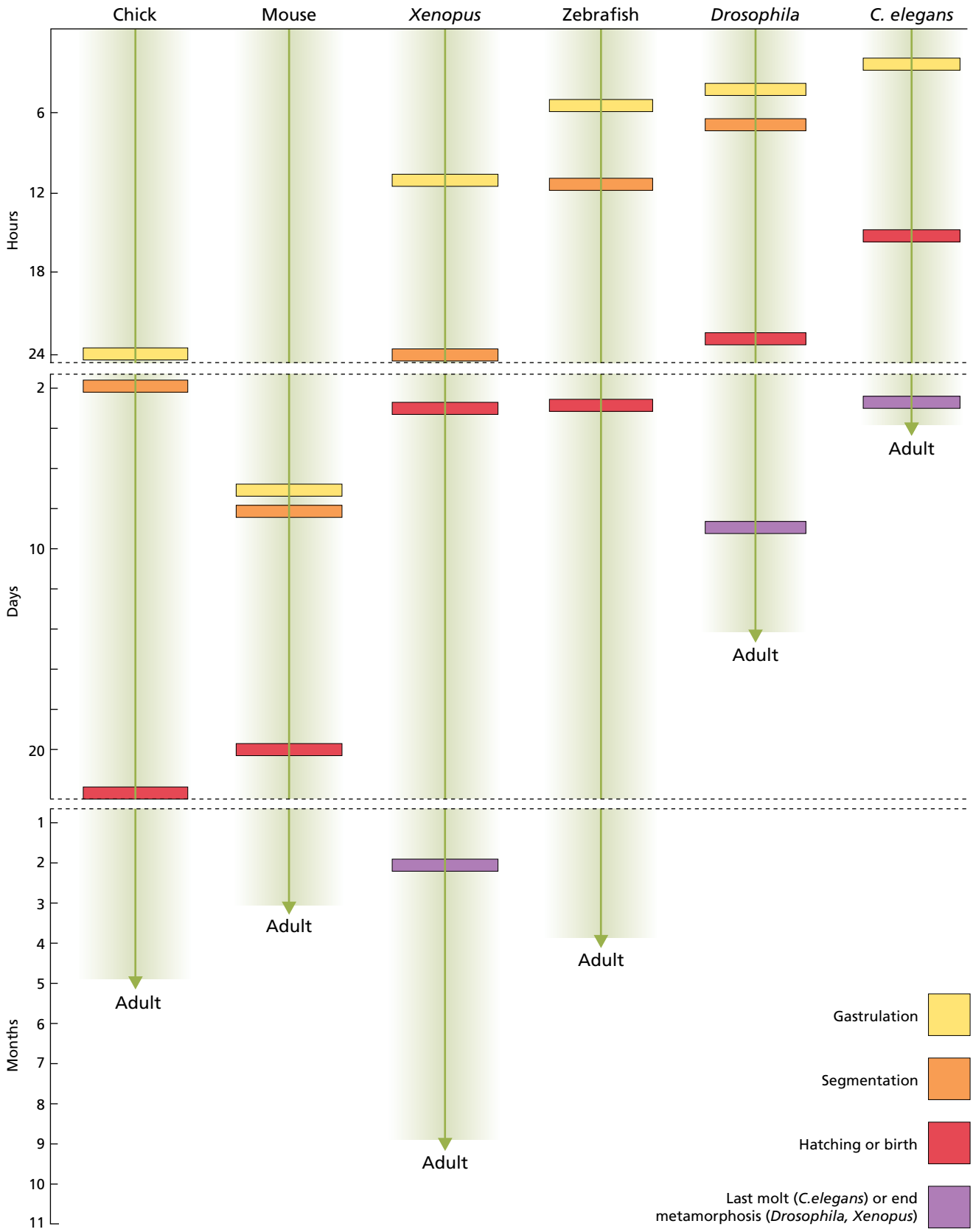


Fig. 6.2 Comparative developmental milestones for the six model organisms. The diagram is spread over three time scales, and times are only approximate since they may depend considerably on temperature for the free-living forms. "Gastrulation" refers to the start of germ layer formation. "Segmentation" refers to the start of visible segmentation. "Adult" refers to reproductive maturity. All times are measured postfertilization, including the chick where this adds approximately 1 day to the usual time scale of days of egg incubation.

The **planarian** worms display some of the most dramatic regeneration behavior in the animal kingdom and might perhaps one day provide us with a seventh model organism. At present however there are rather few labs engaged on the work and several different species are still being studied.

Certain lower eukaryotes including the cellular slime mold *Dictyostelium*, the acellular slime mold *Physarum*, and the alga *Volvox*, have been studied in relation to particular characteristics that they display clearly, respectively chemotactic cell aggregation, synchronous nuclear division, and morphogenetic movements. But all of them are a big evolutionary distance away from the human, substantially more so than even the invertebrate animals.

Then there are the ancillary organisms that lost out historically to the front running models but have some particular technical advantage that keeps them in use in a niche market. The rat is better than the mouse for whole embryo culture over the early postimplantation period and is often used for teratogenicity testing. The quail is easier to keep and breed in the lab than the chicken so can be used for things that require treatment of the mother, like vitamin A depletion studies. It is also used in conjunction with the chick for the labeling of grafts with quail-specific antibody. As mentioned above, *Xenopus tropicalis* offers a simpler genome and the possibility of experimental genetics not shared by *Xenopus laevis*. Also the **urodele** amphibians such as the axolotl and various newts show regenerative behavior greatly surpassing that of *Xenopus*. The medaka is another fish that has been domesticated to laboratory life in Japan and has been better for making transgenics than the zebrafish.

Finally there are some that have been studied not because they are thought to be models for the human but rather from the perspective of trying to understand animal evolution. Here the key issue is not experimental convenience but rather the position in the phylogenetic tree. For example, amphioxus is a cephalochordate thought to resemble to some extent the common ancestor of vertebrates. The features that it displays such as a single Hox cluster are felt to be primitive in the vertebrate lineage. Cnidarians, including the familiar freshwater *Hydra*, have a comparable position insofar as they may resemble the common ancestors of all animals. They also display a high degree of regeneration behavior. Many insects other than *Drosophila* have been used for developmental research, including the dragonfly *Platycnemis*, the cricket *Acheta*, the beetle *Tenebrio*, and the silk moth *Bombyx*. Some of these display a mode of development very different from *Drosophila*, in which the blastoderm grows as it produces posterior parts. Their main interest today is also for evolutionary comparison because of the exquisite level of molecular detail with which *Drosophila* development is understood.

All of these organisms have something to offer, but the big six account for most contemporary work and the convenience of using a standard model organism is such that the research funding agencies expect to see good reasons presented for working on something different.

Further reading

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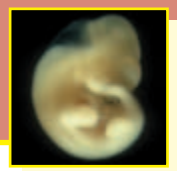
Chapter 7



Xenopus

Although other amphibian species have been used for experimental work in the past, the African clawed frog *Xenopus laevis* has been the world standard for many years. This is because of its ease of maintenance, ease of induced spawning, and robustness of the embryos. The experimental production of *Xenopus* embryos is very simple. The male and female are both injected with chorionic gonadotrophin, are put together overnight, and the next morning there are normally embryos. Nowadays it has become more common to perform *in vitro* fertilization which generates smaller numbers of embryos, but ones whose time of fertilization is known precisely and whose development shows a high degree of synchrony. *Xenopus* embryos are about 1.4 mm in diameter, which is large enough for quite discriminating microsurgery. Because the egg contains a high content of yolk granules and other reserve food materials it is possible for small fragments of embryos to survive and differentiate for several days in very simple media, and the use of embryo fragments (explants) is the basis of many experiments. The introduction of cell lineage labels such as fluorescent dextrans has enabled the accurate identification of cells in graft–host combinations. Methods have been introduced for overexpressing genes by making synthetic mRNA *in vitro* and injecting it into the fertilized egg. It is also possible to make **transgenic** *Xenopus* by incorporating DNA into the sperm and injecting the sperm into the egg.

Many *Xenopus* gene names are prefixed with an "X" to indicate their species of origin, for example *Xbra* for the *Xenopus brachyury* gene, or *Xgsc* for the *Xenopus goosecoid* gene. However, the convention is not uniformly adhered to, and in this book the "Xs" are omitted, partly for simplicity and partly to emphasize the similar developmental functions of homologous genes from different vertebrate species.

Oogenesis, maturation, fertilization

The frog ovary consists of large numbers of **oocytes** surrounded by layers of follicle cells and blood vessels. The **oogonia** in the frog ovary persist and continue to divide throughout life. Oogonia become oocytes following their last mitotic division. Oogenesis then takes several months, during which time the oocytes grow enormously, acquiring the food reserves needed to support the embryo over the several days of development before larval feeding can commence (Fig. 7.1). The oocyte nucleus is very large and is known as the **germinal vesicle**. The chromosomes are the four-stranded **bivalents** characteristic of meiotic prophase, but they remain active during oogenesis and display numerous protruding loops of chromatin. Because of this appearance they are called **lampbrush chromosomes**.

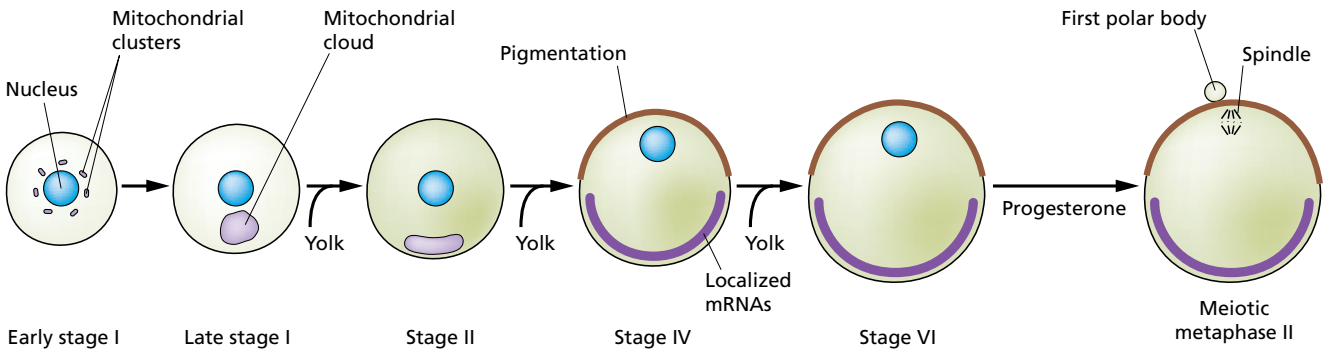


Fig. 7.1 Oogenesis in *Xenopus*. Roman numerals are used for staging. There is considerable increase in size from stage I to VI.

To support protein synthesis in early development, the oocytes need to accumulate a large store of ribosomes and transfer RNA. For this reason the gene cluster coding for ribosomal RNA becomes amplified at an early stage into many additional extrachromosomal copies, all of which are active as templates for ribosomal RNA synthesis. During the growth phase the oocyte also acquires a large amount of **yolk** proteins. These are not made by the oocytes themselves, but by the liver of the mother, and are absorbed by the oocytes from the bloodstream. The early previtellogenic (= preyolky) oocyte is transparent, but it becomes opaque as the yolk granules begin to accumulate.

During oogenesis the **animal–vegetal polarity** of the oocyte arises. In the previtellogenic stage a special cytoplasmic region becomes assembled. This is rich in mitochondria and called the mitochondrial cloud. It is the precursor of the **germ plasm**, and its location determines the future vegetal pole of the egg. A number of mRNAs including *vegT* (see below) are associated with the cloud, and they become localized to the vegetal cortex as the cloud attaches to the cortex in early vitellogenesis. The hemisphere containing the germinal vesicle is called the **animal hemisphere**. In mid-oogenesis this becomes dark due to an accumulation of pigment granules, while the vegetal hemisphere remains light colored. At about the stage that the pigmentation difference appears, a second group of mRNAs including *vg1* (see below), also become localized to the vegetal cortex.

The fully grown primary oocyte is about 1.2 mm diameter, and its synthetic activity has waned. Maturation is achieved in response to gonadotrophins secreted by the pituitary gland of the mother. These travel through the bloodstream and provoke release of progesterone from the ovarian follicle cells. This binds to steroid receptors in the oocyte and activates translation of an oncoprotein, c-mos, which activates the phosphatase cdc25 and hence activates maturation promoting factor (MPF, also referred to as M-phase promoting factor, the complex of cdk and cyclin required to initiate M phase, see Chapter 2). The germinal vesicle breaks down and the first meiotic division takes place, resulting in the formation of the first polar body. The result is often called an **unfertilized egg**, although it is strictly a secondary oocyte arrested in second meiotic metaphase. The metaphase arrest is due to the fact that cyclin breakdown is inhibited by a complex of c-mos with cdk2, known as cytostatic factor. The eggs are shed into the body cavity, enter the oviducts through the fimbriae at the anterior end, and travel down the oviducts where they become wrapped in jelly.

In a normal mating the male would be clasping the female and would fertilize the eggs as they emerge from the cloaca. On fertilization the secondary oocyte/egg becomes a **fertilized egg** or **zygote**. The rise in intracellular calcium caused by sperm entry brings about the destruction of cytostatic factor, leading to the breakdown of cyclin and progression into the second meiotic division, with release of the second polar body. The calcium also causes exocytosis of **cortical granules** near the egg surface whose contents lift the **vitelline membrane** off the egg surface and allow the egg to rotate freely under the influence of gravity to bring the animal hemisphere uppermost.

Embryonic development

Development up to the general body plan stage may be subdivided into **cleavage**, **gastrulation**, and **neurulation**, and in *Xenopus* these stages are completed within 24 hours at 24°C. A numerical stage series was devised by Nieuwkoop and Faber, according to which stage 8 is the mid-blastula, stage 10 the early gastrula, stage 13 the early neurula, and stage 20 the end of neurulation (Fig. 7.2).

The sperm enters the animal hemisphere and initiates a cytoplasmic rearrangement called the **cortical rotation** (Fig. 7.3). This is a rotation of the egg cortex relative to the interior which is associated with the transient appearance of an orientated array of microtubules in the vegetal hemisphere. It leads to a reduction in the pigmentation of the animal hemisphere on the prospective dorsal side, opposite the sperm entry point. Internally a dorsal determinant is moved from the vegetal pole to the dorsal side, ensuring that the dorsal structures will develop opposite the point of sperm entry. In some other frog species a similar pigmentation change gives rise to a surface feature known as the **gray crescent**.

Cleavage

The first cleavage is vertical and separates the egg into right and left halves. The second cleavage is also vertical, and at right angles to the first, separating prospective dorsal from ventral halves. The third cleavage is equatorial, separating animal from vegetal halves. Subsequent cleavages vary between individuals, but it is usually possible to obtain some embryos showing four tiers of eight cells at the 32-cell stage. As in other species, the large cells resulting from early cleavage divisions are called **blastomeres**. As cleavage takes place a cavity called the **blastocoel** forms in the center of the animal hemisphere and the embryo is referred to as a **blastula**. The outer surface of the blastula consists of the original oocyte plasma membrane. A complete network of tight junctions around the exterior cell margins seals the blastocoel from the exterior and renders the penetration of almost all substances highly inefficient. This is why radiochemicals and other substances need to be introduced into the embryo by microinjection. Internal cells are connected by cadherins and are readily dissociated by the removal of calcium ions from the medium. Desmosomes are not found until the neurula stages, but all the cells of early cleavage stages are connected by gap junctions.

Cleavage continues rapidly for 12 divisions after which an important transition occurs, known as the **midblastula transition** or **MBT**, although it is actually in the *late* blastula. The rate

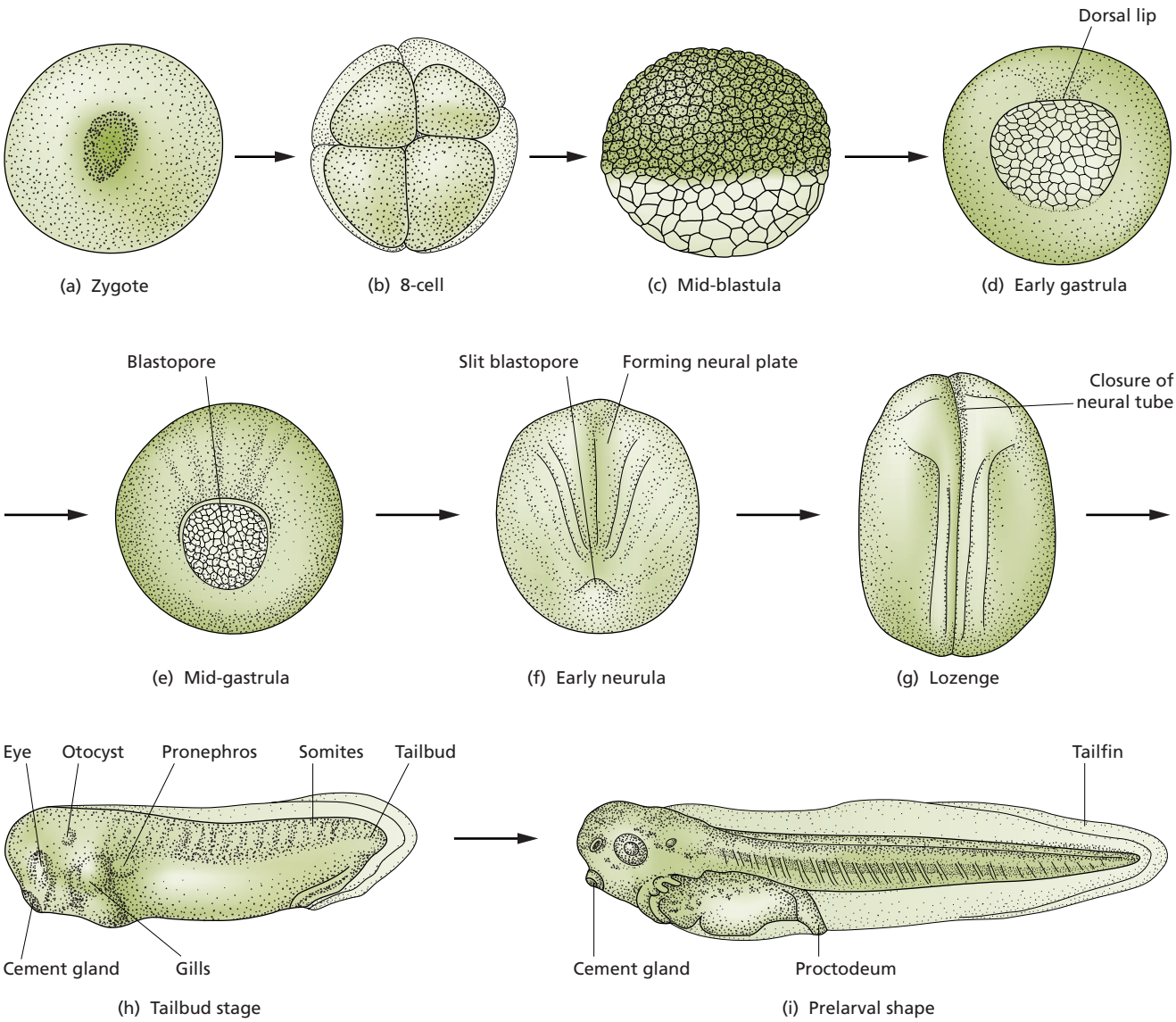


Fig. 7.2 Stages of *Xenopus* development. Here the numbers of the Nieuwkoop–Faber series are used for staging. (a) Zygote, from animal pole; (b) eight cells, stage 4, from animal pole; (c) mid-blastula, stage 8, from side; (d) early gastrula, stage 10, from vegetal pole; (e) mid-gastrula, stage 11, from vegetal pole; (f) early neurula, stage 14, from dorsal side; (g) lozenge, stage 22, about 1 day old, from dorsal side; (h) tailbud, stage 30, about 2 days old; (i) prelarva, stage 40, about 3 days old.

of cleavage slows down, the synchrony of cell divisions is lost, and the strength of intercellular adhesion increases so the blastula appears as a smooth instead of a knobbly ball. The MBT is the time at which significant transcription of the zygotic genome commences, although it is possible to detect low level transcription of some genes during the cleavage stages.

The onset of significant zygotic transcription makes it possible to visualize the early domains of commitment by *in situ* hybridization for specific transcription factors (Fig. 7.4). Only a few examples can be given here of the large number of genes that have been studied. The entire mesoderm can be visualized by the expression of the T-box gene *brachyury*, which is needed to activate later mesodermal genes and to control gastrulation movements. The dorsal sector, which will form the **organizer** region (= **Spemann's organizer**), is characterized by the expression of a variety of transcription factor genes including *siamois*, *goosecoid*, *not*, and *lim1*. All these are required for axial differentiation and, in addition, *siamois* is involved in the original formation of the organizer, and *goosecoid* is needed for gastrulation movements. The ventral mesoderm expresses the homeobox genes *vent1* and *vent2* in nested pattern. These act in a combinatorial way to specify the lateral plate (vent1+2) and somitic (vent2 only)

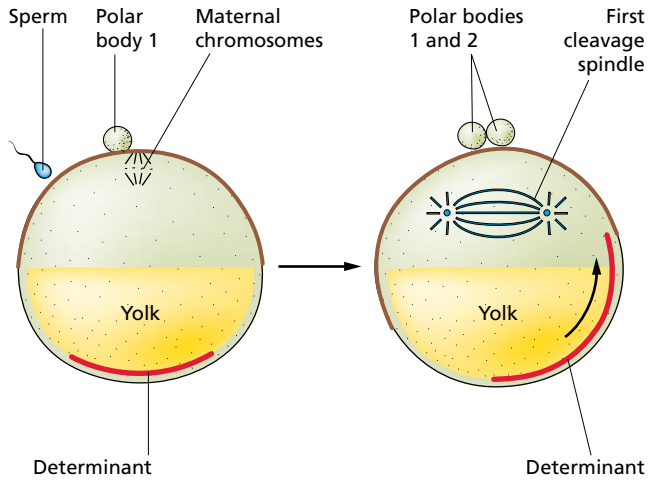


Fig. 7.3 Cortical rotation. The egg cortex moves about 30 degrees relative to the internal cytoplasm, in the direction of the sperm entry point. The vegetally located dorsal determinant moves to the dorsal side.

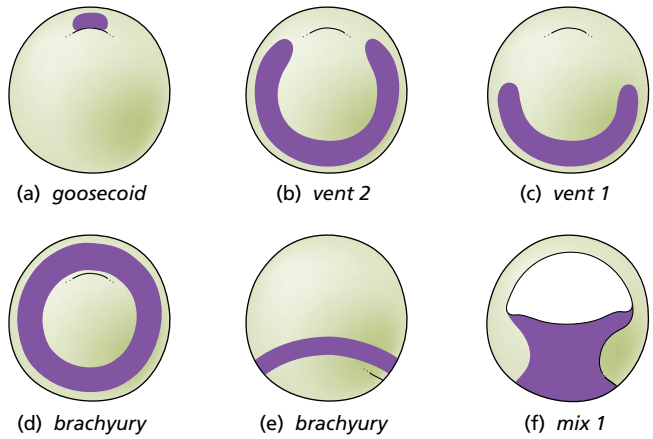


Fig. 7.4 Early domains of zygotic genes. (a–d) From vegetal pole; (e) from side; (f) section from side.

regions. The endoderm can be visualized as the domain of expression of several transcription factors including *mix1* and *sox17*; these are later required for activation of various genes of particular endoderm-derived tissues.

Gastrulation

Gastrulation is a phase of morphogenetic movements in the course of which the belt of tissue around the equator, called the **marginal zone,** becomes internalized through an opening called the **blastopore** (Fig. 7.5). This establishes the typical three-layered structure of an animal body, with outer **ectoderm**, middle **mesoderm**, and inner **endoderm**. The start of gastrulation is marked by the appearance of a pigmented depression in the dorsal vegetal quadrant. This is the **dorsal lip** of the blastopore. The blastopore becomes elongated laterally and soon becomes a

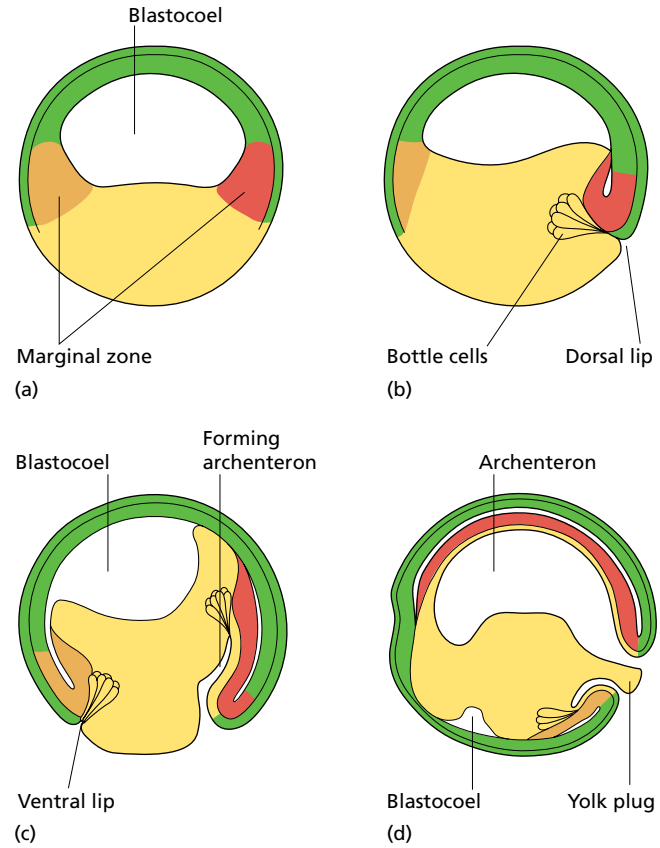


Fig. 7.5 Gastrulation. (a) Blastula; (b) early gastrula; (c) mid-gastrula; (d) late gastrula. All views medial section. In this and subsequent figures, ectoderm is green, mesoderm orange, and endoderm yellow.

complete circle. When it is circular the part referred to as the dorsal lip is the dorsal segment of the complete circle and the part referred to as the ventral lip is the ventral part of the complete circle. Elongated cells called bottle cells are present around the blastopore, although their precise mechanical role is not understood. Tissue invaginates all round the circular blastopore but the invagination is much more extensive on the dorsal side where it proceeds until the leading edge of invaginating tissue is well past the animal pole. In the lateral and ventral parts of the blastopore there is only a small extent of invagination.

Several components of the gastrulation movements may be distinguished. The mechanics of these movements are still poorly understood but it is known that they are to some extent independent of each other:

1 Spreading of the animal hemisphere (**epiboly**) which leads to it eventually covering the whole embryo surface.

2 **Invagination** of the marginal zone. This starts on the dorsal side and spreads to the lateral and ventral side until the blastopore is circular. The cavity formed by the invagination is called the **archenteron** and expands at the expense of the blastocoel as gastrulation proceeds. It has the form of a cylinder which is very much longer on the dorsal than the ventral side. When the yolk plug becomes internalized at the end of gastrulation it becomes

part of the archenteron floor. In the course of the invagination the leading edge of the endoderm literally crawls up the inside of the blastocoel, and requires a layer of fibronectin on the blastocoelic surface to do so.

3 The prospective mesoderm is internal from the start of gastrulation. As the invagination proceeds, the mesoderm separates from the endoderm and **involutes** as a separate tissue layer between the ectoderm and endoderm.

4 Elongation of the dorsal axial mesoderm in the anteroposterior direction. This occurs by an active process of cellular intercalation in all three germ layers, called **convergent extension**, which helps drive the internalization of the marginal zone and the closure of the blastocoel.

5 Expansion of the ventrolateral mesoderm towards the dorsal midline.

The cellular mechanisms of gastrulation are still poorly understood. Most attention has been devoted to convergent extension. This requires the small GTP exchange proteins Rho and Rac, as introduction of dominant negative versions of these proteins will block convergent extension. These are thought to be activated by the Wnt planar polarity pathway (see Appendix and Chapter 17). In addition there is evidence that the Wnt Ca pathway regulates cell adhesion during gastrulation. As explained in Chapter 2, differential cell adhesion is critical for cell sorting behavior. In *Xenopus* embryos it is known that stimulation of this pathway does reduce adhesiveness. In addition, in intact embryos, the dorsal cells undergoing convergent extension show an increase in intracellular calcium. Finally, if synthesis of the Wnt receptor Frizzled7 is blocked using an antisense **morpholino** (see Chapter 3), then the tissue layers adhere to each other instead of remaining separate.

By the end of gastrulation the former animal cap ectoderm has covered the whole external surface of the embryo and the yolky vegetal tissues have become a mass of endoderm in the interior. The former marginal zone has generated a cylindrical layer of mesoderm extending on the dorsal side from the slit-shaped blastopore right the way to the anterior end, and on the ventral side just a limited distance from the blastopore. The mesodermal layer remains incomplete in the anteroventral region for some time.

The fates of the three germ layers in terms of tissue type are as follows:

Ectoderm becomes epidermis, nervous system, lens and ear, cement gland;

Mesoderm becomes head mesoderm, notochord, somites, kidney, lateral plate, blood, blood vessels, heart, limbs, gonads;

Endoderm becomes epithelial lining of gut, lungs, liver, pancreas, bladder.

New Directions in Research

The main opportunity in early *Xenopus* is the study of *morphogenetic movements*, which remain poorly understood. The advantage is the ability to set up simple *in vitro* systems such as the animal cap or the dorsal marginal zone, which can be observed in real time and which can easily be modified by injection of mRNA into the fertilized egg, or by treatment with biologically active substances.

Later stage *Xenopus* can now be used for research into *organogenesis*, *metamorphosis*, and *regeneration*, because transgenic methods can be used to modify gene expression, and can be combined with microsurgical procedures such as isolation or grafting experiments.

In the course of the invagination of the marginal zone, the archenteron has become the principal cavity at the expense of the blastocoel, and the embryo has rotated so that the dorsal side is uppermost. It now has a true **anteroposterior** axis which runs from the leading edge of the mesoderm at the **anterior** to the residual blastopore at the **posterior**.

If embryos are placed in too strong a salt solution, the gastrulation movements are seriously deranged and instead of invaginating into the interior, the endomesoderm evaginates from the ectoderm to form a dumbbell-like structure (Fig. 7.6). This is known as **exogastrulation**. Both the ectoderm and the endomesoderm of the exogastrula is remarkably normally patterned, although the central nervous system is substantially defective and there is no tail.

Although superficially similar, the gastrulation movements of *Xenopus* differ somewhat from the urodele species that were used for classical studies on amphibian embryology. This means that older textbook accounts may differ somewhat from this one.

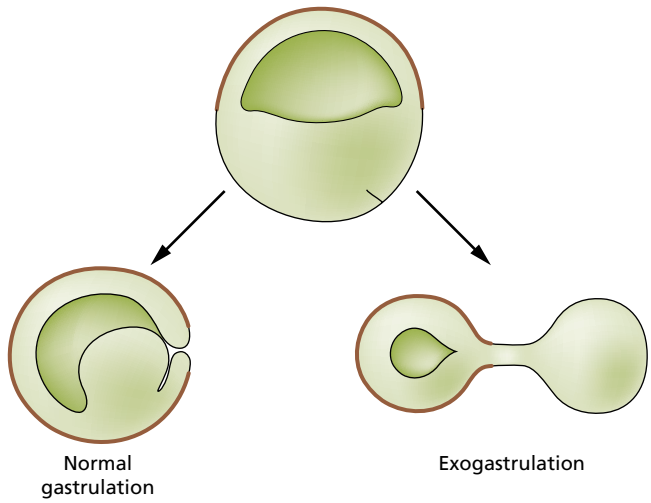


Fig. 7.6 Exogastrulation.

Neurulation and later stages

The next stage of development is called the **neurula**, in which the ectoderm on the dorsal side becomes the central nervous system. The **neural plate** becomes visible as a keyhole-shaped region delimited by raised neural folds and covering much of the dorsal surface of the embryo. Quite rapidly the folds rise and move together to form the **neural tube** which, after closure, becomes covered by the ectoderm from beyond the folds, now known as **epidermis** (Fig. 7.7). During and after neurulation there is a striking elongation of the body, which means that the whole trunk and tail region are derived from the posterior quarter of the neurula. This is driven by a continuing process of cellular intercalation both in the notochord and in other tissues.The neural tube, notochord, and somites are collectively known as the **axis**, not to be confused with the geometrical axes used for anatomical description.

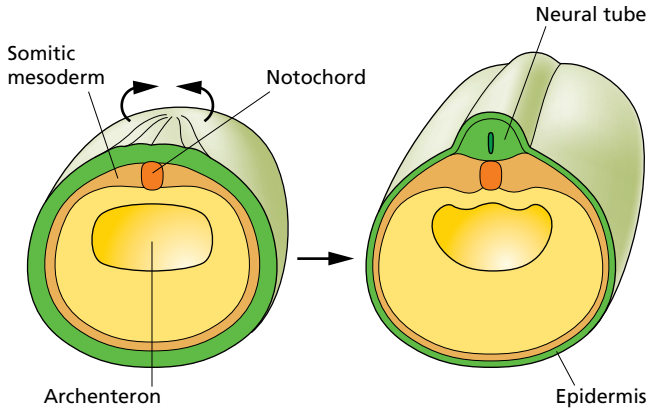


Fig. 7.7 Neurulation. Section through mid-body region.

By the **tailbud** stage all major body parts are in their final positions (Fig. 7.8a,b). The **notochord** forms from the dorsal midline of the mesoderm, rows of segmented **somites** appear on either side, the lateral plate mesoderm later gives rise to limb buds, kidney and coelomic mesothelium, and in the ventroposterior region of the mesoderm is a string of blood islands which provide the early tadpole with its erythrocytes. Another population of blood cells later form in the dorsolateral mesoderm (equivalent to the AGM region of higher vertebrates; see Chapter 9). A complex region at the posterior end, comprising the posterior neural plate and the mesoderm beneath it, becomes the **tailbud** (Fig. 7.8c), which generates the notochord, neural tube, and somites of the tail over the next 1–2 days. Because the neural folds close over the blastopore there is a connection created between the neural tube lumen and the gut, called the **neuroenteric canal**. This persists for about a day and is then blocked. The epithelia of the gut, comprising pharynx, lungs, stomach, liver, pancreas, and intestine, develop from the endoderm, although differentiation occurs much later than for the

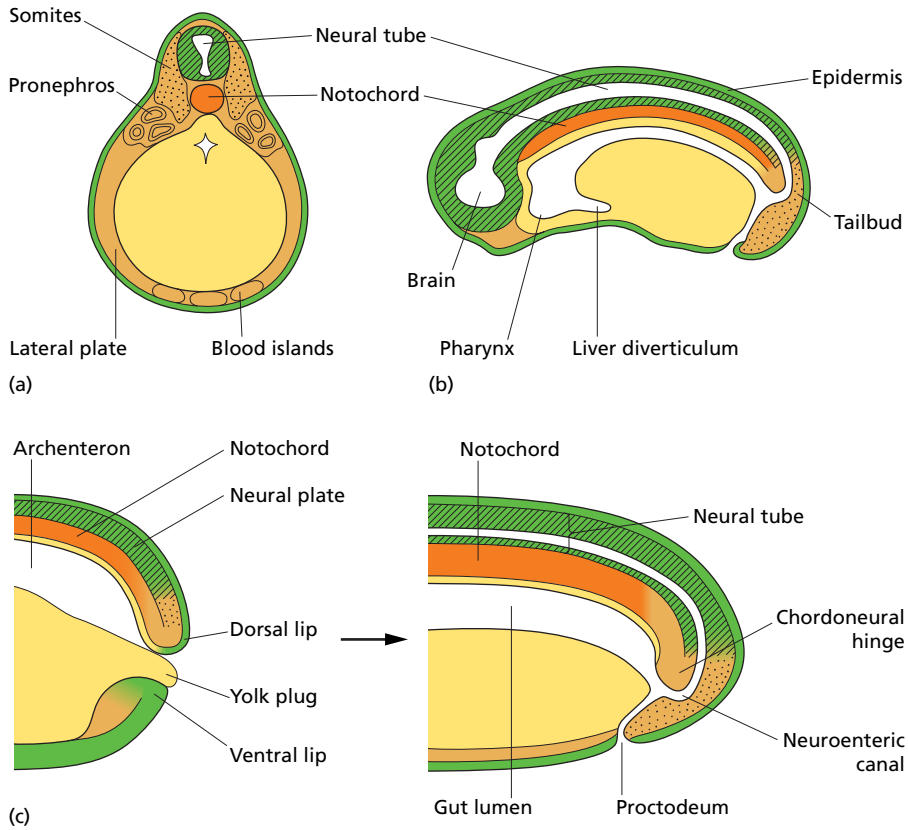


Fig. 7.8 Tailbud stage. (a) Transverse section through trunk; (b) median section; (c) formation of the tailbud by closure of the neural plate over the residual blastopore.

ectodermal and mesodermal tissues. The **proctodeum**, terminating in the anus, forms from the channel between neuroenteric canal and exterior formed by the process of neural tube closure. The mouth develops somewhat later from a new aperture formed at the anterior end.

In the head the anterior neural tube forms three vesicles that become the **forebrain**, **midbrain**, and **hindbrain**. The epidermis forms various columnar thickenings called **placodes.** These include the nasal placode, consisting of sensory cells from which originate the axons of the olfactory nerve connecting to the telencephalon; the lens placodes forming the lens of the eye; and the otic placodes that form the ear. The eyes develop as outgrowths of the forebrain (optic lobes) that invaginate into a cup shape such that the **pigment epithelium** forms from the outer layer and the retina forms from the inner layer. The lens invaginates from the surface epidermal lens placode and becomes surrounded by the **optic cup**. The optic nerve grows back from the retina down the optic stalk and projects to the **optic tecta** in the midbrain (see also Chapter 14).

Some cells from the folds of the neural plate which come to lie on the dorsal side of the neural tube later become the **neural crest**. This is a migratory tissue that forms a variety of tissue types. In the head the neural crest forms most of the skeletal tissues of the skull. In the trunk it forms the dorsal root ganglia, the sympathetic ganglia, and the **melanocytes** (pigment cells). In the vagal and sacral regions of the trunk it also forms the parasympathetic (= enteric) ganglia.

Anteriorly, the head mesoderm forms part of the jaw muscles and branchial arches. A structure known as the cement gland develops from the anterior epidermis, ventral to the future mouth. This is a prominent external feature of the head from the late neurula. The heart is formed from the ventral edges of the anterior lateral mesoderm that move down and fuse in the ventral midline around the end of neurulation.

Fate maps

Numerous **fate maps** have been published for amphibian embryos at stages from the fertilized egg to the end of gastrulation. Until 1983 all studies were by localized vital staining with the dyes neutral red or nile blue, applied to the embryo surface from a small fragment of impregnated agar.

More recently, injectable **lineage labels** have been preferred such as horseradish peroxidase (HRP) or fluorescein-dextran-amine (FDA) (see Chapter 5). These can fill whole cells and do not diffuse. For surface marking **DiI** is now preferred. Because there is little increase in size of the early embryo, passive labels of these sorts do not become diluted and so remain clearly visible for several days. The lineage labels have revealed a certain degree of local cell mixing and this means that the fate map cannot be quite precise since the mixing causes some overlap between prospective regions. As this feature is not so apparent with vital dyes, maps shown in older textbooks imply a spurious degree of precision which does not exist in reality. A modern fate map is shown in Fig. 7.9a and b. Figure 7.9c shows results of filling a dorsal blastomere (C1) and a ventral blastomere (C4) with a lineage label.

Important features of the fate map are:

1. The neural plate arises from the dorsal half of the animal hemisphere and the epidermis from the ventral half.
2. The mesoderm arises from a broad belt around the equator of the blastula, much from the animal hemisphere.
3. The endoderm arises from the vegetal hemisphere.
4. The somitic muscle arises from most of the marginal zone circumference, much from the ventral half of the blastula.
5. Both dorsal and ventral structures at the anterior end of the body come from the dorsal side of the blastula.

Experimental methods

As discussed in Chapter 4, when attempting to establish the function of any gene product in development it is necessary to know at least the following:

1. the expression pattern;
2. the biological activity;
3. the effect of specific inhibition *in vivo*.

The biological activity and inhibition experiments are particularly easy in *Xenopus* because of the ease of injecting materials into embryos and the use of ancillary techniques such as microsurgical isolation of explants or UV irradiation.

For biological activity measurement the material to be injected will usually be a mRNA made *in vitro* (Fig. 7.10a,b). The RNA synthesis is performed using plasmids carrying promoters for RNA polymerases of bacteriophage such as Sp6, T3, or T7, and a poly(A) addition site to ensure *in vivo* addition of poly(A) to stabilize the message. The mRNA can be injected into a whole fertilized egg, or into a specific blastomere during cleavage, which gives control over its location. The mRNA will be translated by the protein synthesis machinery of the egg and is likely to persist and remain active throughout early development, although it will eventually be degraded. Usually the same plasmid can be used for preparation of the *in situ* probes required for expression studies.

As many developmentally active molecules have different effects at different times, it may be desirable to inhibit activity of the introduced gene product until a desired stage. A useful method for doing this for transcription factors involves adding the hormone-binding domain from the glucocorticoid receptor to the protein of interest. This then causes it to be sequestered in the cytoplasm by binding to the cytoplasmic protein hsp90, until such time as a glucocorticoid, ususally dexamethasone, is added. As a lipid-soluble substance, dexamethasone can penetrate into the embryo and will bind to the receptor, liberating it from the hsp90, and allowing it to move to the nucleus where the transcription factor part of the molecule can exert its biological activity (Fig. 7.10c).

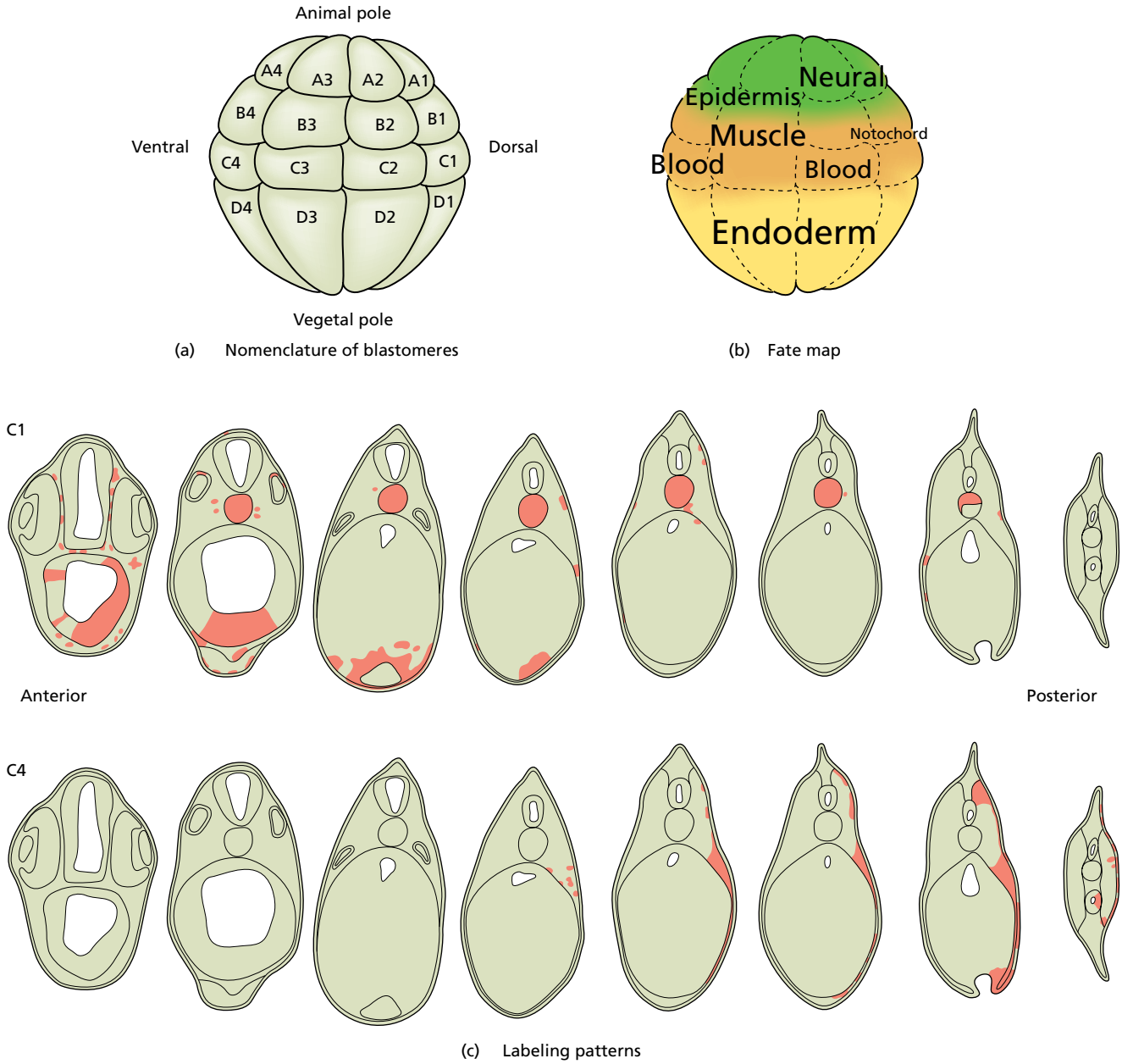


Fig. 7.9 A *Xenopus* fate map for the 32-cell stage. (a) Nomenclature of blastomeres at the 32-cell stage; (b) fate map of tissue types projected onto the 32-cell stage; (c) reconstruction of labeling pattern from blastomeres C1 and C4. The figure shows transverse sections taken at equal intervals along the body from anterior to posterior.

It is also possible to introduce genes by **transgenesis**, particularly important for studying events in late development by which time injected RNA may have been degraded. To make transgenic embryos the transgene DNA is added to sperm heads that have been decondensed in egg extract to make their own DNA accessible, and these are then injected into unfertilized eggs (Fig. 7.10d). It is usual to incorporate a green fluorescent protein (GFP) coding sequence into the transgene so that the transgenic embryos can be identified by their green fluorescence. Each individual transgenic embryo will have a different insertion site and copy number, and this may cause some variability of biological behavior.

In *Xenopus* it is not practical to study the effects of mutating genes to inactivity, but for inhibition experiments there are three standard protocols which are very effective. The most commonly used is the injection of **antisense morpholino** oligos into fertilized eggs (Fig. 7.11a). These hybridize to their complementary mRNA and block translation. It is necessary to have an antibody to the target protein in order to show that the morpholino has been effective and really prevented synthesis of the

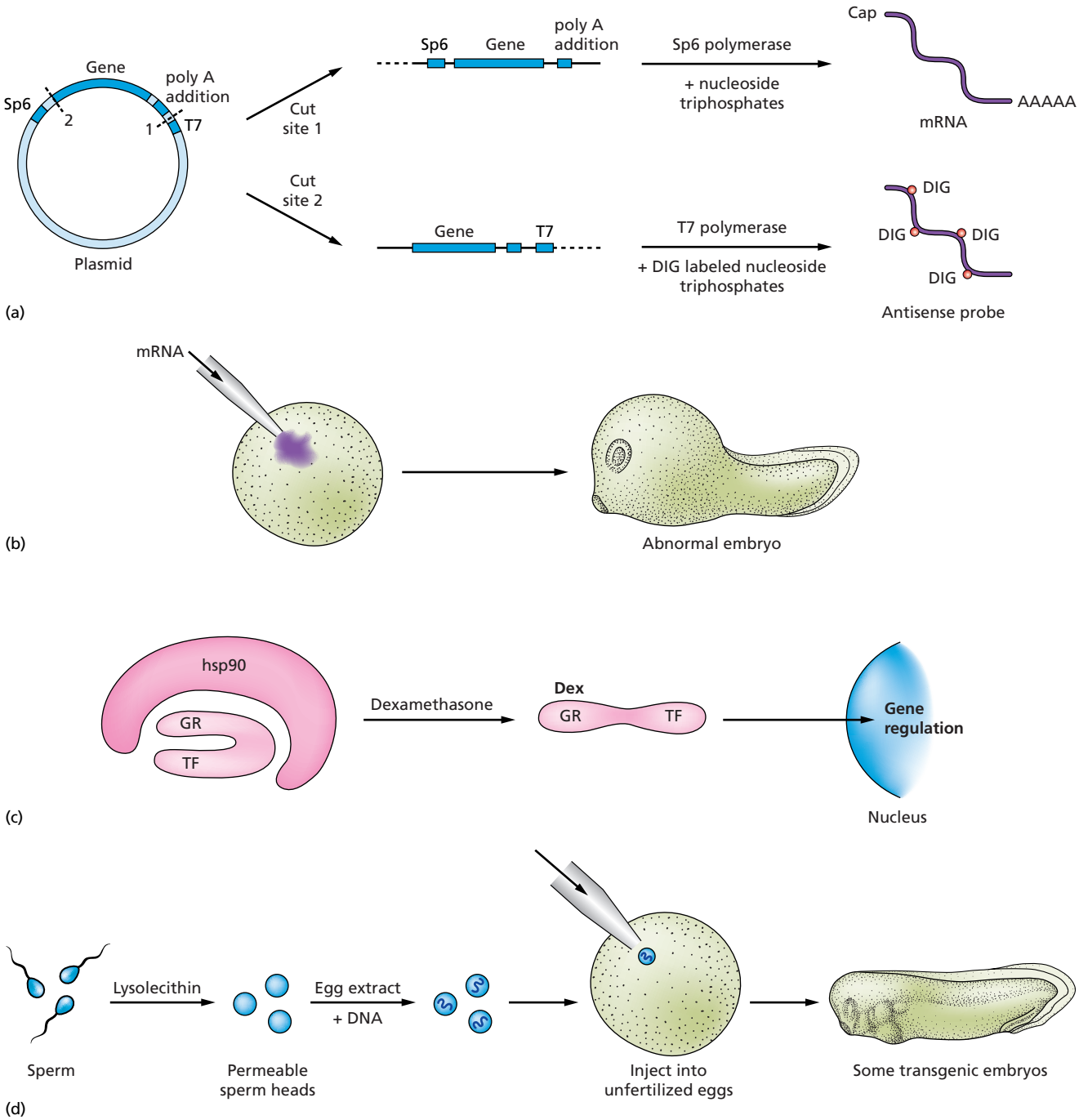


Fig. 7.10 (a) *In vitro* preparation of sense mRNA or antisense hybridization probe from a plasmid. The poly A tail becomes added *in vivo*. (b) Injection of synthetic mRNA into a fertilized egg. (c) Activation of a glucocorticoid receptor fusion protein by dexamethasone. (d) Introduction of genes by transgenesis.

protein. If it is desired to inhibit a maternally acting gene, then antisense deoxy-oligonucleotides are also effective (Fig. 7.11b). It is often very important to be able to deplete the oocyte of a specific mRNA since it is during these stages that various maternal components are laid down that are essential to later, zygotic, development. The antisense oligo is injected into the oocyte and, like the morpholinos, hybridizes to the target messages. But in this case the resulting RNA–DNA hybrid is a target for the nuclease RNAseH which destroys the message. It is of course necessary to confirm destruction of the specific mRNA by one of the biochemical methods described in Chapter 5. In order to establish later developmental effects of these experiments it is

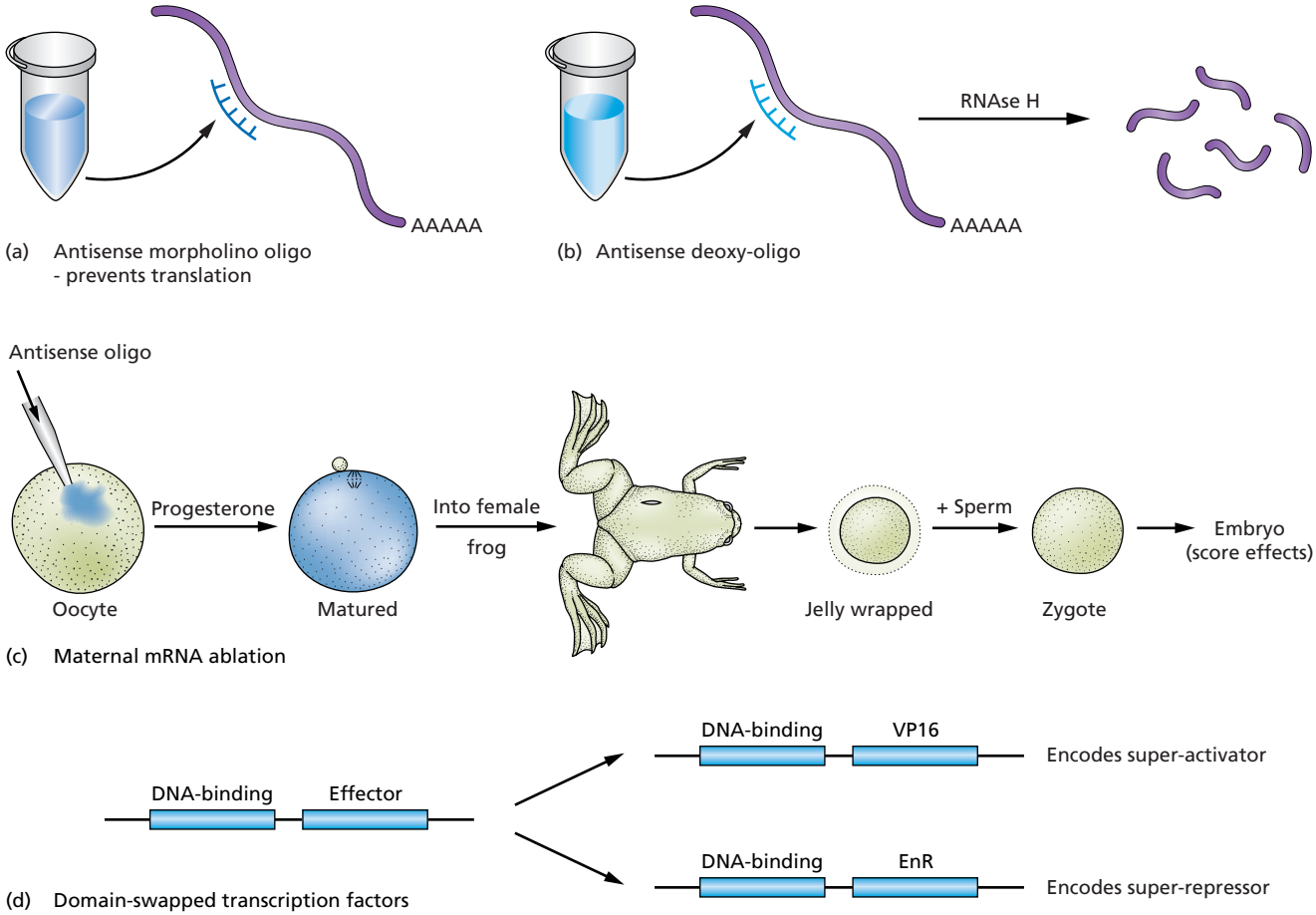


Fig. 7.11 Methods for inhibition of specific gene activity. (a) Antisense morpholino oligo. (b) Antisense deoxy-oligo. (c) Making an oocyte into an embryo after ablation of a specific maternal mRNA. (d) Domain swapped transcription factors.

then necessary to make the treated oocytes into embryos. This is achieved maturing the oocytes *in vitro* with progesterone, coloring them with a vital dye, and reimplanting them into the abdomen of an ovulating frog so that they pass down the oviduct and become wrapped in jelly. They will then be laid by the female, can be fertilized, and the course of development of the colored embryos observed (Fig. 7.11c).

The third protocol involves the design of **dominant negative** versions of gene products which can specifically inhibit the normal protein on overexpression. There are many possible types of dominant negative reagent. In *Xenopus*, particularly extensive use has been made of **domain swapped** versions of transcription factors (Fig. 7.11d). Here, the effector domain of the factor is replaced by a strong activator (e.g. VP16) or a strong repressor (e.g. engrailed repressor). mRNA is made from each of these constructs and is injected into fertilized eggs. If the effect of, say, the VP16 fusion is similar to the normal factor this shows that the normal factor must be an activator. The phenotype of the engrailed repressor fusion, in which the normal targets of the transcription factor are repressed, then gives an indication of the functions for which the normal factor is needed. Conversely if the EnR version gives a normal phenotype then the transcription factor is likely to be a repressor, and the phenotype obtained with the VP16 fusion will indicate what functions require the normal gene product.

The effects of overexpression of a specific RNA on the development of the whole embryo are often not very informative because of the nonspecific nature of the defects observed. But two procedures combining overexpression with another technique have been particularly useful. One is the animal cap autoinduction assay (Fig. 7.12a). If an embryo is injected with a component of the mesoderm-inducing or neural-inducing systems and then the animal pole region of the blastula, called the **animal cap**, is explanted, it will autonomously undergo the induction. This is because some or all of the cells in the cap make and secrete the factor and all the cells are competent to respond to it. Untreated animal caps develop as spherical balls of epidermis. Those induced to form axial tissues undergo a convergent extension process and become very elongated. Those induced to form ventral-type tissues may elongate a little, but will swell to

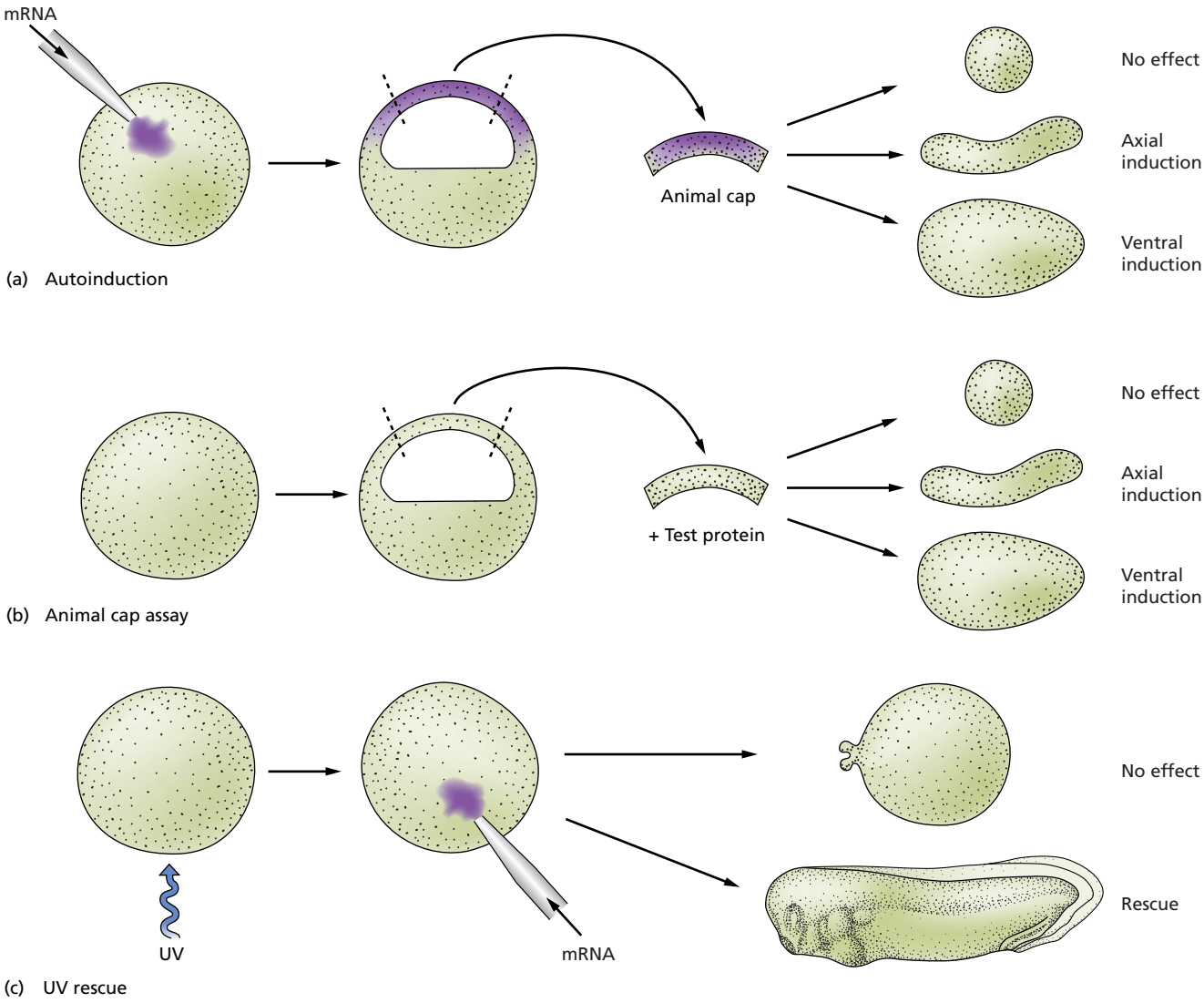


Fig. 7.12 Use of animal caps and UV embryos.

form translucent bodies on the second and third day of culture. As these changes can be observed under the dissecting microscope, this offers a very simple and convenient assay method.

For the study of extracellular factors it is possible simply to apply proteins to explants from embryos and observe the change in developmental pathway (Fig. 7.12b). Since the outer surface of the embryo is impermeable, these treatments must be applied to explants before they round up and become resealed. Whether the assay depends on mRNA injection or on treatment with a protein, the visual scoring of the results can be supplemented by conventional histology, *in situ* hybridization or immunostaining for specific markers, or by biochemical methods for specific mRNAs (see Chapter 5).

Another important combined protocol is the UV rescue method (Fig. 7.12c). As we shall see, it is possible to create embryos lacking all axial structures by UV radiation of the zygote. Injection of an mRNA coding for any component of the axial induction system can restore part or all of the axis to such embryos, and this is easily scored by visual inspection. Because the head is formed mainly from the dorsal part of the blastula, the degree of head formation provides a semiquantitative measure of the degree of dorsal rescue.

Regional specification

Summary of processes

The fertilized egg contains two determinants, a **vegetal** and a **dorsal** determinant (Fig. 7.13). The vegetal determinant becomes established during oogenesis as a result of mRNA localization to the vegetal cortex and causes formation of the **endoderm.** This is the source of a **mesoderm-inducing** signal that induces a ring of tissue around the equator to become the **mesoderm**. The dorsal

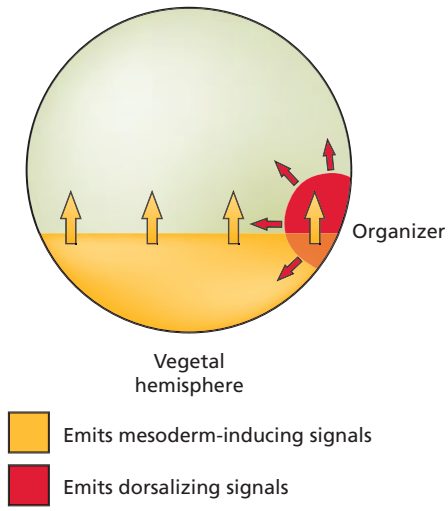


Fig. 7.13 Two signaling centers in the early embryo. The vegetal hemisphere emits a mesoderm-inducing signal (yellow arrows) and the organizer emits a dorsalizing signal (red arrows).

determinant causes formation of the **organizer** in the region that will become the **dorsal lip** of the blastopore. It is initially localized at the vegetal pole, and shifts to the dorsal side during the cortical rotation. The organizer is the source of later dorsalizing signals that both induce the neural plate, and pattern the mesoderm into zones forming different tissues.

Half embryos

Evidence for the existence of the two determinants comes from the effects of early ablation and isolation experiments, which show that formation of a complete body pattern requires the presence of both the vegetal and the dorsal regions. Complete twins can be produced by separation of the first two blastomeres, or the equivalent lateral subdivision of a blastula into right and left halves (Fig. 7.14a). In this case, some vegetal and some dorsal material is present in each half. However, if early cleavage stages or blastulae are divided frontally (separating prospective dorsal and ventral halves) then the dorsal half forms a slightly hyperdorsal whole embryo while the ventral half forms a "belly piece" of extreme ventral character (Fig. 7.14b). Hyperdorsal embryos have a large head and small tail, while ventralized ones have a small head and large tail. Although these effects may seem to

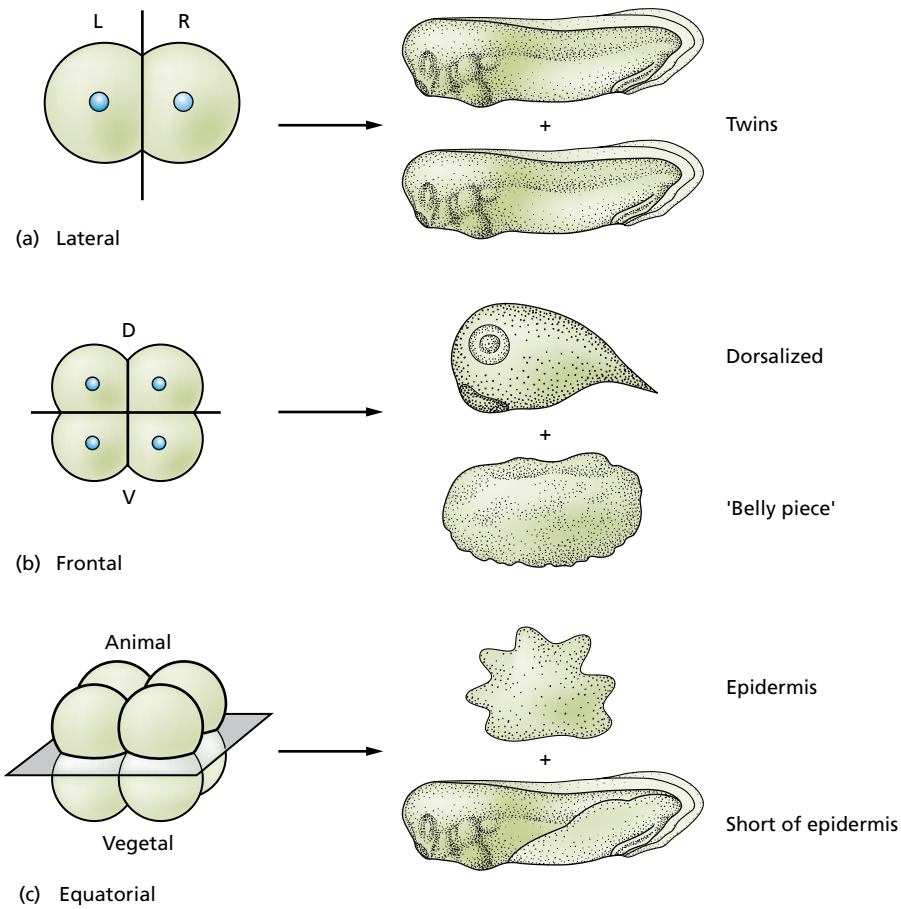


Fig. 7.14 Separation of an early embryo into two halves.

concern the anteroposterior rather than the dorsoventral pattern, it should be remembered that in the fate map the dorsal part of the blastula projects largely to the anterior of the later body and the ventral part projects mainly to the posterior of the later body, so these are the expected results from changing the dorsoventral proportions of the blastula. In the frontal separation, the dorsal half has both vegetal and dorsal tissue, but the ventral half does not. Finally, an equatorial subdivision, into animal and vegetal halves, carried out on the eight-cell to early blastula stage, yields a bag of epidermis from the animal hemisphere and an ectoderm-poor but otherwise well-patterned embryo from the vegetal half (Fig. 7.14c). Again, the explanation is that the vegetal half will contain both dorsal and vegetal material but the animal half will not.

Early dorsoventral patterning

The dorsal determinant is moved from the vegetal pole to the dorsal side by the cortical rotation occurring after fertilization. This depends on an array of parallel microtubules that forms transiently in the sense of the rotation. The microtubules grow from the sperm aster and when they meet the vegetal cortex become anchored to the cell surface by kinesin-related proteins and are aligned with their + ends in the direction of the future cortical rotation. The rotation movement is probably due to the relative displacement of the surface-tethered microtubules relative to internal dynein protein. The cortical rotation can be prevented by microtubule-depolymerizing drugs such as nocodazole or by injection of antibodies to the kinesin-related proteins. It can also be prevented if the vegetal hemisphere is irradiated with UV light before the rotation starts. The embryos that subsequently develop following any of these treatments are radially symmetrical and extreme ventral in character, with the mesoderm mainly consisting of blood islands (Fig. 7.15a,b), showing the importance of the cortical rotation for the development of dorsal structures. The fact that the dorsal side forms opposite the site of sperm entry is explained by the relationship of the microtubule array to the sperm aster.

There is now good evidence that the dorsal determinant consists of inhibitors of glycogen synthase kinase 3 (gsk3), which is a repressive component of the canonical Wnt signal transduction pathway leading to stabilization of β-catenin (see Appendix). Gsk3 is present in eggs and is constitutively active. It phosphorylates, and thereby inhibits, β-catenin. If gsk3 is inhibited, then β-catenin becomes stabilized and active and is free to enter the nucleus and combine with the transcription factor Tcf-3 to activate the target genes that are needed to form dorsal structures. Injection of additional gsk3 mRNA into normal embryos will inhibit formation of the dorsal axis, and, conversely, injection of a dominant negative version of gsk3 can rescue the formation of a dorsal axis in UV embryos or induce a second axis in normal embryos. Evidence for the essential role of β-catenin has been obtained by antisense oligonucleotide-mediated ablation of the β-catenin mRNA from oocytes. When such oocytes are matured and fertilized they develop as ventralized embryos. Although the intracellular components of the Wnt pathway are essential for axis formation, there is no evidence that the extracellular Wnt factors themselves are involved, which is why the pathway in Fig. 7.15e is shown as commencing with dishevelled. In normal development, gsk3 becomes locally inhibited just on the dorsal side as a consequence of the cortical rotation. This means that β-catenin enters the nuclei just on the dorsal side, and this can be observed by immunostaining for β-catenin protein. It extends to some extent above the equator where it is important in the initial formation of the neural plate. One of the targets of the β-catenin-TCF3 complex is the gene encoding the transcription factor siamois, a homeodomain factor important in the subsequent formation of the organizer.

The dishevelled protein, and a protein called GBP (gsk3 binding protein), both inhibit gsk3. Both of these proteins are found in eggs. If fusion proteins containing the dishevelled or GBP sequence joined to GFP are introduced into the egg, then they can be visualized *in vivo* by fluorescence microscopy, and it can be seen that they become sequestered into small vesicles and become moved to the dorsal side by the cortical rotation. GBP binds to kinesin, providing a direct mechanism for this transport process. Both dishevelled and GBP can rescue formation of an axis if injected into UV embryos. It has also been shown that depletion of GBP from oocytes, by the injection of specific antisense oligonucleotide followed by conversion of the oocytes into embryos, results in the production of ventralized embryos in a similar way to removal of maternal β-catenin.

It is possible to produce **hyperdorsalized** embryos by treating early blastulae with lithium salts. These have a structure that is the opposite of the UV embryo and resemble radially symmetrical heads (Fig. 7.15a). They arise because the entire mesoderm has been caused to develop as organizer tissue. UV-ventralized embryos can be rescued back to a normal pattern by a localized injection of lithium (Fig. 7.15d), and a localized injection of lithium on the ventral side of a normal embryo will induce a secondary axis (Fig. 7.15e). Lithium is an inhibitor of gsk3, and may also exert additional effects through inhibition of the inositol phosphate pathway (see Appendix).

Inductive interactions

Isolation experiments

Explantation studies on small pieces taken from **blastulae** show three important features (Fig. 7.16):

1 Explants from anywhere in the animal hemisphere form epidermis but not mesodermal or neural tissues.

2 Ventral or lateral explants containing marginal zone form extreme ventral structures (mesenchyme, blood cells).

3 Only a restricted dorsovegetal region, about 60° of circumference, will form axial structures (notochord, somites, neural tube).

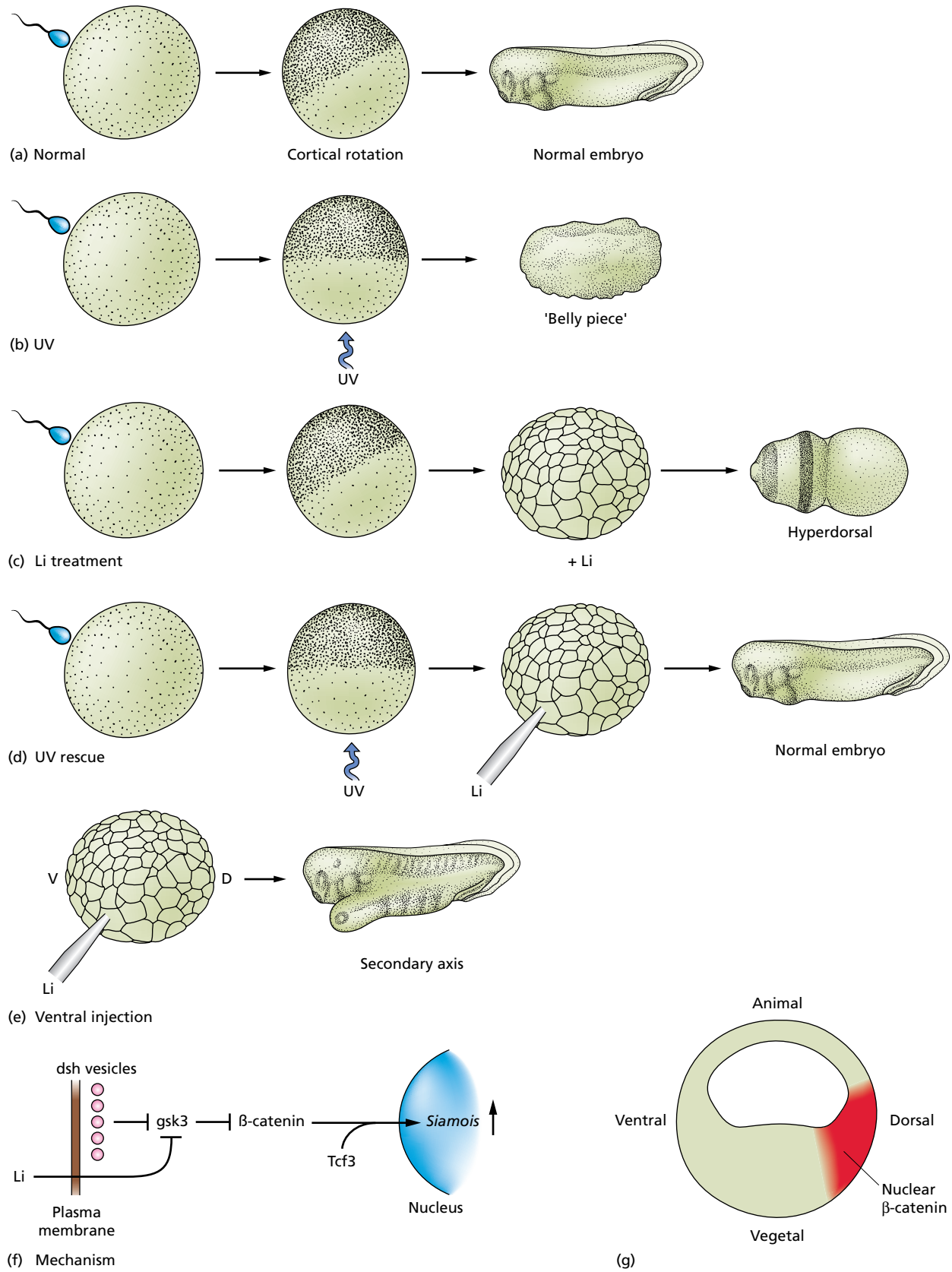


Fig. 7.15 Experiments on dorsoventral axis specification. (a) Normal development. (b) Ventralization by UV irradiation. (c) Formation of hyperdorsal embryo by lithium treatment. (d) Rescue of UV embryo by localized injection of lithium ion. (e) Induction of secondary axis in normal embryo by localized injection of lithium ion. (f) The Wnt pathway in the *Xenopus* egg. gsk3 will normally be repressed by the determinant on the dorsal side, but will be active elsewhere. Lithium can also inhibit gsk3. (g) Location of nuclear β-catenin in the late blastula.

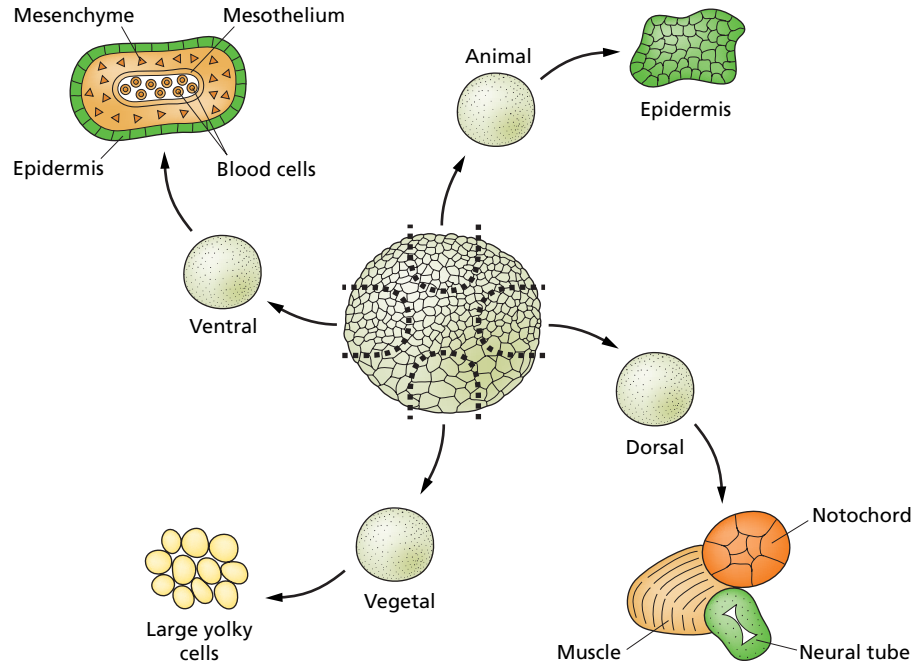


Fig. 7.16 Differentiation of explants from different parts of the blastula.

The differentiation of explants represents the specification of the tissues at the time of isolation. The results differ from the fate map, showing that inductive interactions must be necessary for achieving the final fates, particularly with regard to the formation of the neural plate and the somites.

Germ-layer formation

The fate map shows that the three germ layers arise from different animal–vegetal levels: the endoderm from the vegetal region, the mesoderm from the equatorial region, and the ectoderm from the animal region. The primary cause for this pattern is the localization of maternal mRNAs to the vegetal hemisphere during oogenesis (see Figs 7.1, 7.3). Among these is the mRNA for a T-box type transcription factor called *vegT* (= *brat*, *apod*, *ombi*). This appears to act as a **determinant** for the **endoderm**. The evidence is:

1 *VegT* mRNA is localized to the prospective endoderm.

2 It will induce endodermal markers if injected into other parts of the embryo.

3 Antisense oligonucelotide ablation of maternal *vegT* mRNA in oocytes, followed by maturation and fertilization, will produce embryos lacking endoderm.

The endoderm is defined by the expression of a group of transcription factors, all of which depend directly or indirectly on vegT. Some, including the SRY-related factor sox17a and the homeodomain factor mix1, are induced directly by vegT. The genes encoding these factors are activated from MBT even if the cells have been dissociated so that no intercellular signaling can take place. Expression of other endodermal transcription factors, including the homeodomain factor mixer and the zinc finger factor GATA4, depend on the signals emitted from the vegT-containing cells and are not induced if these signals are blocked by cell dissociation. Later on endoderm becomes regionalized into zones that will form the different tissue types of the gut and respiratory system, and this requires the activation of transcription factor genes of the Parahox cluster: *XlHbox8 (=Pdx1)* in the future foregut and *cdx* genes in the future intestine.

The **mesoderm** arises from the equatorial region of the blastula and is characterized by the expression of various transcription factors including brachyury. The part on the vegetal side of the equator may be formed by the endodermal determinants acting at lower concentration than required to form endoderm. However at least the part of the mesoderm arising from the animal hemisphere is formed by **induction** (Fig. 7.17a). Explants from the animal hemisphere develop into **epidermis** after isolation. If an **animal cap** (an explant from the center of the animal hemisphere) is combined with endoderm, then substantial amounts of mesodermal tissues are induced in the cap (Fig. 7.17b).

Only a small dorsovegetal region of the endoderm will induce organizer mesoderm, characterized by expression of transcription factors such as goosecoid, lim1, and not. The remainder of the endoderm induces **ventral-type** mesoderm characterized by expression of transcription factors such as vent1 and vent2. The region of the endoderm with organizer-inducing ability is sometimes called the **Nieuwkoop center**, after the great Dutch embryologist of that name. It is the region in which expression of *siamois* has been activated by the dorsal determinant.

There is also a difference of **competence** within the animal hemisphere such that it is much easier to obtain axial inductions

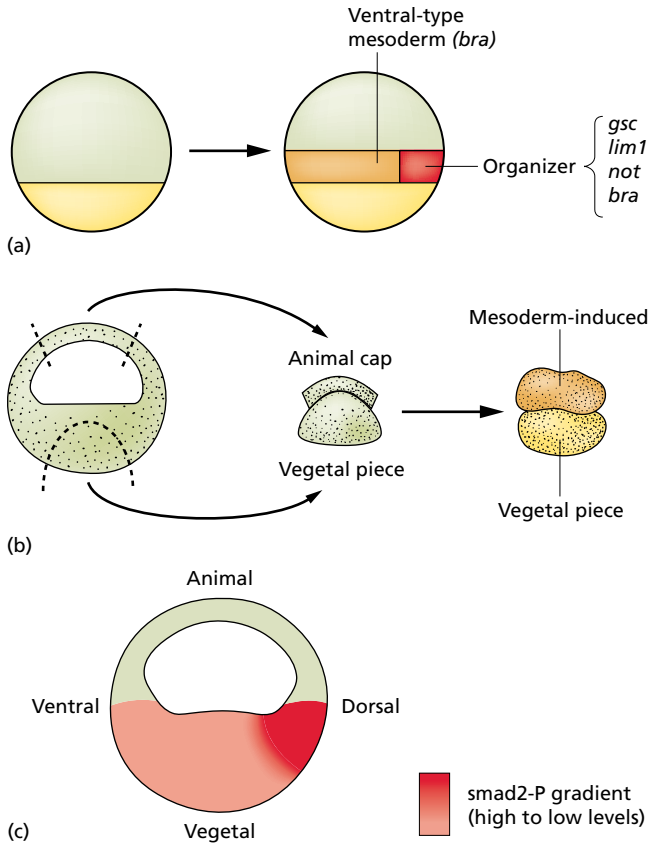


Fig. 7.17 Mesoderm induction: (a) in normal development; (b) mesoderm induced in animal cap combined with vegetal explant; (c) location of phosphorylated smad 2 in late blastula.

from the dorsal region. This is because the β-catenin activation caused by the dorsal determinant extends into the animal hemisphere. The competence of the ectoderm to respond to the mesoderm-inducing signals rises in the early blastula and falls in the early gastrula. The signaling capacity of the vegetal hemisphere is mostly exerted following MBT, as shown by immunostaining for the activated form of smad2 in intact embryos.

The factors responsible for mesoderm induction are members of the transforming growth factor (TGF)-β superfamily, especially the nodal-related factors (see Chapter 10 for the original *nodal*). This subset of factors activates cell surface receptors that phosphorylate smad2 and smad3 proteins in the cytoplasm, and the activated smads can then move into the nucleus and activate target genes (see Appendix). The evidence for the importance of nodal-related factors is as follows:

1 Nodal-related factors will induce mesoderm if applied to animal caps.

2 They are expressed in the vegetal hemisphere after MBT.

3 Overexpression of a dominant negative receptor that inhibits this group of factors will prevent mesoderm formation.

4 Overexpression of Cerberus-short, a peptide from the Cerberus molecule (see below) that inhibits only the nodal-related factors, will also inhibit mesoderm formation.

5 Smad2 becomes activated after MBT, in the vegetal and equatorial region. This can be visualized by using antibodies specific for the phosphorylated forms (Fig. 7.18c).

6 Reporter constructs, containing a TGF-β-signaling sensitive promoter linked to a *luciferase* gene, are activated when injected into blastomeres in vegetal or equatorial positions, but not blastomeres in animal positions. This shows that the signal *in vivo* affects the equatorial region but does not reach to the animal pole.

Transcription of some of the nodal-related factors is activated directly by vegT, and of others indirectly via the initial signaling process. The nodal-related factors acting by themselves induce pan-mesodermal genes such as *brachyury*, and ventral genes such as the *vents*. In the dorsal quadrant where β-catenin is also activated, there is a synergistic response to both pathways resulting in the activation of genes for goosecoid, not, lim1, and other transcription factors whose activity defines the organizer region.

Dorsalization and neural induction

Mesoderm induction leads to the creation of a belt of mesoderm around the equator of the blastula with a large ventrolateral region of extreme ventral character, and a small organizer region at the dorsal side. The organizer region is often called **Spemann's organizer** after the great German embryologist who originally discovered its properties. It is a key signaling center for the subsequent stages of development, and it acts by secretion of factors that *inhibit* the action of bone morphogenetic proteins (BMPs, see Appendix).

During gastrulation the ventrolateral mesoderm becomes partitioned into zones forming, respectively, somites, kidney, lateral plate, and blood islands, as a function of distance from the organizer (Fig. 7.18a). Isolates from the ventrolateral mesoderm retain an extreme ventral character. But if they are experimentally combined with organizer tissue, they will be dorsalized and form large muscle blocks and pronephric tubules (Fig. 7.18b).

Also during gastrulation the neural plate becomes induced from the ectoderm under the influence of the organizer. When gastrula ectoderm is brought into contact with the organizer it will form **neuroepithelium**, characterized by expression of various transcription factors including sox2**,** and the cell adhesion molecule N-CAM. There are various ways of achieving this experimentally. The most straightforward is a combination of competent ectoderm with organizer tissue (Fig. 7.19b). Another is the Einsteckung procedure, where bits of organizer tissue are inserted into the blastocoel of an early gastrula and become pressed against the ventral ectoderm by the gastrulation movements (Fig. 7.19c). Finally, in the organizer graft (see below) a secondary neural plate is formed from the ectoderm overlying the secondary mesodermal axis.

If blastulae are placed in a salt solution isotonic to the embryo rather than the usual very dilute solution, they will **exogastrulate**: the endomesoderm becomes extruded from the animal hemisphere instead of invaginating into it (Fig. 7.6). The elongation

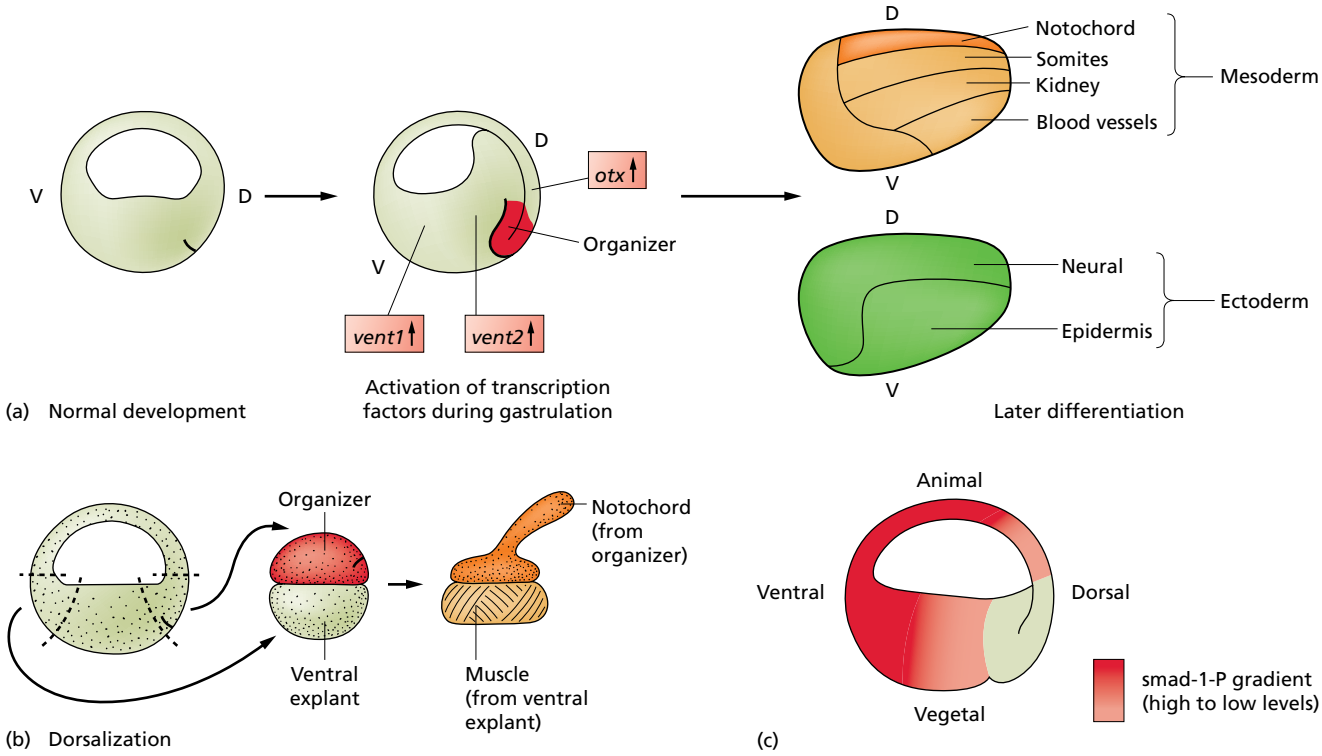


Fig. 7.18 Dorsalization of mesoderm: (a) in normal development; (b) muscle induced in ventral explant combined with organizer; (c) location of phosphorylated smad1 in early gastrula.

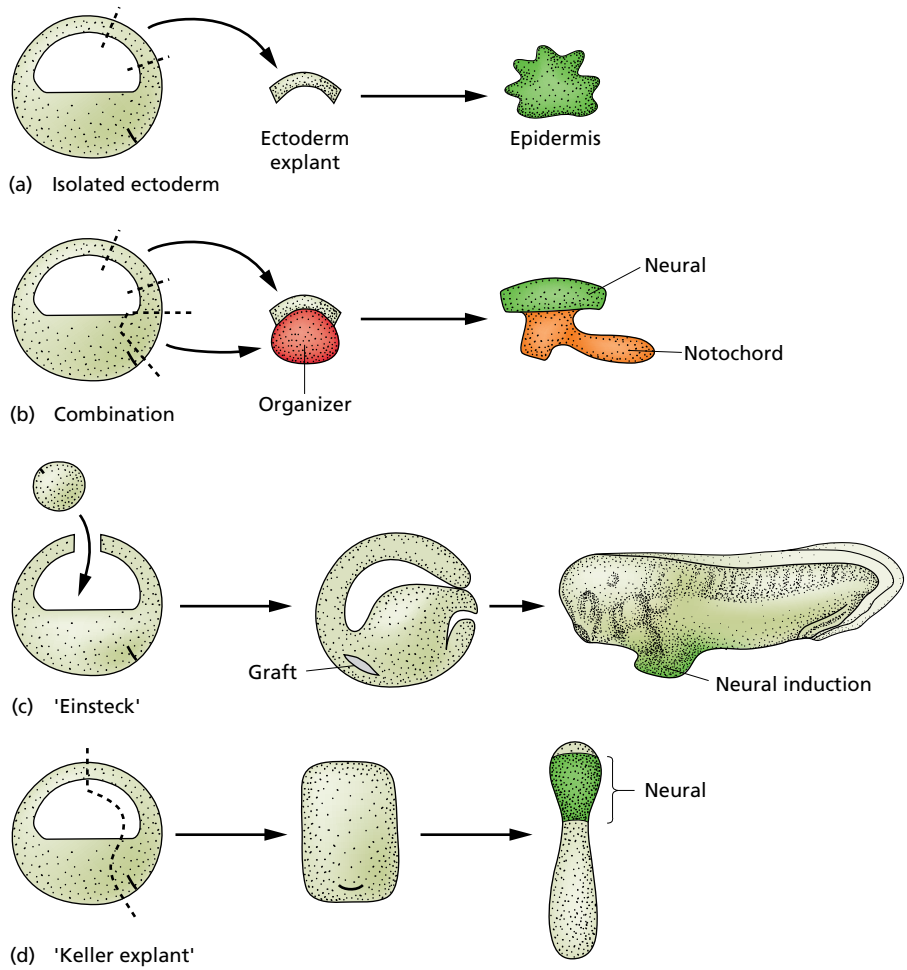


Fig. 7.19 Neural induction: (a) isolated ectoderm from prospective brain turns into epidermis; (b) ectoderm is neuralized in combination with the organizer; (c) ventral ectoderm can be neuralized in the Einsteck procedure; (d) Keller explant.

Classic Experiments

Discovery of inducing factors

The inducing factors that control embryonic development were discovered in the 1980s using *Xenopus*. The key step was the ability to apply purified protein solutions to isolated animal caps and to observe the consequences down the dissecting microscope by changes in shape of the explant. This assay led to discovery of the inducing properties of the FGFs and the activin-like factors (now known to be nodals).

The other vital technique was the injection of messenger RNA, prepared *in vitro*, into fertilized eggs, or into UV-ventralized eggs. This led to the discovery of the role of the Wnt pathway in the formation of the primary axis. Further, it allowed the introduction of expression cloning protocols whereby a whole library of expression clones could be divided into subsets, transcribed, and the mixed mRNA assayed by injection. This led to the discovery of noggin and eventually to the mode of action of the organizer.

First inducing factors

Kimelman, D. & Kirschner, M. (1987) Synergistic induction of mesoderm by FGF and TGF-beta and the identification of a messenger-RNA coding for FGF in the early *Xenopus* embryo. *Cell* **51**, 869–877.

Slack, J.M.W., Darlington, B.G., Heath, J.K. & Godsave, S.F. (1987) Mesoderm induction in early *Xenopus* embryos by heparin-binding growth-factors. *Nature* **326**, 197–200.

Smith, J.C., Price, B.M.J., VanNimmen, K. & Huylebroeck, D. (1990) Identification of a potent *Xenopus* mesoderm-inducing factor as a homolog of activin-A. *Nature* **345**, 729–731.

Axis duplication from Wnt

Smith, W.C. & Harland, R.M. (1991) Injected XWnt-8 RNA acts early in *Xenopus* embryos to promote formation of a vegetal dorsalizing center. *Cell* **67**, 753–765.

Sokol, S., Christian, J.L., Moon, R.T. & Melton, D.A. (1991) Injected Wnt RNA induces a complete body axis in *Xenopus* embryos. *Cell* **67**, 741–752.

Discovery of Noggin

Smith, W.C. & Harland, R.M. (1992) Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in *Xenopus* embryos. *Cell* **70**, 829–840.

of the embryo proceeds normally but the mesoderm is joined to the ectoderm end-to-end rather than being apposed as layers. Another situation in which mesoderm and ectoderm are adjacent, but not apposed as layers, is the **Keller explant**. This is an explant of dorsal tissue extending from the dorsal lip to the animal pole and displays the convergent extension movement without the other features of gastrulation (Fig. 7.19d). In both these situations where the axial mesoderm does not underlie the ectoderm there is still some neural induction, resulting in a correct anteroposterior patterning of structures. This shows that the signals can propagate in the plane of the tissue (tangential induction) as well as from one layer to the other (**appositional induction**).

In normal development, neuralization and mesodermal dorsalization are both caused by inducing factors secreted from the organizer which function as inhibitors of the BMPs. They are chordin, noggin, and follistatin. BMP4 is produced by the embryo in all regions except those dorsal regions with high nuclear β-catenin. The intracellular signal transduction pathway for BMPs is similar to that for the nodal-related factors, but uses different receptors and smads (smad1 and 5; see Appendix). Evidence that inhibition of BMP signaling is the cause of neural induction and mesoderm dorsalization is as follows:

1 The prospective epidermis makes BMPs and smad1 and 5 becomes phosphorylated over the whole ventrolateral region of the embryo (Fig. 7.18c).

2 The organizer region secretes the BMP inhibitors.

3 Treatment of isolated animal caps with BMP inhibitors causes neuralization.

4 Treatment of isolated ventral mesoderm explants with BMP inhibitors will dorsalize them.

5 Injection of mRNA for dominant negative BMP receptors on the ventral side induces secondary axis formation (i.e. dorsal-type mesoderm on the ventral side).

6 Morpholinos to chordin will suppress dorsal pattern, both in normal embryos and in lithium-dorsalized embryos.

Chordin, noggin, and follistatin act by direct binding to and inhibition of BMP protein and thereby bring about a gradient of BMP activity from ventral to dorsal. The *vent1* gene is activated at high BMP and the *vent2* gene at lower BMP concentration (see Figs 7.4, 7.18a). The proteins coded by the *vent* genes are themselves transcriptional repressors as may be shown by the fact that VP16 fusions have a dorsalizing effect (i.e. opposite to the native factors). The region in which both *vent* genes are on later becomes the lateral plate. The region in which *vent2* but not *vent1* is on later becomes the somites, and soon shows activation of genes for **myogenic** factors such as *Myf5*. In the mid-ventral region that forms the blood islands, BMP activates SCL (stem cell leukemia factor), a bHLH-type transcription factor which later activates hematopoietic differentiation.

Experimentally, neuralization can be provoked by disaggregation of gastrula ectoderm. This is because when cells are disaggregated the ERK signaling pathway becomes activated as a general "wounding" response. This leads to inhibition of the action of smads 1 and 5 by phosphorylation at a second site (the linker region – see Appendix).

In addition to the inhibition of BMP, an element of Wnt inhibition is also involved in the normal dorsalization process. After MBT, the *wnt8* gene becomes activated in the nonorganizer part of the mesoderm and the Wnt pathway now has a *ventralizing* effect. Although the Wnt pathway has a dorsalizing function in the egg, after MBT the transcription factor Tcf3 becomes replaced by Lef1. Both of these factors cooperate with β-catenin, but they show a different spectrum of specificity in relation to

their target genes: Tcf3 is dorsalizing while Lef1 is ventralizing. This can be demonstrated because dominant negative constructs for these two transcription factors show the opposite effects when overexpressed in embryos: dominant negative Tcf3 is ventralizing and dominant negative Lef1 is dorsalizing. This change of competence explains the hitherto puzzling fact that treatment with lithium after MBT is ventralizing rather than dorsalizing. The dorsalizing signals from the organizer include at least one Wnt inhibitor called Frzb (pronounced "frizzbee"), which resembles the extracellular part of the Wnt receptors, and will inhibit the action of Wnt-8.

Anteroposterior patterning

During gastrulation not only is the dorsal to ventral pattern of territories specified, but also the anterior to posterior pattern. Neural-inducing activity is shown both by the organizer and by the axial mesoderm into which the organizer develops. In both cases the inductions show regional specificity. Anterior organizer or anterior axial mesoderm induces brain structures, while posterior organizer or posterior axial mesoderm induces both brain and spinal cord. Since a complete neural axis can be induced by a posterior inducer it is thought that a posterior signal controls anteroposterior patterning during the formation of the central nervous system (CNS).

The initial anteroposterior pattern exists in the form of two domains within the organizer prior to gastrulation, which are derived from the different ratio of β-catenin and nodal-related signaling arising from cortical rotation and mesoderm induction respectively. The anterior part will later become the anterior endoderm and the prechordal mesoderm. During gastrulation this moves towards the animal pole by crawling up the blastocoelic surface of the ectoderm. It is characterized by expression of transcription factor genes including *goosecoid* and *hex*. It will induce expression of anterior-type genes from ectoderm, such as *otx2* (fore/midbrain) and *XAG1* (cement gland); and its anterior inductive activity is due to secreted factors including Cerberus, which is an inhibitor of Wnts, BMPs, and Nodal-related factors, and Dickkopf, which is a Wnt inhibitor. The posterior part will later become the notochord and somites and, during gastrulation, it elongates considerably by convergent extension. It is characterized by expression of a different group of transcription factor genes including the homeobox gene *not* and the T-box gene *brachyury*. It will induce both anterior- and posterior-type genes from the ectoderm (e.g. both *otx2* and **Hox genes**), and its posteriorizing activity is at least partly due to secretion of fibroblast growth factors (FGFs). These act through the ras–raf–ERK signal transduction pathway (see Appendix) and activate a group of homeobox transcription factors coded by *cdx* genes and these in turn activate the posterior **Hox genes** of paralog groups 6–13 (Fig. 7.20). This subset of Hox genes specify the trunk–tail part of the pattern and they are normally turned on sequentially during gastrulation.

Evidence that FGF signaling is required to induce the trunk–tail region is as follows:

1 Several FGFs are expressed around the blastopore (i.e. the posterior) during gastrulation.

2 The ras–raf–ERK signal transduction pathway is activated in the prospective trunk in the late gastrula, as shown by immunostaining for the activated form of ERK.

3 If animal caps are treated with the BMP inhibitor noggin, then only anterior-type neural genes are induced, but noggin+FGF will also induce Hox gene expression.

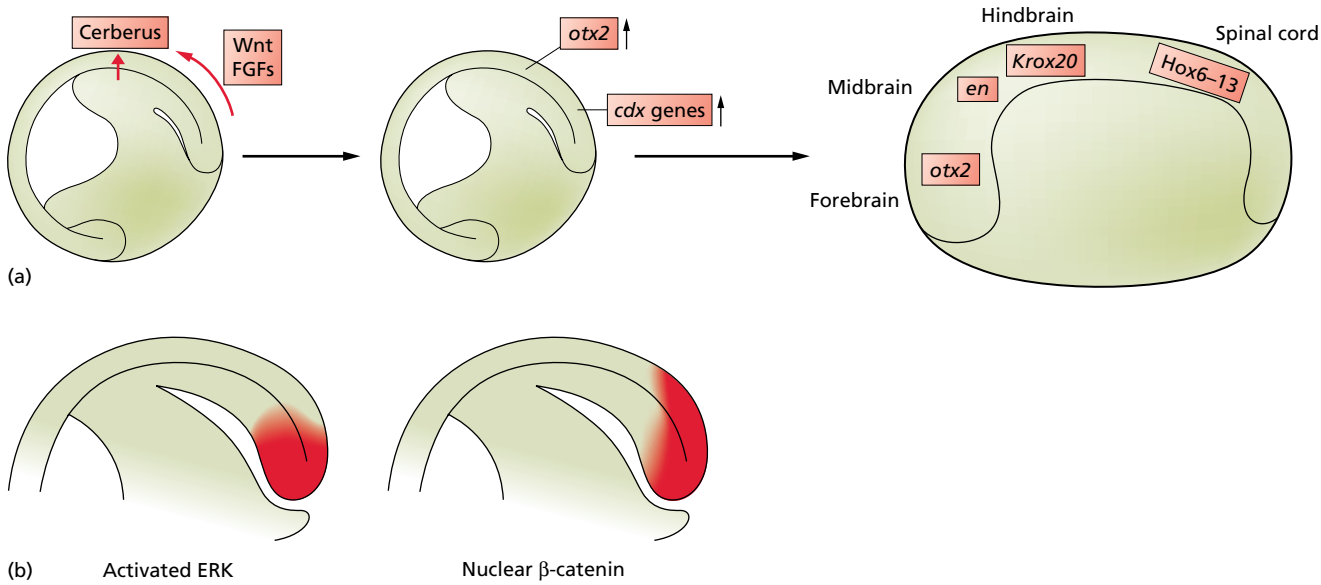


Fig. 7.20 Anteroposterior patterning of the CNS. (a) Action of Cerberus from the anterior part of the organizer, and of FGFs and Wnts from the posterior part. (b) Location of phosphorylated ERK (FGF target) and nuclear β-catenin (Wnt target) in late gastrula.

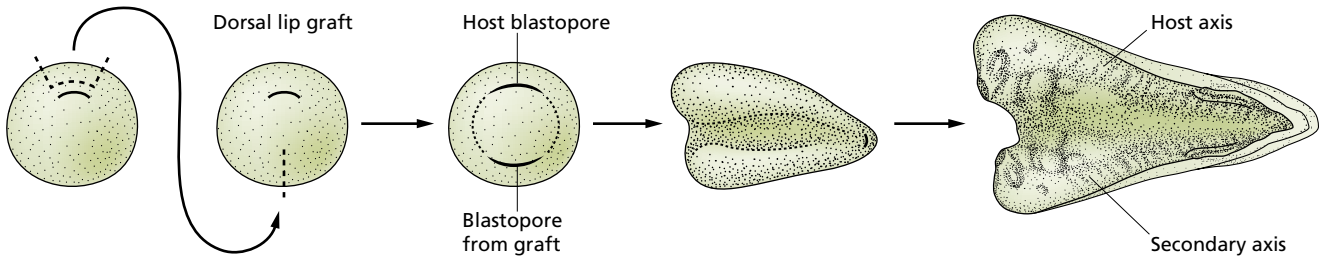


Fig. 7.21 The organizer graft. The graft forms the notochord and head mesoderm of the secondary axis and induces the other parts from the host.

4 Overexpression in embryos of a dominant negative FGF receptor that inhibits endogenous FGF signaling will prevent formation of the trunk and tail.

5 The same phenotype arises from overexpression of a domain-swapped, repressive, version of a *cdx* factor, cdx-EnR, designed to inhibit expression of *cdx* target genes.

Additional components that have been postulated as part of the posteriorizing signal include the Wnt factors and retinoic acid. Evidence for a posteriorizing role for Wnt factors is also good, especially in relation to the hindbrain. A posterior to anterior gradient of nuclear β-catenin can be observed in the early neural plate, and Wnt factors will induce hindbrain markers in animal caps that have been neuralized.

The organizer graft

All three of the processes, dorsalization, neural induction, and anteroposterior patterning, are shown in the organizer graft first performed by Spemann and Mangold in 1924. This is the most famous experiment in embryology, although its true significance has only recently become clear now that the various component processes have become understood. In the graft, a piece of tissue from above the dorsal blastopore lip is implanted into the ventral marginal zone (Fig. 7.21). It leads to the formation of a double dorsal embryo in which the notochord of the secondary embryo is derived from the graft and the remainder of the axis from the host. The ectoderm above the graft becomes induced to form a second neural tube. As gastrulation proceeds, both host and graft axes form progressively more posterior parts. In favorable cases the final result is a symmetrical pair of embryos joined belly to belly.

The movements of the grafted organizer are autonomous and preprogrammed. Therefore it invaginates and undergoes active elongation by convergent extension. The graft emits dorsalizing signals (BMP and Wnt inhibitors). These diffuse to the neighboring mesoderm, and the reduction in BMP signaling suppresses activation of *vent1* and so causes activation of myogenic genes to form a file of somites on either side of the graft. The same substances diffuse to the neighboring ectoderm and activate pan-neural genes. During gastrulation the graft emits FGFs and Wnts which induce *cdx* and Hox genes needed for development of the trunk–tail region. The secondary axes arising from organizer grafts often lack a head, but if the graft includes the deep anterior region of the organizer then this will emit Cerberus and Dickkopf and induce a head in the overlying ectoderm. The net result is the formation of the secondary embryo.

Key Points to Remember

- The descriptive embryology of *Xenopus* is typical of animals generally although details are specific. Cleavages are rapid and synchronous leading to blastula formation. Gastrulation movements lead to the formation of the three germ layers with regional pattern in dorsoventral and anterioposterior axes.
- Accurate fate maps have been constructed by injection of fluorescent dextrans into individual cells.
- Overexpression of gene products is usually carried out by injection of mRNA into fertilized eggs. Inhibition of specific gene products is usually carried out by injection of antisense morpholinos or of mRNA for dominant negative constructs.
- The pattern of the embryo is specified in relation to two determinants in the egg. The vegetal determinant includes the mRNA for the T-box transcription factor vegT which becomes localized to the vegetal cortex during oogenesis. The dorsal determinant comprises dishevelled protein and other Wnt pathway components which become moved from the vegetal to the dorsal side in the sperm-induced cortical rotation.
- VegT protein activates expression of genes encoding endoderm-specific transcription factors (e.g. Sox17) and also the genes encoding nodal factors. The nodal factors are secreted and induce the expression of mesoderm-specific transcription factors (e.g. brachyury) in an equatorial belt of neighboring cells.
- On the dorsal side the Wnt pathway activates expression of dorsal-specific transcription factors (e.g. siamois). The region containing both mesodermal and dorsal transcription factors becomes the organizer. Here genes for BMP inhibitors (noggin, chordin) are activated. These are secreted and create a gradient of BMP activity from ventral to dorsal. Inhibition of BMP activity induces transcription factors such as Sox1 in the animal (ectodermal) region, leading to formation of the neural plate. It also induces transcription factors such as myf5 in the equatorial (mesodermal) region, leading to the formation of the myotomes.
- Anteroposterior patterning depends on a posteriorizing signal emitted from the blastopore region during gastrulation. This consists of FGFs, Wnts, and retinoic acid, and causes activation of Hox genes in a nested manner such that each gene is activated at a particular anteroposterior level and remains on posterior to this.

Further reading

Website

http://www.xenbase.org/

Normal development

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Oocyte maturation

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Gastrulation

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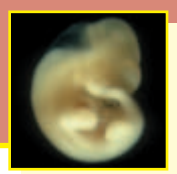
Chapter 8



The zebrafish

This chapter may seem rather short because the overall course of early development in the zebrafish (*Danio rerio*) is quite similar to *Xenopus* and the chapter focuses on the zebrafish-specific features rather than repeating the description of the common features. In addition to the study of early development described here, the zebrafish is becoming increasingly important in organogenesis research and will feature again in later chapters. In contrast to *Xenopus* the main approach to research with the zebrafish has been through mutagenesis, and this means that mutations are available in many of the important genes. A short list of some developmental genes is provided in Table 8.1. These are homologs of the corresponding genes in *Xenopus* and the mouse, but because most were originally discovered by mutation, and named before the identity of the gene was known, the names are specific to the zebrafish.

Normal development

Zebrafish eggs are about 0.7 mm in diameter and surrounded by a transparent chorion. The eggs and sperm are shed by the parents into the water and fertilization is external. As in *Xenopus,* the animal hemisphere is cytoplasm-rich and the vegetal hemisphere is yolk-rich. The course of development is shown in Fig. 8.1. Cleavage is **meroblastic** as it involves only the animal pole region. The first three cleavages are all vertical, generating an eight-cell stage composed of two rows of four blastomeres. The outer parts of the cell contacts are enriched in the proteins vasa and nanos (homologs of the corresponding *Drosophila* proteins, see Chapter 11) which may be determinants for germ cell formation. During this early phase the blastomeres remain connected to the main yolk mass by cytoplasmic bridges. This yolk

Table 8.1 Some developmental genes in the zebrafish.

Gene	Homolog	Developmental function	Gene product*
headless	*tcf3*	early dorsalization	HMG TF
cyclops	*nodal*	mes-endoderm induction	IF
squint	*nodal*	mes-endoderm induction	IF
one-eyed pinhead	*cripto*	needed for nodal action	EGF-CFC factor
dharma/nieuwkoid/bozozok		defines organizer	paired homeo TF
notail	*brachyury/T*	defines posterior mesoderm	T-box TF
spadetail	*tbx6/vegT*	defines trunk mesoderm	T-box TF
acerebellar	*fgf8*	posteriorising	IF
swirl	*bmp2b*	ventralizing	IF
snailhouse	*bmp7*	ventralizing	IF
vent	*vent1/PV1*	defines ventral	homeodomain TF
vox	*vent2/xom*	defines ventral	homeodomain TF
bonnie-and-clyde	*mixer*	defines endoderm	T-box TF
faust	*gata5*	heart/endoderm	Zn finger TF

*TF, transcription factor; IF, inducing factor.

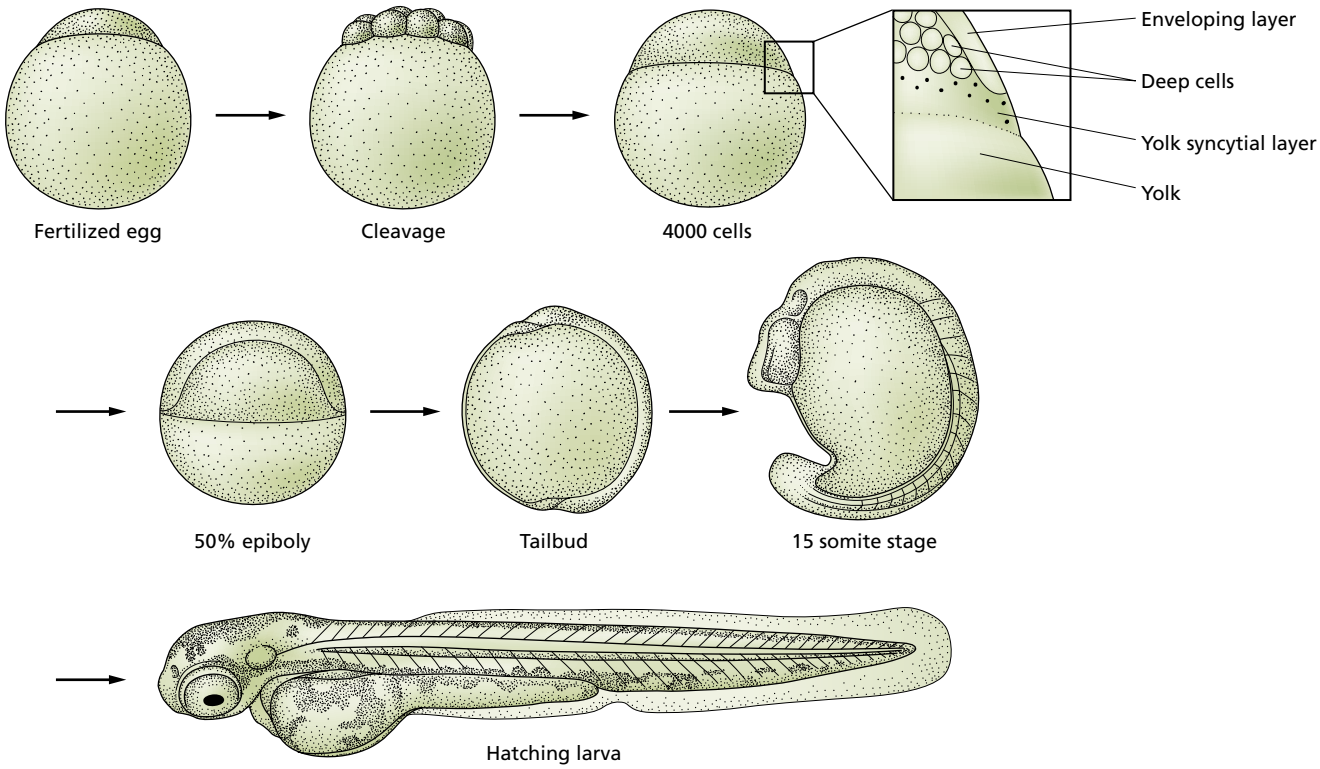


Fig. 8.1 Normal development of zebrafish.

mass becomes known as the **yolk** cell. Succeeding cleavages occur about every 15 minutes, until after about 10 divisions (approximately 3 hours) the **midblastula transition** (**MBT**) occurs. This is similar to *Xenopus* in that the synchrony of divisions breaks down, the average cell cycle duration increases, cells become more motile, and the transcription of the zygotic genome commences.

By this stage the embryo consists of four regions. The majority of cells form a mass in the animal pole region and are called **deep cells**, which are covered by a thin epithelial **enveloping layer**. The vegetal part of the embryo is occupied by the yolk cell, and the region near the blastoderm contains a number of nuclei and is called the **yolk syncytial layer**. These nuclei arise from the cells at the edge of the blastoderm after MBT, which fuse with the adjacent yolk cell to form the syncytium. The overall phase of morphogenetic movements is quite similar to that in *Xenopus*, although the mechanics may be somewhat different. Starting shortly after MBT, the blastoderm commences an active expansion called **epiboly** such that the margin moves down progressively to cover the yolk cell. Stages of zebrafish development are identified as percentage of epiboly, depending on how much of the yolk cell has been surrounded. This movement is driven by the yolk syncytial layer and depends on the activity of microtubules within the yolk cell. At 50% epiboly (approximately 5.5 hours) the margin of the blastoderm begins to involute. In the zebrafish the term **gastrulation** is normally reserved specifically for the involution movement of the mesoderm as distinct from the epibolic spreading process. Involution takes place all around the blastoderm margin, but, as in *Xenopus*, the dorsal involution is much more pronounced than the ventral. The involution means that the blastoderm is thicker around the margin than elsewhere and this thickening is called the **germ ring**. The involution movement is carried out only by the deep cells. The outer enveloping layer cells do not participate and go on to become the outer layer of the larval epidermis, or periderm. Simultaneous with the involution, the dorsal region starts to elongate in an anteroposterior sense by **convergent extension**, drawing in cells from more lateral regions. This process causes the dorsal marginal zone to thicken relative to the remainder of the circumference and it then becomes known as the **embryonic shield**. Epiboly reaches completion at about 9.5 hours when the yolk cell is completely covered by the blastoderm. The outer layer of the shield becomes the **neural plate**. This sinks into the interior as a solid mass of cells, and a lumen forms by cavitation to create a neural tube. The mesodermal layer forms a midline notochord and paraxial somites, segmenting from anterior to posterior. The basic body plan structures becomes visible by about 14 hours, and the axis straightens by 24 hours. Hatching occurs after about 48 hours, and feeding commences about 5 days after fertilization.

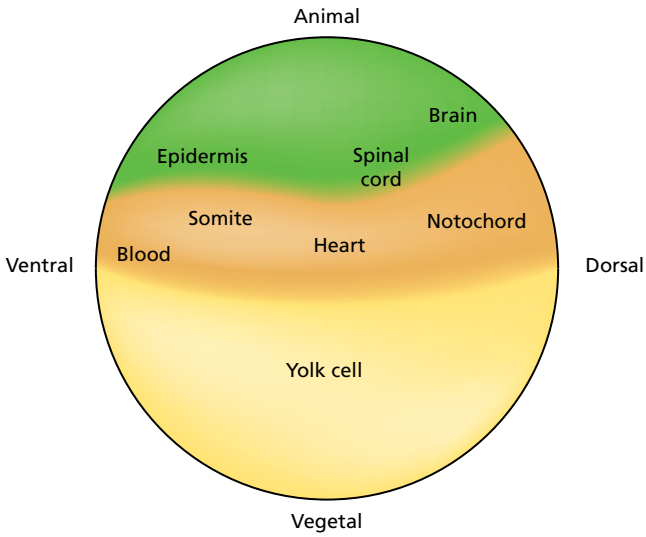


Fig. 8.2 Fate map of zebrafish at 50% epiboly.

Fate map

The fate map for the zebrafish has been constructed by injection of individual blastomeres with fluorescent dextrans. As there is considerable mixing of the deep cells, the fate maps from the cleavage stage are only statistical. Each individual blastomere contributes to a very wide region of the later embryo. By the beginning of gastrulation the range of random cell movement is much reduced and a fate map can be produced with similar resolution to that of *Xenopus* (Fig. 8.2). The fate map shown is for the beginning of gastrulation and concerns just the deep cells. Individual cells may populate more than one tissue type or even more than one germ layer if labeled in the early stages of epiboly. But by the start of involution all clones have become restricted to tissue types such as neuroepithelium or somite. This suggests that the timing of the inducing signals responsible for regional specification is similar to *Xenopus*.

Mutagenesis

Recessive screen

The zebrafish has been the subject of several mass mutagenesis screens to isolate mutations in developmental genes. The most common protocol is the identification of recessive mutations, often lethal ones, by breeding for three generations. Mutations are induced in a small number of founder males by treatment with the chemical **mutagen** ethyl nitrosourea (ENU). This induces point mutations at high frequency in dividing cells, up to about one mutation per 500 gametes for a particular gene. The males are allowed to recover and during this period the sperm already undergoing spermatogenesis are lost and new ones are produced from the mutated spermatogonia. The type of breeding schedule used to generate and identify homozygous mutants is described in Chapter 3 (see Fig. 3.6).

The initial screens were carried out by simple dissecting microscope examination of the F3 embryos with the intention of isolating all possible mutations in all developmentally significant genes. But it has become clear that more mutations can be identified if the screen is more focused, so more recent screens have tended to examine one particular organ system in detail. Some of these have used immunostaining or *in situ* hybridization to highlight the structures of interest, and in other cases the parent line is a transgenic line in which the structures of interest express GFP and can easily be visualized under the fluorescence dissecting microscope. When an interesting-looking phenotype is found among the F3 generation, the F2 parents are outcrossed to wild-type fish to reduce the burden of other, nondevelopmental, mutations and to set up a permanent line. The majority of mutations in developmentally important genes are lethal in the homozygous state, therefore the line has to be maintained by identifying heterozygotes and mating them together when mutant embryos are required.

The results of a screen are initially classified according to the phenotype and then **complementation analysis** is carried out for mutations with similar phenotypes to find whether they are different alleles of the same gene. This is done by mating the heterozygotes of the two lines. If the mutations are indeed in the same gene then there will be 25% offspring with the phenotype, whereas if they are in different genes the offspring will not show the phenotype at all. Then the mutation will be mapped by conventional genetic mapping. The map position will be the starting point for **positional cloning** of the gene. Positional cloning used to be a slow and arduous procedure, but nowadays with a near complete genome sequence and a large number of molecular polymorphisms available to use in mapping, it is relatively easy (see Chapter 3, Fig. 3.8). However, many zebrafish mutants have also been identified without positional cloning by the testing of likely candidates based on the similarity of the phenotype to those found in the other model organisms.

Classic Experiments

Because of the simplicity of the genetic methods employed, the original mutagenesis screen performed in the early 1990s represents a paradigm for a zygotic mutagenesis screen for developmental mutants.

Haffter, P. and 16 others (1996) The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development* (Suppl. 123), 1–36.

Evidence from the results of genome sequencing suggests that the zebrafish, and probably all bony fish, contain up to two similar genes for every one found in higher vertebrates. These related copies have a high level of sequence divergence suggesting a rather ancient duplication of the entire genome. It is estimated that this would have occurred in the ancestor of bony fish shortly after it had separated from the vertebrate main line of descent about 230 million years ago. The effect is that a gene in mouse that has more than one expression domain or developmental function may be represented by two genes in the fish, and these often share out the domains and functions between them such that their combined expression and function resembles that of the single mouse gene. This fact has advantages and disadvantages to the experimenter. On the one hand there are somewhat more genes to sift through and analyze. On the other hand, a gene whose null mutant is an early lethal in mouse may appear as two genes each of whose null phenotype is milder, allowing development to a more advanced stage or even long-term viability.

Other methods

Apart from the normal type of recessive screen, the zebrafish allows two other techniques for mutational screening, which have advantages in particular circumstances (Fig. 8.3). One is the generation of **haploid** embryos. Female F1 fish are taken and squeezed gently to obtain unfertilized eggs. These are fertilized *in vitro* with sperm heavily irradiated with UV light to render them inviable. Such sperm can activate development but the sperm nucleus does not participate and so the embryo is a haploid, arising from the maternal pronucleus alone. In haploid embryos the effects of recessive mutations will be immediately apparent and so an interesting mutation can be identified without the need for the F2 family. However, the haploid embryos are not viable in the long term. They die after a few days and so a mutation needs to be maintained by conventional breeding starting from the mother. Moreover, haploid embryos themselves are not normal even in the absence of mutations, and therefore screens are only feasible for characteristics that would not be masked by the typical haploid syndrome.

The haploid syndrome can be avoided if the eggs are converted into **gynogenetic diploids**. Again, the eggs are squeezed from the female fish and fertilized with inviable sperm. Then they are subjected to a pressure pulse to drive the second polar body back into the egg and cause it to fuse with the maternal pronucleus. This is now a diploid composed entirely of maternal genetic material. However, loci will only be homozygous if there has been no meiotic recombination. If recombination has occurred at the bivalent stage then one mutant copy can be lost into the first polar body and the gynogenetic diploid will potentially be heterozygous for all loci distal to the crossover point. Thus, this technique can be quite useful for genes located near the centromeres, which are unlikely to be lost by recombination, but it does not have a general validity.

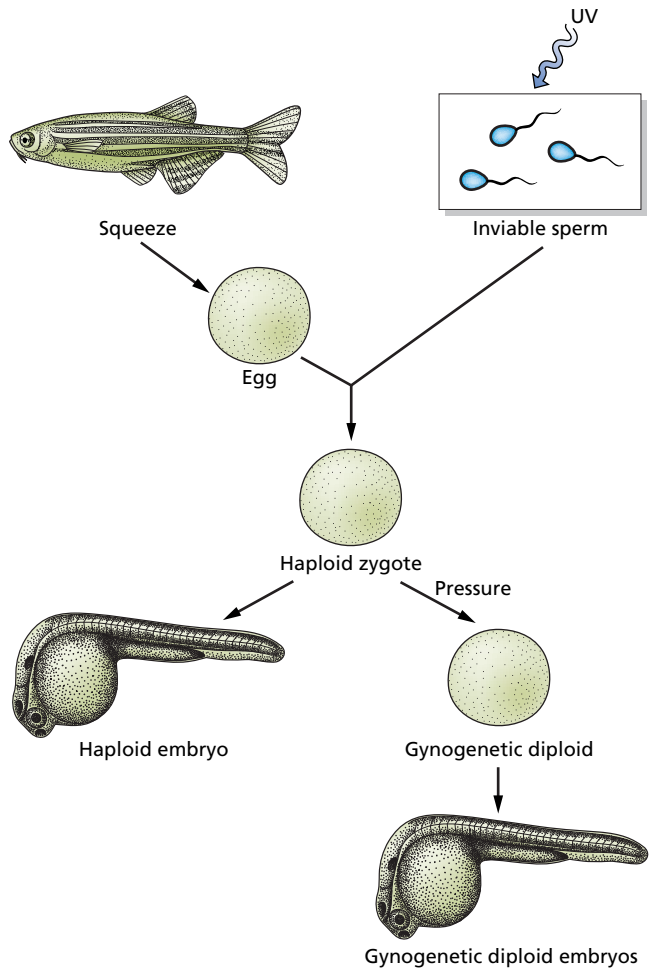


Fig. 8.3 Production of haploid embryos and gynogenetic diploids.

The cloning of genes is rendered easier if mutations are induced by a procedure that results in the insertion of some molecular probe. There are methods for **insertional mutagenesis** based on the introduction of defective retroviruses, but the frequency of mutation is much lower than with ENU, so the number of F2 families to screen is correspondingly higher.

Regional specification

Techniques

As discussed in Chapter 4, the association of a particular molecule with a particular function requires assessment of the expression pattern, the biological activity, and the effects of ablation. Once a zebrafish gene is cloned, the expression pattern is established by *in situ* hybridization or immunostaining. Biological activity is often established, as in *Xenopus*, by injection of specific mRNA into the fertilized egg or into specific blastomeres. Because the starting point for the study of a gene is often a null

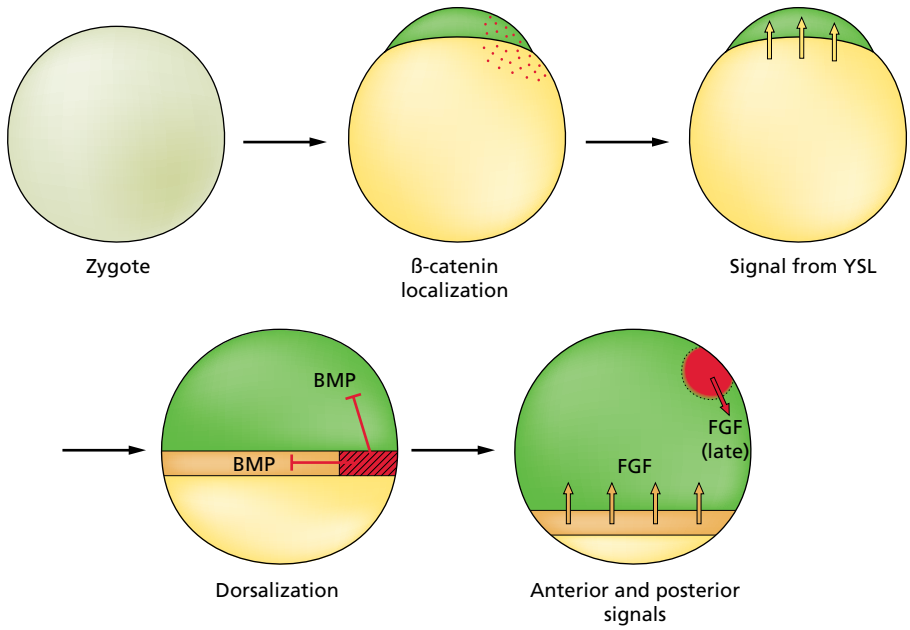


Fig. 8.4 Sequence of inductions in the zebrafish.

mutant, the effect of ablation may already be known. If it is not, then similar methods can be used as in *Xenopus*, involving the injection of morpholino antisense oligos or of mRNA coding for dominant negative variants of the gene under study. The microsurgical possibilities in zebrafish fall short of those in *Xenopus* because the embryo is considerably smaller. However, using micromanipulation equipment, it is possible to isolate "animal caps" from gastrulae and to transplant groups of cells from one region to another. Transgenesis can be achieved by injection of DNA into the fertilized egg. Normally it is injected into the yolk and cytoplasmic streaming carries it into the nuclei of the early blastomeres, which remain attached by large cytoplasmic bridges. Transgenesis often yields mosaic individuals in which only some cells carry the transgene, although germline integration can be achieved and it has now become routine to produce transgenic lines of fish.

Dorsoventral polarity

There is no obvious cortical rotation in the zebrafish, but treatment with the microtubule depolymerizing drug nocodazole does suppress axis formation, suggesting a role for microtubules in the establishment of the dorsal center. The Tcf3 transcription factor is encoded by the gene *headless*, whose loss of function phenotype is anterior reduction, suggestive of a role for the Wnt pathway. Formation of the dorsal axis can be suppressed by overexpression of gsk3, and a dominant negative gsk3 can induce a secondary axis. There is a migration of β-catenin to the nuclei on the dorsal side, visible by immunostaining. Overexpression of β-catenin will activate transcription of a gene of the paired-homeobox class, *dharma* (also called *nieuwkoid* or *bozozok*). *dharma* is necessary for formation of the organizer as the loss-of-function mutant lacks notochord, prechordal plate, and neural tube. This set of evidence suggests that events are comparable to *Xenopus*, with an activation of the intracellular Wnt pathway on the dorsal side .

Meso-endoderm induction

The sequence of inductive events that build up the body pattern also seems very similar to those in *Xenopus* with a few variations (Fig. 8.4). There is an initial mesoderm induction, involving the formation of a ring of mesoderm, with an organizer region (the embryonic shield) on the dorsal side. The organizer region then emits signals leading to neural induction of the ectoderm and to dorsalization of the lateral mesoderm.

One variation concerns the action of vegT. In zebrafish this is encoded by the gene *spadetail*, which is expressed zygotically in the mesoderm and whose mutant phenotype is a defect in the trunk rather than loss of all mesoderm and endoderm. *spadetail* is not expressed maternally. However the yolk cell, which is the zebrafish equivalent of the *Xenopus* vegetal hemisphere, does emit a meso-endodermal inducing signal. If the yolk cell is removed from the embryo before the 16-cell stage, then formation of the mesoderm is prevented. If the yolk cell, with its associated yolk syncytial layer, is recombined with an animal cap, then mesoderm is induced around the junction, as shown by expression of markers like *notail* (the zebrafish homolog of *brachyury*) in the responding tissue.

There are two zebrafish homologs of *nodal*: *cyclops* and *squint*. The double loss-of-function mutant has no germ ring and forms little mesoderm. A similar phenotype is shown by loss-of-

function mutants of the gene *one-eyed-pinhead*. This encodes the zebrafish homolog of cripto, an extracellular factor whose action is required for nodal signaling (see also Chapter 10). Furthermore, a similar loss of mesoderm follows overexpression of Cerberus-short, the fragment of the *Xenopus* Cerberus factor that antagonizes nodal. Conversely, the overexpression of nodal mRNA or of a mRNA for a constitutive nodal receptor, will induce mesoderm in zebrafish animal caps. These experiments make up good evidence that mesoderm induction is carried out by nodal signals.

Transcription factor genes characteristic of the early endoderm can be also activated by high concentration of nodal. These include *bonnie and clyde* (=*mixer*), *faust* (=*GATA5*), and *casanova* (*sox* related).

The organizer

The organizer region in zebrafish is called the embryonic shield. Grafting of the shield to a ventral position can induce a secondary axis containing somites and a neural tube. As in *Xenopus*, dorsalization of the mesoderm by signals from the organizer involves inhibitors of BMPs.

Two BMP genes, *swirl* (BMP2b) and *snailhouse* (BMP7), are expressed in the ventral part of the gastrula. Their loss-of-function mutants show dorsalization, showing that BMP signaling is needed for ventral development. The transcription factor dharma, expressed in the organizer region, normally suppresses expression of *swirl*. The loss of function mutant of *dharma* is ventralized, and this can be rescued by injection of mRNA for the EnR domain swap version of dharma, showing that the factor normally acts as a transcriptional repressor. The action of dharma also leads to activation of the *chordin* gene, presumably indirectly since dharma itself is a repressor. The zebrafish chordin homolog of *chordin* is called *dino* or *chordino*, and, as expected, the loss-of-function mutant of *chordino* causes ventralization.

The ventral state is defined by expression of two homeodomain transcription factors, Vent and Vox, which are the homologs of Vent 1 and 2 in *Xenopus*. These have a redundant action, but loss-of-function mutants of both genes together produce a dorsalized phenotype. Their normal expression is dependent on BMP signaling, and overexpression of Vent or Vox has a ventralizing effect.

Thus, as in *Xenopus*, it seems that the dorsalizing action of the organizer arises from inhibition of BMPs by direct transcriptional inhibition in the organizer itself and by secreted BMP inhibitors in the surrounding regions.

Anteroposterior patterning

The anteroposterior patterning mechanism shows some similarity and some difference to that in *Xenopus*. During gastrulation, the blastopore region emits a posteriorizing signal in a similar way. There is good evidence that FGFs are an important constituent of the signal. Overexpression of FGFs causes anterior truncation, or induction of posterior markers in anterior explants. Conversely overexpression of a dominant negative FGF receptor causes posterior truncation. In terms of loss-of-function mutation, if the *fgf8* (*acerebellar*) mutant is combined with injection of a morpholino oligo directed against *fgf24*, then formation of posterior structures is inhibited. This suggests that these two FGFs make up most of the normal posteriorizing signal.

As in *Xenopus*, there is also some evidence for a role for Wnt and retinoic acid signaling since both types of factor will posteriorize on overexpression. It has been argued that the ventral marginal zone is a "tail organizer," as it will form a tail if combined with an animal cap, much of the tail arising from the animal cap cells. This effect can be mimicked by injection of a combination of mRNAs encoding nodal + BMP + Wnt, and since the combination of nodal + BMP might be expected to generate ventral mesoderm, this is further evidence for the posteriorizing activity of Wnt.

There is also a group of anterior ectoderm cells, fated to form anterior telencephalon, pituitary, and nasal placodes, which has an anteriorizing influence when grafted to other parts of the neural plate. Removal of the embryonic shield ablates the notochord and prechordal plate, together with the ventral midline structures of the central nervous system (see Chapter 14), but it does not greatly affect the oveall anteroposterior pattern of forebrain–midbrain–hindbrain–spinal cord. This is now known to be a source of FGF at a later stage.

New Directions in Research

The zebrafish will probably make most contribution in the following two areas:

1 For research into late, organogenesis, stages of development, there will be further mutagenesis screens targeted to particular organs. Individual organs or cell types can be highlighted by transgenic markers or by antibody staining. Such screens should reveal previously unknown genes with key roles in the formation of specific organs.

2 For research into morphogenetic movements, the transparency of the embryo will enable high-resolution studies of cell movement in real time. Again, individual cell populations can be labeled by transgenic markers or microsurgical methods.

Key Points to Remember

- The zebrafish is a vertebrate well suited to genetic experimentation. Embryonic development is rapid, the generation time is short, and large numbers of fish can be kept in a facility. Transparency of the embryos enables visualization of cell behavior *in vivo*.
- Many developmental mutants have been isolated from mutagenesis screens. The genes within which the mutations lie have been identified either by positional cloning or by testing candidates.
- In general the fate map and sequence of inductive steps in early development is similar to *Xenopus*. The Wnt signaling pathway is required for dorsal patterning, nodal factors are required for mesoderm induction, and BMP inhibitors for the effects of the organizer. Anteroposterior patterning involves FGFs, Wnts, and retinoic acid.
- However there are also some differences. In particular *spadetail*, the homolog of *vegT*, is not expressed maternally in the yolk cell but is expressed at blastula stage in the mesoderm. So this is unlikely to be playing a similar role as a vegetal determinant.

Further reading

Website

http://zfin.org

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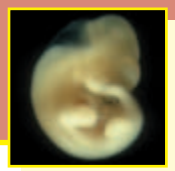
Chapter 9



The chick

The visible course of development of the chick is superficially very different from the lower vertebrates and is much closer to the mammalian type. But even though the visible morphogenetic movements in the early embryo can appear quite different, at a molecular level it is clear that essentially the same basic processes are taking place in all vertebrates. Because the chick has been extensively used for work on later stages of development, a general outline of vertebrate organogenesis is given in this chapter. In section 3 this will be revisited in greater detail and with incorporation of relevant evidence from other vertebrate species.

Fertilized eggs are usually obtained from commercial hatcheries, and, at the stage of laying, the embryo is a flat **blastoderm** of about 60,000 cells. Development is arrested at low temperatures and therefore eggs can be stored for some days at 10°C during which time embryonic development remains suspended. It recommences when the eggs are incubated at 37.5°C. Unlike *Xenopus* and the zebrafish, the chick undergoes extensive growth during embryonic life because it has at its disposal the food reserves of the egg.

For the experimentalist the chick has the great advantage over the mouse that the embryo is accessible at all stages following laying of the egg. Early blastoderms can be cultured *in vitro* for long enough to form a recognizable primary body plan. Alternatively, a hole can be cut in the egg shell and the embryo can be manipulated *in ovo*, then the hole is resealed with adhesive tape and the whole egg incubated until the embryo has reached a later stage of development. Furthermore it is possible to explant small pieces of tissue onto the **chorioallantoic membrane (CAM)** of advanced embryos, where they become vascularized and will grow and differentiate in effective isolation. Culture of some organ rudiments is also possible *in vitro*. For the labeling of grafts, extensive use has been made of interspecies combination between chick and quail. Quail embryos are anatomically very similar to chick although they are slightly smaller and develop a little faster. Originally they were used because all quail cells possess a condensation of **heterochromatin** associated with their nucleolus, and this is easily visualized as a dark blob by staining for DNA using the Feulgen histochemical reaction. Nowadays the quail cells are normally visualized by staining with a species-specific antibody.

The chick is not well suited to genetic work. The life cycle is long, the existing mutants limited in number, and there is no routine protocol for transgenesis or targeted mutagenesis. However there are now several useful methods for overexpression of genes in chick embryos. Much use has been made of **retroviruses** carrying the gene in question. These are often called **RCAS viruses** for **r**eplication-**c**ompetent, **a**vian-**s**pecific. The virus can be injected locally in the region to be modified, and the effect will spread as new virus particles are produced by the infected cells. If the range of infection is to be limited, this can be achieved by making an orthotopic graft of tissue from a sensitive strain of chick into an embryo of a resistant strain. Then just the tissue of the graft will become infected and overexpress the gene. More recently, **electroporation** has been used. This involves injecting DNA into the region of interest and then subjecting the embryo to a series of low voltage electric pulses which can drive DNA into the cells without doing too much damage. The DNA is negatively charged and so moves towards the anode, hence the tissue on the cathode side of the injection will be untransformed and can serve as an internal control for the effect of the gene. Localized treatment of embryos with **inducing factors** was originally achieved by implantation of pellets of **tissue culture** cells expressing and secreting the factor in question. With the greater availability of pure factors, they are now usually absorbed to affinity chromatography beads which are implanted into the embryo in the desired position. Such beads can bind a large amount of the factor and then release it slowly for 1–2 days.

As for *Xenopus*, gene ablation studies need to be performed by overexpression of dominant negative reagents. Morpholino oligos are much less useful than for *Xenopus* or zebrafish, because they do not readily penetrate cell membranes and in the chick there are no large blastomeres to inject.

Normal development

The hen's egg is a familiar object, with its shell, albumen layer ("white"), and yolk. But the true egg consists of just the yolk, and, in a fertilized egg, an inconspicuous **blastoderm** of cells, which is surrounded by a vitelline membrane. The yolk corresponds to the mature **oocyte**. It was formerly supposed that avian, like mammalian, oogenesis occurred only during fetal life and that the complement of oocyte present in the newborn represented a lifetime's supply. A question has arisen about whether this is really true for mammals, but in birds the issue has not been recently reinvestigated. Up to about 1–2 weeks before ovulation, the hen's oocyte remains quite small, but it then puts on a tremendous growth spurt and over a few days acquires a weight of about 55 g. On ovulation it is released from the ovary and enters the **oviduct** where, if the hen has recently mated, it will be fertilized. Passage down the oviduct takes about 24 hours, in the course of which the egg is invested successively with the albumen layer, the shell membranes, and the shell itself.

Cleavage is highly **meroblastic** and involves just the patch of cytoplasm 2–3 mm in diameter which is present in the zygote (and oocyte) at the edge of the yolk mass (Fig. 9.1a). The early cleavages take place in the oviduct producing a circular blastoderm initially one cell thick, and later several cells thick. The cleavage pattern is very variable from one embryo to the next and the blastomeres at the ventral and lateral faces of the sheet

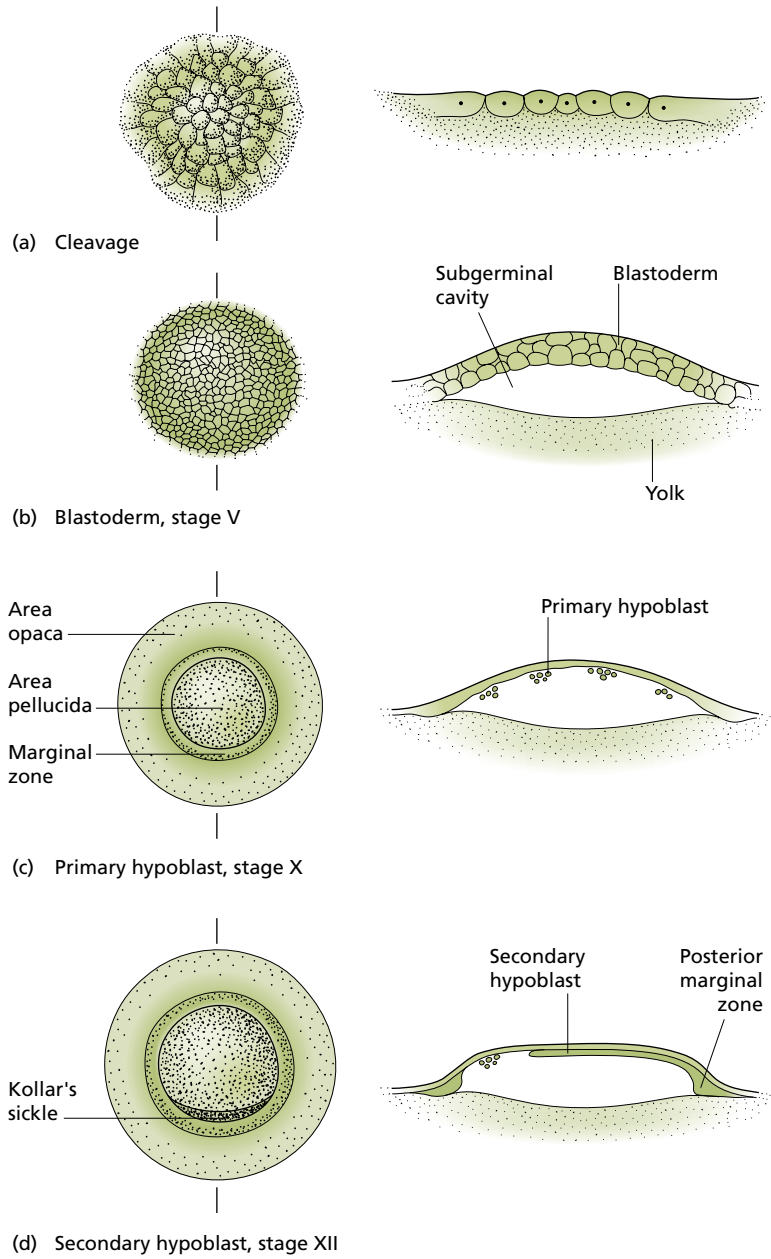


Fig. 9.1 Development of the chick blastoderm up until the time of egg laying. Vertical lines indicate planes of section of right side figures.

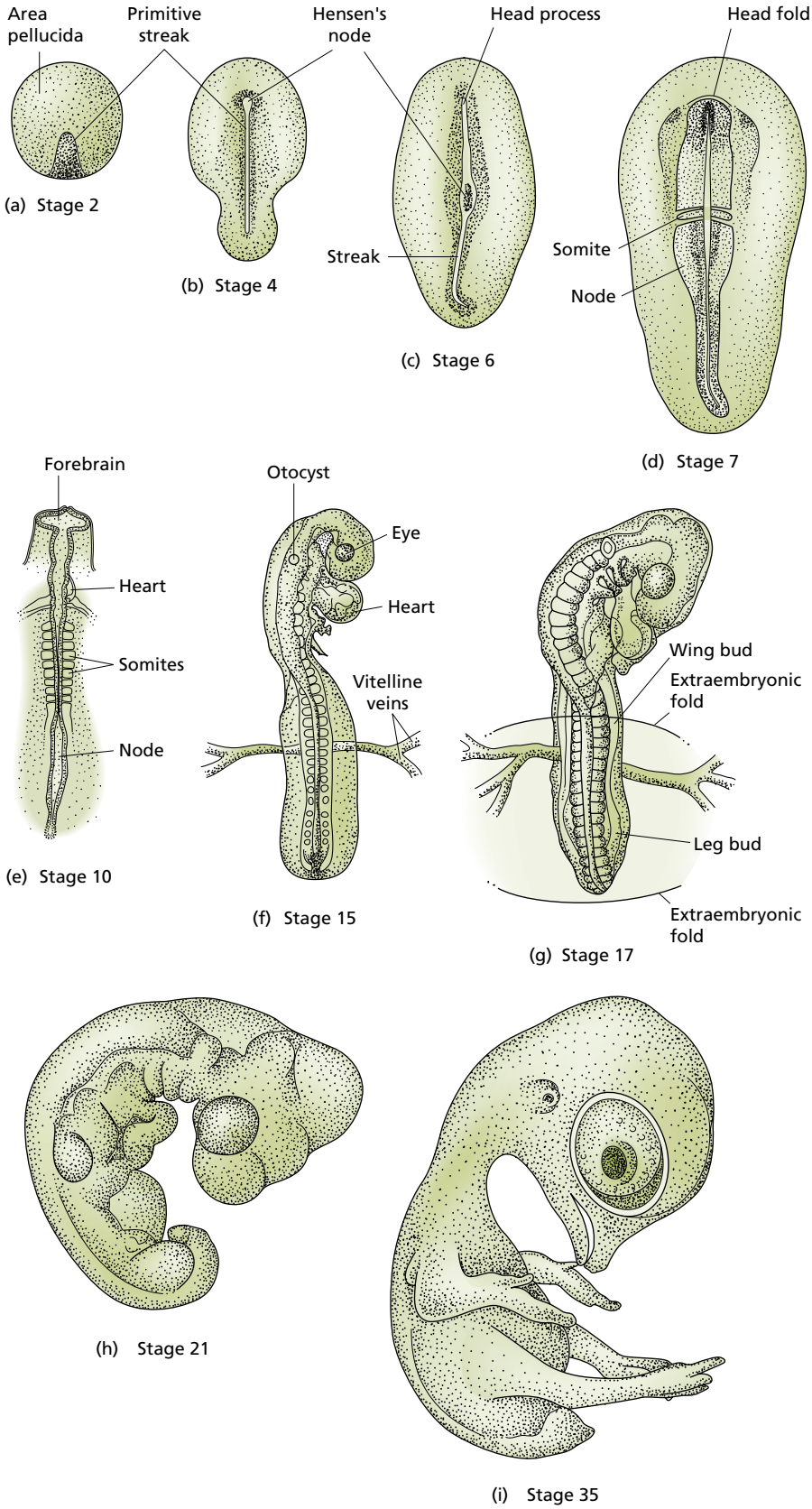


Fig. 9.2 Normal development of the chick. Stage 7 is reached after about 1 day of egg incubation, stage 12 after 2 days, stage 17 after 3 days, stage 24 after 4 days, and stage 35 after 9 days. Time from fertilization is about 1 day more than egg incubation time.

remain connected to the yolk for some time by large cytoplasmic bridges. The egg spends about 20 hours in the lower part of the oviduct, called the uterus, undergoing slow rotations driven by uterine peristalsis while the calcareous shell forms around it. When the blastoderm consists of a few hundred cells, a space called the subgerminal cavity opens beneath it (Fig. 9.1b). Cells are shed from the lower surface of the blastoderm into this cavity and probably die, so that by the end of the uterine period the central region of the blastoderm has thinned to an organized epithelium one or a few cells thick. Because of its translucent appearance this is known as the **area pellucida**. The outer, more opaque, part of the blastoderm is called the **area opaca** and the junctional region the **marginal zone**. Note that the region called the "marginal zone" of the chick embryo is not a homologous structure to the marginal zone of an amphibian embryo.

A lower layer of cells, the **hypoblast**, then develops, partly by ingression of small groups of cells all over the area pellucida (the primary hypoblast) and partly by spreading of cells from the deep part of the posterior marginal zone (the secondary hypoblast). A thickening of the epiblast at the posterior margin is known as **Kollar's sickle** (Fig. 9.1c,d). The hypoblast contributes only to extraembryonic structures and may perhaps be homologous to the visceral endoderm of the mouse egg cylinder (see Chapter 10). The upper layer of cells now becomes known as the **epiblast**. At the time the egg is laid it will usually just have commenced secondary hypoblast formation, and the total blastoderm consists of approximately 60,000 cells. The early developmental stages are described by the stage series of Eyal-Giladi and Kochav which uses Roman numerals. In this series, I represents the fertilized egg, X represents the single-layered blastoderm stage, and XIII represents the complete two-layered blastoderm stage. The stage of egg laying, after which the embryos become accessible for experimentation, is about stage X–XI. Subsequent development is described by the stage series of Hamburger and Hamilton, which uses Arabic numbers and is indicated in what follows.

The stages of body plan formation are depicted in Fig. 9.2 showing top views, and Fig. 9.3 showing transverse sections. A condensation of cells called the **primitive streak** arises at the posterior edge of the area pellucida (stage 2) and elongates until it reaches the center (Figs 9.2a, 9.3a). The streak expresses the T-box transcription factor gene *brachyury*, already encountered in *Xenopus* and zebrafish. Cells from the epiblast migrate into the streak and pass through it to become the **mesoderm** and the **definitive endoderm** part of the lower layer. This process is regarded as **gastrulation** in the chick, although the future gut lumen, or **archenteron**, is not a new cavity but is the pre-existing space below the endoderm. The area pellucida gradually changes from a disc to a pear shape and a further condensation called **Hensen's node** appears at the anterior end of the primitive streak (stage 4; Fig. 9.2b). This expresses various transcription factor genes characteristic of the organizer region in the lower vertebrates, including *goosecoid*, *not*, and *FoxA2*(*HNF3β*) (chick genes are sometimes given a C prefix, as in *C-goosecoid* or

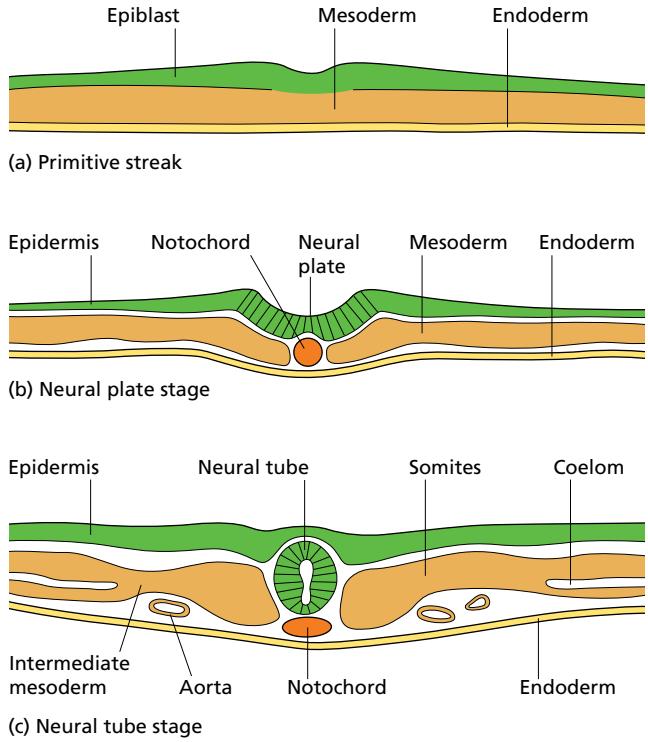


Fig. 9.3 Normal development of the chick. Transverse sections during formation of main axial structures.

C-not). The node contains the presumptive notochord cells, some of which migrate anteriorly to form the head process, or part of the notochord lying within the head (stage 6; Fig. 9.2c). The remainder of the node moves posteriorly and as it does so the principal structures of the body plan appear in its wake: the **notochord** in the midline, the **somites** on either side of it, and the **neural plate** in the epiblast (Fig. 9.3b). The **primordial germ cells** appear at the extreme anterior edge of the area pellucida, outside the embryo proper.

By about 1 day of incubation the anterior end of the embryo is marked by an uplifting of the blastoderm called the head fold, and one somite and the anterior neural folds have appeared in the track of the regressing node (stage 7; Fig. 9.2d). From this stage the embryo proper becomes progressively separated from the surrounding extraembryonic tissue. This is achieved by the appearance of folds involving all three germ layers that appear around the embryo and undercut it such that initially the head, and later the tail and trunk, project above the surface of the extraembryonic tissue (Fig. 9.4).

Early on the second day, blood islands appear in the outer extraembryonic part of the blastoderm, and the heart primordium forms by fusion of the rudiments on the right and left side of the anterior mesoderm (Fig. 9.2e). The heart is able to form in this anterior mid-ventral position because the formation of the head fold has now lifted the head above the level of the surroundings (Fig. 9.4). The formation of the head fold has also caused the foregut to become enclosed as a pocket, while the rest

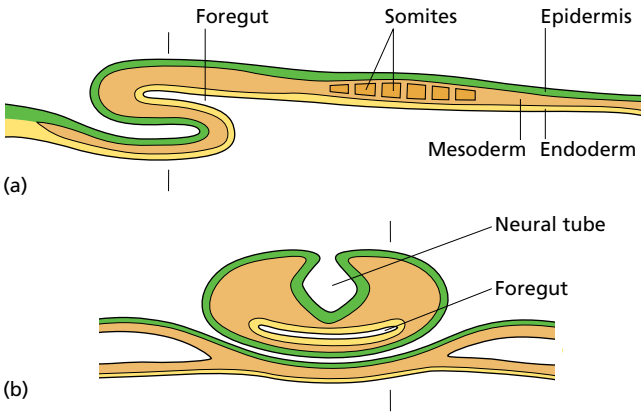


Fig. 9.4 The head fold at stage 8. (a) Parasagittal section; lines indicate plane of section of (b). (b) Transverse section; lines indicate plane of section of (a).

of the presumptive gut endoderm is still a lower layer of cells facing the yolk. The **neural tube** closes first over the midbrain and then progressively in both directions. **Somites** continue to arise in anteroposterior sequence from the segmental plates of mesoderm which flank the notochord and neural tube. By about 36 hours there are 10 somites and the neural tube has closed to form forebrain, midbrain, and hindbrain vesicles (stage 10). The **lateral plate mesoderm** becomes divided into a **somatic** layer, adhering to the epidermis, and a **splanchnic** layer, adhering to the endoderm. The space in between is the **coelom** (pronounced "see-loam": Fig. 9.3c). A further subdivision of mesoderm appears as a longitudinal strip in between the presomitic mesoderm and the lateral plate. This is the **intermediate mesoderm** that later forms the kidney, adrenals, and gonads. Although node regression and the formation of the posterior part of the body continues for some time, this stage marks approximately the junction between early and late development since the general body plan has been laid down and the formation of individual organs is about to begin.

Extraembryonic membranes

In embryos that have an external food supply the formation and arrangement of the **extraembryonic membranes** is essential to their survival. Amniotes (reptiles, birds, and mammals) are so called because they all have an **amnion**, and in fact many of the extraembryonic structures are obviously homologous. The arrangement in the chick embryo is shown diagrammatically in Fig. 9.5.

From the time of gastrulation onwards, the outer **area opaca** expands over the surface of the yolk as a membrane consisting of extraembryonic ectoderm and endoderm. The mesoderm has a more restricted spread and is coincident at any one time with the region of extraembryonic vasculature (see below). Initially the coelom is continuous between the embryonic and extraembryonic regions. The inner extraembryonic layer, which is composed of splanchnic mesoderm, blood-forming tissue, and endoderm, is called the yolk sac. This gradually surrounds the entire yolk mass and serves as a digestive organ, the products of digestion of the yolk being absorbed into the blood vessels and conveyed to the embryo. The outer extraembryonic layer consists of somatic mesoderm and ectoderm and is called the **chorion**. During the third day, a fold of the chorion starts to cover the anterior end of the embryo, and a corresponding fold also grows from the posterior. These folds are shown as lines on

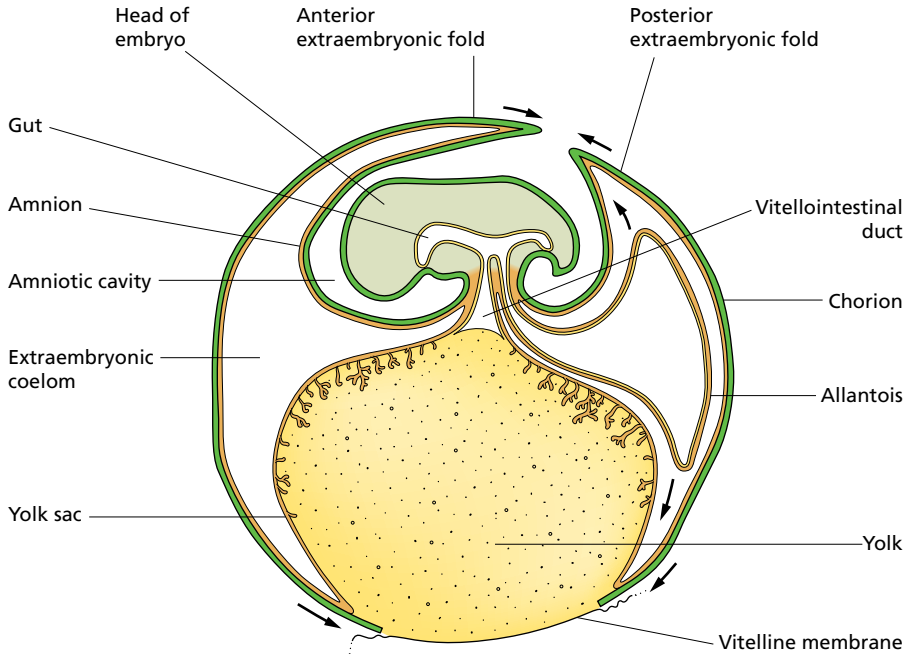


Fig. 9.5 Formation of the extraembryonic membranes in the chick. (After Hildebrand 1995. *Analysis of Vertebrate Structure*. New York: Wiley.)

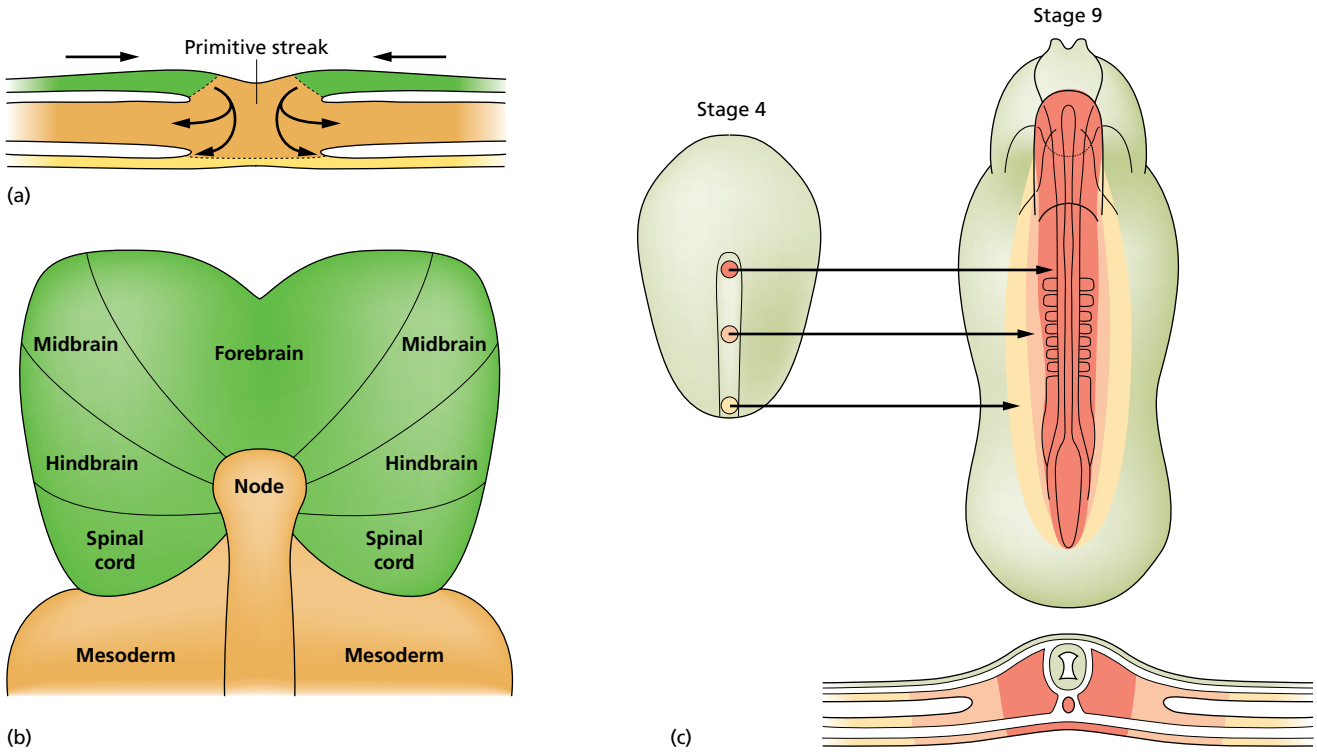


Fig. 9.6 Fate map of the chick embryo. (a) Movement of cells through the primitive streak. (b) The prospective region for the central nervous system about stage 4. (c) The origin of mesodermal and endodermal levels from the primitive streak.

Fig. 9.2g. The folds fuse in the middle to form two complete membranes covering the embryo, the outer still being called the **chorion** and the inner being called the **amnion**.

The **allantois** consists of a layer of endoderm covered by splanchnic mesoderm. It grows out from the hindgut into the extraembryonic coelom and expands to fuse with the undersurface of the chorion. The allantois serves two functions, one being as an excretory receptacle in which uric acid accumulates, the other as the main respiratory organ of the embryo. The **chorioallantoic membrane**, as it is called after fusion, is located just under the shell and is richly supplied with blood vessels for gaseous exchange. It is often used by experimentalists as a site for "*in ovo*" culture of isolated organ rudiments taken from the embryo.

Fate map

Only a small proportion of the blastoderm cells become incorporated into the embryo itself, the rest forming extraembryonic structures. The **fate map** of the early embryo has been extensively studied, most recently using small localized marks of **DiI** and **DiO** which can be visualized at later stages. These studies show that the primitive streak originates from the most posterior part of the area pellucida and from the posterior marginal zone. Like the area pellucida itself, this region contains two cell layers; the upper layer making a substantial contribution to the ectoderm of the streak, and the lower layer to the secondary hypoblast. The streak extends to the center of the area pellucida by active stretching. Although some cells remain resident in the streak, it consists mainly of cells in the process of moving through it, as there is a migration of epiblast cells from both sides which enter and move through to form both the mesoderm and endoderm (Fig. 9.6a). As a result of the entrance of new cells into the midline of the lower layer, the original hypoblast cells become pushed to the outer rim.

Labeling experiments show that the node, like the rest of the streak, has a continuous flux of cells moving in and out. During the phase of node regression the node itself is the prospective region for the notochord along the entire length of the body. The neural plate arises from epiblast around the node, with a conservation of anteroposterior levels (Fig. 9.6b), the somites arise from the region just around the node, and the lateral plate from more posterior parts of the streak. The origin of the endodermal layer closely follows the mesoderm, with medial parts from the anterior streak and lateral parts from the posterior streak (Fig. 9.6c) It is important to note that the primitive streak does not, as is often thought, map in a one-to-one manner onto the later anteroposterior body plan. Instead the anteroposterior axis of the streak maps to the mediolateral axis of the later embryo and this means that the posterior half of the streak is destined to become extraembryonic tissues.

Regional specification of the early embryo

Anteroposterior polarity and fragmentation of the blastoderm

As long ago as 1828, von Baer propounded a rule that enabled the anteroposterior axis to be predicted in the majority of eggs. This states that if the egg is horizontal with the pointed end to the right then the tail of the embryo should be towards the observer. The rule arises because the egg undergoes a continuous rotation when it is in the uterus and this is usually in the same direction relative to the sharp and blunt ends of the egg (Fig. 9.7). The embryo and yolk do not rotate along with the outer surface of the egg but are nevertheless tipped in the direction of rotation and, in fact, a simple tipping of the blastoderm at the critical period of 14–16 hours of uterine life is enough to establish the polarity such that the posterior end forms at the uppermost end of the blastoderm.

The early blastoderm can be cut into two or three parts, each of which will form a complete embryonic axis. So long as such fragments contain part of the original posterior of the blastoderm, then this will remain the posterior, showing the importance of the early polarization.

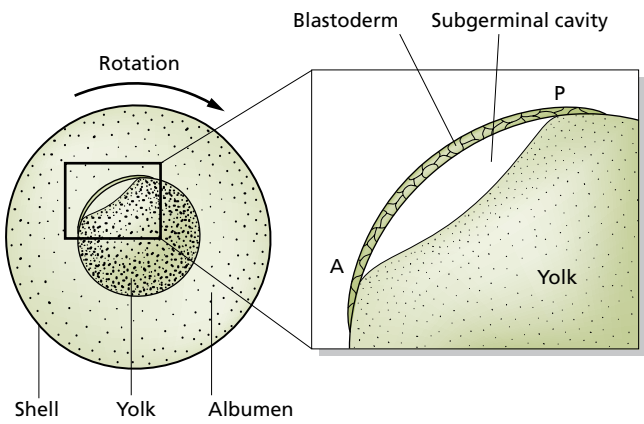


Fig. 9.7 Acquisition of anteroposterior polarity by the chick blastoderm, as a result of intrauterine rotation of the egg. A = anterior; P = posterior.

Early inductive interactions

It is possible to identify processes of mesoderm induction and the organizer effect by embryological experiments. To some extent the same molecules seem to be involved as in *Xenopus*, but there may also be some differences.

The primitive streak expresses the T-box transcription factor *brachyury*, and its formation corresponds to **mesoderm induction** in *Xenopus*. Normally the streak arises from the extreme posterior part of the area pellucida epiblast. If an anterior piece of epiblast is combined with a piece of posterior marginal zone (PMZ), then a new streak can be induced with polarity such that its posterior end abuts the PMZ (Fig. 9.8). Competence for streak induction by the PMZ is lost at stage XI, when formation of the secondary hypoblast is commencing, although a streak can still be induced to a substantially later stage (Hamburger & Hamilton (H&H) stage 3) by the secondary hypoblast itself. Interestingly, the secondary hypoblast and germ ring show a nuclear localization of β-catenin, like the dorsal side of the *Xenopus* egg, and lithium treatment can induce axial structures from the epiblast. It is possible to induce a streak from the area pellucida by application of vg1 (a nodal-like molecule) together with Wnt. This suggests a similar mechanism to *Xenopus*, but the evidence is incomplete as there is no satisfactory inhibition experiment at present.

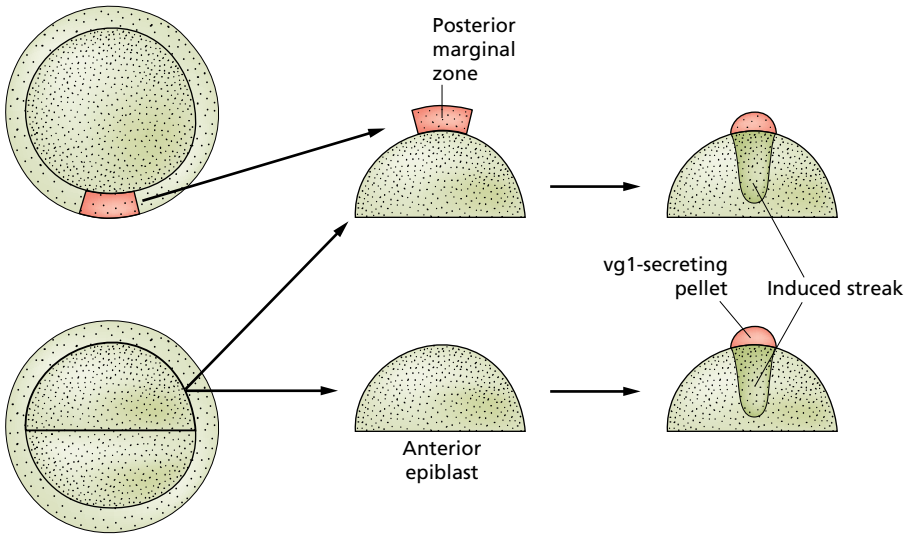


Fig. 9.8 Induction of a primitive streak by the posterior marginal zone or a vg1-secreting cell pellet (vg1 is a nodal-like substance).

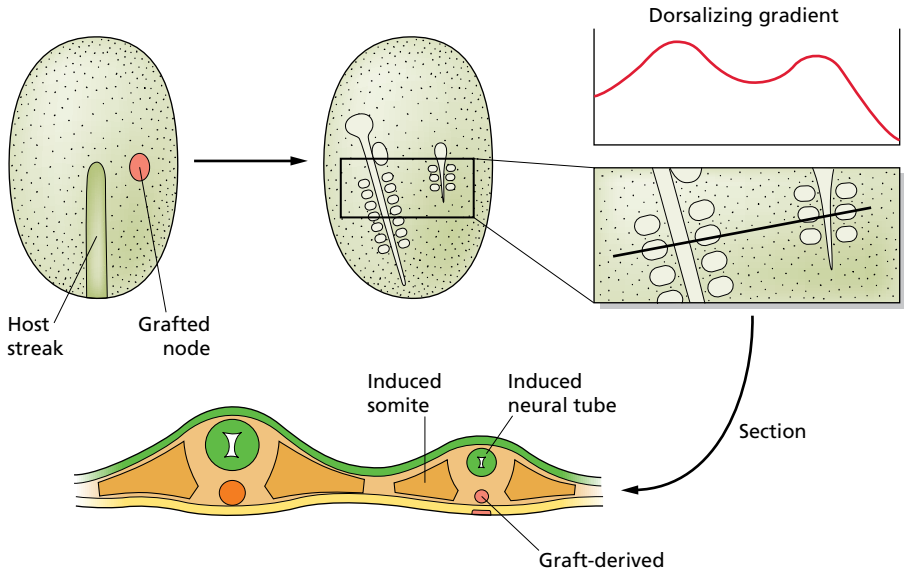


Fig. 9.9 A node grafted to the area pellucida will induce a partial second axis in which the notochord is graft-derived and the neural tube and somites are host-derived.

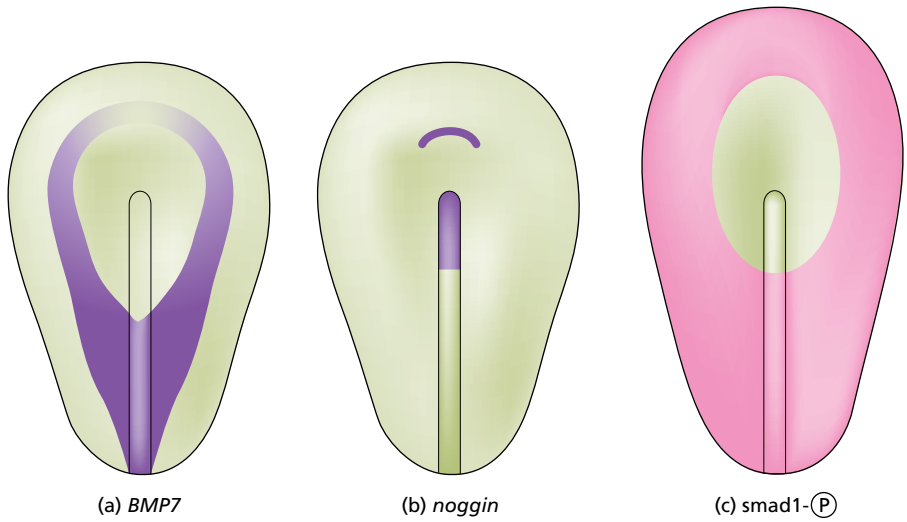


Fig. 9.10 Expression domains of: (a) a BMP; (b) a BMP inhibitor; (c) visualization of BMP signaling by detection of smad1-phosphate.

Interestingly this method can be used to show that formation of one streak represses formation of any others. An isolated anterior half blastoderm will usually form one streak at the posterior margin. If a pellet releasing vg1 is grafted laterally then it will induce a streak locally and suppress the host streak. If two pellets are grafted then they will both induce streaks unless the time interval between them exceeds 5 hours, in which case only the first induces a streak. This shows clearly that a new streak emits a signal inhibiting the formation of additional streaks in its vicinity.

The next inductive interaction is the "organizer effect": the regionalization of the ectoderm and mesoderm under the influence of Hensen's node. A node grafted into the area pellucida not too far from the host streak can induce a secondary axis in which the notochord is derived from the graft but the neural tube and somites are derived from the host (Fig. 9.9). This closely resembles the behavior of the *Xenopus* organizer and shows that there is an inductive signal emitted by the node. The activity of the node persists until about H&H stage 8 (four somites), with younger nodes inducing anterior neural structures and older nodes inducing posterior structures. The competence of the epiblast disappears before this, after H&H stage 4 (definitive streak). It is tempting to suppose that the organizer effect is due to BMP inhibition, as several BMPs are expressed in the peripheral part of the blastoderm and the BMP inhibitors noggin and chordin are expressed in the node. There is also a reduction of smad1-phosphate detectable by immunostaining in the prospective neural plate region (Fig. 9.10). However, there has been some controversy over this because, in general, the BMP inhibitors do not have the expected neuralizing activity when applied to explants of epiblast. Probably more than one factor is involved, as it is also known that FGFs have some neuralizing activity, FGF inhibitors can suppress neural induction, and Wnts are also antagonistic. As far as effects on the mesoderm are

concerned, applied BMPs can suppress somite formation in favor of the lateral plate, suggesting that mesodermal patterning does depend on a graded inhibition of BMP activity, as in *Xenopus*.

Note that it is unwise to refer to the organizer effect in the chick as "dorsalization." Because the chick blastoderm is flat, the axis homologous to the dorsoventral axis of *Xenopus* and zebrafish runs, at this stage, from medial to lateral. In the early chick embryo the "dorsoventral axis" is often referred to as the axis running through the blastoderm from the dorsal surface to the subgerminal cavity. This is homologous to the animal–vegetal axis and not to the dorsoventral axis of *Xenopus*. After ventral closure, the notochord, somites, and lateral plate do indeed run from dorsal to ventral and the nomenclature becomes the same for the chick and the amphibian.

A complete axis can still be produced following extirpation of Hensen's node at an early stage. This is a good example of embryonic regulation (see Chapter 4) whose mechanism remains unknown. The node reforms from the posterior margin of the hole and the process requires the presence of the mid-primitive streak. Once a node has been formed it suppresses formation of other nodes in the vicinity.

Formation of the trunk-tail parts of the anteroposterior pattern may be due to FGF, as in *Xenopus* and zebrafish. *Fgfs* are expressed in the primitive steak, and an FGF-impregnated bead will induce posterior neural tube from the epiblast. FGFs will induce expression of Cdx genes and these will in turn induce expression of the posterior Hox genes (Hox 6–13).

Left–right asymmetry

As mentioned in Chapter 2, vertebrates are not exactly bilaterally symmetrical. In the chick, the deviation arises at an early stage with a tilt of Hensen's node to the left. This is soon followed by the S shape of the heart tube and the flexion of the whole embryo, with the head lying to the right when viewed from above. When the stomach and intestine develop, they are markedly asymmetrical. Several of the gene products involved in this process have been identified in the chick because of their asymmetrical expression patterns, and the sequence of events has been worked out from experiments in which these factors are applied locally, either as RCAS virus or as protein on beads, and the effects on expression of other factors is observed. The process involves four steps. Firstly a breakage of the basic bilateral symmetry of the embryo in the node or midline structures. Secondly an amplification to create different regimes of gene expression on either side of the midline. Thirdly a spread of the information out to the lateral mesoderm which is the tissue layer most involved in the formation of the asymmetrical organs; and finally the control of the events of cell adhesion and movement that actually bring about the asymmetrical morphology.

In the chick the original symmetry-breaking event is still unclear, although in the mouse there is good evidence that it depends on the asymmetrical structure of the cilia in the node (see Chapter 10). The key player among the asymmetrically expressed genes is believed to be *nodal*, since it is preferentially expressed on the left side in all types of vertebrates, and application of nodal to the right side will randomize the situs of multiple organ systems. The components upstream and downstream of *nodal* do, however, vary between vertebrates. In the chick, *nodal* appears in a small domain on the left of the regressing node at about H&H stage 6 and then spreads to a much wider domain in the left lateral plate mesoderm. Its expression on the left side is preceded by *sonic hedgehog*, and on the right by *activinβB fgf4* and *fgf8* (Fig. 9.11). Shh will activate nodal expression when applied to the right side and activin or FGFs will repress nodal if applied to the left side, indicating that they are upstream regulatory components.

Spread of the *nodal* zone to the lateral plate mesoderm seems to involve partly an autocatalytic loop whereby nodal signaling activates *nodal* transcription, and partly a Cerberus-like BMP inhibitor called Caronte whose transcription is activated by nodal. BMPs are expressed on both sides and can suppress *nodal* expression. But Caronte suppresses BMP signaling on the left and thereby allows the spread of nodal expression on this side. The limitation of spread is controlled by another TGF-β-like factor called lefty, which becomes expressed on either side of the nodal domain. The lefty protein is an inhibitor of nodal and reduces its signaling activity. The end product of the gene cascade is expression of the homeodomain transcription factor pitx2 on the left side. This is controlled by nodal signaling through

Classic Experiments

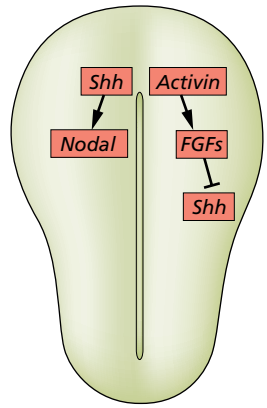
Left–right asymmetry

Vertebrate embryos are more or less bilaterally symmetrical, and so for many years the nature of the mechanism producing asymmetry was mysterious. In 1995 it was shown that some key genes (*nodal*, *sonic hedgehog*, and *activin receptor IIA*) have asymmetrical expression patterns in the early chick, and regulated each other to form a linked pathway from initial breaking of lateral symmetry to final morphological asymmetry of the heart and viscera.

Levin, M., Johnson, R.L., Stern, C.D., Kuehn, M. & Tabin, C. (1995) A molecular pathway determining left–right asymmetry in chick embryogenesis. *Cell* **82**, 803–814.

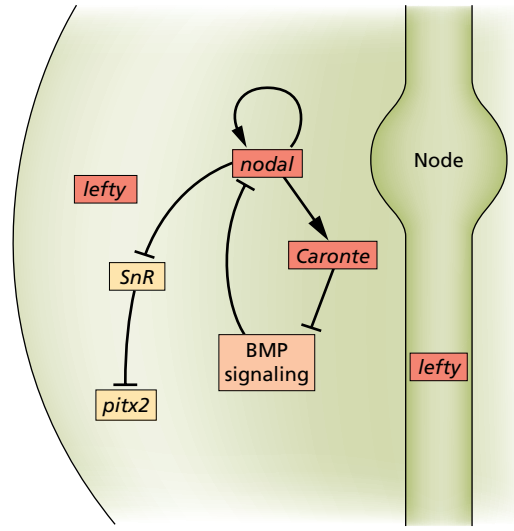
Subsequently it was shown in the mouse that the node bears cilia which, because of their inherent molecular asymmetry, drives fluid preferentially to the left.

Nonaka, S., Tanaka, Y., Okada, Y., Takeda, S., Harada, A., Kanai, Y., Kido, M., & Hirokawa, N. (1998) Randomization of left–right asymmetry due to loss of nodal cilia generating leftward flow of extraembryonic fluid in mice lacking KIF3B motor protein. *Cell* **95**, 829–837



	L	R
Activin ßB	–	+
FGFs	–	+
Shh	+	–
nodal	+	–

(a) Early events



(b) Later events

Fig. 9.11 Development of left–right asymmetry in the chick embryo.

transcriptional repression of snail-related, a zinc finger transcription factor, which itself represses *pitx2* transcription. The sequence of events is shown in Fig. 9.11.

Although some steps of this mechanism are not shared by other vertebrates, nodal and pitx2 are always expressed on the left and seem to represent the principal signaling step and final controller of cell differentiation.

Description of organogenesis in the chick

The later stages of chick embryo development have been very well known for a long time and for this reason serve as the basic resource for our knowledge of vertebrate organogenesis in general. As the experimental work on several organ systems is described in more detail in later chapters, this section contains just a brief summary of some of the major morphological events.

Whole embryo

By the second day the heart is bent to the right and the **optic vesicles** appear from the forebrain (see Fig. 9.2e). The head turns to face the right and at this time (stage 13) an anterior extraembryonic fold rises to cover the head. This fold moves progressively posteriorly and will later become the chorion and amnion (see Fig. 9.5). The head becomes sharply flexed between the region of the forebrain and the hindbrain. The first three **pharyngeal pouches** appear, the optic vesicles invaginate, and the lenses appear in the adjacent epidermis. On day 3 the limb buds appear in the lateral plate mesoderm (stage 17) and after a further day are as long as they are broad. A posterior extraembryonic fold appears and moves anteriorly, eventually meeting the anterior fold and fusing shortly after the appearance of the limb buds to enclose the embryo within an amniotic cavity (see Fig. 9.5). Eye pigmentation appears from about 3.5 days.

By the third day the original head fold has deepened into an anterior body fold, such that the anterior half of the body has become elevated above the surroundings (see Figs 9.4, 9.5). This results in the formation of a closed tube of **foregut** running anteriorly from an anterior intestinal portal connecting it to the subendodermal space. Over the fourth day, a corresponding posterior body fold lifts the rear part of the embryo and results in the formation of a **hindgut**. The residual ventral opening of the gut becomes progressively smaller and eventually becomes narrowed to a **vitellointestinal duct** joining the yolk mass to the midgut (see Fig. 9.5). The **allantois** arises from the hindgut and expands rapidly into the space between chorion and amnion.

A mouth is formed at the anterior end of the foregut. The face is formed by the fusion of a set of paired processes: above the mouth are the frontonasal and maxillary processes and below the mouth the mandibular processes. Each pair fuses in the midline to make up the face. In birds, a beak appears from about 5.5 days, the upper part from the maxillary and the lower part from the mandibular processes. The outer **epidermis** of the embryo is often called **ectoderm** for several days because of its undifferentiated appearance, but this is a misnomer as it is no longer able to form neural tissues after the primitive streak stage. In the chick, the feather germs start to appear in the epidermis from about 6.5 days.

From 3 to 4 days the posterior extremity of the embryo consists of a **tailbud**. This consists of a juxtaposition of the various axial tissue types: the notochord, neural tube, somites, and hindgut. In the chick it is only responsible for producing the most posterior four to five somites, although in other vertebrates it can produce many more. Hatching of the chick occurs on the 20th or 21st day.

Nervous system

The early brain is shown in Fig. 9.12. The three primary brain vesicles, visible from the second day, are the **forebrain**, **midbrain**, and **hindbrain**. The anterior part of the forebrain is the **telencephalon**, later forming the cerebral hemispheres, and the posterior part is the **diencephalon**, which produces the optic vesicles. The midbrain later forms the **optic tecta**, which are the receptive areas for the optic nerves. The hindbrain forms the **cerebellum**, controlling the body's movements, and the medulla oblongata, site of control centers for various vital functions. The remainder of the neural tube forms the spinal cord. Ten pairs of **cranial nerves** leave the brain to innervate various muscles and sense organs, and the spinal cord produces pairs of spinal nerves in between the vertebrae.

The dorsal part of the neural tube gives rise to a migratory population of cells called the **neural crest**. In the head this contributes to the cranial nerve ganglia and a large proportion of the skeleton of the skull. In the trunk it forms the spinal ganglia, autonomic ganglia, adrenal medulla, and pigment cells.

Further details of development of the nervous system will be found in Chapter 14.

Pharyngeal arch region

At the level of the hindbrain the body has an obviously segmental arrangement (Fig. 9.13). This is made up of elements from different germ layers. In the endoderm of the pharynx, paired lateral pouches develop. These are the famous "gill slits," or branchial clefts, which all vertebrate embryo possess in rudimentary form, although in the chick they do not become fully patent. The hindbrain itself is divided into seven **rhombomeres**. Rhombomere 1 becomes the cerebellum. Each pair of rhombomeres 2–7 produces the neural crest cells that migrate to form one cartilaginous **branchial arch** surrounding the pharynx. The first of these, lying anterior to the first cleft, is the mandibular arch, which subsequently becomes the mandibular and maxillary processes which form the lower half of the face. The second arch is called the hyoid arch. Each pair of rhombomeres also produces one cranial nerve running into its associated arch. The cranial ganglia are composed partly of cells from the neural crest and partly of the corresponding epibranchial placodes, which form in the adjacent epidermis. Each pharyngeal arch is associated with a vascular aortic arch connecting the ventral aorta

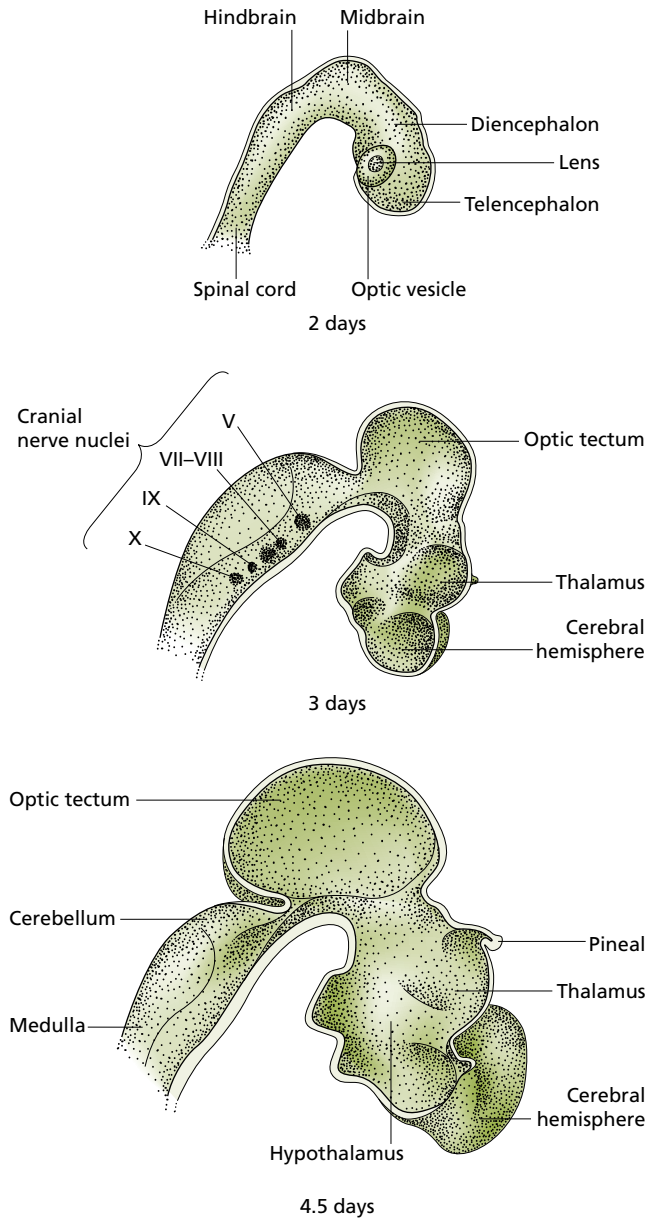


Fig. 9.12 Change in shape of the chick embryo brain from 2 to 4.5 days of development.

from the heart with the paired dorsal aortas (see below). In the endoderm, the thyroid forms in the ventral midline from second pouch tissue, and paired thymus and parathyroid rudiments form from the third and fourth pouch. This whole segmental arrangement is transitory, so the clefts are only open for a short time and the aortic arches are not all functional simultaneously.

Heart and circulation

The heart originates at the hindbrain level from paired endothelial condensations between the splanchnic mesoderm and the

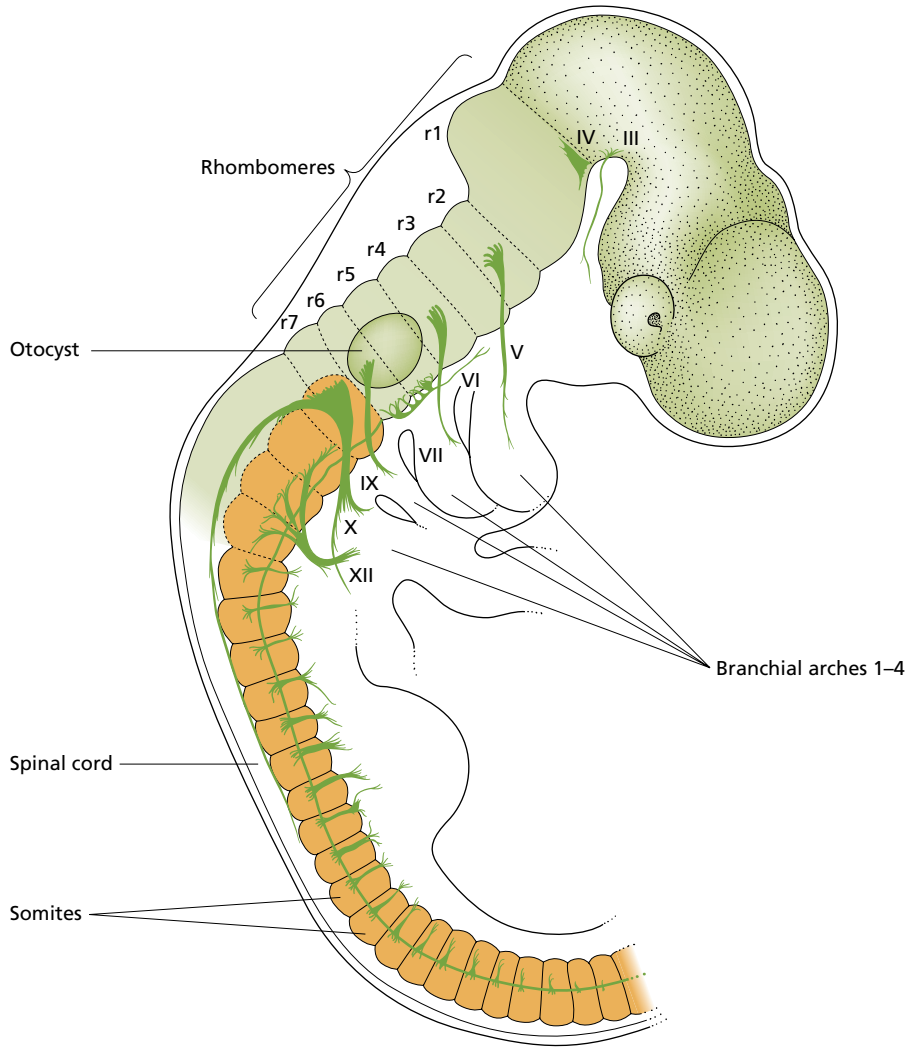


Fig. 9.13 Pharyngeal arch region of a 3-day chick embryo. The rhombomeres are labeled r1–r7 and the associated cranial nerves V–XII. Each pair of rhombomeres innervate one arch. The otocyst is at the level of r5–6. (After Lumsden 1991. *Philosophical Transactions of the Royal Society* **331**, 281–286. Figure 1.)

gut. These initially form separate tubes and then early on the second day, from about seven somites, they fuse in the ventral midline to form a single tube of **endocardium**. The **myocardium**, or muscular wall of the heart, originates from the adjacent splanchnic mesoderm. As the fusion is taking place, the heart moves posteriorly, following the progress of the anterior body-fold. By 48 hours the heart is a coiled tube consisting of, from posterior to anterior, sinus venosus, atrium, ventricle, and outflow tract.

During the early stages of heart formation, a system of blood islands and blood vessels appears in the area opaca. The vessels grow into the area pellucida and join up with vessels arising from the mesoderm within the embryo. The heart starts to beat about the middle of the second day, and by the third day establishes a blood circulation between the embryo and the yolk mass. From the heart, blood flows into the short ventral aorta, through the aortic arches, initially one for each of the first three pharyngeal arches, into the dorsal aortas (Fig. 9.14). These are initially paired but become progressively united, from anterior to posterior, into a single aorta. From these, blood flows to all parts of the embryo and also out of the embryo through paired vitelline arteries located at the level of the trunk. It then becomes oxygenated in the extraembryonic capillary bed and returns via the vitelline veins, which approach the embryo from both anterior and posterior directions, and, with the embryonic venous system, join up at the sinus venosus. As the body folds reduce the connection between the embryo and the yolk mass, the vitelline arteries and veins are moved together into the **umbilical tube**. Later on, from the sixth day, most of the blood flow becomes directed through the **allantois** (see Fig. 9.5), as this becomes the principal respiratory organ.

Mesoderm

Somites arise from the mesoderm on either side of the notochord, often called the **paraxial mesoderm**. From the end of day 1 the somites appear in anterior-to-posterior sequence. The

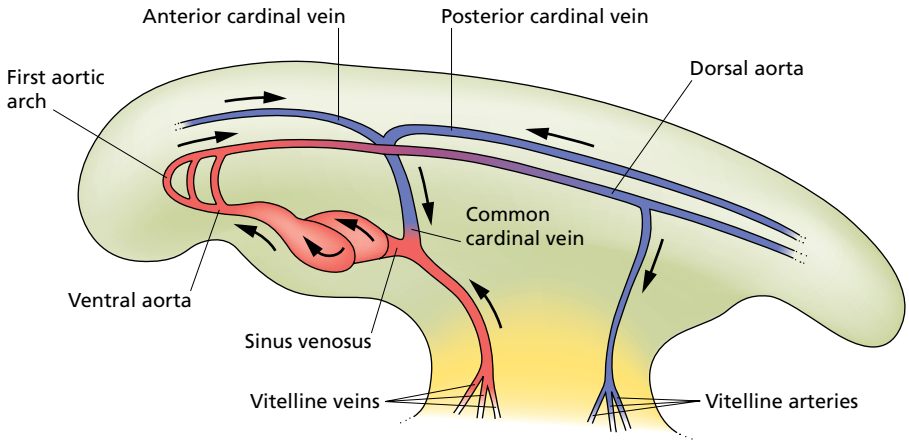


Fig. 9.14 The basic circulation of an amniote embryo.

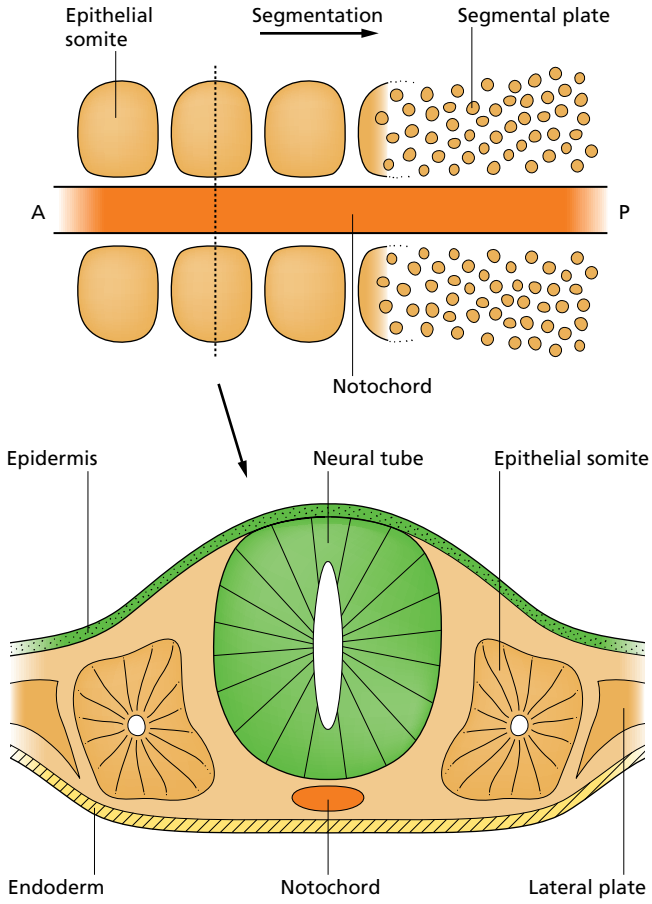


Fig. 9.15 Somitogenesis. Somites are formed sequentially from anterior (A) to posterior (P).

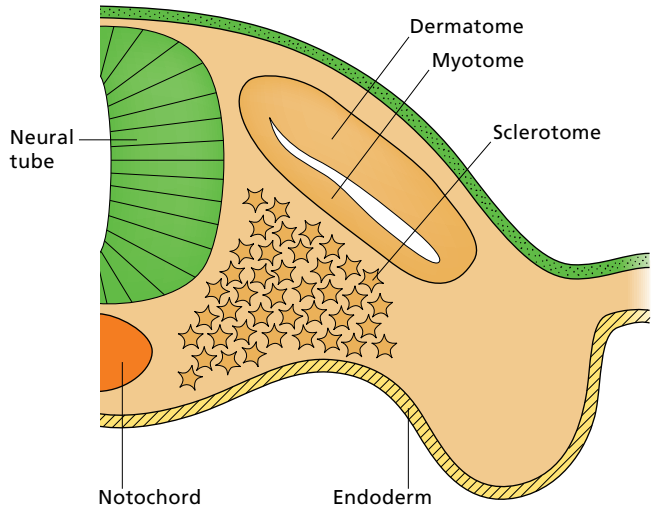


Fig. 9.16 Formation of dermatome, myotome and sclerotome from the somite.

visible event of segmentation corresponds to a transition of each somite from a mesenchymal morphology to epithelial spheres of tightly apposed cells (Fig. 9.15). Somites continue to form until there are 45 by 4 days. They will later form three types of structure (Fig. 9.16). The inner part, flanking the notochord, is called the **sclerotome** and later becomes the vertebrae. The formation of vertebrae is out of phase with the somites, so each vertebra is formed from the posterior sclerotome of one somite and the anterior sclerotome of the next. The middle part of the somite is the **myotome**, which will form segmental striated muscles. The outer part is the **dermatome**, which will contribute to the dermis of the skin. In the chick, the most anterior two somites disperse shortly after their formation and the next four are occipital somites, which contribute to the occipital part of the skull rather than forming vertebrae.

The kidney develops from the intermediate mesoderm (Fig. 9.17). First the **pronephros** develops on day 2–3 at the level of the seventh to 15th somite. A nephric duct grows posteriorly from the pronephric area down to the cloaca. In fish and amphibians the pronephros is functional, but in amniotes it soon degenerates as a **mesonephros** appears from the 16th to 27th somite. In the chick this develops on the third and fourth

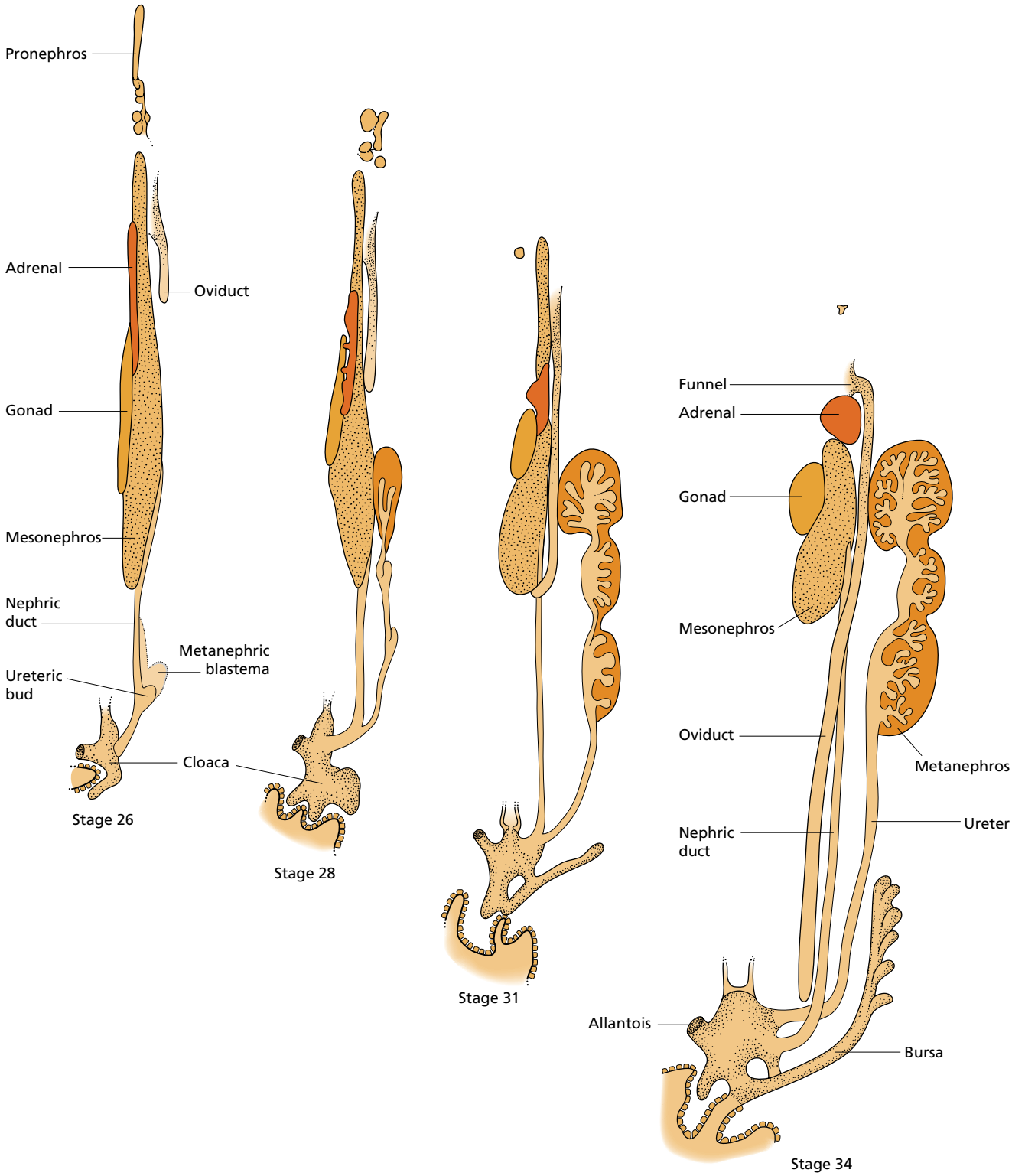


Fig. 9.17 Development of the kidney, gonads and adrenals from the intermediate mesoderm. (After Witschi 1956. *Development of Vertebrates.* Saunders.)

New Directions in Research

The early chick offers a good opportunity to study the as yet mysterious property of embryonic regulation. In most embryo types regulation requires the persistence of the signaling centers but in the chick the main signaling center, Hensen's node, can itself be replaced following extirpation.

The later chick embryo will continue to provide material for research on organogenesis, because of the ease of micromanipulation. Research programs often combine microsurgical experiments on the chick with the use of tissues from knockout mouse lines.

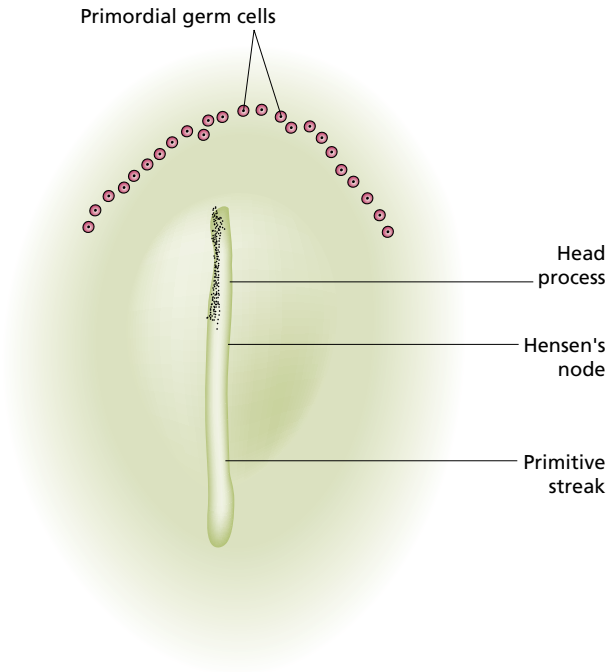


Fig. 9.18 Extraembryonic origin of the primordial germ cells.

day, and consists of glomeruli and tubules that attach to the nephric duct. The mesonephros is the functional kidney in birds during embryonic life. It is superseded after hatching by the **metanephros**. This develops from about 5 to 8 days from the posterior end of the intermediate mesoderm. A branch from the nephric duct, the **ureteric bud**, grows into the neighboring mesoderm and provokes the formation of the metanephric tubules in a mesenchymal to epithelial transition.

The strip of intermediate mesoderm ventromedial to the kidney forms the adrenal gland and the gonads. The adrenal gradually becomes a compact body over days 4–8, with the outer cortex formed from intermediate mesoderm and the inner medulla formed from the neural crest. The gonads arise both from the mesenchyme and from the overlying coelomic epithelium, which together form a protrusion into the coelom called the **genital ridge**. The **primordial germ cells** arise from an anterior extraembryonic position (Fig. 9.18) and enter the germinal ridge after a long migration. On the fourth day the gonads are still of similar appearance in males and females, but subsequently they differentiate as testes or ovaries, respectively. The region of mesoderm forming the aorta, gonads, and mesonephros is sometimes called the **AGM region** and is important in hematopoiesis.

The limbs appear on day 3 as buds formed from the lateral plate mesoderm and overlying epidermis. They elongate, and from the fourth day start to differentiate in proximal to distal sequence. In birds the forelimb buds become the wings and the hindlimb buds the legs.

Key Points to Remember

- The chick is an amniote with a generally similar morphology to mammalian embryos.
- Early development occurs as a flat blastoderm. The primitive streak is induced at the posterior margin and elongates to the anterior. During gastrulation cells pass through the streak and become the major body parts of the embryo.
- The chick possesses a set of extraembryonic membranes needed to support the embryo and to transfer nutrients and oxygen to the embryo. These are the yolk sac, the chorion, the amnion, and the allantois.
- The fate map of the primitive streak shows that the anteroposterior axis of the streak becomes the mediolateral axis of the primary body plan.
- The sequence of inductive interactions is comparable to *Xenopus*. The induction of the primitive streak corresponds to mesoderm induction in *Xenopus* and the behavior of Hensen's node is similar to the *Xenopus* organizer. However some of the molecular components underlying these processes may be different.
- The formation of left–right asymmetry of the embryo depends on the expression of nodal on the left side of the axis.
- The chick is very important in research on organogenesis (see later chapters).

Further reading

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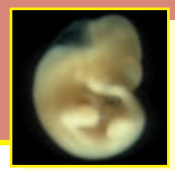
Chapter 10



The mouse

Unlike the other model organisms considered here, mammals are **viviparous** and the resulting inaccessibility of the post-implantation stages of development means that microsurgical procedures are used less than in *Xenopus*, zebrafish, or chick. For this reason the developmental biology of the mouse has depended to a much greater extent on genetic manipulation (transgenesis or knockout). There are many laboratory strains of mice that have been inbred to almost total homozygosity at all loci and each have their own advantages and disadvantages for different types of experiment. Each strain has a characteristic coat color (e.g. black, albino, agouti), and in experiments involving embryos of more than one strain these differences can serve as a visible indication of the genetic constitution.

Mice mate in the night and so the age of the embryos is often expressed as days and a half, for example a 7.5-day embryo (= E7.5 embryo) is recovered on the eighth day after the mice mated. A solid white deposit, or "plug," that is formed in the female vagina after mating allows determination of the mating night. There is a stage series by Theiler, in which stages 1–5 are preimplantation, 6–14 are early postimplantation, comprising body plan formation and turning, and 15–27 cover organogenesis and fetal growth phases up to birth, which occurs at about 20 days after fertilization.

Ovulation occurs a few hours after mating and fertilization takes place at the upper end of the oviduct. For the early, preimplantation, stages the embryos are located first in the oviduct and then the uterus of the mother. During this time they can be collected and kept *in vitro* in reasonably simple media and it is possible to do microsurgical manipulation of these early stages, including those required to make transgenics and knockouts. In order to turn a modified preimplantation embryo into a late embryo or into an adult mouse, it must be transplanted into the uterus of a **foster mother** which has been made **pseudopregnant**, and thus receptive to the embryos, by previous mating with a vasectomized, and therefore sterile, male. So long as the transplanted embryos implant, they should develop and be born in the normal way.

At late stages it is possible, as in the chick, to explant individual organ or tissue rudiments from embryos into *in vitro* culture, where they are accessible to manual intervention. The scope of these experiments is greatly increased in the mouse by the ability to use tissues from transgenic and knockout strains.

Because of their viviparity, mammalian embryos have a considerable external nutrient supply and undergo extensive growth during development. In this respect the

Classic Experiments

The main contribution of the mouse to developmental biology has been through transgenic and knockout technology. These techniques are now fundamental to virtually every investigation of early development or organogenesis. They have also been used to create many mouse models for human diseases which are used to investigate both the molecular mechanisms of the disease and to test potential therapies.

First transgenesis

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Invention of basic knockout procedure

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mouse resembles the chick and differs from *Xenopus* and the zebrafish.

Mammalian fertilization

From a biological standpoint, the fertilization mechanism needs to bring the male and female gametes together in a productive union, while avoiding both cross-species fertilization (**hybridization**) and fertilization of the egg by multiple sperm (**polyspermy**). Fertilization is also a topic of great practical importance in human reproduction. The mechanisms have been studied in a variety of animal models, including especially marine invertebrates such as the sea urchin. However, the molecular mechanisms of fertilization are much more diverse than those of other developmental processes, and it has turned out that there are rather few features in common between the sea urchin and mammals. So in this case the value of invertebrate models for building a general picture is somewhat reduced. To maintain coherence the following account relates just to the mouse, although most features apply also to other mammals. Some of the evidence depends on the use of mice in which specific genes have been "knocked out," or "knocked in" (i.e. replacing an endogenous gene). The techniques for making such strains are described later in the chapter.

The sperm is a highly specialized cell (Fig. 10.1). The nucleus is haploid and the DNA is highly condensed with protamines instead of histones making up much of the protein content of the chromatin. In front of the nucleus lies a large Golgi-like body called the **acrosome**. Behind the nucleus lies a centriole, a mid-piece rich in mitochondria, and the tail which is a reinforced flagellum having the usual 9+2 arrangement of microtubules. The swimming movements of the sperm are driven in an ATP-dependent process by dynein arms which are attached to the microtubules.

In most mammals the sperm are not capable of fertilization immediately after release. They need to spend a period in the female reproductive tract during which they become competent to fertilize, a process known as **capacitation**. The details of capacitation are not fully understood but it can be brought about *in vitro* in simple synthetic media containing albumin, calcium, and bicarbonate. One element of capacitation is the loss of cholesterol, as a medium rich in cholesterol will inhibit capacitation. It is thought that the loss of cholesterol makes the membrane permeable to the Ca^{++} and HCO_3^-, which can directly activate an adenylyl cyclase, resulting in the production of cAMP and activation of protein kinase A. This has various consequences including an increase of membrane potential from about −30 to −50 mV, which may make the subsequent opening of Ca^{++} channels easier. Capacitation also involves the loss of glycoproteins that prevent the sperm–zona interaction (see below).

The "egg" of most mammals, including the mouse and the human, is strictly speaking an **oocyte** arrested in second meiotic metaphase. It is released from the ovary, together with ovarian follicle cells (**cumulus** cells), as an oocyte–cumulus complex. The oocyte itself is surrounded by a transparent layer of extracellular material called the **zona pellucida**, secreted by the follicle cells. Outside this lie cumulus cells, which are embedded in an extracellular matrix rich in hyaluronic acid. The oocyte–cumulus complex is picked up by the funnel (infundibulum) at the entrance to the **oviduct**, a process which depends on adhesion of the cilia of the infundibulum to the extracellular matrix of the complex. It is then "churned" to compress the matrix and allow it through the narrow neck (ostium) into the oviduct itself.

Although chemotaxis of sperm towards eggs occurs in some other types of animal, the evidence for this in mammals is not clearcut. The transport of the sperm certainly depends to some extent on muscular movements of the female reproductive tract which assist its passage up the vagina, through the uterus and into the oviducts. The main steps of sperm–egg interaction are shown in Fig. 10.2. The sperm carry a membrane-bound hyaluronidase that assists their passage through the extracellular matrix of the oocyte–cumulus complex. The next stage is the binding of sperm to the zona pellucida and this is the stage at which species specificity is controlled. If the zona is removed, then cross-species fertilization is possible, and this is the basis for the routine assay of the effectiveness of human sperm using hamster eggs from which the zona has been removed. The zona is composed of three glycoproteins called ZP1, ZP2, and ZP3, which share a common "ZP" peptide sequence motif at the C-terminus. Of these, ZP3, with its specific O-linked oligosaccharide, is the specific sperm receptor. Low concentrations of ZP3, or the oligosaccharide alone, will prevent sperm-zona binding. Female mice in which the gene for ZP3 has been knocked out will form normal oocytes but they do not have a zona and the mice are infertile. If the human ZP3 gene is "knocked in" to replace the mouse gene then zona formation and fertility is restored. Interestingly, eggs from these mice do not acquire a

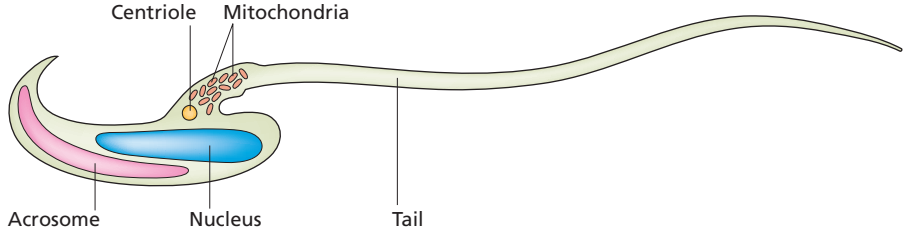

Fig. 10.1 Diagram of a mouse sperm.

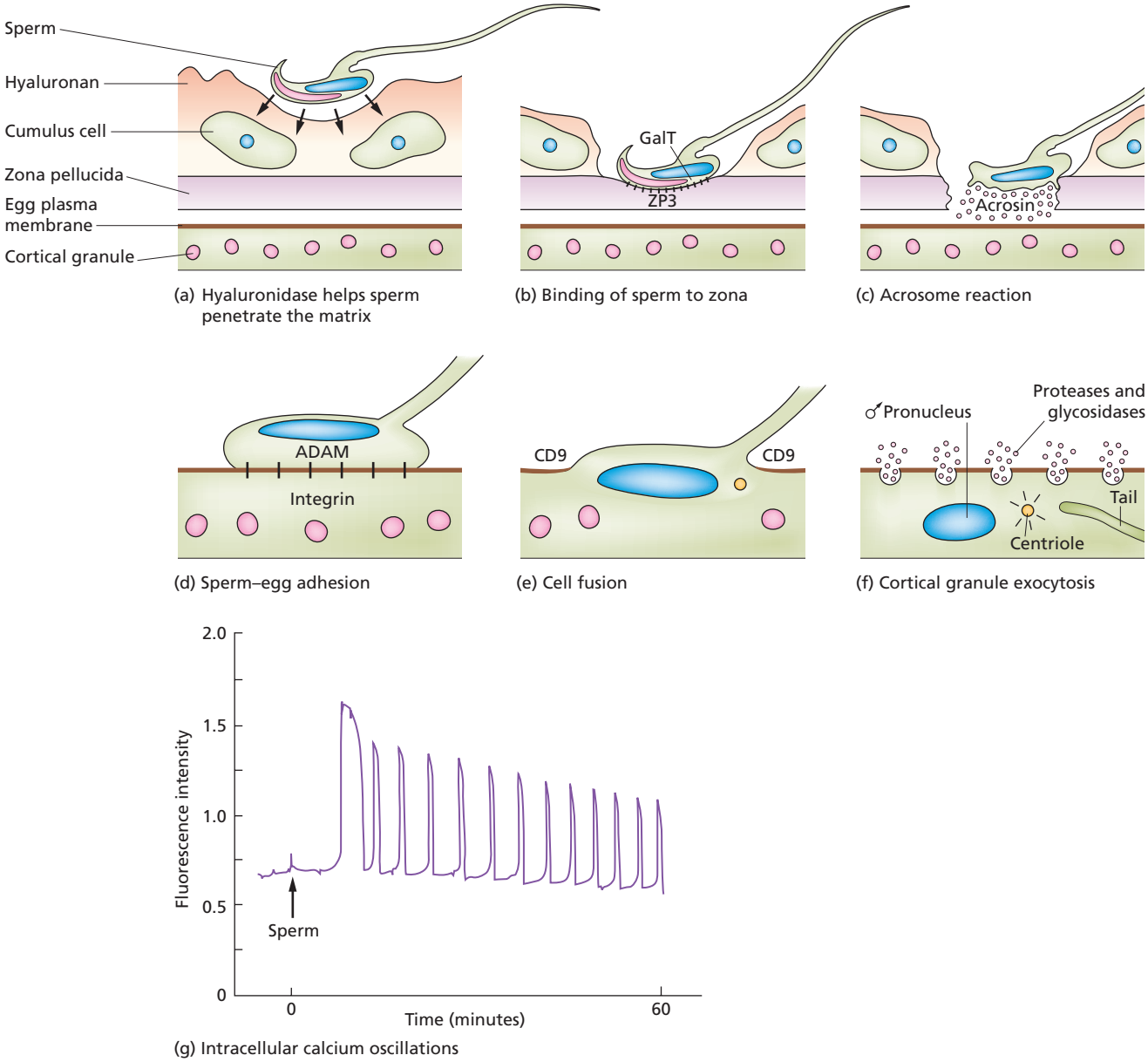

Fig. 10.2 Events of fertilization in the mouse.

competence to be fertilized by human sperm. This is because the species specificity resides in the carbohydrate attached to the ZP3 polypeptide, and this has the murine structure even though the polypeptide is of human origin, because it is assembled by the murine glycosyl transferases.

The recognition protein for ZP3 on the sperm is a cell surface β-1,4-galactosyl transferase (GalT) and binding of ZP3 to this provokes the acrosome reaction. The evidence for this is that GalT will bind tightly to the ZP3 oligosaccharide, and that sperm from mice in which the GalT gene is knocked out show reduced binding to the zona and are not provoked into the acrosome reaction. The acrosome reaction is a rapid exocytosis of the acrosomal vesicle and the released products are needed for the later events of fertilization. The coupling between GalT and the exocytosis is thought to proceed by the sequence: G-protein activation, less negative membrane potential, opening of voltage-gated Ca^{++} channels which cause a rise in intracellular Ca^{++}. There is also a rise in intracellular pH. GalT itself is a single pass membrane protein with a G-protein activation domain in the cytoplasm. If GalT is expressed in *Xenopus* oocytes, then they will bind ZP3, and ZP3 will stimulate G-protein activation and activation events such as cortical granule exocytosis. Here the *Xenopus* oocyte is used simply as an experimental test system, because it is a large cell into which it is easy to inject mRNA. The fact that it is an oocyte is not relevant since the normal location of GalT is the sperm.

The materials released by the acrosome reaction include hydrolytic enzymes, such as the serine protease acrosin, that help digest a path through the zona and enable the sperm to reach the egg surface. At this stage there is a second recognition process. The sperm–egg recognition is thought to be carried out by ADAM proteins on the sperm binding to integrins on the egg surface. ADAM stands for a disintegrin and metallprotease domain, and proteins of this class will bind tightly to integrins. The sperm contains three ADAM proteins: fertilin α, fertilin β, and cyritestin. All probably play some role in the interaction. Peptides from fertilin β will block sperm–egg binding, and sperm from mice in which the genes for fertilin β or cyritestin have been knocked out show greatly reduced fertility. The best candidate for the target integrin on the egg is integrin α6 because binding is prevented by a monoclonal antibody to this protein. However as the knockout mouse for this integrin has normal fertility there must be some other components also involved which have a redundant function. The binding of sperm to egg is the first stage in fusion of the plasma membranes, which occurs at a domain on the side of the sperm head. Fusion requires a four-pass membrane protein on the egg called tetraspanin or CD9. Evidence for this is that female knockout mice for CD9 show no fusion and are infertile; but the fusion ability of the eggs can be restored by injection of CD9 mRNA. In the course of fusion the whole sperm, including the tail, enters the egg.

Cell fusion causes a rise of intracellular calcium ion concentration which is responsible for all the subsequent events of fertilization. This calcium rise is also the aspect of fertilization that does seem to be universal across the animal kingdom. In many mammals including the mouse the initial calcium spike is followed by a series of others making up an oscillatory pattern over several hours (Fig. 10.2f). Calcium transients of this sort are observed by loading the eggs with calcium-sensitive reagents such as the phosphorescent protein aequorin or the fluorescent dye fura2 and measuring the resulting light emission or fluorescence. It is thought that the Ca^{++} release is caused by activation of the inositol trisphosphate (IP_3) pathway by a specific phospholipase C introduced by the sperm. The evidence for this is as follows:

1 Injection of IP_3 will provoke Ca^{++} release.
2 Inhibitors of phospholipase C or of IP_3 receptor will inhibit Ca^{++} release.
3 Injection of whole sperm, or demembranated sperm heads, or sperm extracts, will provoke Ca^{++} release.
4 Sperm contains a specific phospholipase C (PLCζ) which itself will cause Ca^{++} release.
5 Immunodepletion of this PLCζ from sperm extract will abolish activity.

Injection of Ca^{++}, or treatment with calcium ionophore, that allows entry of Ca^{++} from the medium, will both cause the same events of egg activation as fertilization by sperm. These events comprise the exocytosis of **cortical granules**, the completion of the second meiotic division, the resumption of DNA synthesis, and a general metabolic activation (Fig. 10.2g). The cortical granules are found just under the plasma membrane and their contents include glycosidases and proteases that modify the zona receptors so that they can no longer bind sperm. In the mouse this process is the main factor preventing polyspermy. The completion of the second meiotic division results in expulsion of the **second polar body** containing the surplus chromosomes. The sperm nucleus itself decondenses, assisted by reduction of protamine disulfide bonds by the – SH containing peptide glutathione in the egg. In addition to the nucleus, the sperm also introduces some mitochondria and a centriole. The mitochondria degenerate and do not participate in later development, but the centriole becomes the **microtubule organizing center** for the sperm **aster**, and later divides to form the first mitotic spindle. The two pronuclei migrate slowly towards each other and undergo DNA replication. In mammals they do not fuse to form a true zygote nucleus, instead the pronuclear envelopes break down as they meet, and the chromosomes become aligned on the mitotic spindle ready for the first cleavage. Athough a variety of treatments that elevate intracellular calcium can cause egg activation, such eggs are **parthenogenetic** (i.e. contain no paternal nucleus) and because of this they cannot develop far (see imprinting below).

Normal embryonic development

Preimplantation

The course of preimplantation development is shown in Fig. 10.3. Following fertilization the first few cleavages are very slow, and in contrast to *Xenopus* and the zebrafish, expression of the zygotic genome commences as early as the two-cell stage. The first cleavage occurs after about 24 hours and the second and third cleavages, which are not entirely synchronous, follow at intervals of about 12 hours. This slow tempo of early development may be related to the time required for the uterus to prepare for implantation. In the early eight-cell stage the shapes of individual cells are still clearly visible but they cease to be visible when the whole embryo acquires a more nearly spherical shape in a process called **compaction** (Fig. 10.3d). This consists of a flattening of blastomeres to maximize intercellular contacts and is mediated by the calcium-dependent adhesion molecule E-cadherin (also called leukocyte cell adhesion molecule (L-CAM) or uvomorulin). At this stage the cells become polarized in a radial direction. This is most obviously apparent from the appearance of **microvilli** on the outer surfaces, but it also involves a variety of changes in the cell interior. **Gap junctions** are also formed at this stage and allow diffusion of low molecular weight substances throughout the embryo.

The embryo is called a **morula** from compaction until about the 32-cell stage. During this period, desmosomes and tight junctions appear, creating a permeability seal between the inside and outside of the embryo, and a fluid-filled **blastocoel** begins to form in the interior. This is about 3 days after fertilization and around the time that the embryo moves from oviduct to uterus. The cavity expands the embryo into a **blastocyst** which

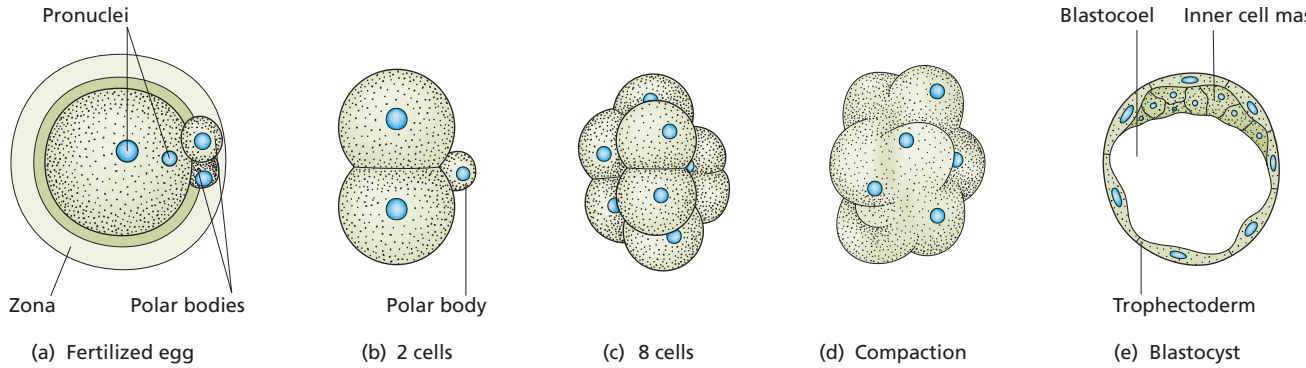

Fig. 10.3 Preimplantation development. The zona remains present but is not shown in (b) to (e).

consists of an outer cell layer of epithelial morphology called the **trophectoderm** and a clump of cells attached to its interior, the **inner cell mass (ICM)** (Fig. 10.3e). At the 60-cell stage, about one-quarter of the cells are found in the ICM and three-quarters in the trophectoderm. The ICM expresses a POU domain transcription factor Oct4, and secretes FGF4. The trophectoderm contains an FGF receptor, FGFR2.

From E3.5 to E4.5 both the ICM and the trophectoderm diversify (Fig. 10.4a). The ICM delaminates a layer of **primitive endoderm** on its blastocoelic surface. This layer contributes to extraembryonic tissues but not to the **definitive endoderm** of the embryo itself. The trophectoderm becomes divided into a polar component, which overlies the ICM, and a mural component which makes up the remainder. While the polar trophectoderm continues to proliferate, the mural trophectoderm becomes transformed into **polyploid** giant cells in which the DNA continues to be replicated but without mitosis.

Early postimplantation stages

At about this stage the embryo hatches from the zona and becomes implanted in a uterine crypt. The uterus is only competent to receive embryos during a short period about 4 days from mating. The uterus is attached to the body wall by a membrane called the **mesometrium**, which carries the uterine blood vessels. When the embryos implant, they are orientated such that the ICM lies away from the mesometrial side of the uterus, which is the side on which the placentas will form.

The course of early postimplantation development is shown in Fig. 10.4b and c. After implantation the trophectoderm becomes known as the **trophoblast** and soon stimulates proliferation of the connective tissue of the uterine mucosa to form a **deciduum** (plural **decidua**). From this stage onward the embryo receives a nutrient supply from the mother and can begin to grow in size and weight. As in the chick, the zygote does not just form an embryo but also a complex of extraembryonic membranes and the whole is referred to as the **conceptus**.

The next stage of development is known as the **egg cylinder**. The cylinder itself can be regarded as homologous to the area pellucida of the chick embryo and consists of an "upper" layer of **primitive ectoderm** or **epiblast**, homologous to the chick epiblast, and a "lower" layer of **primitive endoderm**, homologous to the chick hypoblast. The terms "upper" and "lower" are in quotes because the embryo is actually the shape of a deep cup with the epiblast on the inside and the endoderm on the outside. It appears U shaped in a sagittal section or O shaped in a transverse section. Cells derived from the primitive endoderm move out to cover the whole inner surface of the mural trophectoderm and start to secrete an extracellular basement membrane known as Reichert's membrane containing laminin, entactin, and type IV collagen. These cells are called the **parietal endoderm**. The remainder of the primitive endoderm remains epithelial and forms a layer of **visceral endoderm** around the epiblast. The cells of this layer somewhat resemble the later fetal liver being characterized by the synthesis of α-fetoprotein, transferrin, and other secreted proteins. In addition to the epiblast, the inside of the egg cylinder also contains extraembryonic ectoderm derived from the polar trophectoderm. This extraembryonic region has now become the ectoplacental cone and as it proliferates it produces further layers of giant cells which move around and reinforce the trophoblast. In contrast to the differentiated extraembryonic tissues, the primitive ectoderm from which the entire embryo will be derived remains visibly undifferentiated.

At about E6.5 the anteroposterior axis of the future embryo becomes apparent with the formation of the **primitive streak** at one edge of the epiblast. The streak marks the posterior end of the future embryonic axis which will extend across the ectoderm toward the distal tip of the cup. It is a region of cell movements similar to that found in the chick embryo and these movements result in the formation of the **definitive endoderm** and mesoderm. By this stage the egg cylinder has become somewhat compressed so that the longer axis, as seen in transverse section, coincides with the anteroposterior axis of the embryo. The streak elongates until it has reached the distal tip of the egg cylinder and the **node** appears at its anterior end. This is homologous to Hensen's node in the chick and consists of two cell layers while the remainder of the streak has three. By E7.5 a head process appears anterior to the node consisting of a forming

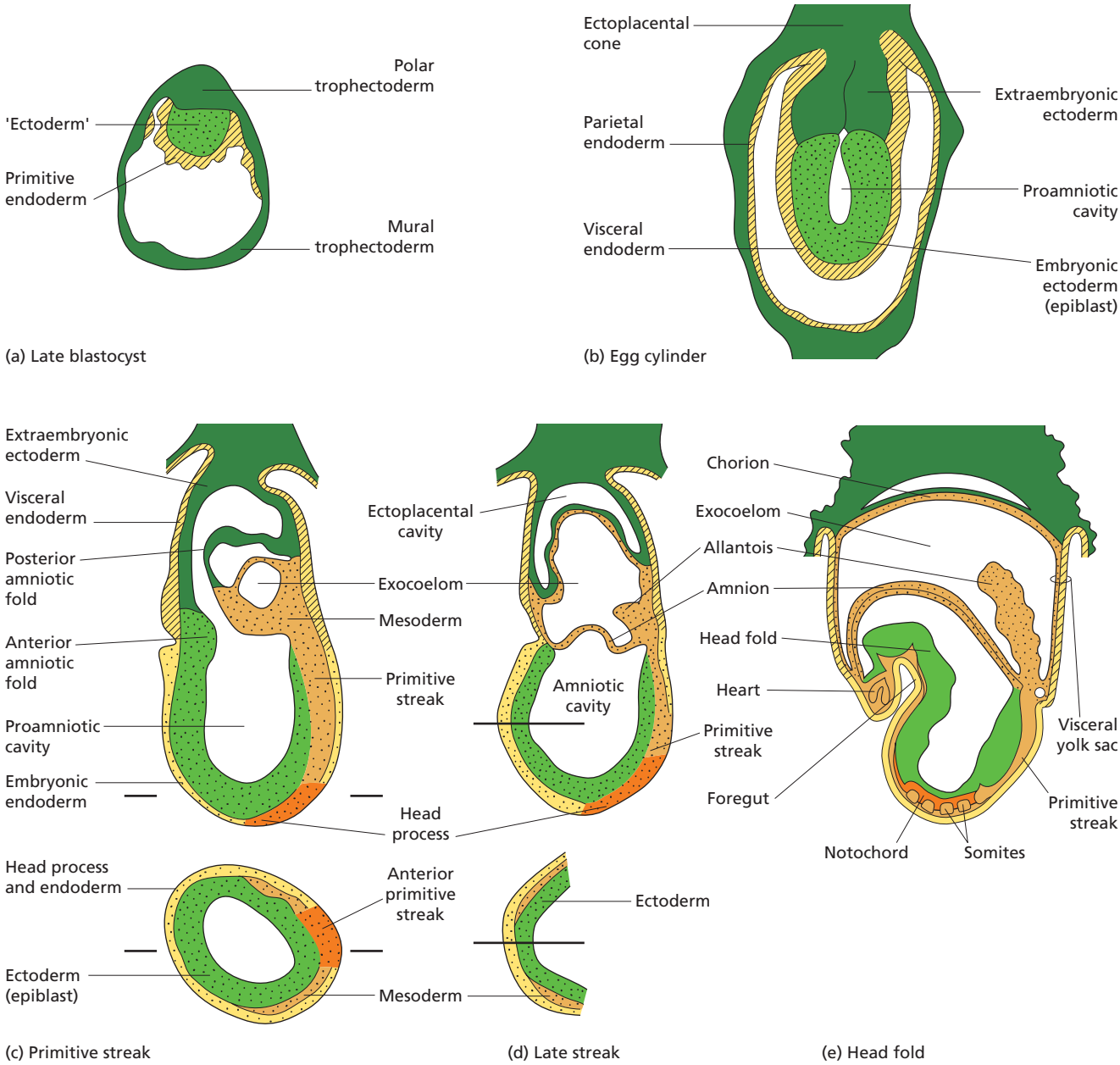

Fig. 10.4 Peri- and early postimplantation development. (c) and (d) show both sagittal and transverse sections at levels indicated by the lines.

notochord flanked by definitive endoderm in the lower layer and **neural plate** in the upper layer. As in the chick the node then moves posteriorly and the axial parts appear in anteroposterior sequence in its track. By E8.5 the embryo has elongated somewhat in length and a massive head fold has formed at the anterior end, mainly composed of the anterior neural tube. The **somites** begin to form from E8 in anteroposterior sequence, at the rate of about one somite per 1.5 hours.

Although at first sight the morphology of the mouse embryo and the lower vertebrates seems rather different, the homology of parts is clearly displayed by the gene expression patterns during gastrulation. The primitive streak and notochord express *brachyury* (usually called *T* in the mouse), with the posterior part expressing *cdx* genes. The node expresses *goosecoid*. The node, notochord, and floor plate express *FoxA2* (*hnf3β*). As far as inducing factors are concerned *nodal* is expressed in the node, *bmp4* in the surrounding ectoderm, and the BMP-inhibitor *follistatin* in the streak (Fig. 10.5).

The amniotic fold forms at about 7 days as an outpushing of the ectoderm and mesoderm at the junction of posterior primitive streak and extraembryonic ectoderm (Fig. 10.4c,d). The side of this fold nearer the embryo becomes the **amnion** and the side nearer the ectoplacental cone becomes the **chorion**. The fold pushes across the proamniotic space and divides it into three: an amniotic cavity above the embryo, an exocoelom separating amnion and chorion, and an ectoplacental cavity lined with

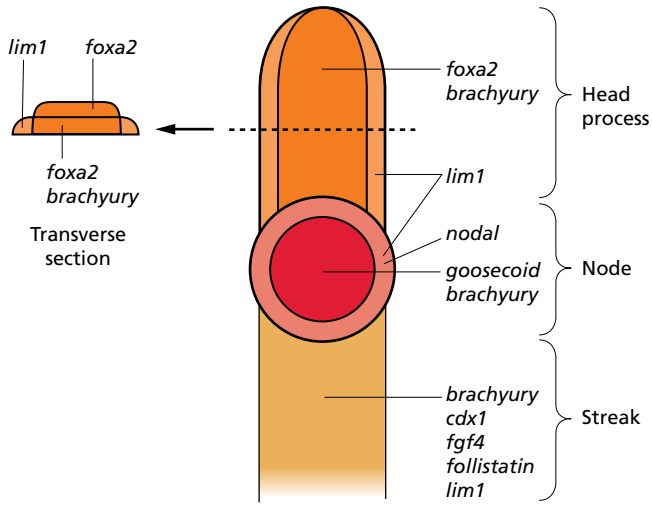

Fig. 10.5 Expression of genes at head process stage.

extraembryonic ectoderm. Into the exocoelom grows the **allantois**, consisting of mesoderm from the posterior end of the primitive streak. This grows to contact the chorion and later forms the embryonic blood vessels of the placenta. The placenta itself forms from the ectoplacental cone region which is directed toward the mesometrium. From the maternal side the mature placenta consists of the following layers: maternal decidual tissues; giant trophoblast cells with maternal blood sinuses; diploid trophoblast with fetal blood vessels; and finally crypts lined with extraembryonic endoderm (Fig. 10.6). The placenta is not only important as the organ supplying nutrients to the fetus, but also has endocrine functions, producing progesterone and estrogen to maintain the pregnancy as well as a number of other important hormones.

Around 8.5 days a most remarkable process takes place known as **turning**, which brings the germ layers into the proper orientation within the embryo. This is best described as a rotation of the embryo around its own long axis. It starts as a U-shaped structure with the dorsal side concave and the rotation

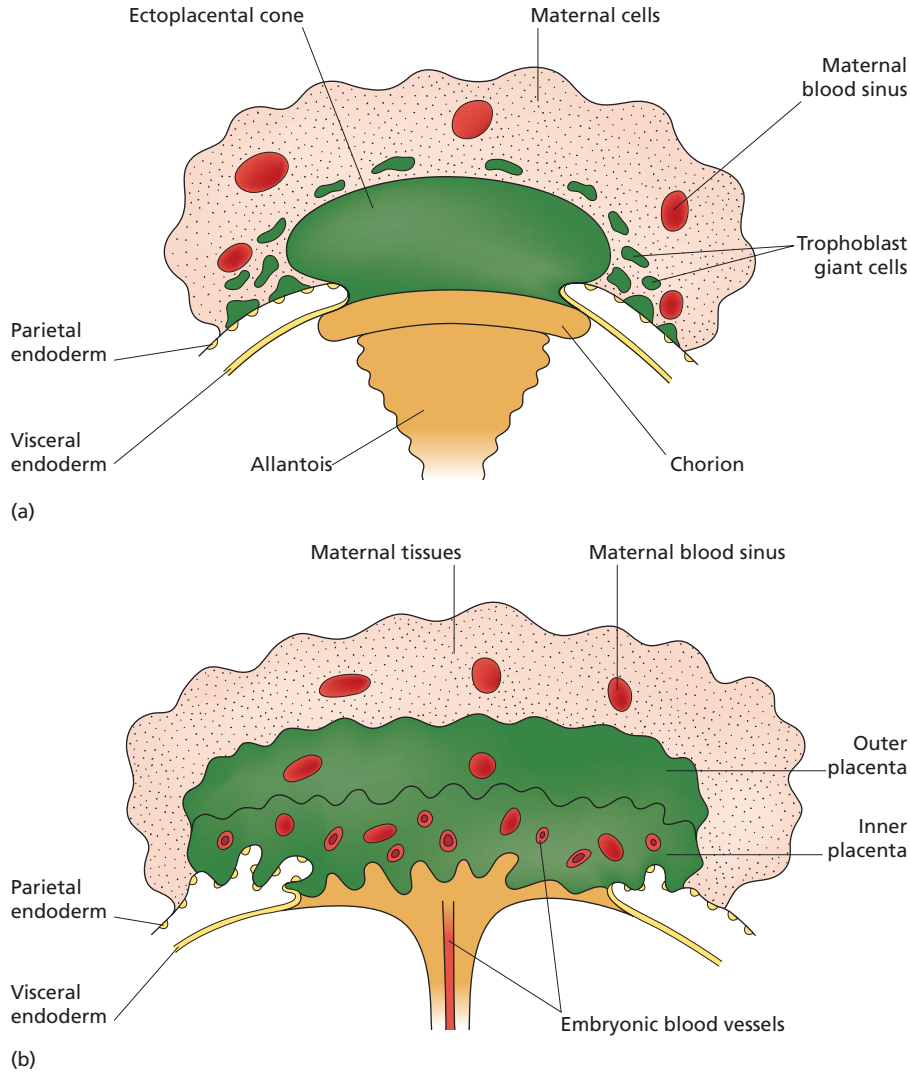

Fig. 10.6 Schematic view of placenta: (a) E8.5; (b) E14.5.

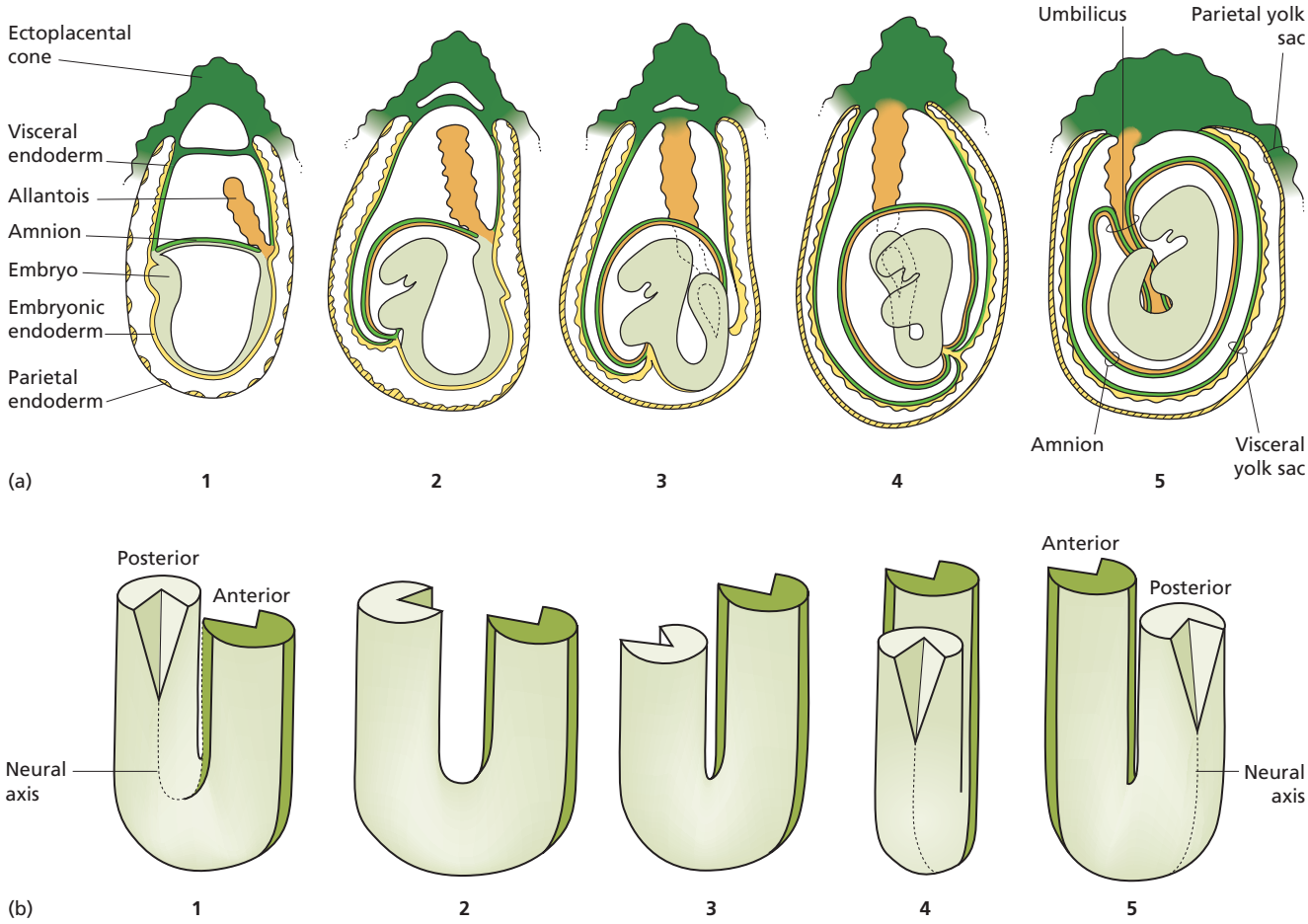

Fig. 10.7 Turning. (a) From about E7.5 to E9.5 the embryo becomes rotated around its long axis leading to ventral closure of the gut. (b) Diagrammatic representation of turning. (Both after M. Kaufman. In: Copp & Cockroft (eds) 1990. Postimplantation Mammalian Embryos. IRL Press, pp. 88–9.)

converts it into an inverted U with the dorsal side convex (Fig. 10.7). This change of orientation has drastic consequences for the arrangement of the extraembryonic membranes. The amnion becomes enlarged so that it surrounds the whole embryo instead of just covering the dorsal surface. The membrane lining the exocoelom, which consists of visceral endoderm and mesoderm, also becomes stretched to cover the entire embryo, and becomes known as the visceral yolk sac. The final arrangement of membranes after turning has the amnion on the inside, the visceral yolk sac next and the parietal yolk sac, formed from the trophectoderm lined with parietal endoderm, on the outside. Turning leads to a rapid closure of the midgut, which starts as a large area of endoderm exposed on the ventral side and becomes constricted to a small **umbilical tube** containing the vitello-intestinal duct, the vitelline vessels, and the allantois.

Organogenesis

By about E9.5, when the axis has formed, the embryo has turned, and the gut has closed, the embryo has reached the junction between the phase of body plan formation and of **organogenesis**. Organogenesis is in most respects very similar to that in the chick so only a brief sketch is given here with Fig. 10.8 showing exterior views of the embryo. Further details of organogenesis will be found in Chapters 14–16.

Neural tube closure takes place simultaneously with turning. It commences in the hindbrain and proceeds both anteriorly and posteriorly, with the anterior neuropore closing at E9 and the posterior neuropore at E10–10.5. The **optic vesicles** form at E9.5 and the lens has been incorporated into the eye by E11.5. The **neural crest** emerges from the neural tube over the period E8.5 to E10.5. As in the chick, it forms the skeletal structures of the head, the dorsal root ganglia, Schwann cells, sympathetic ganglia, pigment cells, adrenal medulla, and enteric ganglia. **Somites** continue to form until E14 by which time there are about 65, many being in the tail which is much longer than that of the chick. As in other vertebrates, the somites form the vertebrae and myotomes and contribute to the dermis. The paired heart primordia fuse at E8.5 and the heartbeat begins at E9. The left and right atria become separated at E11.5 and the ventricles at E14. The gut originates from fore- and hindgut pockets as in

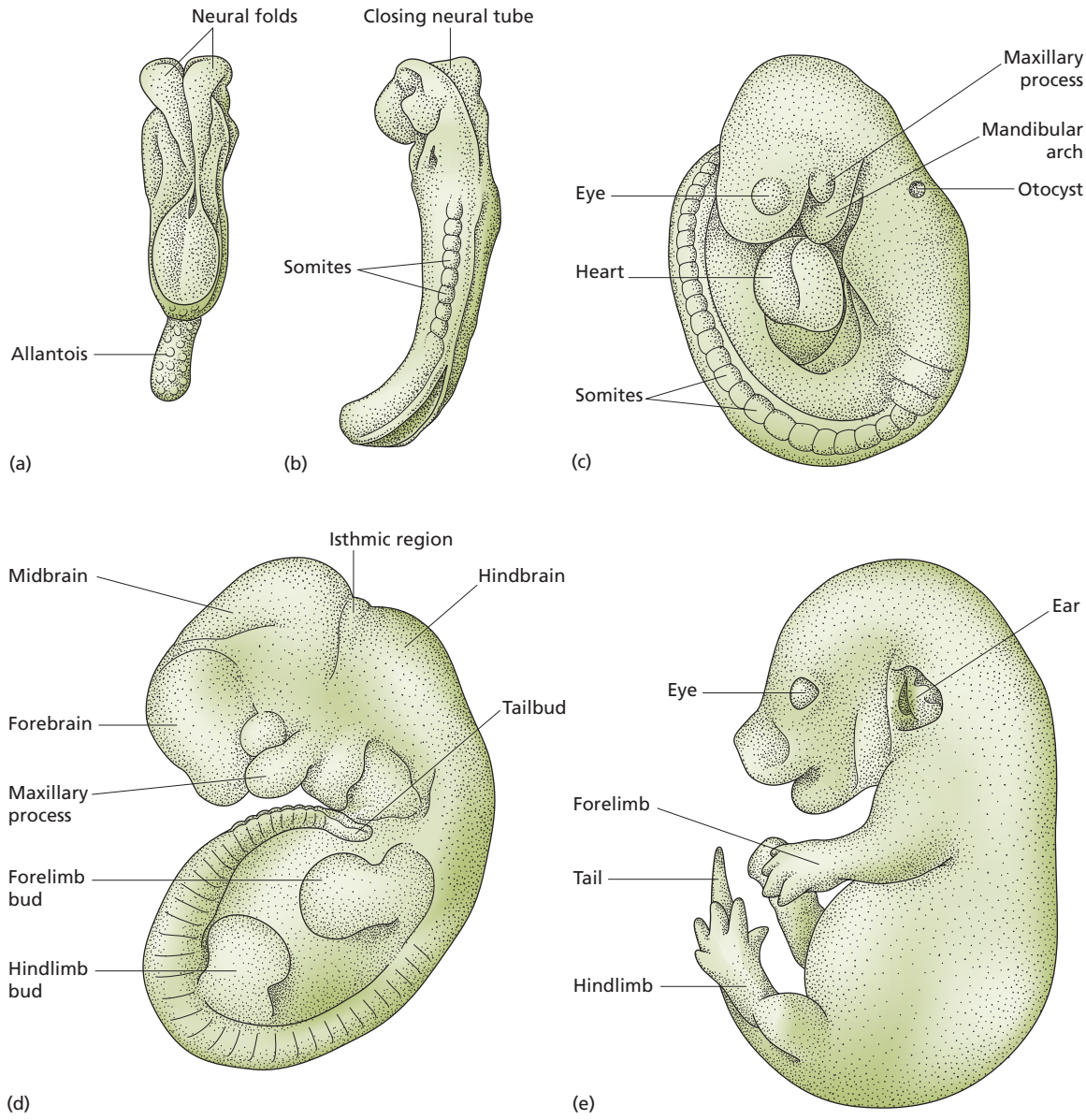

Fig. 10.8 Organogenesis stages: (a) E8; (b) E8.5; (c) E9.5; (d) E11.5; (e) E15.5.

the chick, although the closure of the midgut is faster because it is driven by the turning process. There are six **pharyngeal arches** forming from E9, although the fifth arch is vestigial. The mouth forms at E9.

The mesoderm lateral to the somites is the **intermediate mesoderm**, which becomes the kidney and gonads. The pronephric primordium arises about E9, but has no function. The **genital ridges** become visible from about E10 from mid-trunk to mid-tail. The lateral part of these ridges forms the mesonephros, although in mammals this too is vestigial and without function. The nephric duct produces the **ureteric bud** at E11.5 and this grows into the posterior nephrogenic mesoderm to produce the **metanephros**, which is the functional kidney. The medial part of the urogenital ridges produces the **gonads**. The **germ cells** originate from the extraembryonic mesoderm in the posterior amniotic fold. They enter the hindgut at E10 and migrate up the mesentery, reaching the gonads between E11 and E13. Lateral to the intermediate mesoderm is the **lateral plate**, which is divided by the coelom into the outer **somatopleure** (epidermis + somatic mesoderm) and the inner **splanchnopleure** (endoderm + splanchnic mesoderm). The limb buds arise from the somatopleure at E9.5–10 with the forelimb bud at the level of somites 8–12 and the hindlimb buds at the level of somites 23–28. The outer epidermis of the mouse, like other mammals, is covered with hair follicles, arising from E14 onwards.

It should be stressed that although the course of organogenesis is similar in most vertebrates, the arrangement of extraembryonic membranes can differ considerably even between

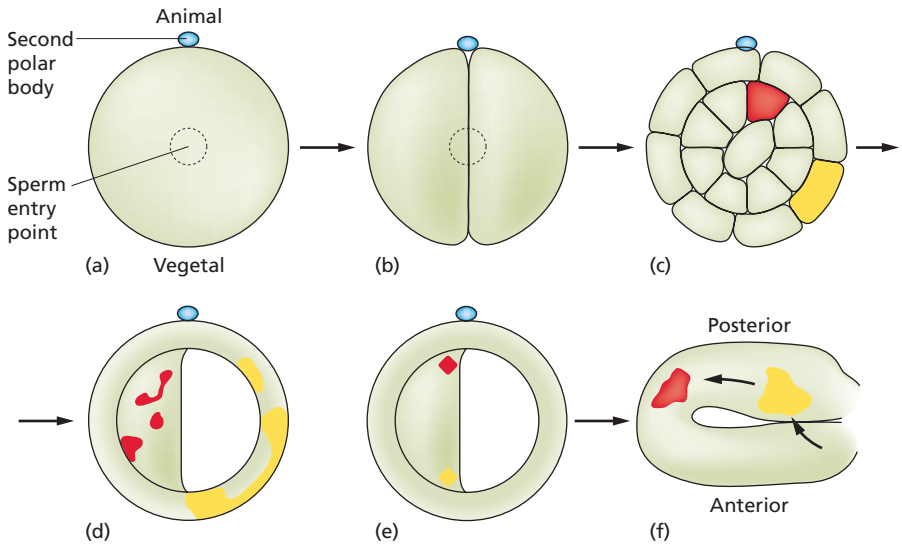

Fig. 10.9 Fate mapping of early stages. (a) The second polar body marks the animal pole of the zygote. (b) The first cleavage is meridional. (c,d) In the compacted blastocyst, labeling of inner cells shows that they enter the inner cell mass, while the outer cells enter the trophectoderm. (e,f) In the inner cell mass, labeled animal cells become distal and labeled vegetal cells become proximal in the egg cylinder.

different groups of mammals. In particular, rodents are unusual in having their deep cup-shaped egg cylinder and the associated turning process. In most mammals the embryo develops more like the chick, from a flat plate of epiblast. Particular features of human development that differ from the mouse are that the amniotic cavity arises precociously within the ICM shortly after the blastocyst has commenced implantation, and that the allantois is vestigial, the embryonic blood vessels being formed from extraembryonic mesoderm lining the chorion.

Fate map

It used to be believed that mammalian embryos, with their high regulative capacity, had no definable fate map at the stage of the fertilized egg. However it is now recognized that the fertilized egg does possess a polarity which is retained through the early embryonic stages (Fig. 10.9a–d). This has been established by a combination of careful observation and the labeling of portions of the egg surface with fluorescent lectin-coated beads and other markers. The original **animal pole** of the egg can be identified by the position of the second polar body. The first cleavage is approximately meridional and separates blastomeres that will preferentially become the embryonic (i.e. the end with the inner cell masss) and abembryonic (the end of the mural trophectoderm) poles of the blastocyst. There is also some evidence for a coincidence of the first cleavage plane with the site of sperm entry.

Labeling of individual blastomeres of early preimplantation embryos by injection of horseradish peroxidase (HRP) or fluorescent dextrans shows that the trophectoderm arises from the polar cells on the exterior of the morula, while the inner cell mass arises from the apolar cells in the interior. By the time the blastocyst expands, there is no further interchange of cells between these two populations. In blastocysts, single labeled cells of the polar trophectoderm give rise to clones of postmitotic cells in the mural trophectoderm, showing that the polar region is a proliferating zone feeding the mural trophectoderm. The ultimate fate of ICM and trophectoderm was shown by reconstitution of blastocysts from ICM and trophectoderm of genetically distinguishable mouse strains followed by reimplantation into foster mothers. This showed that the ectoplacental cone, the giant cells, and the extraembryonic ectoderm of later stages were derived solely from the trophectoderm. The entire fetus, the amnion, allantois, and extraembryonic endoderm were derived from the inner cell mass.

There is also a predictable relationship between the axes of the **blastocyst** and the later **egg cylinder** stage (Fig. 10.9e,f). If the cells on the animal pole end of the blastocyst are labeled by injection of mRNA for green fluorescent protein (GFP) it is found that they end up distally in the visceral endoderm of the egg cylinder. Conversely if ICM cells at the vegetal pole end are labeled, they end up proximally in the egg cylinder. This distal–proximal distribution of cells arises from the early stages of the morphogenetic movements which form the primitive streak of the embryo. It follows from this that the original animal pole of the zygote is likely to end up as the posterior side of the embryo, i.e. the side of the forming primitive streak, when it becomes visible in the egg cylinder stage.

Fate mapping of postimplantation stages mostly involves labeling the embryo by injection of cells with HRP, or extracellularly with DiI, then culturing them *in vitro* for up to 48 hours (Fig. 10.10). These studies show that the primitive streak and the mesoderm of the amnion and allantois all arise from the posterior edge of the epiblast. The streak pushes distally and the extraembryonic tissue expands into the proamniotic space, mainly driven by growth but also by some lateral movement of cells around the cup of epiblast. The anterior part of the streak becomes the node, which forms the notochord and part of the somites along the entire length of the body. Cells also enter the somites and the neural plate from positions lateral to the midline. The middle part of the streak populates mainly lateral plate

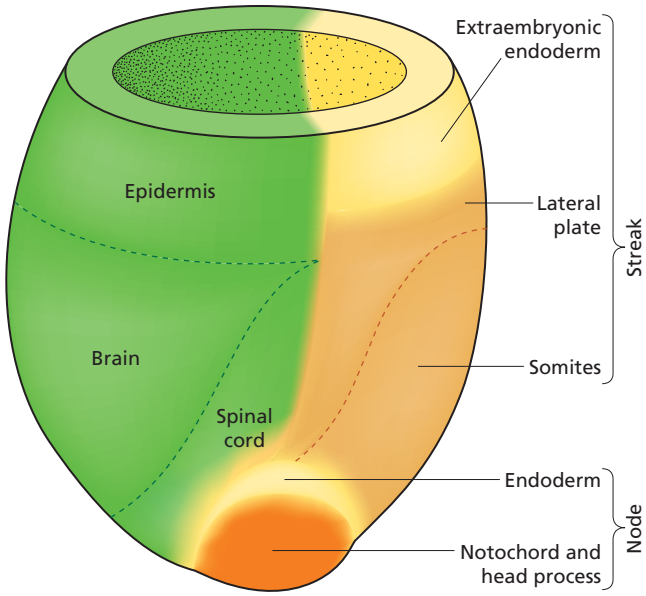

Fig. 10.10 Fate map of the late streak stage. The boundaries are much fuzzier than shown, because of cell mixing.

mesoderm in the posterior half of the body. The posterior part of the streak populates mainly mesoderm of the amnion, visceral yolk sac, and allantois. It seems that the primitive streak in the mouse works in a similar way to that of the chick with cells from the surface layer moving laterally towards and then through the streak to become definitive endoderm and mesoderm. But all the studies show a substantial degree of mixing between labeled and unlabeled cells showing that the fate map is a statistical construction and that the boundaries between prospective regions are not precise.

Technology of mouse development

Transgenic mice

The creation of mice with an engineered genetic constitution has become a substantial industry, with applications in many branches of biology. Sometimes the term **transgenic** is used to include all forms of modification, here it will be used more narrowly to indicate introduction of new genes into the germ line. The standard method is to inject the DNA directly into one **pronucleus** of the fertilized egg (Fig. 10.11). This leads to a reasonable yield of transgenics with a good probability that the germ line will be transgenic. Normally integration is at a random position in the genome and comprises many copies of the transgene joined head to tail in a tandem array. Better levels of gene expression are achieved if the gene to be injected is purified away from plasmid DNA as the prokaryotic sequences are usually inhibitory to transgene expression. It is also important to use genomic DNA containing introns, as this gives better expression

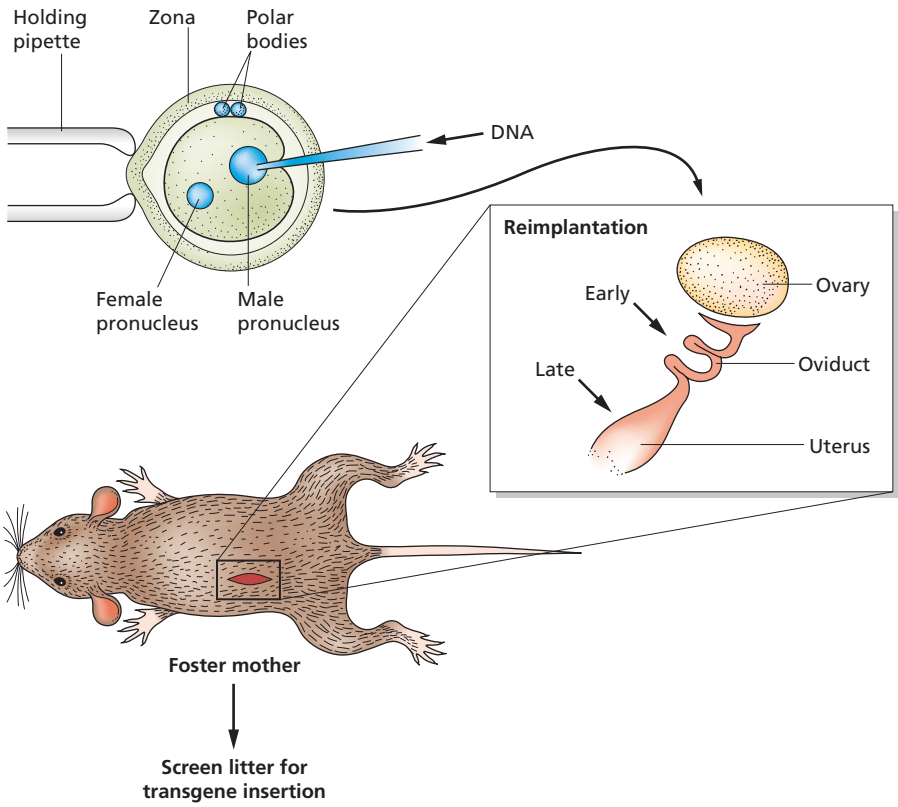

Fig. 10.11 Making transgenic mice by injection of DNA into a pronucleus of the fertilized egg.

levels than complementary DNA (cDNA; i.e. DNA copied from mRNA, without introns). Normally, linear rather than circular DNA is preferred for injection. In addition to the simple pronuclear injection method, it is possible to introduce genes into embryos via embryonic cells, following the methods used to knock out genes (see below), and gene introduction has also occasionally been achieved using retroviruses.

The newborn mice arising from a transgenesis experiment will be screened for incorporation of the gene, by performing a genomic **Southern blot** or **polymerase chain reaction** (**PCR**) on a DNA sample from the tail tip of each individual. A transgene will usually behave as a simple dominant gene in subsequent breeding. It may be desirable to breed it to homozygosity so that all embryos from the line are known to possess the gene.

It is known that, despite the abnormal chromosomal location of transgenes, they can show correct temporal and regional expression so long as sufficient flanking DNA sequences are included. A very large amount of work has been performed in which the flanking sequences of the genes are modified in order to identify the particular regulatory sequences responsible for control of their expression. For this type of work it may be sufficient to use transient transgenics. This means that the injections are done, the embryos reimplanted, and then later recovered and analyzed directly without any breeding to set up a permanent line of animals. Instead of monitoring the normal gene product, such studies are usually done using a **reporter gene** whose product is more easily detected. Commonly used reporter genes are ***lacZ*** and ***luciferase***. The β-galactosidase coded by the *lacZ* gene can easily be detected in wholemounts or sections using the X-Gal reaction. Luciferase is an enzyme from an insect that will convert a specific substrate, luciferin, into a phosphorescent product. It can be measured in tissue samples with very high sensitivity using a luminometer.

By making the appropriate molecular constructs it is possible to express the coding region of one gene under the control of the promoter of another, and this enables **ectopic** expression of specific genes, and the resulting modification of the course of development. Sometimes uniform expression is required, which can be achieved using promoters for housekeeping genes such as *cytoskeletal (β) actin* or *histone H4*. Sometimes a particular stage- or region-specific promoter is required to drive expression in just that tissue or position. It is also possible to ablate a particular region of the embryo by expressing a toxin, such as diphtheria toxin, under the control of a suitable promoter. Transgenes may also be made **inducible**, so they can be activated at a particular stage by a treatment given by the experimenter (see below).

Mosaicism and chimerism

Among mammals an animal composed of cells which are genetically dissimilar is called a **mosaic** or a **chimera**. Usually the term "mosaic" is used if the animal has arisen from a single zygote, and "chimera" if it has arisen from some experimental or natural mixture of cells from different zygotes. One type of naturally occurring mosaic is the **X-inactivation** mosaic**,** described below. Genetic mosaics should not be confused with **mosaic** development, an entirely different concept discussed in Chapter 4.

There are two experimental methods for making a chimera (Fig. 10.12). **Aggregation chimeras** are made by removing the zona pellucida of four- to eight-cell-stage embryos, then gently pressing them together so that the cells adhere. The fused embryo is reimplanted into the uterus of a pseudopregnant recipient. Such embryos show normal development and consist of a mixture of the cell types of the two components. Injection chimeras are made by injecting cells into the blastocoelic cavity of expanded blastocysts, with zona intact. Again they are implanted in foster mothers for further development. It is often possible to detect chimerism by examination of the coat pattern, if the two mouse strains used have different coat colors. Although the pigment cells themselves are derived from the neural crest, the expression of pigment in the hair follicles will depend also

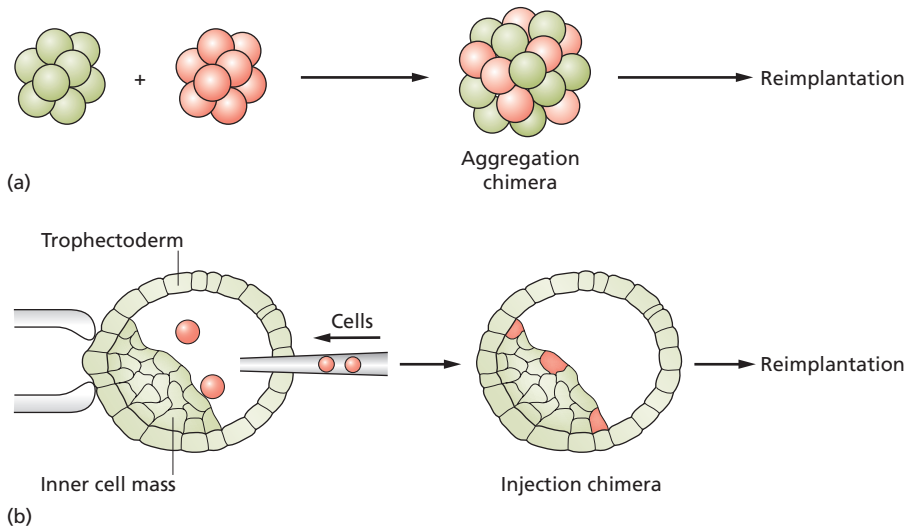

Fig. 10.12 Making chimeras: (a) by aggregation; (b) by injection of cells into the blastocyst.

on the local genetic composition of the epidermis. Therefore chimeras will often be blotchy in coat pattern, or at least have some admixture of the hair colors of the two contributing strains. Injection chimeras have enormous practical importance because they represent an important step in the production of knockout mice.

In addition both types of chimera, and also X-inactivation mosaics, can be used for the **clonal analysis** of structures in the mature mouse. If a particular type of structure is always entirely composed of one of the two genotypes, then it must normally be formed by a single cell. If it contains patches of the two genotypes, then it is possible to calculate the probable number of progenitor cells, allowing for the fact that some similar clones will be adjacent. This type of analysis has been carried out for hair follicles and for intestinal crypts (see Chapter 13).

Clonal analysis can also be carried out using transgenics for a modified form of the *lacZ* gene called *laacZ*. This gives an inactive product due to the insertion of a repeated sequence into the coding region. Intragenic recombination can restore the functional *lacZ* sequence and this happens spontaneously at low frequency. Once the recombination event has occurred all progeny cells will express β-galactosidase permanently so this can be used as a method for random labeling of clones.

Embryonic stem cells

Cell lines showing developmental **pluripotency** can be produced by putting mouse blastocysts into culture. After the blastocyst hatches from the zona it will adhere to the substrate and, in a suitable medium, the ICM cells will proliferate. The resulting cells are **embryonic stem cells** (**ES cells**). These resemble the ICM and the epiblast of the normal embryo, but microarray analysis shows that they are slightly different from both. They can be cultured for many passages and be frozen for later use. They are grown on **feeder layers** of irradiated fibroblasts or in the presence of leukemia inhibitory factor (LIF). When the feeder cells or the LIF are removed, the ES cells will differentiate into **embryoid bodies** in which the outer layer of cells resembles the primitive endoderm. In the normal embryo, LIF is expressed in the trophectoderm and the receptors LIFR and gp130 are present in the inner cell mass.

ES cells can be established from any strain of mouse, although most are from strain 129. The stem-cell property depends on the transcription factors Oct4 (POU class) and Nanog (homeodomain), as knockouts of their genes result in cells that can form differentiated extraembryonic cell types but will not self-renew. ES cells are usually of normal karyotype, although this can become abnormal on prolonged culture. When implanted into immunologically compatible adult mice, ES cells form tumors containing several differentiated tissue types; and, more significantly from the developmental point of view, when ES cells are injected into blastocysts they will colonize the resulting embryos giving a high frequency of chimerism. In many cases the chimerism extends to the **germ cells**, making it possible to breed intact mice from cells that have been grown in culture (Fig. 10.13). The existence of ES cells shows that it is possible to disengage growth from developmental commitment, as lines have been passaged as many as 250 times without loss of the ability to repopulate embryos.

The main practical importance of ES cells is that they offer a sophisticated route for the reintroduction of genes into an embryo. Although genes can be introduced by simple injection of DNA into the zygote, this offers no control over the number of copies introduced or the location of the insertion site. By contrast, with ES cells in culture it is possible to select for rare events, particularly **homologous recombination** of exogenous DNA into the complementary site in the genome. This has mainly been used to **knock out** individual genes by replacing them with an inactive variant, but there are many other possibilities such as replacement of one gene by another, or the assembly of complex binary and conditional gene regulation systems.

Knockouts

In developmental biology, the main object of knocking out individual genes is an expectation that the null phenotype will reveal the gene's function. Usually the product of the gene in question is believed to be important for some reason, such as its biochemical activity, or expression pattern, or the existence of a developmental function for the homolog in another organism, such as *Drosophila*. But knockouts are also useful to create mice mutant for particular gene variants responsible for human genetic diseases. This can create an **animal model** of the human disease, so that further information can be gained about the pathology of the disease, and strategies for therapy can be tested. An example would be the creation of mice mutant for the cystic fibrosis transmembrane conductance regulator (CFTR protein) to serve as a model of human cystic fibrosis. Another is a mouse lacking the gene for apolipoprotein B, which develops atherosclerosis over 2–3 months. A further application in medical research is the creation of mice especially susceptible to cancer, for example by removal of tumor-suppressor genes such as the *p53* gene.

Most genes have been knocked out using the positive–negative method, which is a selection procedure for homologous rather than random integrations. A targeting construct is assembled that consists of genomic DNA for the region to be replaced, with an essential functional region of the gene replaced by an antibiotic-resistance gene, usually neomycin resistance (*neo*^{r}). Flanking this is a copy of a viral gene coding for thymidine kinase (*tk*). The construct is transfected into the ES cells and if it integrates by homologous recombination, only the *neo*^{r} will be incorporated (Fig. 10.14a). If it integrates at random, in the wrong place, both *neo*^{r} and *tk* will probably be incorporated (Fig. 10.14b). Then the cells are subject to selection using two drugs. **Neomycin** will kill the cells that have not incorporated the targeting vector at all, as the host ES cells are sensitive to it (the feeder cells are resistant). **Ganciclovir** will kill cells that have incorporated the construct in such a way that the *tk* gene is

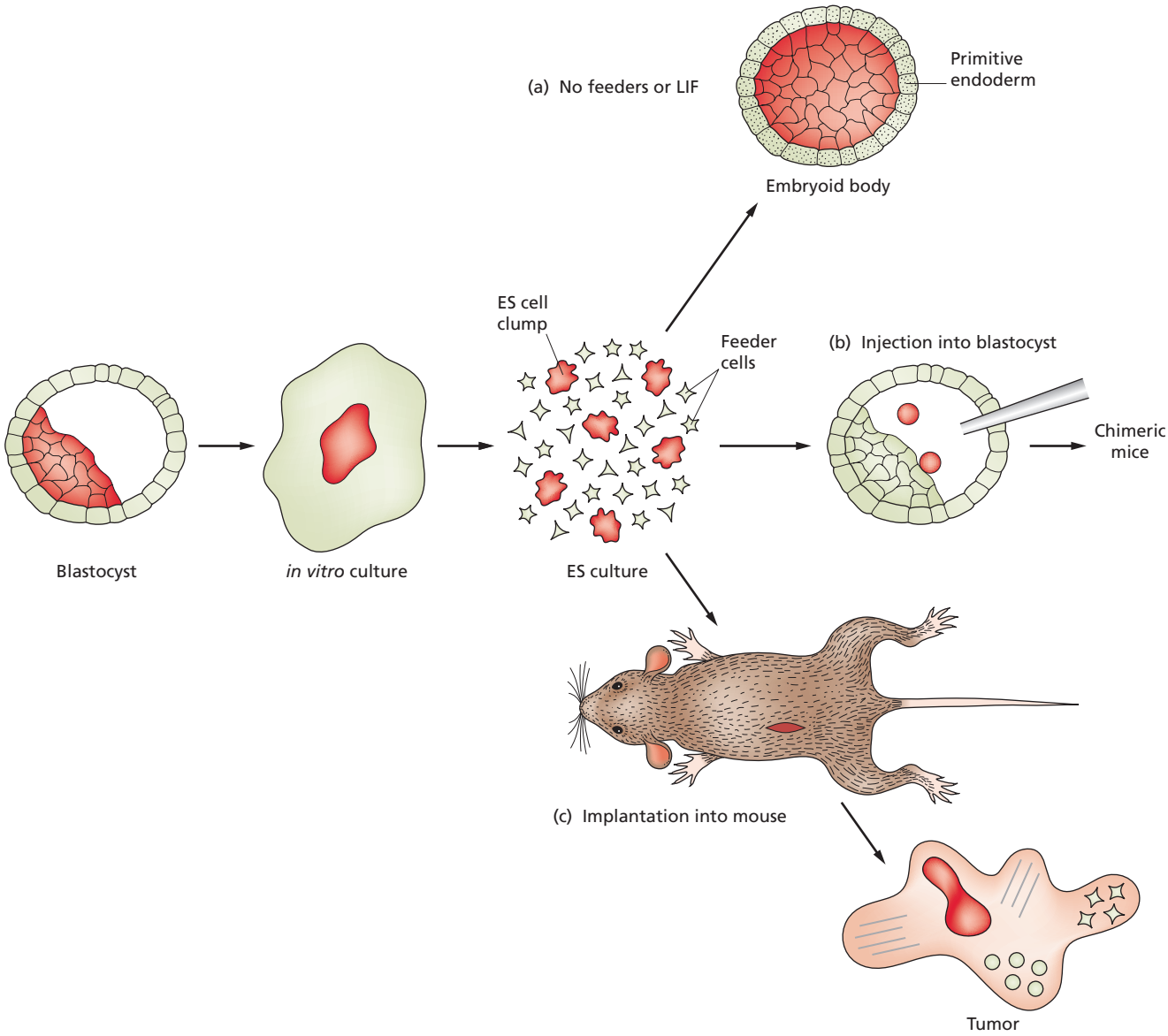

Fig. 10.13 ES cells. (a) Removal of feeder cells will cause differentiation *in vitro* into "embryoid bodies." (b) Injection into blastocysts can generate chimeric mice. (c) Implantation under the kidney capsule can produce a teratocarcinoma.

present. This is because the thymidine kinase converts the drug into a cytotoxic product. The net effect is that the surviving cells are often ones that have undergone homologous recombination such that the target gene has been exactly replaced by the inactive version. These cells are grown up as individual clones and are screened by Southern blotting or PCR to ensure that the targeting construct has indeed integrated at the predicted position (Fig. 10.15). Then the cells are injected into host blastocysts and the embryos reimplanted into foster mothers. The strain of the host embryos will be chosen such that the coat color differs from that of the parent strain of the ES cells. Most ES cells are from strain 129 mice, which have an agouti coat color (mixed black/yellow, like many wild mammals), so a black or albino strain is preferred for the host blastocysts. The chimerism should then be indicated by a patchy coat color, and this is confirmed by analysis of DNA from tail tips. Chimeric mice may or may not have any of the donor cells in their germ line, so they are mated and the resulting offspring also subject to tail-tip DNA analysis. If the F1 generation do contain the targeting construct, then they can be mated together to generate homozygotes, and the phenotype of the null mutation can be established.

Exactly the same technology can be used to perform "**knock-ins**." Here instead of replacing the endogenous gene with an inactive version, it is replaced by another gene, maybe a modified version of itself. The knock-in method has an advantage over traditional transgenesis because the insertion site is defined and so there should be no unpredictable consequences of random insertion.

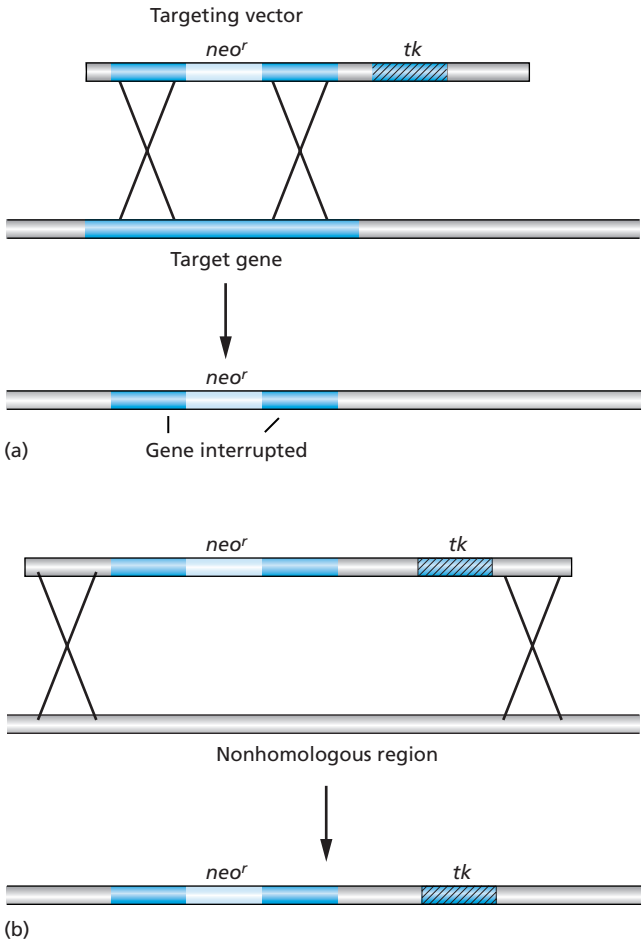

Fig. 10.14 Gene targeting. (a) Recombination at the homologous site disrupts the host gene and introduces *neo*^{r} but not *tk*. (b) Recombination at a nonhomologous site introduces both *neo*^{r} and *tk*.

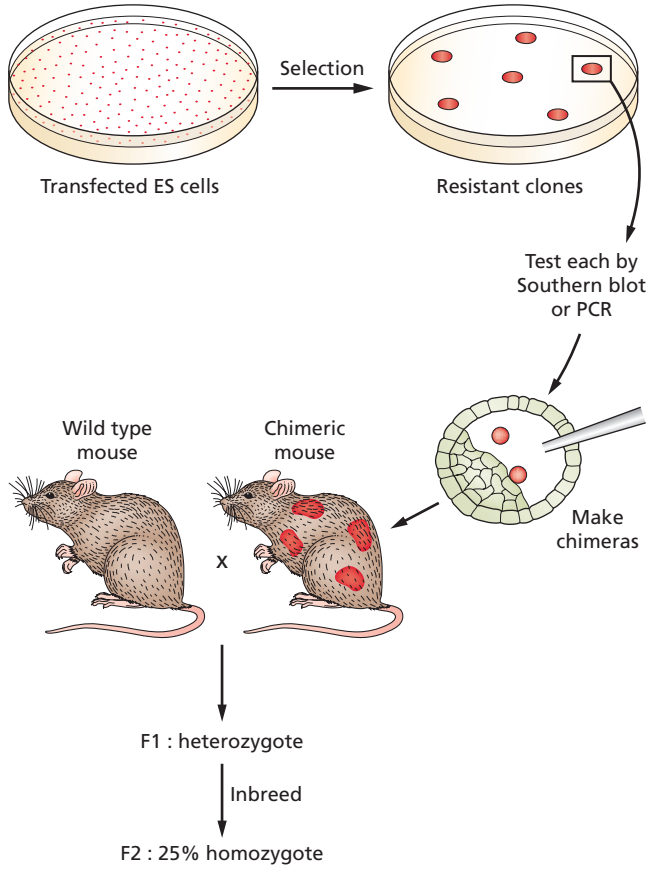

Fig. 10.15 Procedure for making a gene knockout via homologous recombination into ES cells.

Various problems have been encountered with the use of knockout mice. Firstly, there may be no abnormality of phenotype, or a barely perceptible abnormality. This is usually ascribed to **redundancy**, or the presence of other genes in the genome with overlapping functions, which is very common in vertebrates because of the large number of multigene families (see also Chapter 3). In order to establish the function of a set of genes showing significant redundancy it is necessary to knock them all out and then assemble a multiple homozygous null by mating of the individual knockout lines together. Considerable work of this sort has been done, for example on **Hox gene paralog** groups and on the various retinoic acid receptors. Secondly, the effects of the knockout may vary considerably depending on the genetic background, or strain of mouse into which the mutation is introduced. Although it may complicate the functional analysis this can be a useful property as it can help to identify other interacting genes important for the process under investigation. Finally, the phenotype may be so severe that the embryos die at an early stage which precludes any examination of later functions of the same gene. There are various solutions to this problem. One is to use a **conditional knockout** strategy as discussed below. Another is to make **chimeras** in which the gene is knocked out in only part of the conceptus.

Chimeric knockouts

The usual application of the chimera method is the case where a gene is required at an early stage in the extraembryonic tissues and at a later stage in the embryo itself, so the early death is due to failure of nutritional support from the extraembryonic tissues. It turns out that when ES cells are injected into a **tetraploid** blastocyst, the ES cells mainly form the embryo and the tetraploid cells mainly form the extraembryonic tissues. Tetraploid embryos are made by electrofusion of the blastomeres at the two-cell stage and are not themselves viable in the long term. To establish the postimplantation function of a gene, homozygous null ES cells, or ICM cells from a homozygous null blastocyst, are injected into tetraploid blastocysts, which are implanted into foster mothers. The tetraploid-derived extraembryonic structures support development until the gene is needed in the embryo itself, and from this stage an identifiable defect will arise which will hopefully reveal something of the normal gene function. An example is the case of *fgf receptor 2*. If

knocked out, this causes preimplantation death, due to failure of proliferation of the polar trophectoderm. But a chimeric embryo, composed of *fgfr2*$^{-}$ cells in a wild-type tetraploid blastocyst, will develop to about E10.5, and at this stage it is clear that the embryos lack both lungs and limbs, showing that FGF signaling through this receptor is needed for the formation of both these organs.

Conditional systems

In a conditional system the desired genetic change occurs only when the appropriate *condition* is achieved. The most commonly used conditional system is the ***cre-lox*** system. Cre (pronounced "cree") is a recombinase enzyme from phage P1 which can excise segments of DNA flanked by binding sequences called *loxP* sites. In order to knock out a gene in just one tissue, and thereby circumvent problems of early lethality, two mouse lines need to be created. The first is a line in which the target gene has *loxP* sites inserted on either side (the gene is said to be "**floxed**"). The second is a transgenic line in which the Cre recombinase is driven by a tissue-specific promoter. When the two types of mice are mated together, the target gene should be excised in the offspring, but only in the tissue containing the Cre (Fig. 10.16). In practice the Cre does not give a 100% effective excision, so it is best if the parent with the floxed target gene is a heterozygote with the second copy of the gene inactive.

The utility of the *cre-lox* system is not confined to tissue-specific knockouts. It can also be used for a tissue-specific activation of a gene if the Cre is used to excise an inhibitory segment. For example, a short polypeptide with termination codon can be inserted at the beginning of the coding region and be flanked with *loxP* sites. When this is removed by the Cre, the gene will become active. A similar effect could be achieved more simply by driving the gene off a tissue-specific promoter in a conventional transgenic, but the *cre-lox* system enables greater sophistication. For example, if it is desired to know what will normally be formed by a particular group of cells that activate a particular promoter at a particular stage, then a mouse can be assembled in which the promoter in question drives the Cre, and the Cre activates a reporter gene, such as *lacZ*, by excision of an inhibitory segment (Fig. 10.17). In such a mouse, the descendant cells of those in which the promoter was turned on will continue to express β-galactosidase thereafter, regardless of subsequent changes in the state of the tissue-specific promoter. An example of this strategy is the proof that all cell types of the pancreas come from endoderm expressing the transcription factor Pdx1 (see chapter 16).

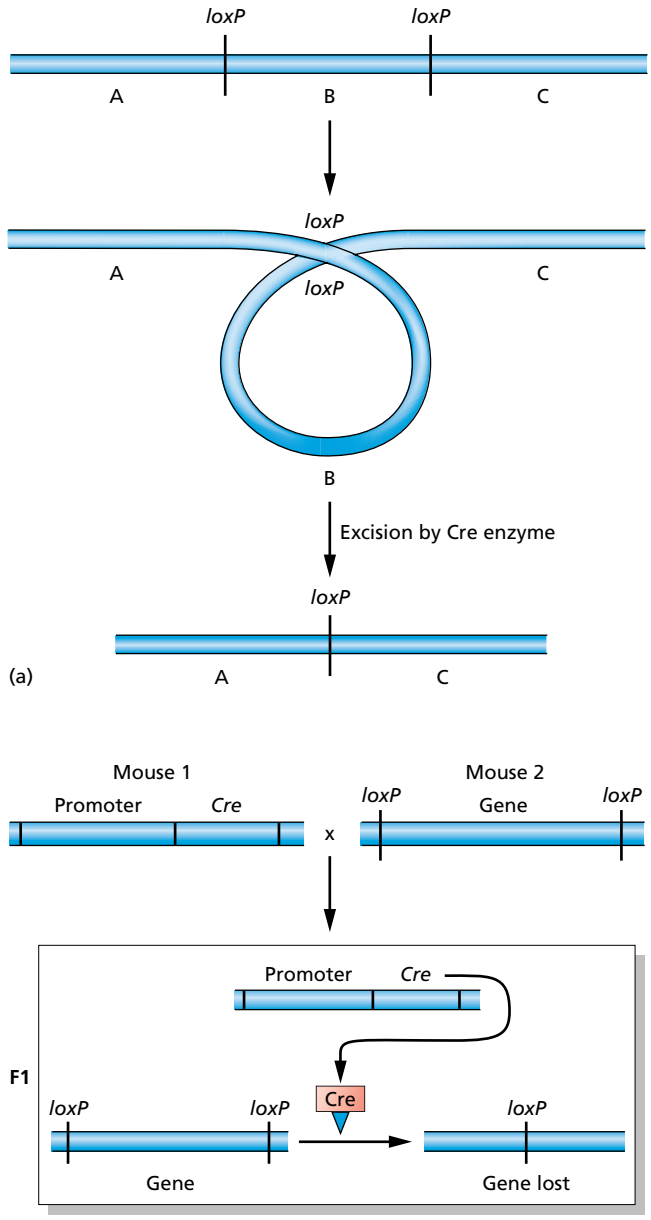

Fig. 10.16 *Cre-lox* system. (a) The *cre* recombinase will excise DNA between two *loxP* sites. (b) A binary mouse. When mouse 1 and 2 are mated, the *cre* recombinase is expressed according to the specificity of the promoter to which it is coupled and will drive excision of the gene between the *loxP* sites.

Inducibility

Another important element of mouse embryo technology that can be incorporated both into the conventional transgenic, or into a binary combination, is inducibility. Early experiments used the *metallothionein* promoter, which is activated by heavy metals such as zinc or cadmium. But this is not tissue specific, and it is often desirable to confer inducibility on an otherwise noninducible, but tissue-specific, promoter. The preferred system uses elements of the tetracycline system from *Escherichia coli*. The enzymes that degrade tetracycline are coded by genes of the *tet* operon. In the absence of tetracycline the operon is repressed, but in the presence of tetracycline it becomes activated. The system works because the tetracycline combines

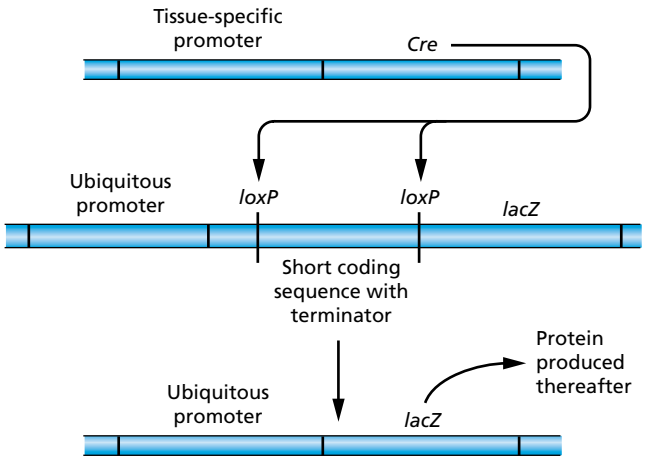

Fig. 10.17 Use of the *cre-lox* system to label permanently a population of cells in which the tissue-specific promoter was active only transiently.

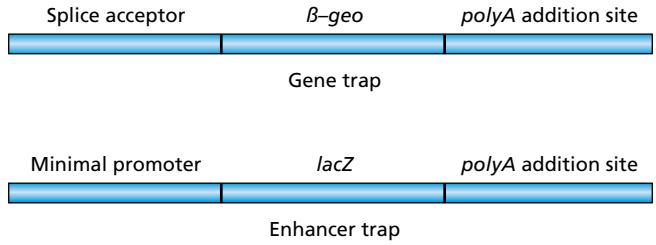

Fig. 10.19 Gene trap and enhancer trap.

with a Tet repressor protein (TetR), and thereby causes it to dissociate from the *tet* operator (*tetO*) sequence in the DNA, enabling transcription to take place.

This system has been modified, and improved in specificity, by converting the TetR into an activator (TetA) in which its normal repression domain is replaced by the VP16 activation domain (Fig. 10.18). In this variant, called "**tet-off**," the gene is inactive in the presence of tetracycline but, when the tetracycline is withdrawn, the TetA will bind to the *tetO* and activate transcription. In a transgenic situation, the *tetO* will be combined with the tissue-specific promoter driving the target gene and the TetA will be expressed from a constitutive transgene elsewhere in the genome. Tetracycline is supplied continuously to the pregnant female mice by including it in their drinking water, so the TetA is sequestered and the target gene remains off. If it is desired to activate the target gene on a particular day of development, then the tetracycline is withdrawn. It becomes cleared from maternal and fetal circulation within a few hours, the TetA is able to bind the *tetO*, and the target gene becomes activated.

It is possible to make the *cre* system inducible by using a fusion of the Cre enzyme with the estrogen receptor (Cre-ER). In the same way as discussed for the glucocorticoid receptor in Chapter 7, the nuclear hormone receptors like ER are sequestered in the cytoplasm by binding to heat shock proteins. When their ligand is supplied this will displace the heat shock protein and allow migration of the ER fusion protein to the nucleus. Cre-ER can still be driven by a tissue-specific promoter but the time at which it becomes active is controlled by injecting the mother with a suitable receptor ligand, usually the synthetic estrogen **antagonist** tamoxifen.

Gene and enhancer traps

The knockout technology is very powerful, but it can only be applied to identified genes. New genes need to be identified by mutagenesis. This may be chemical mutagenesis, as described in Chapters 3 and 8, followed by positional cloning of the genes. It can also be useful to be able to carry out **insertional mutagenesis** with a suitable DNA vector, as this allows easy identification of the modified locus. The relevant constructs are called **gene traps** and contain a splice acceptor site, a reporter gene, and a selectable marker (Fig. 10.19). Often the reporter and selectable marker are a single gene called ***β-geo***, which is a fusion of *E. coli lacZ* and the neomycin-resistance gene, having the activities of both. The construct is transfected into ES cells. If it integrates outside a gene it remains inactive because it has no promoter of its own. If it integrates within a gene it should function as the last exon, with the splice acceptor enabling incorporation into the coding sequence. Because the endogenous gene is disrupted by the insertion, it is likely to be mutated to inactivity or reduced activity. The β-geo will be expressed as a fusion on the C-terminal end of the truncated endogenous protein, and if the splicing is in frame then the cells should be selectable by

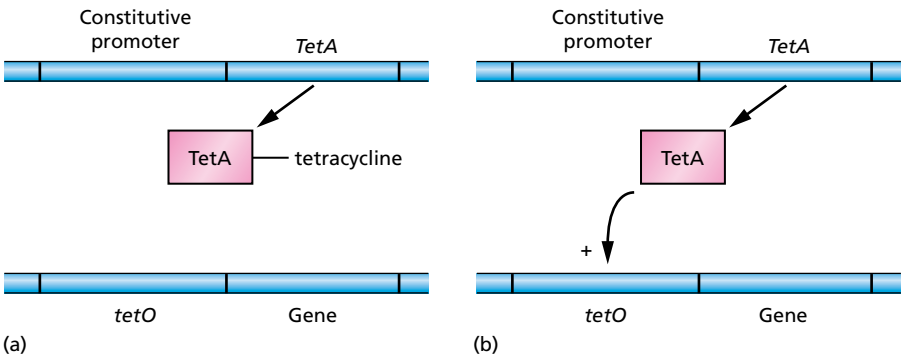

Fig. 10.18 Tet-off system. In the presence of tetracycline the target gene is inactive. When tetracycline is removed, the target gene is activated.

neomycin resistance, and should also express β-galactosidase. There are more sophisticated versions of this method that will work even if the insertion is not in frame.

The gene trap is transfected into ES cells, then introduced into mice by the same procedure as used for knockouts. It is often possible to examine the normal expression pattern of the gene by doing X-Gal staining on the chimeras themselves. Although only some of the cells will carry the construct, it may be enough for this purpose. If the expression pattern looks interesting, the chimeras will be bred to establish an F1 generation heterozygous for the gene trap, and these will be mated with each other to establish an F2 with 25% homozygosity. At this stage it will be clear whether the trap has produced an interesting recessive phenotype. If so the gene will be cloned by making a genomic DNA library from the gene trap line and probing it for the gene trap vector sequence. The 5′ junction sequence should be within an intron of the trapped gene and enable rapid cloning of the remainder.

One important gene trap mouse strain is called **Rosa26**. In this case the trapped gene encodes an untranscribed RNA of unknown function. But it has the property of being reliably expressed in a ubiquitous manner, at all stages and in all tissues. For this reason mouse lines designed for constitutive ubiquitous expression of a transgene are now often made by "knocking in" the required gene to the *rosa26* locus, using the techniques of homologous recombination. For example a ubiquitous Cre expressing line is of this type. Also the locus has been modified by insertion of a *lacZ* gene containing a floxed termination sequence (R26R strain of mice). This is useful for testing transgenic Cre lines. Once the Cre line has been made, it can be mated to R26R and the offspring will express β-galactosidase permanently in all regions where the Cre was active. This enables the tissue specificity and level of expression of the Cre line to be established before experiments are undertaken with functional target genes.

An **enhancer trap** is a gene trap that lacks the splice acceptor and carries its own minimal promoter, which provides a basal RNA polymerase II binding site but is not sufficient for detectable transcription (Fig. 10.19). If it enters the genome within range of an endogenous promoter or enhancer then this will complement the minimal promoter and activate significant transcription of the *lacZ*. Enhancer traps are usually not mutagenic as they may be activated anywhere within effective range of an endogenous enhancer. Because they may be at some distance from any endogenous gene they are also not so useful for cloning purposes. Their main use is providing lines of mice in which particular tissues or cell types are highlighted by expression of β-galactosidase and are therefore very easy to visualize.

Regional specification in development

Embryo vs. extraembryonic structures

The brief sketch given here concerns only body-plan formation, as mammalian organogenesis will be considered further in Section 3.

The early blastomeres of a mouse embryo are known to be **totipotent**. It is possible to obtain formation of a complete blastocyst from each single blastomere isolated from the two- or four-cell stage. These blastocysts tend to have a higher proportion than normal of trophectoderm but they will form complete normal embryos after reimplantation. From the eight-cell stage it is no longer possible to obtain a complete embryo from one blastomere, although blastomeres from the eight-cell stage can still integrate into host blastocysts and contribute to all tissues, both embryonic and extraembryonic.

In normal development the formation of ICM and trophectoderm depends on the cell polarization that occurs at the eight-cell stage (Fig. 10.20). It is known that the polarization depends on cell contact and that the microvillous region always appears at the external surface, but the identity of cytoplasmic determinants responsible for initiating the genetic programs for the two cell types is still not known. Although the normal specification commences at the eight-cell stage, it is still possible for a period to force polar cells to become ICM, or apolar cells to become trophectoderm, by putting them, respectively, on the inside or the outside of a cell aggregate. By the 64-cell stage the two cell types have stabilized and are no longer interconvertible.

After implantation the ICM becomes divided into an outer layer of primitive endoderm and an inner core of epiblast. This probably depends on the cell layer in contact with the blastocoel being induced to form primitive endoderm. The trophectoderm becomes divided into the polar trophectoderm, neighboring the

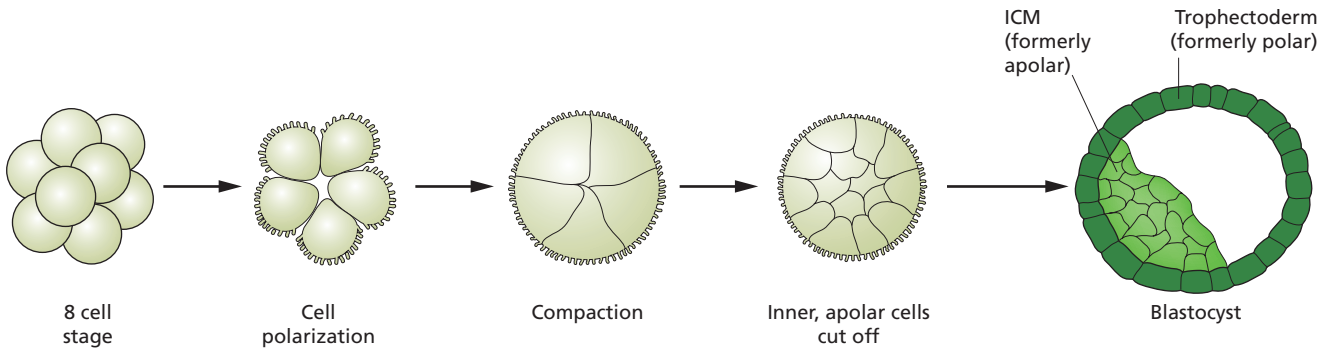

Fig. 10.20 Origin of ICM and trophectoderm from cell polarization at the eight-cell stage.

New Directions in Research

Mice will continue to be used to make transgenic and knockout lines to address a huge range of biological issues, by no means confined only to mechanisms of development. For example these include problems in cell biology, immunity, and neurobiology, as well as the study of disease mechanisms.

The phenomena of imprinting and X-inactivation will continue to be used as models to try to understand the mechanisms of **epigenetic** inheritance. The role of chromatin structure in gene regulation, and of inheritance of chromatin modifications through cell division are still poorly understood.

ICM, and the mural trophectoderm surrounding the rest of the blastocyst. The maintenance of cell division in the polar region depends on the proximity of the ICM, and, among other factors, requires FGF4. It is possible to grow trophectoderm cells *in vitro* in the presence of FGFs. A knockout of *fgf4*, or of *oct4*, expressed in the ICM, or of *fgfr2*, expressed in the trophectoderm, will terminate development at this stage.

The primitive endoderm becomes divided into visceral endoderm, in contact with the epiblast, and parietal endoderm, in contact with the mural trophectoderm. There is good evidence from isolation and recombination experiments that these distinctions are also due to a difference in cellular environment, but the molecular nature of the signals is not known.

Embryonic body plan

The whole embryo derives from the epiblast of the egg cylinder stage. Even until late primitive streak stage it is possible to induce twinning by treatment with cytotoxic drugs, presumably by killing large numbers of cells and causing regulation to occur from small nests of survivors. This shows that regional determination has not become irreversible before this stage. By comparison, it is thought that human identical twins usually arise from division of the ICM (70–75%), less often from blastomere separation (25–30%), and most infrequently from division of the primitive streak (1%). These figures are arrived at on the basis of whether the twins share a common placenta and amnion. In cases of blastomere separation the placentas will be separate, in the case of ICM division the placenta will be common but the amnions separate, and in the case of primitive streak division, both placenta and amnion will be common. The fact that cells are still able to contribute to more than one individual up to primitive streak stage is the basis for the law in the UK permitting some experimentation on human embryos up to this stage, which is reached at about 14 days.

As it is very difficult to do microsurgical experiments in mice, much of the investigation of early postimplantation development relies on the interpretation of knockouts. These have underlined the critical importance of the nodal factor, a member of the TGF-β superfamily. Nodal was originally discovered by retroviral mutagenesis of the mouse, and found to be expressed in the node, although it is in fact expressed earlier throughout the epiblast. The homozygous null embryos are unable to form any embryonic pattern, and many of the developmentally important genes active during formation of the primitive streak and the node are not expressed. As we have seen, *nodal* homologs are also important for mesoderm formation and patterning in *Xenopus*, zebrafish, and chick. The knockout of the so-called *activin receptor IIA* and *B*, which are receptors for nodal, or of *smad2*, which is required for signal transduction of nodal-like factors, or *foxh1*, which is a partner of smad2 in transcriptional regulation, all abolish the anteroposterior polarity of the embryo, such that the mesoderm that forms is entirely extraembryonic.

The anteroposterior pattern of the embryo derives initially from the proximodistal pattern in the early egg cylinder. The transcription factor gene *hex* and the inducing factor genes *cerberus-like, dickkopf*, and *lefty1* are expressed at the distal tip in the visceral endoderm and a number of genes including that encoding the T-box transcription factor brachyury (usually called *T* in the mouse) are expressed at the proximal end in the epiblast (Fig. 10.21). This proximo–distal pattern is thought to be due to signals emitted from the extraembryonic ectoderm. Two good candidates for components of this signal are Wnt3 and BMP4. Genes for both these inducing factors are expressed in the proximal part of the egg cylinder, abutting the future epiblast. Knockouts for both are early lethals, the *wnt3* knockout having no primitive streak or mesoderm, and the *bmp4* knockout having no allantois and being defective in embryonic mesoderm.

As the cup-shaped egg cylinder elongates and the proamniotic cavity forms, morphogenetic movements shift the *hex* domain to one side and the *T* domain to the other. These, respectively, become **anterior** and **posterior** ends of the embryo. As indicated above, there is a statistical association between the position of the original animal pole of the zygote, and the posterior side of the early embryo, so it is possible that the breakage of the radial symmetry of the egg cylinder arises because of small asymmetries derived from the fertilized egg. The anterior visceral endoderm (**AVE**) then expresses a group of genes for transcription factors (including *otx2*, *foxA2*, and *lim1*) and genes for secreted factors (including *nodal* and *cerberus-like*) that are associated with anterior development. A little later the same genes are activated in the overlying epiblast that becomes the head fold. By this time

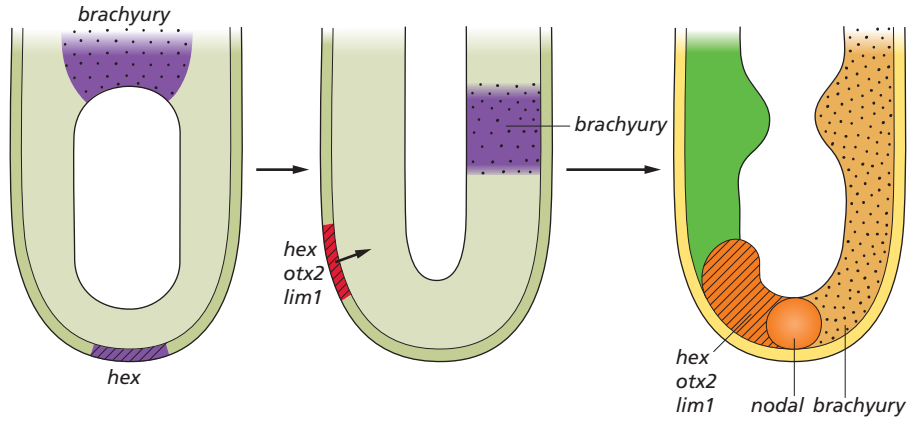

Fig. 10.21 Anteroposterior patterning of epiblast in the egg cylinder to primitive streak stage.

the primitive streak has formed in the posterior, and the node lies in between the head domain and the streak. Microsurgical recombination experiments show that the head structures can be induced in the epiblast by the action of the AVE. Knockouts of the genes expressed in the AVE tend to lead to anterior truncations. The relative requirement for gene function in the AVE and in the future head itself can be established by the chimera protocol described above. When chimeras are made of mutant ES cells in wild-type 4n blastocysts, the phenotype will reflect the requirement for the gene in the epiblast. If chimeras are made by injecting normal ES cells into mutant blastocysts, then the phenotype will reflect the requirement in the visceral endoderm. Such experiments have shown that some of the anterior genes, such as *otx2* and *foxA2*, are required just in the visceral endoderm at this stage, and others, such as *lim1 (=lhx1)*, are required in both the visceral endoderm and in the epiblast. At the same time the signals from the AVE suppress formation of a streak in the adjacent epiblast. This is probably through the inhibitory action of Cerberus-like and Lefty-1, as a double knockout of both factors leads to the formation of ectopic streaks.

The node behaves like the organizer in *Xenopus*, as it is able to induce a second axis containing host-derived neural tube and somites if transplanted to another part of the epiblast. Mouse nodes will also induce second axes in chick blastoderms. It is currently unclear whether BMP inhibitors are the principal signals involved, although both chordin and noggin are expressed in the node. Moreover the double knockout of chordin and noggin prevents formation of the forebrain and gives defects in notochord and sclerotome, indicating at least some role in neural induction and mesodermal dorsalization.

As in *Xenopus*, the FGF–Cdx–Hox pathway probably controls patterning of the posterior region as *fgf4* and *fgf8* genes are expressed in the streak, activated ERK can be detected by immunostaining, and knockouts of *fgf8* or *fgf receptor 1* or *cdx* genes show posterior defects or anteriorizations associated with reduction of Hox activity. A gradient of FGF8 protein can be seen by immunostaining in all three germ at tailbud stages. This arises by transcription of *fgf8* in the tailbud followed by gradual decay of the mRNA after the cells have left the tailbud. This mechanism depends on growth from a posterior zone, and would not work in *Xenopus* or zebrafish where there is little growth at early stages. However the end result of a gradient of FGF activity seems similar. Some *wnt* genes are also important in posterior body formation in the mouse. *wnt3A* in particular is expressed in the posterior and the knockout for *wnt3A* lacks posterior parts.

The available information suggests that body patterning in the mouse is comparable to *Xenopus*, although not identical. In both species, the initial regionalization is into just two parts, a head and a trunk region, and depends originally on the pattern in the original animal–vegetal axis of the egg. In the mouse, the head pattern depends on a group of genes activated initially in the anterior visceral endoderm, with an inductive signal required to induce a corresponding anterior territory in the epiblast. The trunk pattern depends on the signaling center associated with gastrulation movements (the dorsal lip or node), which is also responsible for neural induction and for dorsalization of the mesoderm. In both species the nodal factors have a key role in mesoderm formation, and the FGF and Wnt systems are needed for formation of posterior parts. But the relative importance of the BMP inhibitors for neural induction in the higher vertebrates is still under debate.

Left–right asymmetry

As in the chick, *nodal* becomes expressed preferentially on the left side of the node from the two- to three-somite stage, along with another TGF-β superfamily member, *lefty2*. Expression then spreads to the left lateral plate mesoderm. The sequence of subsequent gene activations is different from the chick but eventually these factors bring about the asymmetries of organogenesis by differential activation of downstream targets on the two sides. For example, within the early heart there are two bHLH type transcription factors, dHAND being mainly on the right side and eHAND mainly on the left.

The original cause of the asymmetry comes from the action of the nodal cilia. Each node cell bears a single cilium and they are motile in the period just before the onset of asymmetric gene

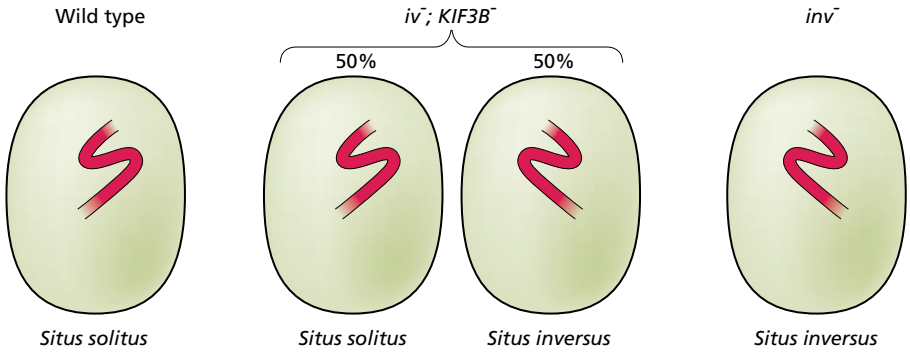

Fig. 10.22 Asymmetry mutants in the mouse.

expression, driving a flow of fluid from right to left. This can be visualized by addition of fluorescent dyes to embryos cultured *in vitro*. The fluid flow is causally related to the later gene expression as may be shown by experimentally reversing its sense, which brings about nodal expression on the right instead of the left. The exact mechanism remains unclear but it is likely that the fluid flow stimulates sensory cilia on the left side provoking an increase of intracellular calcium, which affects gene expression.

Cilia are intrinsically asymmetrical structures based on the standard 9+2 arrangement of microtubules together with motor proteins to generate the movement. In the mouse there are numerous mutants which perturb the normal left–right asymmetry and a high proportion of these cause defects in cilia. Three distinct classes of mutant phenotype can be distinguished (Fig. 10.22). First are mutants that cause randomization of *situs*. This means that half the embryos have the normal ***situs solitus***, and half have ***situs inversus***. Such an outcome suggests that the breakage of bilateral symmetry still occurs but that the bias that normally guarantees the usual *situs solitus* has been removed. In such mutants the expression of *nodal* and *lefty2* may be on either side, but not both, indicating that the mutant genes are upstream of these factors. The mutations are in **motor proteins**, for example the knockouts of the kinesin components KIF3A and KIF3B, or the naturally occurring *iv* mutant, which is in a dynein (lrd). These motor proteins are needed either for the assembly of the cilia, in the case of kinesin, or for ciliary motion, in the case of dynein. The second class of mutants leaves the embryo in a state of bilateral symmetry. This is known as an **isomerism** (not, of course, to be confused with isomerism of molecules). An example is the *TG737* mutant. Here, the gene product, called polaris, is also involved in the assembly of cilia and the mutant has no cilia and shows symmetrical *nodal* expression. The third class of mutant has a reversed asymmetry (*situs inversus*). An example is *inv*, which arises from a transgene insertion but, like *iv* and *kif3b*$^{-}$, is genetically recessive. Its homozygotes all have *situs inversus*. In *inv*$^{-}$ embryos, *nodal* and *lefty* are expressed on the right side. The *inv* product, inversin, is a cytosolic protein with ankryn repeats. It is expressed early and affects the direction of the ciliary beat. In the mutant the fluid flow is reduced but rightwards instead of leftwards and expression of nodal is on the right side.

The human genetic disease Kartagener's syndrome involves a randomization of *situs*. Genetically it is somewhat heterogeneous but many cases involve dynein mutants. Individuals also suffer from male infertility, because their sperm are immotile, and from lung diseases because the cilia of their bronchial epithelia are immotile and cannot remove the accumulated mucus from the lungs.

Hox genes

In the mouse the four **Hox gene** clusters contain a total of 39 genes. Most of the paralog groups have two or three members. As in other vertebrates, the genes are maximally expressed at the **phylotypic** stage, they tend to have sharp anterior expression boundaries in the central nervous system (CNS) and mesoderm and to fade out in the posterior, and members of the same paralog group have similar anterior boundaries (Fig. 10.23). Extensive work has been done on Hox function by making knockouts of the Hox genes, or by expressing them ectopically by making transgenics in which a Hox gene is driven by a foreign promoter.

In general a knockout results in an anterior transformation. The effects are usually strongest at the anterior expression boundary of the gene in question, because it is there that the particular gene distinguishes the combination of active transcription factors from that in more anterior positions. Often the knockout of just one Hox gene has only a modest effect, but when the whole paralog group is knocked out then the effect becomes substantial. For example, knockout of the paralog group 10 causes all lumbar vertebrae to convert to thoracic character, and knockout of the paralog group 11 causes all sacral vertebrae to convert to lumbar. This is because the members of a paralog group are quite similar and are expressed in similar domains, so there will normally be some redundancy of function between them.

Ectopic expression of Hox genes generally results in a posterior transformation. For example if *hoxa7*, normally with its anterior boundary in the thoracic region, is expressed in the head, then the basal occipital bone of the skull is transformed into a pro-atlas type vertebra. Activation of Hox genes can also be provoked by treatment of the mothers with retinoic acid,

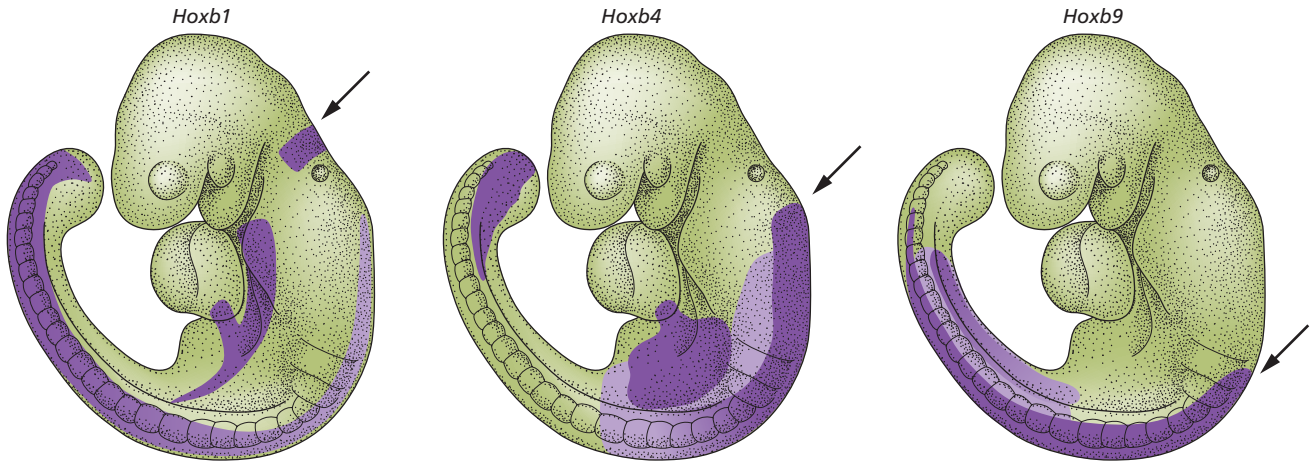

Fig. 10.23 Expression of three Hox genes, showing the different anterior boundaries.

and this produces multiple posteriorization events due to simultaneous ectopic expression of several Hox genes.

Other topics in mouse development

Nuclear transplantation and imprinting

Nuclei transplanted from early blastomeres to enucleated eggs can support development, although the ability to do so falls off rapidly with stage, perhaps associated with the early onset of zygotic transcription in the mouse. An essential condition for normal development is for the egg to contain one paternally derived and one maternally derived pronucleus. **Parthenogenetic** diploid embryos, made by activation of the egg and suppression of second polar body formation, develop poorly because of inadequate formation of extraembryonic tissues. **Androgenetic** diploids, made by removing the female pronucleus and injecting a second male pronucleus, have abundant extraembryonic tissue but the embryo arrests at an early stage. This suggests that there is some difference between the state of homologous genes on chromosomes from the two parents such that the paternal copy is more readily activated in the trophoblast and the maternal copy in the embryo.

The reason for this is the existence of **imprinted** genes on several chromosomes. These are genes that are only expressed from either the maternal or the paternal chromosome and there are probably about 100 such loci altogether. Many are concerned with growth control and one explanation for the phenomenon is an evolutionary one, as natural selection will always favor traits that maximize the number of offspring. For the father the maximum offspring are achieved by mating with many females and by the embryos achieving the maximum growth rate relative to any other male's offspring. For females the maximum offspring are achieved by devoting equal resources to each embryo and by surviving the birth so that another reproductive cycle can take place. There is thus a potential evolutionary conflict between the traits that will be favored in males and females. Put crudely, the males "want" fast growth of the embryos while the females "want" uniform and controlled growth. An example supporting this principle is the insulin-like growth factor (IGF)-2 system. IGF2 is a growth factor promoting prenatal growth of the embryo and its gene is active only on the paternal chromosome. There is an inhibitor of IGF2 called (misleadingly) the IGF2 receptor, and this is active only on the maternal chromosome. In such cases the effects of genetic heterozygosity depend on which chromosome (maternal or paternal origin) the mutation lies on. If an inactive allele is present on the nonexpressed chromosome then there will be no effect, while if it is present on the expressed chromosome the effect will be similar to that of a homozygous mutant. Conversely, it is possible to have mutations that lead to loss of imprinting on the normally nonexpressing chromosome and this may have effects by doubling the normal gene dosage. For example in humans the Beckwith–Widemann syndrome is a condition involving embryonic overgrowth and predisposition to cancer. It involves the disruption of the gene for a voltage-gated K^+ channel, but also a high proportion of the cases show loss of imprinting of IGF2. IGF2 also shows loss of imprinting in most cases of the pediatric Wilm's tumor.

Although there are many other imprinted genes, the *igf2* system itself seems the most significant in terms of prevention of parthenogenetic development. If an oocyte is reconstructed to contain two haploid female nuclei, one of which is modified to enable expression of *igf2* like a paternal nucleus, then the embryo can develop to term. The establishment of imprints can also be assayed by nuclear transplantation into eggs, followed by analysis of whether both maternal and paternal alleles at an imprinted locus are expressed. This has shown that the imprints are set up during gametogenesis (Fig. 10.24). Whole embryo clones made with nuclei from primordial germ cells show an erasure of the original imprints from E11.5, probably associated with a genome-wide demethylation of DNA in these cells. The

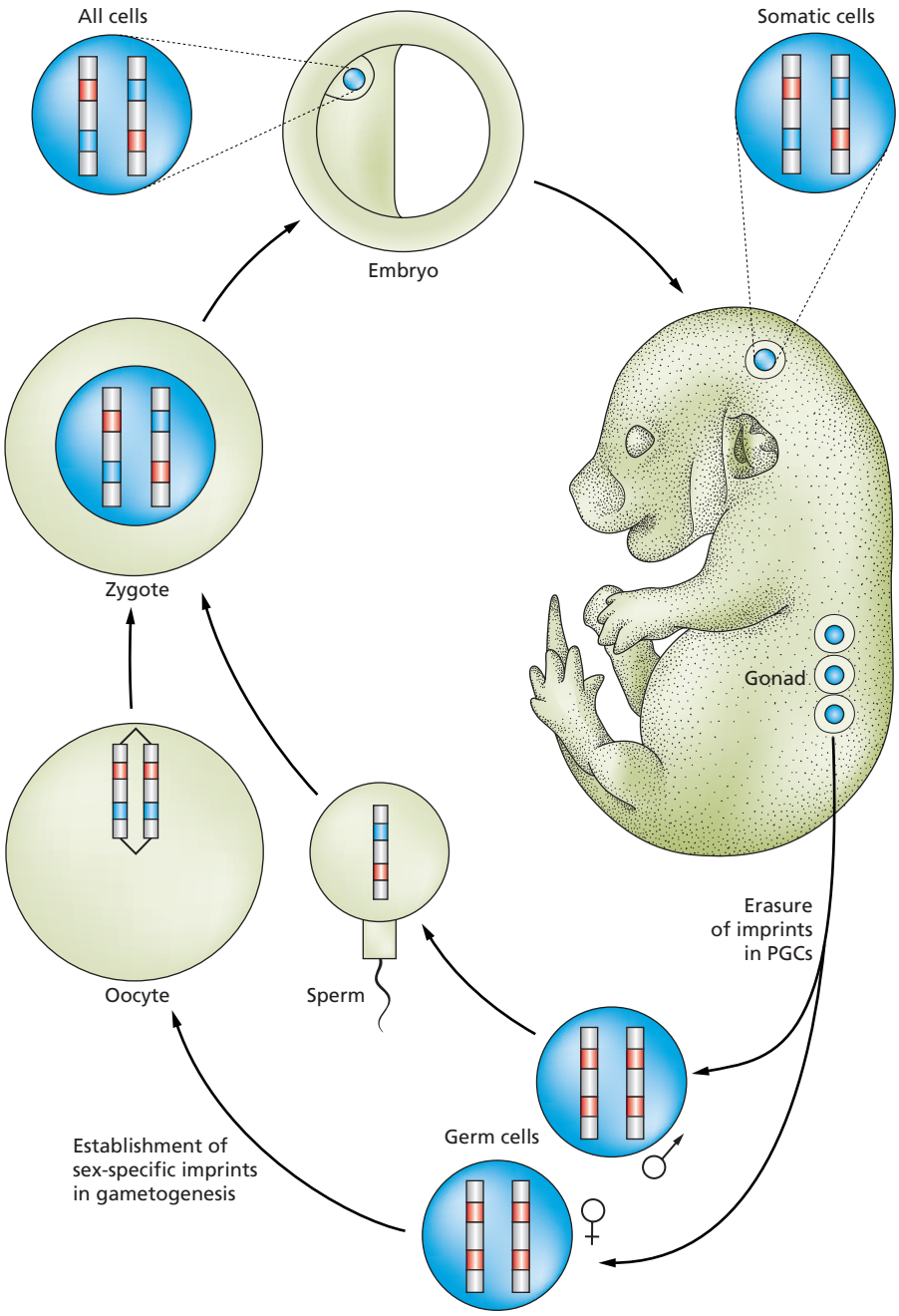

Fig. 10.24 "Life cycle" of imprinting. Two loci are shown, with red for an expressed gene and blue for an imprinted, inactive, gene. Imprints are erased in the primordial germ cells and reset during gametogenesis.

imprints become re-established late in female gametogenesis, during the growth phase of the oocyte, and earlier in male gametogenesis. It remains unclear how they are initially established, although their maintenance generally correlates with the DNA methylation state of the genes (see Appendix). Several experimentally introduced transgenes have been found to become imprinted, and are differentially methylated in sperm and eggs. In the early stages of embryonic development there is another general demethylation of the genome but this does not affect the imprints, which must be protected in some way.

X-inactivation

In all female mammals, one copy of the **X-chromosome** becomes **heterochromatic** and inactive, and is visible as a Barr body within the nucleus. This occurs at random at the primitive streak stage such that either the maternally derived or the paternally derived X becomes inactive. The inactivation of one copy of the X-chromosome produces a **mosaic** with respect to any heterozygous locus, since some cells will express the gene in question and others will not, and this ensures that all female

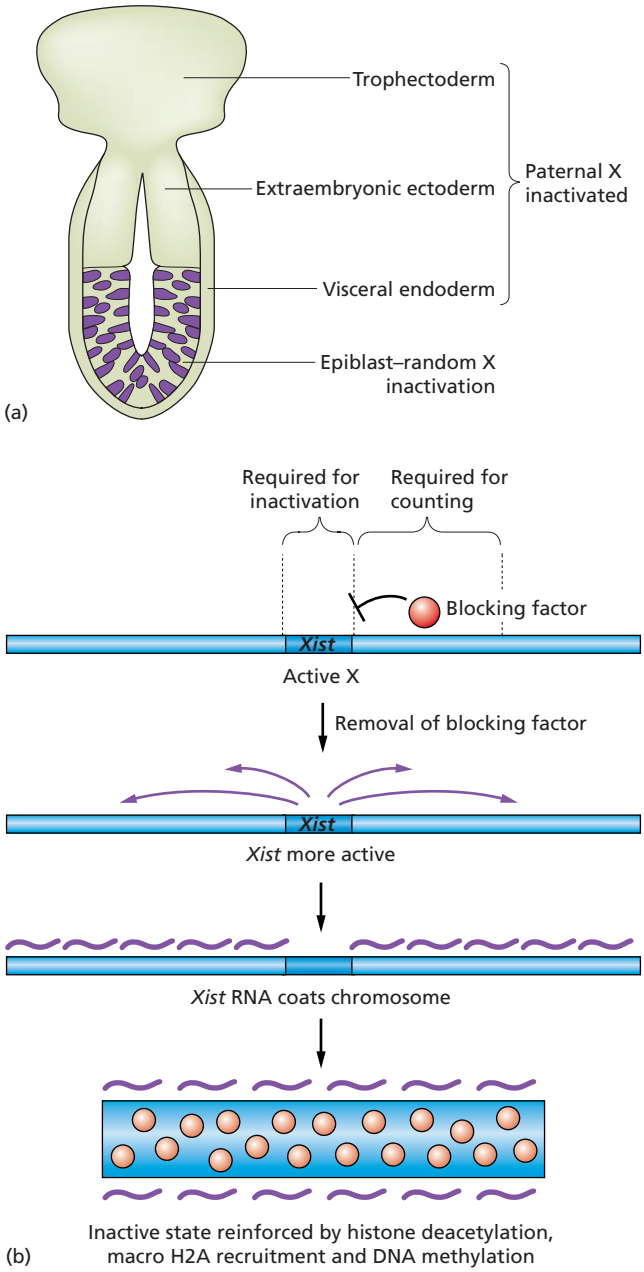

Fig. 10.25 Inactivation of the second X-chromosome in females. (a) Expression of beta-galactosidase in a heterozygous H253 mouse embryo which has *lacZ* on the paternal X. (b) Events of X-inactivation.

mammals are naturally mosaic for all X-linked heterozygous loci. X-inactivation is accompanied by late replication of the DNA relative to the rest of the genome, and by hypoacetylation of the histones on the inactive chromosome. In mouse, X-inactivation occurs earlier in the extraembryonic tissues than in the epiblast itself, and in these tissues the paternal X-chromosome is inactivated (Fig. 10.25a). This is known as imprinted X-inactivation and shows some differences from the random X-inactivation found in the embryonic tissues. X-inactivation can be easily visualized in the H253 strain of mice, which carry a number of copies of *lacZ* on the X-chromosome. In female heterozygotes the chromosome carrying the *lacZ* is inactivated in 50% of cells, so when a specimen is stained with X-Gal individual cells are either blue or colorless. This enables some types of **clonal analysis**, as any structure formed from a single cell must become either all blue or all colorless but cannot be of mixed composition.

X-inactivation depends on the presence in the chromosome of an X-inactivation center (Xic). This contains a gene called *Xist* (pronounced "exist") which encodes a nontranslated RNA. *Xist* is critically important for X-inactivation and works only on its own chromosome, not on others within the same nucleus. It is active at a low level in all X-chromosomes of the early embryo. In the chromosomes that become inactivated the activity increases and the *Xist* RNA coats the whole of the chromosome. If the *Xist* locus is deleted then the chromosome cannot become inactivated. Conversely, a transgene for *Xist* can cause inactivation of the chromosome where it lies, even if this is an autosome. Experiments with an inducible *Xist* transgene in ES cells have shown that it will cause inactivation of its own chromosome when induced and that its presence is required for 48 hours for maintenance of inactivation. After this it is no longer required, as the inactivation becomes stabilized by other mechanisms, such as recruitment of a special histone, macroH2A, and histone hypoacetylation (Fig. 10.25b). The promoter for *Xist* is differentially methylated during gametogenesis, such that it is demethylated in sperm and methylated in oocytes. This pattern is maintained in the extraembryonic tissues, correlating with the preferential inactivation of the paternal X. In the epiblast, the imprint is erased and replaced by random methylation of the promoter. There is an antisense gene called *Tsix* that overlaps the *Xist* gene on the other DNA strand. This is active in maternally derived X-chromosomes and helps make the maternal X resistant to early inactivation.

X-inactivation must involve a counting mechanism that senses the ratio between the number of X-chromosomes and the number of autosomes. This is because individuals that contain multiple X-chromosomes inactivate all but one. The region of the Xic that "counts" lies at the 3′ end of the *Xist* locus. Deletion of this region causes preferential inactivation. A model consistent with these data involves a blocking factor, produced by autosomes, which is produced in limiting amount so that it can only block *Xist* expression on one X-chromosome. The binding sites for the blocking factor would lie in the "counting" region (Fig. 10.25b).

Teratocarcinoma

Most of the thoughts about the nature of ES cells and the uses to which they might be put were anticipated by earlier work on **teratocarcinomas**. These are malignant and transplantable tumors which consist of several types of tissue and also contain undifferentiated cells. The undifferentiated cells, which will grow

in tissue culture, are considered to be the stem cells for the tumor and are often called embryonal carcinoma (EC) cells. Three types of teratocarcinoma can be distinguished: spontaneous testicular, spontaneous ovarian, and embryo derived. Spontaneous testicular teratocarcinomas arise in the testes of fetal male mice of strain 129. They are thought to arise from primordial germ cells and the well-known F9 cell line is of this type. Spontaneous ovarian teratocarcinomas arise in females of LT mice at about 3 months of age. They are derived from oocytes that have completed the first meiotic division and undergo approximately normal embryonic development as far as the egg cylinder stage. Embryo-derived teratocarcinomas can be produced by grafting early mouse embryos to extrauterine sites in immunologically compatible hosts, usually the kidney capsule or testis. The embryos become disorganized and produce various adult tissue types together with proliferating EC cells.

Different teratocarcinoma cell lines differ greatly in their properties: some need feeder cells to grow and others do not. Some will grow as dispersed cells in the peritoneal cavity (called ascites tumors) and others will not. Some will differentiate *in vivo* or *in vitro* while others will not. Most lines have an abnormal karyotype, although a few are normal or nearly normal. Most of the differences have probably arisen by selection of variants during establishment of the tumor and during culture, although it may to some extent also reflect a heterogeneity of the parent cell type. Chimeric mice can be produced by injection of some types of teratocarcinoma cells into blastocysts, but this does not work nearly as well as with ES cells. It does, however, have some theoretical interest since it shows that at least one sort of tumor can be made to revert to normal behavior if it is placed in the appropriate biological environment.

Key Points to Remember

- Fertilization comprises several distinct steps. Firstly, recognition of the zona pellucida by the sperm. Secondly, the acrosome reaction leading to release of hydrolytic enzymes that digest a path through the zona. Thirdly, the fusion of the sperm and oocyte plasma membranes with introduction of a phospholipase C into the oocyte cytoplasm. Fourthly, the elevation of intracellular calcium, which provokes the release of cortical granules, the completion of the second meiotic division of the oocyte, and the resumption of DNA synthesis ready for the first cleavage division.
- During the first 4 days of development the mouse embryo is in the preimplantation phase. It lies free in the oviduct or uterus and develops up to the blastocyst stage. Preimplantation embryos can easily be cultured *in vitro* and manipulated. Subsequent postimplantation development occurs anchored to the uterus by the placenta.
- The overall course of development resembles the chick, but there are distinct features associated with the egg cylinder arrangement found in rodents. Fate mapping shows that the animal pole of the early embryo becomes the distal tip of the egg cylinder.
- Transgenic mice are those containing an extra gene. This can be introduced by injection of the DNA into a pronucleus of a fertilized egg. The injected eggs are implanted into the reproductive tract of a "foster mother" and reared to term. Then they are bred to create a transgenic line of mice. The specificity of transgene expression can be controlled by the promoter to which it is attached.
- Knockout mice are those in which a gene has been inactivated. They are made by homologous recombination of a targeting construct containing the defective gene into ES cells. The cells are injected into mouse blastocysts to create chimeric embryos. They are reimplanted into foster mothers and reared to term. Offspring that transmit the mutation through their gametes are used to breed a knockout line.
- The embryo body plan is established in the egg cylinder epiblast by inductive signals from the extraembryonic ectoderm and the anterior visceral endoderm. Nodal is essential for formation of the mesoderm and the node region has similar properties to the organizer in *Xenopus*. FGF and Wnt signaling are required for formation of posterior body parts.
- The breakage of symmetry that leads to left-right differences depends on the nodal cilia that drive fluid to the left.
- Some genes are expressed only from the maternal or the paternal chromosome (imprinting).
- In early embryos of females, one copy of the X-chromosome becomes inactivated.

Further reading

Website for gene knockouts

http://www.bioscience.org/knockout/knochome.htm

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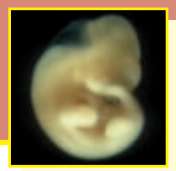
Chapter 11



Drosophila

The first organism whose development was understood in molecular detail was the fruit fly *Drosophila melanogaster*. *Drosophila* is highly suited to genetic experimentation because of its small size and its short life cycle of 2 weeks. A number of very sophisticated techniques have been developed for constructing stocks and carrying out mutagenesis screens. Work on *Drosophila* has tended to start with mutagenesis to produce mutants with interesting-looking phenotypes. Then the genes are cloned and the expression patterns studied by *in situ* hybridization. The interactions between genes are then deduced by examining the expression of one gene in embryos in which there is loss of expression, or ectopic expression, of other genes. Finally more detail about the molecular biology may be obtained by *in vitro* studies of interactions between transcription factors and regulatory regions in the DNA.

Insects

All adult and larval insects are built up of an anteroposterior sequence of segments which fall into the three principal body regions of head, thorax, and abdomen. The prototype body plan is most clearly seen at the embryonic stage which is called the **extended germ band** and is the phylotypic stage at which all insect species display their maximum morphological similarity. The head may consist of as many as six segments: three **procephalic** and three **gnathal** (pronounced "naythal"), the gnathal segments bearing leg bud-like appendages which later become the mouthparts. The middle part of the body is the thorax which always consists of three segments: the **prothorax (T1)**, **mesothorax (T2)**, and **metathorax (T3)**. All of these develop a pair of legs on the ventral side and the meso- and metathorax also produce a pair of wings on the dorsal side. The number of abdominal segments varies with species but is usually in the range 8–11.

Drosophila, like other Diptera (two-winged flies), follows the general insect pattern except that it does not display the procephalic head segments even in the embryo, the three gnathal segments appear only transiently, and only the mesothorax bears wings, the metathoracic wings being represented by small balancing structures called **halteres**. *Drosophila* has the usual three thoracic segments (T1–3) and has eight abdominal segments (A1–8). Although the conventional segments are

Classic Experiments

The breakthrough from mutants to gene function

Homeotic mutants had been known in *Drosophila* since the 1920s but few investigators were interested in them. One was Ed Lewis who published a classic study of the bithorax complex in 1978 and proposed a model for body-plan development. The full range of developmental genes was revealed by the mutagenesis screens of Christiane Nüsslein-Volhard and Eric Wieschaus which provided the raw material for the work of most labs in the 1980s. These three workers were awarded the Nobel Prize for Physiology in 1995.

The newly invented techniques of molecular biology made it possible to clone the genes that gave rise to developmental mutants and to study their expression patterns. This showed that the homeotic genes were indeed expressed in spatial domains, and that genes with periodic phenotypes, like the pair-rule genes, were expressed in periodic patterns.

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dominant in the larval and adult body plan, during early development the most important repeating units are the **parasegments**, which have the same period but are out of phase with the later segments (see below).

Inside the insect body the principal nerve cord is on the ventral side. The heart is on the dorsal side, and its action moves the **hemolymph** around the body cavity, there being no specialized vascular system as in vertebrates. Oxygen is brought to the tissues by diffusion through **tracheae**, which are long, branched ingrowths of the epidermis.

Drosophila is a holometabolous insect, meaning that it undergoes an abrupt and complete metamorphosis, so the egg hatches into a larva which is quite different in structure from the adult. The larva grows and passes through two molts before becoming a resting stage called a **pupa** in which the body is remolded to form the adult. Much of the adult body is formed from the **imaginal discs** and the abdominal histoblasts which are present as undifferentiated buds in the larva. Imaginal disc development is described in Chapter 17. Some other insect orders are **hemimetabolous**, meaning that the larva resembles the adult and acquires the final adult form via a series of nymphal stages separated by molts.

Normal development

Oogenesis

Events during **oogenesis** are very important for regional specification in the *Drosophila* embryo, and the egg is laid with a considerable amount of its pattern already specified. At the start of oogenesis, one germ cell divides four times to produce 16 cells, one of which becomes the **oocyte** and the other 15 all become **nurse cells**. Interestingly, the *par1* gene, important for cytoplasmic asymmetry in *C. elegans* (see Chapter 12), is also essential for oocyte formation in *Drosophila*, and without it all 16 cells become nurse cells. The whole cluster of oocyte and nurse cells is surrounded by ovarian follicle cells to form the **egg chamber** (Fig. 11.1). The follicle cells are derived from the gonads and are thus of **somatic** rather than **germ-line** origin. As the egg chamber enlarges, the follicle cells become divided into three populations. Those over the nurse cells are **squamous** in form and those over the oocyte are columnar. At both ends of the oocyte there is a special group of follicle cells called **border cells**, which are important in the determination of anteroposterior pattern. The nurse cells become polyploid and export large amounts of RNA and protein into the oocyte, contributing to its increase in size. In the later stages the oocyte becomes visibly polarized in both dorsoventral and anteroposterior axes and a granular **pole plasm** forms at the posterior end. The follicle cells secrete both the **vitelline membrane** and the **chorion** which is a tough outer coat surrounding the egg. The major production site for the **yolk** of the egg is the fat body of the female fly, the yolk proteins being carried to the ovary through the hemolymph.

Embryogenesis

Fertilization occurs in the uterus, the sperm entering at the anterior end through a hole in the chorion called the micropyle. Development of *Drosophila* is very fast compared with most other insects and the larvae hatch after less than 24 hours at normal laboratory temperatures. The early embryonic stages are depicted in Fig. 11.2. The initial period is called "cleavage" but as in most insects it is actually a period of rapid synchronous nuclear divisions without cellular cleavage. This is sometimes called **superficial cleavage** (see Chapter 2) and at this early stage the whole embryo forms a **syncytium**, in which all the nuclei lie in a common cytoplasm. After the first eight divisions, the **pole cells** are formed at the posterior end, incorporating those nuclei lying within the pole plasm. These later become the **germ cells**. After nine divisions most of the nuclei migrate to the periphery to form the syncytial blastoderm, those remaining internally are later incorporated into vitellophages which end up in the gut lumen. After four more nuclear divisions, during the third hour, cell membranes grow inwards from the plasma membrane to separate the nuclei and the **cellular blastoderm** is formed. At this stage there are about 5000 surface cells, 1000 yolk nuclei, and 16–32 pole cells. The division rate of the blastoderm cells slows dramatically, and the pole cells divide just once more

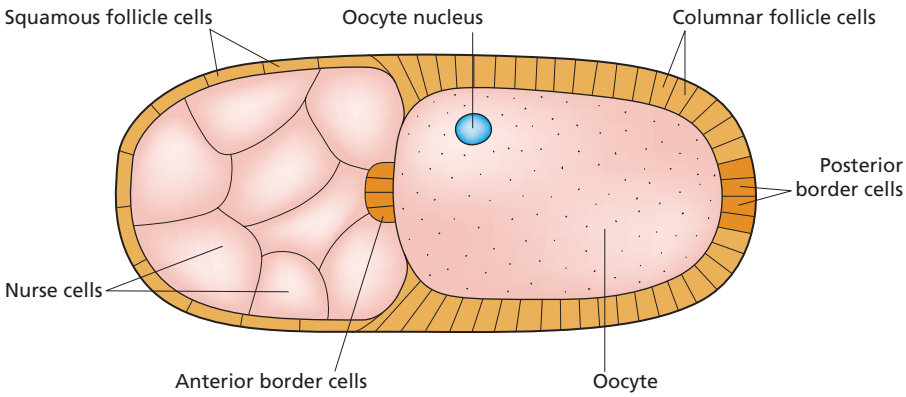

Fig. 11.1 The *Drosophila* egg chamber.

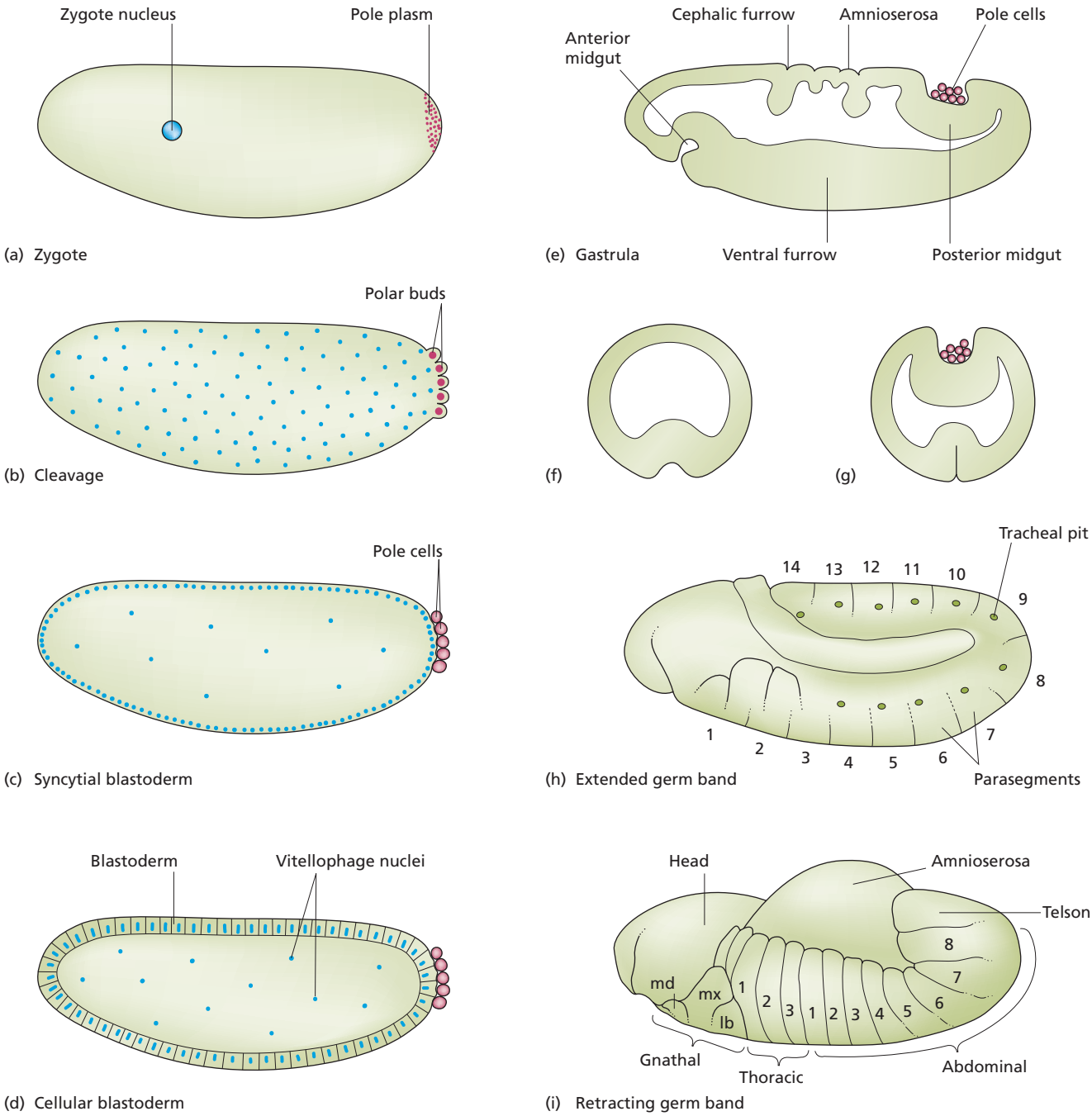

Fig. 11.2 Early development of *Drosophila*. (f) and (g) are, respectively, middle and posterior transverse sections of the gastrula.

before gastrulation. In insect embryology, positions along the anteroposterior axis of the early stages are often expressed as percentage egg length (%EL) from the posterior pole. So, for example, the region designated 100–50%EL is the anterior half, and the region 10–0%EL is the posterior tenth of the embryo.

The columnar epithelium of the blastoderm is quite thick and most of it is destined to become part of the embryo, only a thin dorsal strip becoming the extraembryonic amnioserosa. Gastrulation commences at about 3 hours with the formation of a deep **ventral furrow** along much of the embryo length (Fig. 11.2e–g). This consists of a mesodermal invagination along the ventral midline, joined shortly later by invaginations of **anterior** and **posterior midgut** at the respective ends. The cephalic furrow appears laterally at 65%EL. Concurrent with gastrulation the germ band begins to elongate, driving the posterior end with the pole cells round to the dorsal side of the egg. By about

4 hours the first **neuroblasts** appear in the neurogenic ectoderm which is now mid-ventral, having closed over the ventral furrow.

Segmentation initially appears at the extended germ band stage. The initial repeating pattern is of **parasegments**. The definitive segments each form from the posterior two-thirds of one parasegment combined with the anterior third of the next. Although their morphological appearance is transient, parasegments are important because they are fundamental units for the construction of the body plan (see below).

At about 7.5 hours the germ band retracts and as it does so the epidermal grooves rearrange themselves so that they separate the definitive segments. The anterior and posterior midgut fuse in the middle and the ventral nerve cord becomes segregated. **Dorsal closure** of the epidermis occurs at 10–11 hours, displacing the amnioserosa into the interior. At about this time the head "involutes" into the interior and is therefore scarcely represented on the outer surface of the larva. This happens also in other Diptera but is unusual for insects in general. In later stages the Malpighian tubules (excretory organs) are formed at the junction of posterior midgut and hindgut; muscles, the fat body, and the gonads arise from the mesoderm, and the central nervous system is formed by the ganglia of the ventral nerve cord.

Larval stages

The *Drosophila* larva has no legs, its head is tucked away in the interior, and it has three thoracic and eight visible abdominal segments. The specializations of the epidermal cuticle which are formed during late development are very important as they are the features used to assess the phenotypes of late embryo lethal mutations. On the dorsal side, the region from T2 to A8 is covered with fine hairs. On the ventral side are **denticle** belts on each of the thoracic and abdominal segments (Fig. 11.3). Each belt occupies mainly the anterior part of a segment but it also straddles the segment boundary and extends slightly into the posterior of the next segment. The thoracic and abdominal segments can be distinguished by the shapes and sizes of the denticle belts. Segment A8 bears the posterior spiracles, which are openings of the tracheal system. The extreme posterior is

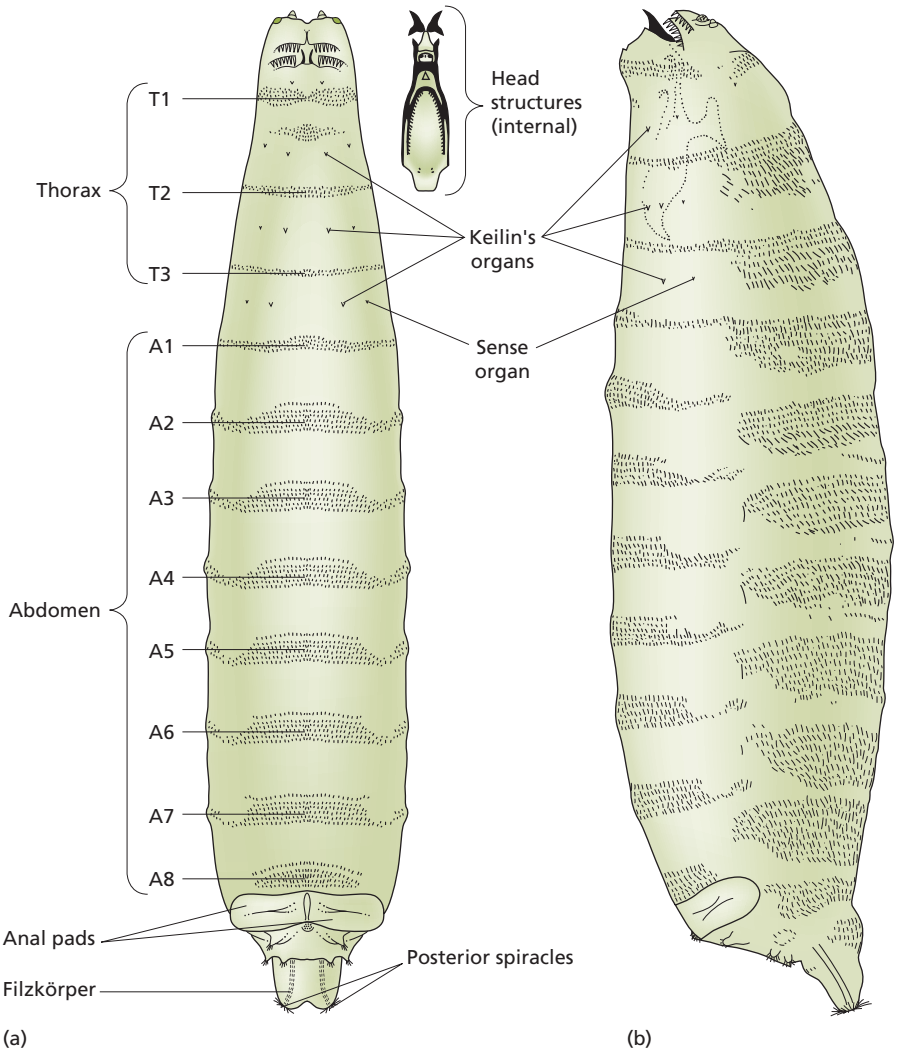

Fig. 11.3 *Drosophila* larva. (a) Ventral view. (b) Lateral view.

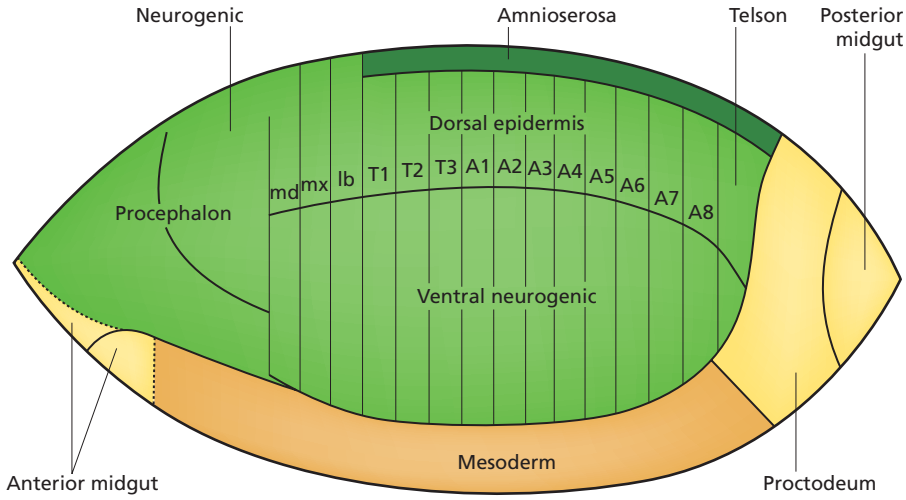

Fig. 11.4 Fate map of the cellular blastoderm stage. (After Hartenstein 1993. *Atlas of* Drosophila *Development*. Cold Spring Harbor Laboratory Press, p. 5.)

unsegmented and is called the **telson**. The structure of the larval head is very complex, but its most prominent component is a horny cephalopharyngeal skeleton secreted by the stomodeal part of the alimentary tract.

The **imaginal discs** are present as small nests of cells in the first-instar larva. They expand slightly during embryonic development, but most of their growth occurs during the larval stages. During pupation they differentiate and form the main epidermal structures of the adult body. The abdominal segments are formed from abdominal histoblasts which grow only in the pupal stage.

Fate map

Fate maps for the cellular blastoderm-stage *Drosophila* embryo have been constructed by localized UV irradiation to produce small defects, and by injection of cells labeled with **horseradish peroxidase** (Fig. 11.4). The principal features of the fate map are as follows: prospective regions exist for all the larval segments, there being no regions of indeterminacy representing later cell mixing or later growth from a small bud. The prospective regions for the recognizable gnathal, thoracic, and abdominal segments are arranged in anteroposterior sequence from 75% to 15% of the egg length. The prospective procephalic head structures occupy the anterior 25%. The prospective anterior midgut also maps to the anterior, while the posterior midgut, Malpighian tubules, proctodeum, and germ cells map to the posterior, as one would expect from the descriptive embryology.

Pole plasm

The pole plasm is formed during oogenesis and contains a **determinant** for germ cells, important components of which are the products of the genes *oskar* and *vasa*. Direct evidence that the pole plasm can program the nuclei to become germ cells was obtained from transplants of pole plasm to the anterior end of host eggs at cleavage stages. Ectopic pole cells arise from those nuclei that are surrounded by the grafted pole plasm. If these ectopic pole cells are then grafted back to the posterior of a second host, they can be incorporated into the gonad and form functional germ cells. A number of maternal-effect mutations prevent the formation of the pole plasm and result in sterility of the resulting offspring.

Drosophila developmental genetics

The spectacular level of understanding of *Drosophila* development depends largely on the sophistication of the genetic methods available. The genome size is small in comparison with vertebrates, both in gene number and in DNA content, and is fully sequenced. The generation time is short and the animals are small, so experiments involving complicated breeding protocols and large numbers of individuals can be completed in a few weeks. The genetic maps are very detailed as are the cytological maps of the giant **polytene** chromosomes from the larval salivary glands. All these features have been vital for the rapid transition from the discovery of a potentially interesting mutant phenotype to the molecular identification and characterization of the gene responsible.

P-element

A transposable element known as the **P-element** is very important both for the creation of transgenic lines and for insertional mutagenesis. For transgenesis, the gene to be inserted is cloned into a P-element that also contains a marker gene to enable transformants to be identified. This is injected into the posterior pole of the egg and if it is incorporated into the genome of one or more pole cells then the resulting flies will produce some offspring containing the integrated P-element, and a stable line can

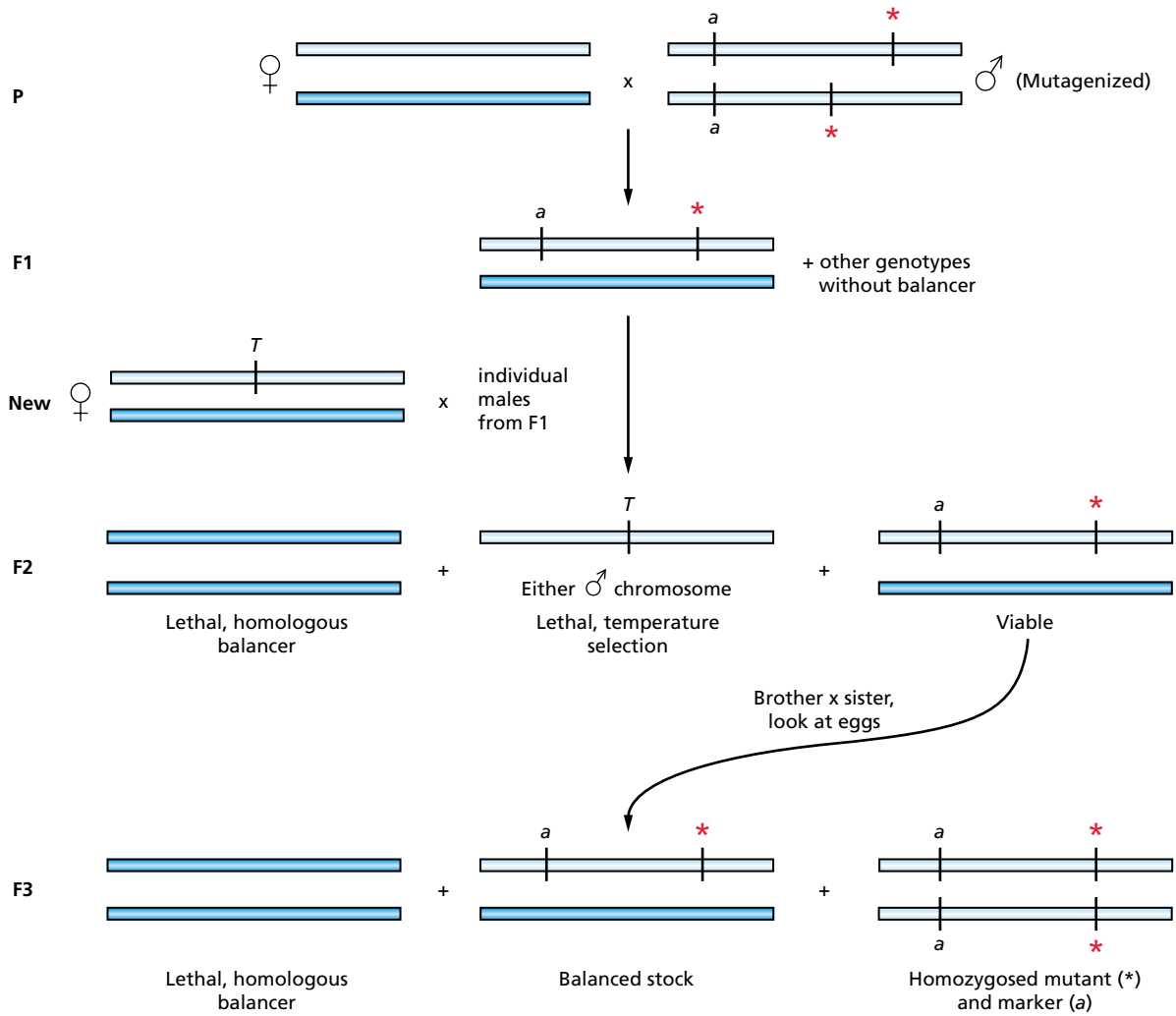

Fig. 11.5 A mutagenesis screen for zygotic autosomal recessive mutants affecting early development. The darker blue chromosome is a **balancer** which suppresses recombination, carries a visible dominant marker gene, and is lethal in homozygous form. "a" is a different visible marker gene, which is recessive. "T" is a dominant temperature-sensitive lethal. "*" represents a new mutation. The matings are carried out as shown and the F3 eggs are examined for abnormalities.

then be bred. The method has been used for several different purposes: to introduce enhancer traps (see also Chapter 10), to create strains carrying reporter constructs, to rescue endogenous mutations, and to express genes in an ectopic manner. For insertional mutagenesis, a line carrying a P-element is crossed to a line carrying a transposase enzyme. In the offspring the transposase can move the P-elements around in the genome, so the gametes contain a range of new insertions.

Identification of relevant genes

The *Drosophila* genome contains about 13,000 genes altogether, including about 5000 genes that can be mutated to lethality. By mutagenesis screening it has been possible to assemble a fairly complete collection of genes which, when mutated, give rise to pattern alterations in the embryo. There are several types of protocol for doing this, of which a simple example is shown in Fig. 11.5. Although the basic principle is the same as the zebrafish screen outlined in Chapter 3, the special tricks available for *Drosophila* select against the unwanted classes of recombinant and greatly reduce the labor involved. The screen illustrated in Fig. 11.5 is for autosomal recessive mutations and like most screens depends on the use of special **balancer chromosomes** which have three important features: they suppress recombination, they carry some visible marker gene, and they are lethal in the homozygous condition. Males carrying a visible marker (*a* – distinct from the balancer marker) are mutagenized and are then mated to females carrying the balancer. Each *individual* offspring fly represents one mutagenized gamete,

so individual males from the F1 generation are isolated and crossed again. For creation of the F2, females are used which carry a dominant temperature-sensitive lethal mutation on the chromosome opposite the balancer and this enables selection against offspring not carrying the mutant chromosome. In the F2 generation each *tube* of flies represents one of the original mutagenized gametes. The viable F2 flies are mated with each other to produce the F3 generation. As the F2s are heterozygous for the mutagenized chromosome and the balancer, 25% of the F3 will have a homozygosed mutant chromosome. If these homozygous mutants are viable, they will display the recessive marker phenotype (*a*/*a*) from the mutagenized males. If this marker is not visible, then the dead embryos or larvae are examined to see whether they have any significant pattern abnormality. In the case where the mutants are lethal the stock is maintained by breeding from the heterozygotes that have one mutant chromosome and one balancer. These will breed true because both the possible homozygous chromosome combinations that can arise from mating are lethal. The original mutagenesis will induce mutations on all the chromosomes, but those that do not lie on the balanced chromosome are mostly lost in the outcrosses, so the method enables the screen to be carried out for one chromosome at a time. Once obtained, mutations are sorted into groups of alleles of the same gene by **complementation tests**, and are genetically mapped.

In *Drosophila*, much pattern information is laid down during oogenesis and mutations in genes required during oogenesis manifest themselves as **maternal effects**, meaning that the structure of the embryo corresponds not to its own genotype but to the genotype of the mother (see also Chapter 3). Strictly, when discussing maternal-effect mutations one should always say "eggs from mutant mothers" rather than "mutant eggs" but this convention is rarely adhered to. Maternal-effect genes are often identified as female sterile mutations in screens similar in principle to those for zygotic lethals. But some important maternal-effect mutations are also zygotic lethals. In other words the gene is needed for embryonic development as well as for oogenesis. In such a case the homozygous individuals do not grow up to become flies whose fertility can be tested, so maternal screens are usually incomplete. It is, however, possible to test individual zygotic lethals for maternal effects by making mosaic embryos in which the germ line is mutant but the somatic tissues are wild type (see below).

Types of mutation

Most mutations are reduced function or **loss of function**, representing the production of a smaller amount of gene product, or a gene product of reduced effectiveness. These are usually recessive. The alleles are called **hypomorphs** and the phenotypes are called **hypomorphic**. In *Drosophila* genetics much effort goes into the creation of several alleles for each locus to obtain at least one that has lost all function, the so-called **null** alleles giving amorphic phenotypes. Frequently the hypomorphic alleles can be arranged in a series of increasing severity with the amorphic phenotype as the limit form. Such **allelic series** can often give useful information about function, particularly where the weaker alleles give something recognizable and the stronger ones do not.

Some alleles may be **temperature sensitive**, usually because the mutation affects the thermal stability of the protein product. In general the protein is active at a low temperature (the **permissive** temperature) but inactive at a high temperature (the **nonpermissive** temperature). Temperature-sensitive mutants are useful because they can help to establish the developmental stages at which a gene product is required. This is done by shifting between the temperatures, and if the shift to the nonpermissive temperature is made before the time of gene function then most cases will be mutant, while if it is made afterwards, most cases will be normal.

Dominant mutations are sometimes loss of function, in those cases where the locus is **haploinsufficient**. This means that a reduction in the level of the product to 50% of the wild-type level is sufficient to cause a mutant phenotype. In such cases the homozygous phenotype will be more severe than the heterozygous one. More often dominant mutations are **gain of function**, resulting in the production of active gene product in positions or at times when it is not normally found, or **dominant negative (= antimorphic)**, where the mutant version of the gene product interferes with the function of the wild-type version.

The names of *Drosophila* developmental genes usually derive from the appearance of the mutant phenotype. This means that they may indicate the opposite to the normal function. For example the *dorsal* gene is so called because null mutants are dorsalized, but this is because the normal function of the gene is to form ventral parts. Some genes are named after the adult phenotypes of viable alleles that were discovered before the big screens for embryonic lethal mutations. For example *Antennapedia* is so named because of a gain-of-function allele that converts antenna to leg, although the null phenotype of *Antennapedia* is a conversion of parasegments 4 and 5 to parasegment 3 and is lethal at embryonic stages.

Cloning of genes

Historically, most of the *Drosophila* developmental genes were cloned by **P-element mutagenesis**. When a P-element integrates into or near a gene it may mutate it to inactivity and it is possible to screen for a particular mutation by methods similar to those of Fig. 11.5. A mutation caused by P-element insertion can then be used as a starting point for cloning the gene. A genomic library from this strain is screened with the P-element probe and the resulting clones are tested by *in situ* hybridization to polytene chromosomes to find which particular P-element they represent. The one lying nearest to the genetic map position of the mutation can be used as the starting point for a

chromosomal "walk" to obtain the whole of the required gene. Proof that the cloned candidate really *is* the required gene is obtained by three criteria:

1 that several known mutants have identifiable sequence changes in the candidate gene;

2 that the candidate gene is not expressed in null mutants (although sometimes null mutants can produce an inactive product);

3 by rescuing the phenotype of the null mutant by P-element-mediated transgenesis with the candidate gene.

Once the gene is cloned and sequenced the next step is to determine the normal expression pattern using *in situ* hybridization to different stage embryos. The next step is to make antibodies to the protein product, usually of a fusion protein expressed in bacteria, and use this to determine the protein expression pattern.

Gene function is studied by finding how the mutation of one gene affects the expression of others. If A turns on B, then removal of A will cause loss of B, and overexpression of A will cause corresponding ectopic expression of B. On the other hand if A normally represses B, then removal of A will cause ectopic expression of B, and overexpression of A will cause repression of B in its normal domains. But such results do not reveal whether the effects are direct, meaning that the protein product of A is actually interacting with gene B to regulate it. It could equally well be indirect, with gene A turning on something else that regulates gene B. If gene A codes for an inducing factor or receptor then the effect is necessarily indirect. If gene A codes for a transcription factor, then there are three methods of establishing directness:

1 It may be possible to demonstrate interactions between gene A–product and gene B–regulatory region, using **band shift assays**.

2 Genes A and B can be transfected together into *Drosophila* tissue culture cells to see whether B is turned on in the absence of other developmental machinery.

3 It may be possible to do a domain swap such that a new DNA recognition domain is put onto A and the corresponding DNA sequence is inserted into the regulatory region of B. If the effects of A on B are maintained under these circumstances then it must be due to a direct molecular interaction.

When the effect of A upon B is examined, it is quite common to look not at the endogenous B gene product but at a construct composed of the regulatory region of B fused to a reporter gene, usually *lacZ*. The reasons for this may be simply improved sensitivity if the endogenous product is hard to detect. Also in many constructs the β-galactosidase protein is quite stable and so its concentration effectively "integrates" the cumulative gene activity up to the time of examination. Most often it is because the regulatory region of B has been dissected into a number of parts to find which DNA sequences are responsible for each component of the expression pattern. When a reporter construct is used it may include just a single enhancer from the original regulatory region of gene B.

Classic Experiments

Discovery of the homeobox

The homeobox was discovered as a DNA sequence that was present in all of the genes of the Bithorax and Antennapedia complexes. It was called the homeobox because of its location in homeotic genes. It was soon found to exist also in the DNA of other animals, including vertebrates. Excitement grew as it was thought that it might label all homeotic genes, or perhaps all genes concerned with segmentation, and be a real "Rosetta stone" for developmental genetics. Things are not quite so simple however, as it turned out that transcription factors containing the homeodomain DNA binding region are not confined to animals and do not have any single biological function. However within the animal kingdom a high proportion of them are indeed concerned with some aspect of development, and the Hox clusters homologous to Antennapedia/Bithorax are homeotic genes concerned with anteroposterior patterning.

Carrasco, A.E., McGinnis, W., Gehring, W.J. & Derobertis, E. M. (1984) Cloning of an x-laevis gene expressed during early embryogenesis coding for a peptide region homologous to *Drosophila* homeotic genes. *Cell* **37**, 409–414.

McGinnis, W., Garber, R.L., Wirz, J., Kuroiwa, A. & Gehring, W.J. (1984) A homologous protein-coding sequence in *Drosophila* homeotic genes and its conservation in other metazoans. *Cell* **37**, 403–408.

McGinnis, W., Levine, M.S., Hafen, E., Kuroiwa, A. & Gehring, W.J. (1984) A conserved DNA sequence in homeotic genes of the *Drosophila* Antennapedia and Bithorax complexes. *Nature* **308**, 428–433.

Scott, M.P. & Weiner, A.J. (1984) Structural relationships among genes that control development – sequence homology between the *Antennapedia, Ultrabithorax* and *fushi tarazu* loci of *Drosophila*. *Proceedings of the National Academiy of Sciences USA* **81**, 4115–4119.

Hox genes

Although the homeobox and the Hox genes were first discovered in *Drosophila*, we have already met them in Chapters 4, 7, and 10. Like other animals, *Drosophila* contains a Hox cluster and also many other non-Hox homeobox genes that are involved in development, but are not part of the Hox cluster. The Hox cluster is on a single chromosome, but is split into two gene groups: the Antennapedia complex and the Bithorax complex. This split is probably quite recent in evolutionary history, as other insects that have been examined maintain a single cluster.

Overview of the developmental program

Because most of the genes involved are known, the genetic program of

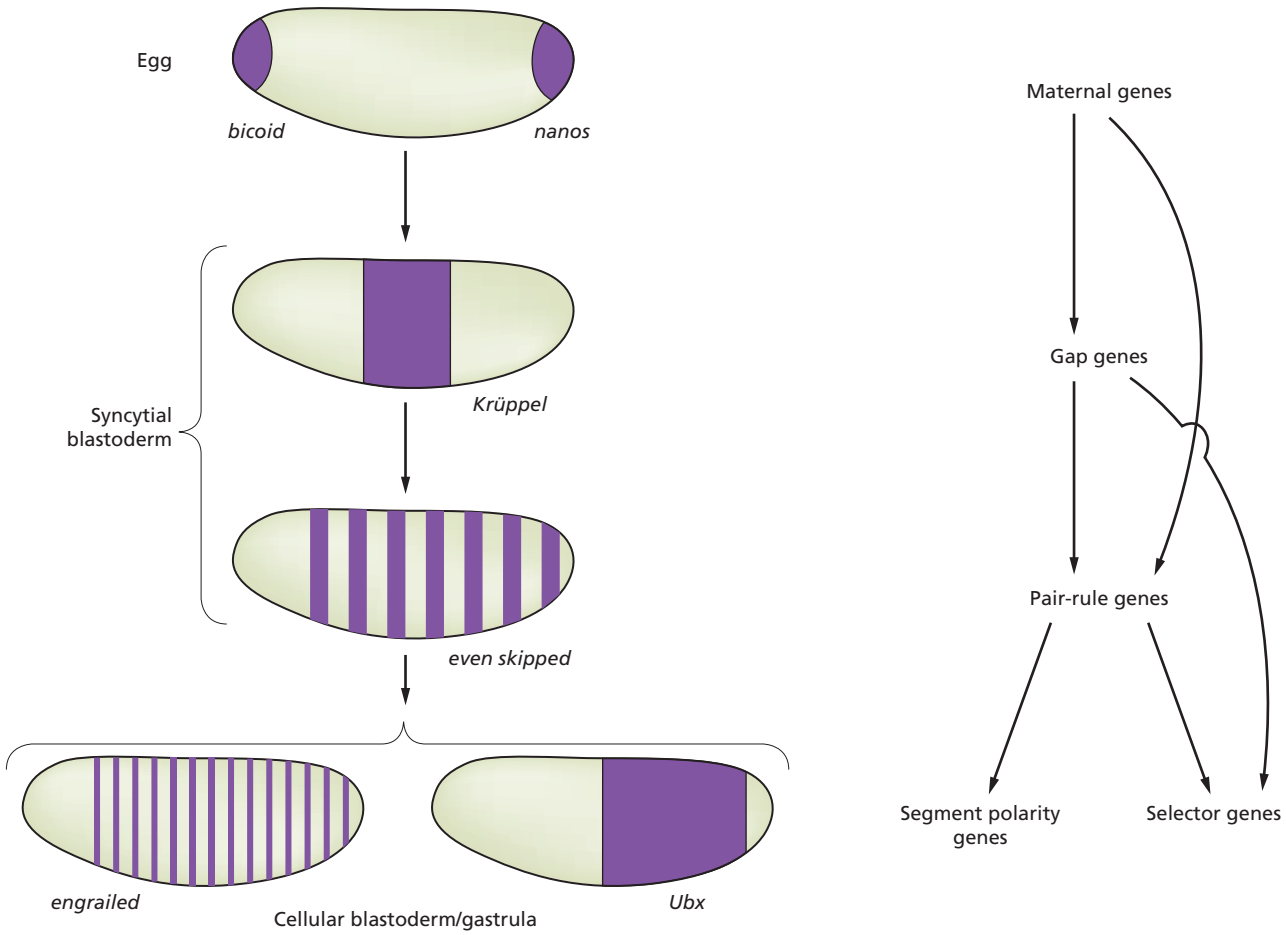

Fig. 11.6 Hierarchy of steps in the development of anteroposterior pattern.

Drosophila appears very complex, although in reality it is simpler than that of vertebrates. The detail can be understood if it is appreciated that the overall program can be regarded as a set of subprograms. One system operates in the **dorsoventral axis** and is responsible for the formation of mesoderm, neurogenic region, and epidermis from ventral to dorsal. As a result of a series of events in the egg chamber of the mother, the product of a maternal gene *dorsal* becomes distributed in the nuclei of the blastoderm in a ventral–dorsal gradient. It regulates a set of zygotic genes including *twist*, *rhomboid*, and *zerknüllt* which control formation of the various bands of tissue from ventral to dorsal.

An independent system operates in the **anteroposterior axis** and is more complex. It is shown in Fig. 11.6, with the expression patterns of a few of the key genes. The first phase of specification occurs in the egg chamber with the establishment of three maternal systems. The anterior system is concerned with the production of a gradient of the bicoid protein from *bicoid* mRNA localized in the anterior. The posterior system, of which the pole plasm is an integral part, deposits the mRNA of a gene called *nanos* in the posterior. There is also a **terminal system**, not shown in Fig. 11.6. The products of the maternal systems divide the embryo into several zones depending on their relative concentrations or activities. Before cellularization, one nucleus can affect the gene activity of nearby nuclei simply by producing a transcription factor; no receptors or signal transduction mechanisms are required. Because of the overlaps between the domains of activity, and because different concentrations of the same substance can have different effects, the maternal systems can activate a spatial pattern of zygotic gene activity which is more complex than their own. The genes activated at this stage belong to the gap class, e.g. *Krüppel*, and to the pair-rule class, e.g. *even skipped.* The gap genes are expressed in one or a few domains while the pair-rule genes are expressed in stripy patterns with a periodicity of two segment widths. Their periodicity arises because many different combinations of maternal and gap genes can activate the same pair-rule gene. The overlapping periodic patterns of pair-rule genes lead to the activation of a repeating pattern of **segment polarity genes**, e.g. *engrailed*, which have single segment periodicity and cause the subdivision of the axis into parasegments. Simultaneously the combined maternal, gap and pair-rule gene product combinations activate the Hox genes, e.g. *Ubx*, which control the character of each parasegment and thus its subsequent pathway of differentiation.

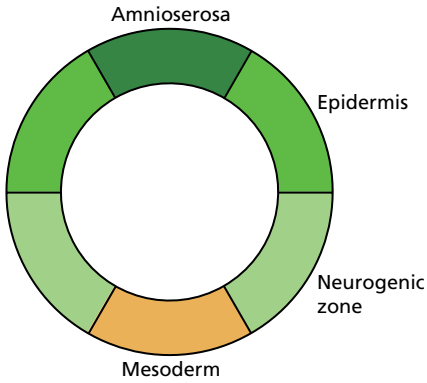

Fig. 11.7 Territories formed during dorsoventral specification of the early *Drosophila* embryo.

The dorsoventral pattern

The pattern along the dorsoventral axis is relatively simple. It consists at the cellular blastoderm stage of four strips committed to become, from ventral to dorsal, the mesoderm, the ventral neurogenic region, the dorsal epidermis, and the amnioserosa (Fig. 11.7). The disposition of these four territories is controlled by a ventral–dorsal nuclear gradient of the *dorsal* gene product, which is a transcription factor. To avoid confusion between dorsal position and the presence of the product of the *dorsal* gene, the gene product will be referred to here as "dorsal protein." The high point of the dorsal protein gradient depends on signaling from the ventral follicle cells (spätzle protein), and this signal exists because it is repressed on the dorsal side by a previous inhibitory signal from the oocyte (gurken protein). The dorsal protein gradient itself works by regulating various zygotic genes such that each ventral to dorsal strip of cells has a different combination of transcription factors active.

Maternal control of dorsoventral patterning

To understand how the dorsoventral system worked, a group of maternal-effect genes affecting dorsoventral pattern were identified from mutagenesis screens. For most of them the loss-of-function phenotype consists of a folded tube of larval cuticle bearing the fine hairs typical of the dorsal epidermis, but lacking structures normally derived from the lateral or ventral territories of the blastoderm. The prototype mutation of this **dorsalizing** class was called *dorsal*. In addition there are three genes, *gurken, torpedo*, and *cactus*, whose loss-of-function phenotype is ventralizing, with denticle bands extending all round the embryo. One gene, called *Toll*, has loss-of-function dorsalizing alleles and also has a gain-of-function allele with a ventralizing effect.

Several types of genetic and embryological experiment were important in understanding how these genes worked. Firstly, several of the genes have dorsalizing alleles with different degrees of function and these can be arranged in allelic series in which structures are progressively lost from the ventral side until, in the amorphic embryos, only the symmetrical tubes of dorsal cuticle remain. This shows that the normal function of these genes is to promote ventral development.

Next, the genes can be arranged in a temporal series by making double mutants of which one has a dorsalizing and the other a ventralizing effect. Whichever predominates is assumed to act later in the developmental program (see also Chapter 3). Some idea of the time of action can also be gained by using temperature-sensitive mutants and shifting between the permissive and nonpermissive temperature at different times.

Next, it can also be asked whether a particular gene is required in the **germ line** (i.e. the oocyte itself and the nurse cells) or in the **soma** (i.e. the ovarian follicle cells). To answer this, embryos were created by grafting mutant pole cells into normal embryos. When these are grown up the females will produce offspring that are mutant if the gene was required in the germ line, or normal if it was required in the soma. Studies of this sort showed that some genes are required in the germ line (e.g. *gurken, Toll, dorsal*) and some in the soma (e.g. *torpedo, pipe*). Nowadays this type of experiment is usually done not by transplantation but by using the **FLP system** (see Chapter 17).

Finally, even before the genes were cloned and the gene products were identified, it was possible to find out something about their function by performing cytoplasmic **transplantations**. Most of the dorsal group mutants can be "rescued" towards a normal appearance by the injection of small amounts of cytoplasm from wild-type embryos before the pole cell stage.

These various types of experiment have led to the following account, in which the term "mutant" will indicate loss of function unless otherwise stated (Fig. 11.8a). The first known gene to act is called *K10*. The mutant has a dorsalizing effect and the function of its product is to sequester the mRNA for *gurken* in the vicinity of the oocyte nucleus. *gurken* and *torpedo* are two genes with similar ventralizing mutant phenotypes. *gurken* codes for a growth factor related to vertebrate TGFα, and is required in the oocyte. *torpedo* codes for a TGFα receptor and is required in the follicle cells. *gurken* mRNA is present just in the vicinity of the nucleus, which is positioned on the dorsal side of the oocyte, while *torpedo* is expressed all over the follicle cells. The protein product of *gurken* is secreted only on the dorsal side, it stimulates the *torpedo* product in the dorsal follicle cells thereby activating the ERK signaling pathway and causing changes of gene expression, in particular the repression of *pipe*, which would otherwise be activated. Mutations in *gurken* and *torpedo* are ventralizing because if the genes are inactive, then *pipe* becomes activated all over.

pipe is one of a group of genes whose function is to create an active extracellular ligand localized on the ventral side of the oocyte. *pipe* itself codes for an enzyme responsible for adding sulfate groups to heparan sulfate, an extracellular glycosaminoglycan. In normal development, *pipe* is expressed just in the ventral follicle cells, since expression on the dorsal side is repressed by the *gurken–torpedo* system. The local sulfation of heparan

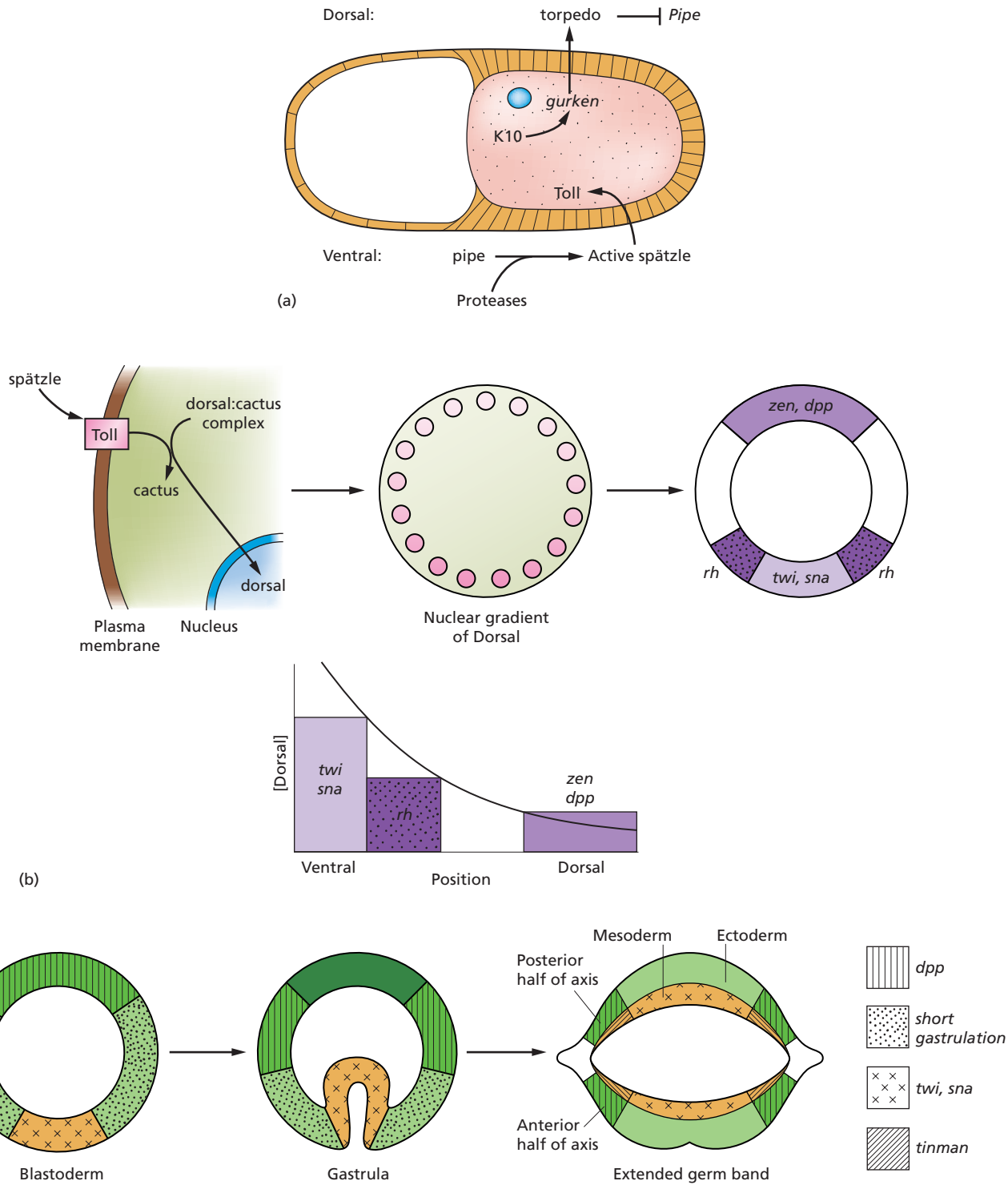

Fig. 11.8 Operation of the dorsoventral system. (a) Dorsally, the Gurken signal causes repression of *pipe*. Ventrally *pipe* is active and enables the activation of Spätzle (actually produced by the oocyte or nurse cells). (b) Spätzle activates Toll, which causes nuclear translocation of Dorsal, and Dorsal regulates zygotic genes. (c) The territories are later refined by means of the gradient of Dpp. *tinman* is maintained in the lateral mesoderm. Abbreviations: *twi*, *twist*; *sna*, *snail*; *rh*, *rhomboid*; *zen*, *zerknüllt*; *dpp*, *decapentaplegic*; *sog*, *short gastrulation*.

sulfate sequestrates a group of proteins on the ventral side which include proteases produced by the genes *snake* and *easter* which can activate the actual ventral signal. This signal is the protein product of the *spätzle* gene, active in the oocyte, and is synthesized as an inactive precursor requiring proteolytic cleavage for activation. The function of spätzle is to activate a receptor coded by the *Toll* gene which is present all over the surface of the oocyte. This has some homology to the vertebrate interleukin 1 receptor, although spätzle does not itself resemble interleukin 1. *Toll* has two types of mutant, recessive dorsalizing mutants in which receptor function is lost, and dominant ventralizing mutants in which the receptor is signaling continuously even in the absence of ligand.

The activation of *Toll* on the ventral side commences after fertilization and the subsequent events occur during the embryonic cleavage stages (Fig. 11.8b). *dorsal* is the final gene in the maternal dorsoventral pathway. The dorsal protein is a transcription factor homologous to the vertebrate factor NFκB. Its mRNA is uniformly distributed in the oocyte and the protein is synthesized after fertilization. The distribution is initially uniform but during the syncytial blastoderm stage it enters the nuclei preferentially on the ventral side, forming a ventral–dorsal gradient of nuclear protein. The entry to the nuclei depends on the proximity of activated Toll which causes dissociation of dorsal protein from a complex formed with another protein, IκB. This releases dorsal protein and allows it to enter the nuclei and regulate its target genes. *Drosophila* IκB is encoded by the gene *cactus*. *cactus* mutants are ventralizing because the normal role of cactus is to inhibit the action of dorsal protein, so in the absence of cactus, dorsal protein can enter the nuclei all over the embryo and make it ventral in character all over.

Zygotic control of dorsoventral patterning

The gradient of dorsal protein works by activating or repressing various transcription factors that are encoded by zygotic genes (Fig. 11.8c).

The **mesoderm** is defined by two transcription factors encoded by the genes *twist* and *snail*. These both code for transcription factors and are activated by dorsal protein at high nuclear concentration. *twist* encodes a bHLH protein and is required for correct mesodermal differentiation. *snail* encodes a zinc-finger transcription factor and is needed for invagination of the mesoderm.

Other genes are turned on at lower concentrations of dorsal protein so they become expressed laterally as well as ventrally. Some of these are repressed by snail, leading to a lateral stripe in the prospective neuroectodermal region, for example *rhomboid*, coding for a putative transmembrane receptor, is activated by dorsal protein and repressed by snail, such that it is expressed as a stripe in the neurogenic region.

The **dorsal ectoderm** is defined by a homeoprotein encoded by *zerknüllt*, normally expressed in about 40% of the embryo circumference. It is repressed by dorsal protein, and therefore becomes expressed all round in dorsalizing mutants. Another gene repressed by dorsal protein and normally expressed in the dorsal 40% zone is *decapentaplegic (dpp)*. This encodes a signaling molecule which brings about the patterning of the dorsal half of the embryo circumference and is a homolog of the vertebrate BMP4. Injection of *dpp* mRNA can induce amnioserosa at high dose and dorsal hairs at lower dose. Although this suggests gradient-like behavior, there is not a gradient of production of dpp protein itself. Instead the induction of dorsal genes is conducted by dpp acting together with another BMP homolog called screw, which is expressed ubiquitously. The graded effect of these factors arises because of the action of a inhibitor encoded by the *short gastrulation* (*sog*) gene. This is expressed in a lateral belt because it is repressed by *snail*, and it inhibits screw, leading to a dorsal–ventral gradient of the overall BMP-like activity. Sog is the homolog of the vertebrate chordin, also an inhibitor of BMP4 (see Chapter 7).

The patterning of the mesoderm also depends on dpp, whose expression later resolves into a pair of broad longitudinal stripes on the dorsal side. *tinman* encodes a homeodomain transcription factor necessary for heart formation. It is initially activated in the whole mesoderm by *twist* but then is turned off except in the lateral region that is adjacent to the *dpp*-expressing epidermis. This lateral region forms the heart while the ventral part forms the body wall muscles.

Thus, the full dorsoventral pattern arises from a maternal gradient of dorsal protein controlling the ventral half, and the zygotic gradient of dpp controlling the dorsal half. Interestingly the dorsal dpp–ventral sog pattern is homologous to the vertebrate ventral BMP4–dorsal chordin pattern, providing evidence that one of the groups must have turned upside down during evolution. Dpp and BMP4 have similar biological activity, as do chordin and sog. In this context it is significant that *tinman* has a vertebrate homolog *Nkx2.5*, and this is also needed for heart development (see Chapter 15).

The dorsoventral system provides examples of several key developmental processes. The gurken and spätzle proteins are both **inducing factors** that cause a regional patterning of their competent tissue. The localized region of activation of Toll is an example of a **cytoplasmic determinant** in the egg, in this case a determinant which is not made up of localized mRNA. The nuclear gradient of dorsal protein, although intracellular, exemplifies the conversion of a simple pattern into a more complex one through the formation of a **gradient**. The various dorsoventral tissue types arise because each is encoded by a combination of transcription factors turned on, directly or indirectly, by the dorsal protein gradient.

The anteroposterior system

The specification of structures along the anteroposterior axis is controlled by maternal systems which are largely, although not

entirely, independent from that controlling the dorsoventral pattern. The basic mechanism is summarized in Fig. 11.9. In the anterior, mRNA for *bicoid* is deposited in the egg and a gradient of bicoid protein activates various genes to generate regional subdivisions (Fig. 11.9a). In the posterior, mRNA for *nanos* is deposited, and its protein product lifts the inhibition on gene activation in the future abdomen (Fig. 11.9b).

Classic Experiments

The first real gradient

Developmental biologists had speculated for some years about the existence of morphogen gradients that might control the pattern of gene expression. This was an element in the theoretical paper by Wolpert which revived the problems of experimental embryology and restated them in modern language. But the first gradient to be detected experimentally was that of the bicoid protein within the early *Drosophila* embryo. This was actually an intracellular gradient since the early embryo is a syncytium. But it did control gene expression and it did follow the predicted behavior quantitatively, so people finally started to believe in gradients.

Wolpert, L. (1969) Positional information and the spatial pattern of cellular differentiation. *Journal of Theoretical Biology* **25**, 1–47.

Driever, W. & Nüsslein-Volhard, C. (1988) A gradient of bicoid protein in *Drosophila* embryos. *Cell* **54**, 83–93.

Anterior system

bicoid is a maternal effect gene coding for a homeodomain transcription factor. Loss-of-function mutations cause a deletion of the head and thorax. Transcription of *bicoid* occurs during oogenesis in both oocyte and nurse cells, and the mRNA becomes localized at the anterior of the oocyte. Study of the bicoid protein by antibody staining showed that it was synthesized during the syncytial stage at 1–3 hours of embryonic development and forms an exponential concentration gradient from anterior to posterior. The localized mRNA is the source and, as the protein appears to have a short half-life, the remainder of the embryo is the sink, and such a system will generate an exponential gradient as explained in Chapter 4. The level of protein can be manipulated by changing the number of active copies of the gene in the female and this displaces in the expected directions features such as the cephalic furrow or stripes of pair-rule gene expression whose formation depends on the gradient.

A number of microsurgical experiments have been done on this system which show exactly the properties expected for a morphogen source at the anterior end of the egg. An effect similar to the *bicoid* mutation can be produced by pricking the egg at the anterior end and extruding about 5% of the cytoplasm, which contains most of the bicoid mRNA. Conversely, eggs

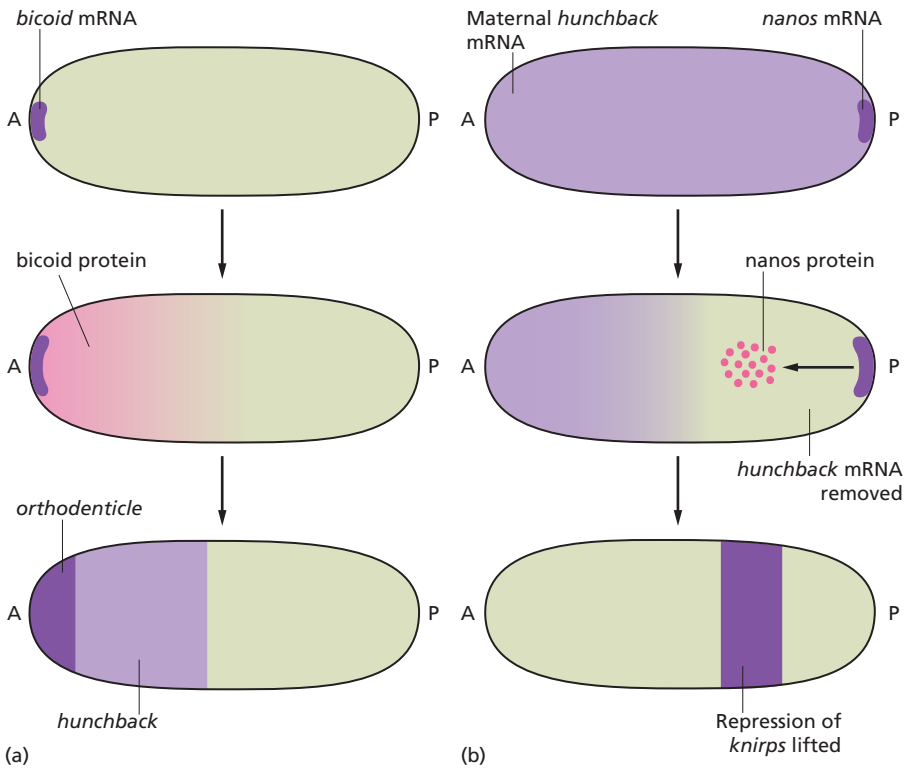

Fig. 11.9 Maternal anteroposterior system: (a) anterior system; (b) posterior system.

lacking *bicoid* can be rescued toward a normal phenotype by injection of *bicoid* mRNA. The position at which the injection is made determines the position of the induced anterior end, so, for example, injection of mRNA to a central position of a *bicoid*$^{-}$ egg produces a head at the injection site, flanked by two thoraxes in mirror symmetrical arrangement.

The function of the bicoid protein gradient is to regulate the zygotic expression of several of the gap genes, such as *orthodenticle*, *hunchback*, and *Krüppel*. *orthodenticle* and *hunchback* both encode homeodomain transcription factors, and *Krüppel* encodes a zinc-finger transcription factor. Each of these genes has a promoter of different sensitivity to bicoid, so they become activated at different anteroposterior levels.

Posterior system

The posterior system depends on the deposition of mRNA for *nanos* in the posterior and the action of its protein product in lifting inhibition on the transcription of gap genes in the future abdomen (Fig. 11.9b). The mechanism of this system has been deduced from a combination of genetic and embryological experiments.

Pricking of the posterior pole and extrusion of a small volume of cytoplasm causes defects in the larva; not in the telson as might be expected, but in the abdomen whose prospective region lies around 50–20%EL. This indicates that something present at the posterior pole is needed for development of the abdomen. There are also a number of mutations in maternal-effect genes that cause loss of the abdomen. Among these are mutations in *nanos*, *oskar*, and *pumilio*. Mutant embryos for all these genes can be rescued toward normality by injection of cytoplasm taken from the pole plasm region of a wild-type embryo and injected into the abdominal region of the mutant, confirming the localization of an "abdomen-forming substance" at the posterior pole of normal embryos. Cytoplasm taken from the nurse cells of mutant egg chambers has a similar rescue activity, except in the case of the *nanos* mutant, from which the cytoplasm has no rescue activity. This shows that *nanos* must encode the functional end product of the pathway. *oskar* is in fact required for the formation of the pole plasm, and sequestration of the *nanos* mRNA, and *pumilio* is required for relaying nanos activity from pole plasm to prospective abdomen.

nanos codes for an RNA binding protein. Its mRNA is normally localized in the pole plasm, but the protein is found in the prospective abdomen. The function is to allow the transcription of the zygotic gap gene *knirps*. The mechanism of action involves a double repression with *knirps* being repressed by hunchback, and translation of *hunchback* being inhibited by nanos. As we have seen, *hunchback* is another zygotic gap gene and is activated by bicoid in the anterior. But *hunchback* is also active during oogenesis such that the egg is normally filled with a uniform concentration of maternally derived *hunchback* mRNA. Translation of this mRNA commences in early cleavage and is normally inhibited in the posterior half of the embryo by the nanos protein. *knirps* becomes turned on in the posterior but not in the anterior because hunchback protein is absent from the posterior. If nanos is missing then the hunchback protein is made all over and *knirps* cannot be turned on anywhere. Since activation of *knirps* depends on a double inhibition it follows that the nanos protein would not be necessary in the absence of the maternal *hunchback* message, and indeed this is the case as embryos from double mutant *hunchback*$^{-}$/*nanos*$^{-}$ mothers are near normal. Nanos is required again at a later stage for germ cell development, and for this it is produced from the zygotic gene.

Initial establishment of anteroposterior and dorsoventral polarity in the oocyte

The initial establishment of the anteroposterior polarity occurs early in oogenesis when the oocyte begins to elongate. At this stage an array of microtubules is formed and is orientated such that the minus end is anterior and the plus end posterior. The mRNAs for *bicoid* and *oskar* are translocated along these microtubules by **motor proteins** so that they end up anterior or posterior, respectively (Fig. 11.10). This tubule array can be demonstrated in flies transgenic for a gene for a fusion protein of kinesin with β-galactosidase. In the egg chambers of these flies, the fusion protein could be located by staining for β-galactosidase, and was found at the posterior end of the oocyte, along with *oskar* mRNA. Kinesin normally migrates to the plus end of microtubules and this experiment shows that the tubule array can serve as a polarized substrate for intracellular localization.

The direction of polarization of the tubules depends on the position of the oocyte in the egg chamber. In mutants of certain genes, such as *spindle-C*, the oocyte is located in the center rather than the posterior of the egg chamber. The oocytes then develop as double anterior in character, with the *oskar* mRNA central, and *bicoid* mRNA at both ends. The key genes required for the tubule polarization process have already been encountered as genes required for dorsoventral patterning, namely *gurken* and *torpedo*. In females lacking either of these, the specialized group of follicle cells called **anterior border cells** develop not just at the anterior but instead form at both ends of the oocyte.

From the behavior of this type of mutant, the course of events in normal development has been deduced. Early in oogenesis the oocyte lies at the posterior of the egg chamber. At this stage the border cells at both ends are committed to develop with an anterior character. *gurken* is expressed uniformly in the oocyte, but, because of the posterior location of the oocyte, gurken protein can only affect the neighboring border cells which thereby become switched to be posterior in character. The posterior state requires activation of torpedo at the cell surface and consequently the ERK signal transduction pathway internally. Following this, the posterior border cells emit another, as yet unknown, signal which brings about the polarization of the

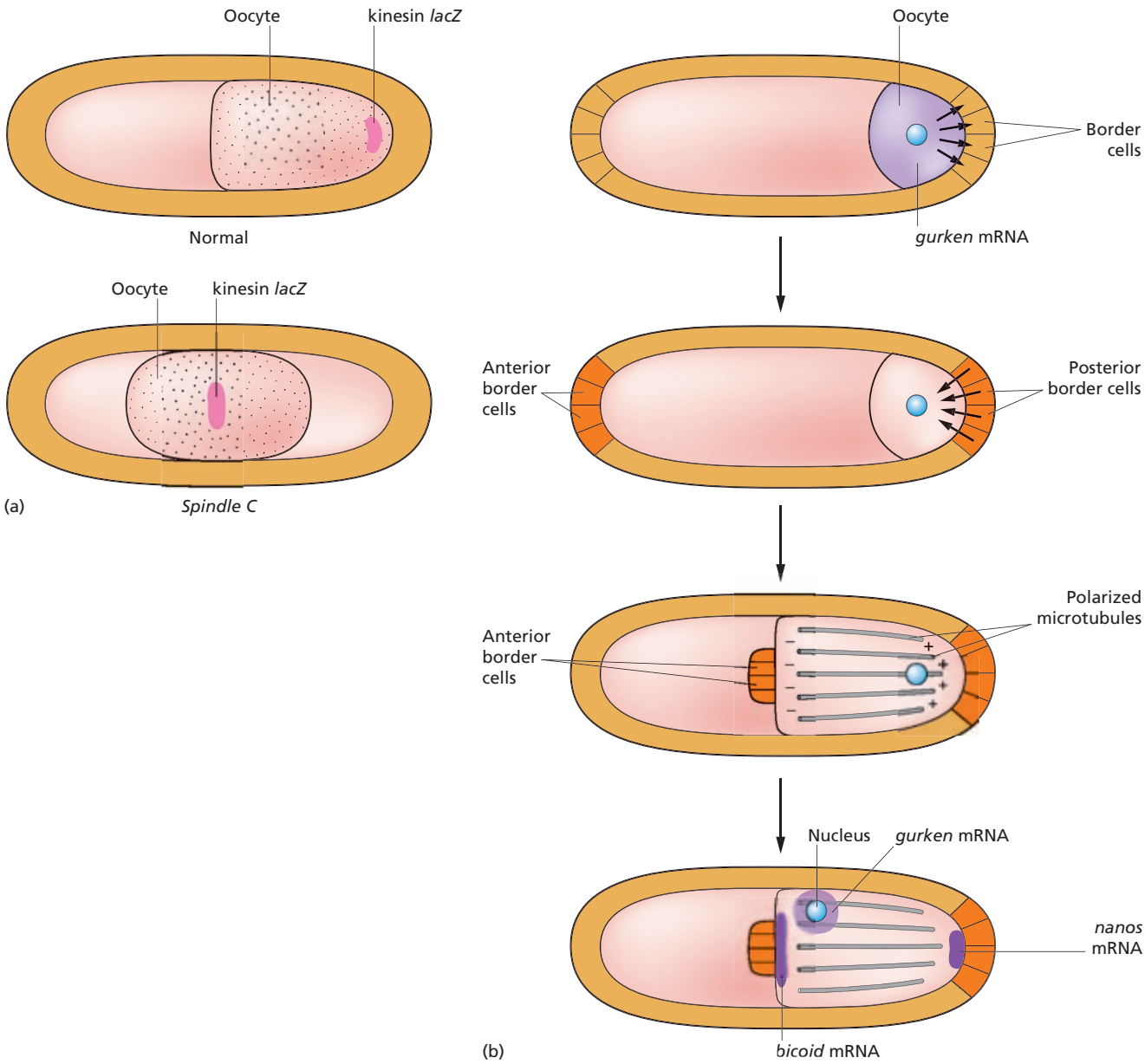

Fig. 11.10 Origin of anteroposterior polarity: (a) *Spindle* C mutant; (b) localization of *oskar* and *bicoid* mRNAs by transport along orientated microtubules.

microtubules in the oocyte such that the plus ends are directed to the posterior. The orientation of the tubules means that plus- or minus-directed motor proteins can, respectively, localize *oskar* mRNA to the posterior and *bicoid* mRNA to the anterior.

As the oocyte grows larger, the nucleus moves back towards the anterior along the microtubule array. The tubules are arranged around the exterior of the oocyte, so the nucleus has to follow a track around the exterior rather than travel straight down the central axis. This movement breaks the former radial symmetry of the oocyte and causes the nucleus to lie closer on one side than the other. Now the *gurken* mRNA, still near the nucleus, causes gurken protein to be secreted just from the side on which the nucleus is present. The adjacent follicle cells become dorsal in character because the gurken signal stimulates torpedo and thereby represses *pipe*, as described above.

Thus, the same genes that are responsible for the dorsoventral patterning of the follicle cells are also, at an earlier stage of oogenesis, responsible for the anteroposterior patterning of the oocyte and border cells. The reason that the same signal can be involved in polarization along two anatomically orthogonal axes is threefold: it acts at different times; the responding populations of follicle cells have different competence; and the intervening growth of the oocyte has changed the effective location of the signal.

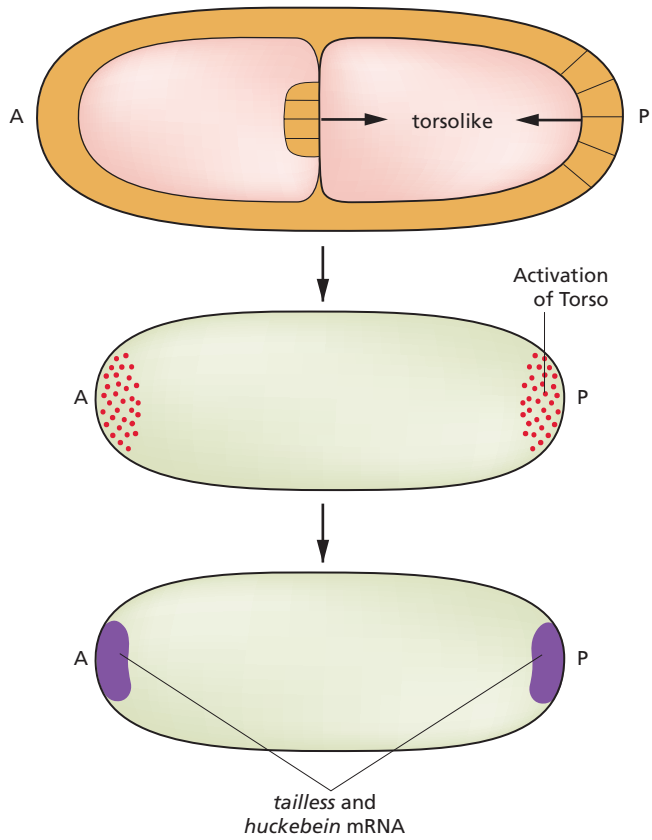

Fig. 11.11 The terminal system.

Terminal system

A third maternal system is concerned with the formation of the embryo termini (Fig. 11.11). It involves a signal from the follicle cells activating a receptor at both termini and thereby turning on zygotic genes.

Embryos resulting from loss-of-function mutations of the terminal system show defects at both ends with a normal pattern in between. The key gene in the pathway is *torso*, which also has a gain-of-function allele causing substantial suppression of segmentation in the thorax and abdomen. Cloning and sequencing of *torso* revealed that it encoded a cell-surface receptor of the tyrosine kinase class. It stimulates the ERK signal transduction pathway and the gain-of-function phenotype arises from a constitutively active form of the receptor. As with *Toll*, expression is uniform in the oocyte and in normal development the receptor becomes activated at the termini. The ligand is encoded by *trunk*, which is active in the oocyte and belongs to the same gene family as *spätzle*. It is activated at the termini by the product of *torsolike*, encoding a novel protein, which is present in the anterior and posterior border cells.

Activation of *torso* leads, via the ERK pathway, to activation of two zygotic gap genes: *tailless* and *huckebein*. *tailless* encodes a transcription factor of the nuclear receptor family and *huckebein* encodes a zinc-finger transcription factor.

Although different molecules are involved, the terminal system has a mechanism remarkably similar to the dorsoventral system, and once again the activated receptor protein can be regarded as a type of **cytoplasmic determinant**.

Gap genes

The system of morphogens and determinants bequeathed by the mother becomes elaborated into increasingly complex patterns of gene activity by successive levels of the developmental hierarchy. The first zygotic level is made up by the **gap genes**, so called because mutant embryos have patterns bearing gaps of up to eight contiguous segments. All the gap genes code for transcription factors, and because the early embryo is a syncytium, these can diffuse from one nucleus to another and exert their effects directly, with no need for cell–cell signaling. Some important members of this group are *orthodenticle*, *hunchback*, *Krüppel*, *knirps*, and *giant*. The regulatory relationships have been deduced mainly by two types of experiment:

1 Examining the expression pattern of one gene in the absence of another. Expansion of a domain indicates repression and reduction of a domain indicates activation in normal development.

2 Examining the effect of uniform overexpression of another gene.

The type of result predicted from these two protocols is shown in Fig. 11.12.

Along with bicoid protein, another important early regulator of gap gene expression is the product of the *caudal* gene. This encodes a homeodomain transcription factor and is homologous to the *cdx* gene family in vertebrates. It is expressed maternally to produce a uniform distribution of mRNA in the oocyte. At the syncytial blastoderm stage, mRNA is differentially lost resulting in a posterior-to-anterior gradient of mRNA and protein. Embryos lacking *caudal* have severe posterior defects. Some gap genes are activated by both bicoid and caudal proteins, and since these two factors form inverse gradients in the early embryo, it means that the activation appears to be autonomous and not spatially regulated. *caudal* also has a posterior zygotic domain in the prospective proctodeum.

orthodenticle mutants have defects in the head. The gene encodes a homeodomain transcription factor. Expression is in the head and is activated by high levels of bicoid and by torso. An important vertebrate homolog of *orthodenticle* is *otx2*, which is required for the formation of the forebrain and midbrain.

Embryos homozygous for null alleles of *hunchback* have a large anterior gap which removes the labium and thorax. The gene codes for a zinc-finger transcription factor. Transcription during oogenesis leaves mRNA uniformly distributed in the egg, but as we have seen its stability and translation is antagonized in the posterior half by the nanos protein and so an anterior to posterior gradient of hunchback protein arises during cleavage.

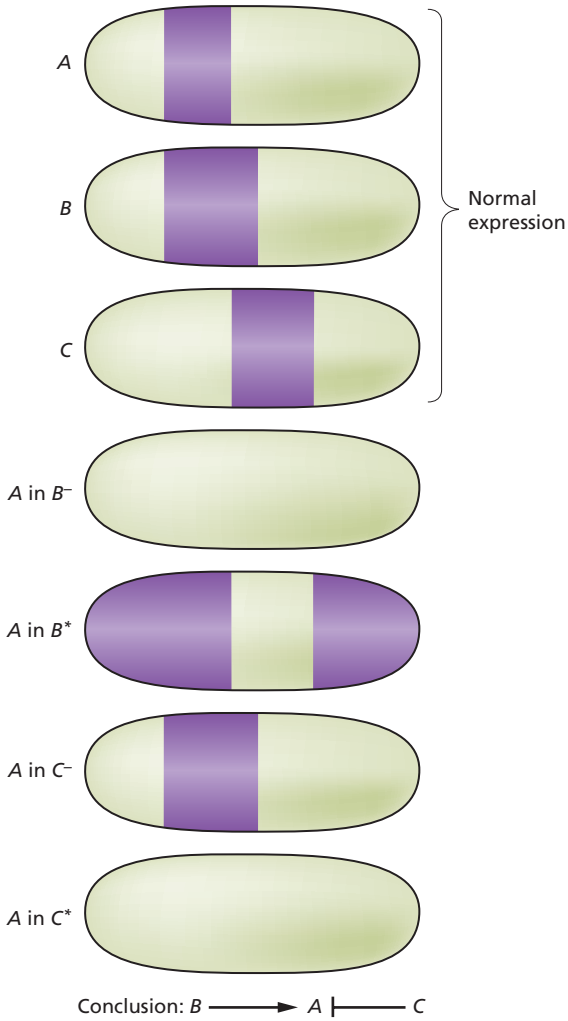

Fig. 11.12 How to work out regulatory relationships between genes. The top three drawings show the normal expression of three genes. The bottom four show expression of gene *A* in embryos mutant (–) or with ubiquitous overexpression (*) of *B* or *C*. The results show that *B* activates *A* and that *C* represses *A*.

Zygotic transcription commences in the syncytial blastoderm in the anterior half of the embryo, and at cellular blastoderm also in a posterior stripe. The anterior, but not the posterior, zygotic domain is activated directly by the bicoid protein at a lower concentration than that required to activate *orthodenticle*. The posterior zygotic domain is activated by torso.

Krüppel is an entirely zygotic gene whose null mutants have a large deletion of the central part of the body, comprising the thorax and abdominal segments 1–5. A second copy of abdominal 6 is often found in inverted orientation. The gene codes for a zinc-finger transcription factor. Expression commences in the syncytial blastoderm as a central band. *Krüppel* is activated by bicoid and hunchback, and repressed by knirps and giant. This ensures its activation as a broad band from about 60 to 50%EL.

Null mutants of *knirps* are similar to the maternal posterior group, having abdominal segments 1–7 replaced by a single large abdominal segment of uncertain identity. The gene codes for a transcription factor belonging to the nuclear receptor family. The expression pattern shows a band between about 45 and 30%EL, coming on at syncytial blastoderm. Activation is constitutive and the position of the main band in normal development is regulated by repression due to hunchback and tailless.

giant mutants have defects in the anterior thorax and in the abdomen at A5–A7. *giant* encodes a leucine zipper transcription factor and expression starts in the syncytial blastoderm in two zones, an anterior zone about 80–60%EL and a posterior zone about 33–0%EL. By the cellular blastoderm the posterior band is fading and the anterior one has resolved into three stripes. *giant* is activated by bicoid and caudal, and repressed in the anterior by hunchback.

tailless and *huckebein* are the zygotic genes activated by torso. The mutants each have terminal defects, which together add up to the phenotype produced by mutants of the maternal terminal genes.

The main regulatory relationships that have been mentioned are summarized in Fig. 11.13.

Pair-rule system

The **pair-rule genes** function as a layer in the developmental hierarchy between the gap genes and the segment polarity genes. They also have a role, along with the gap genes, in controlling the expression of Hox genes and aligning their domains with

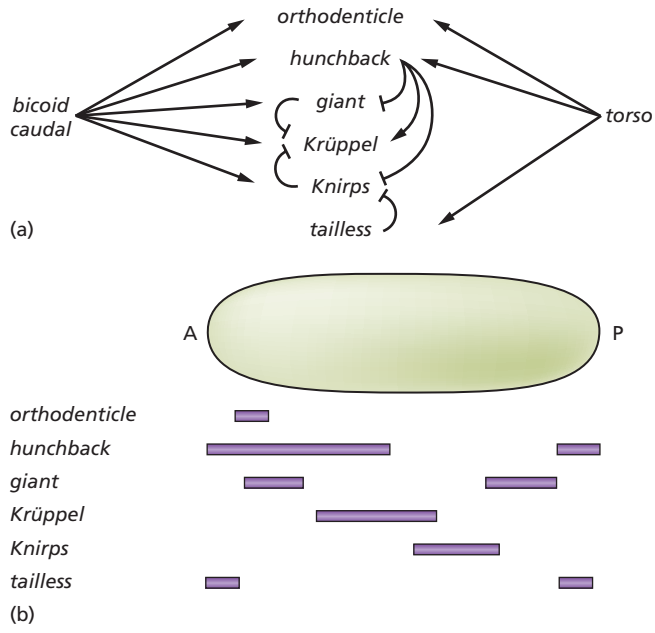

Fig. 11.13 Gap genes: (a) some regulatory relationships; (b) simplified expression domains of six gap genes.

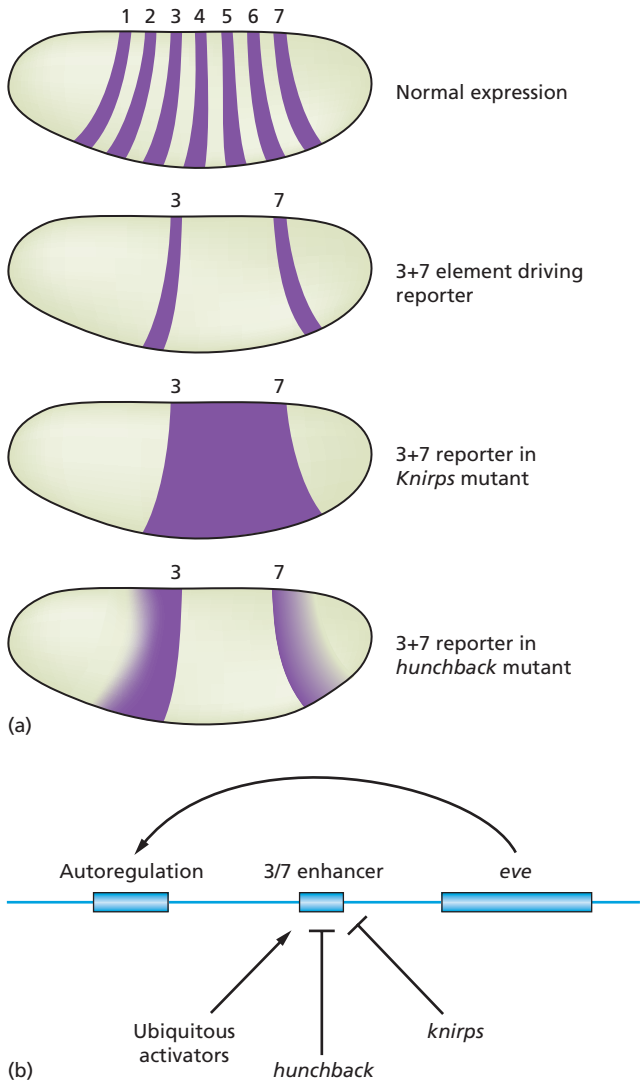

Fig. 11.14 Regulation of *even-skipped*: (a) normal expression pattern, reporter driven by 3+7 enhancer, effects of mutations on pattern of reporter expression; (b) two enhancers controlling *even-skipped* expression.

the segmental repeating pattern. The activation of the pair-rule genes represents the first formation of a reiterated pattern in the embryo.

All the pair-rule genes code for transcription factors and the expression patterns consist of seven stripes corresponding to two segment-wide bands of the syncytial blastoderm (Fig. 11.14). The mutant phenotypes typically show deletions with a periodicity of two segments, although more complex and more severe phenotypes can be found. For example *even-skipped* gets its name from the fact that the even numbered segments are reduced or lost in the original mutant. But this is now known to be a hypomorphic mutant, and in the null mutant all the segments are lost.

Those pair-rule genes designated primary are regulated mainly by the maternal and gap genes, while those designated secondary are regulated mainly by the primary genes. Primary pair-rule genes are *hairy*, *even-skipped (eve)*, and *runt*, and they have complex regulatory regions containing several enhancers. Among the secondary pair-rule genes are *paired, odd-paired (odd), sloppy-paired (slp)*, and *fushi-tarazu (ftz)*. *ftz* is regulated to a large extent by repression due to *hairy* and so the *ftz* stripes appear in between the *hairy* stripes.

In general the rule is "one enhancer, one stripe." Each enhancer contains overlapping binding sites for activators and repressors such that it will be on if the activators prevail and off if the repressors prevail. To form a stripe it is necessary that the gene be turned on at one anteroposterior level and turned off again at a slightly different level. The *even-skipped* gene has been subject to very detailed analysis. It encodes a homeodomain protein which acts as a transcriptional repressor and the gene has large regulatory regions containing 12 enhancers. As an example, the stripe 2 element is activated by bicoid and hunchback and is repressed by giant and Krüppel, so in normal development stripe 2 is formed in the thin strip in between the *giant* and *Krüppel* domains. Two of the enhancers control pairs of stripes. The 4+6 and the 3+7 enhancers are both activated by ubiquitous components and repressed by hunchback and knirps. hunchback and knirps repress each others' transcription, ensuring that the *knirps* domain lies in between the two zygotic *hunchback* domains. The two enhancers show different levels of sensitivity to repression so each enhancer controls two expression stripes and they are nested such that stripes 3 and 7 form outside stripes 4 and 6 (Fig. 11.14). In addition to the stripe enhancers, there is an autocatalytic element which stabilizes the seven-stripe pattern once it is formed, and there are other elements active at later stages driving expression in the mesoderm or in subsets of neurons.

The way that the pair-rule transcription factors activate the 14 stripes of the segment polarity genes is very complex indeed. But in

New Directions in Research

As the development of *Drosophila* is rather well understood, its main role in the future is likely to be in the area of cell biology. The genetic tools for ablating genes in specific regions or labeling specific cell types are extremely powerful. These are increasingly enabling the investigation of phenomena such as cell polarity, cell movement, and intracellular trafficking of materials, in a way which is complementary to the continued use of mammalian tissue culture cells.

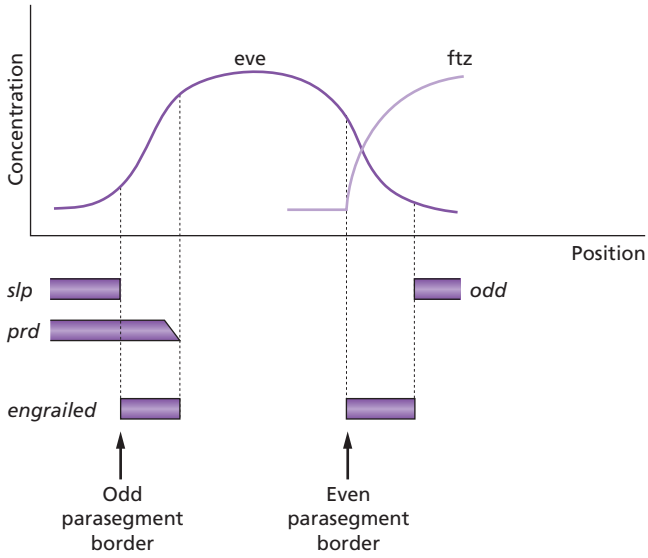

Fig. 11.15 Initial establishment of *engrailed* stripes. Low levels of eve repress *slp* and *odd*, thus enabling *engrailed* to be activated by prd and ftz.

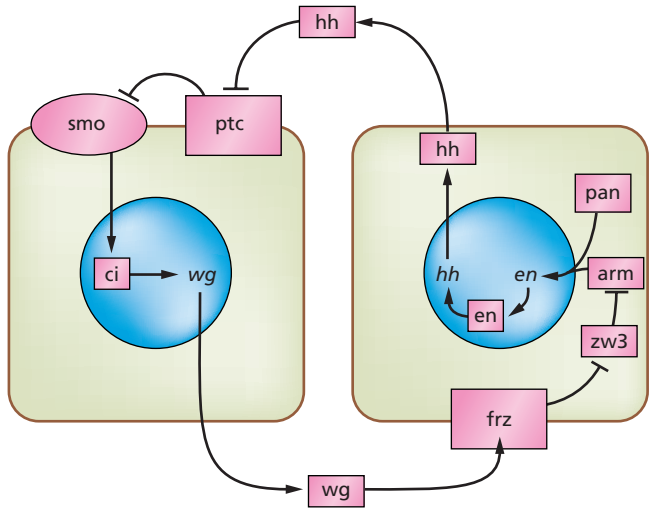

Fig. 11.16 Maintenance of the pattern by the action of Hedgehog and Wingless systems. Abbreviations: *en*, *engrailed*; hh, hedgehog; ptc, patched; smo, smoothened; ci, cubitus interruptus; *wg*, *wingless*; frz, frizzled, zw3, zeste white-3; arm, armadillo; pan, pangolin.

principle it is a matter of creating 14 narrow bands in which activators predominate over inhibitors. For example the *engrailed* stripes of the odd-numbered parasegments are activated by *paired*, and *eve* enables this to occur by repressing the expression of *sloppy-paired*, such that there is a domain in which *paired* predominates over *sloppy-paired*. The *engrailed* stripes of the even-numbered parasegments are activated by *ftz* in the region where there is a slight overlap with *eve* (Fig. 11.15). This is because here *eve* represses expression of *odd-paired* and enables *ftz* to prevail. The fact that the odd- and even-engrailed stripes are regulated by different mechanisms explains the origin of the "pair-rule" phenotypes when expression of one pair-rule gene is altered. Note that the gene names *even-skipped* and *odd-skipped* relate to visible segment defects. Because the initially formed parasegments are out of phase with the final segments the *eve* mutants give odd parasegmental defects and *odd* mutants give even parasegmental defects!

Segment polarity system

The **segment polarity genes** function to create the **parasegment** boundaries of the early embryo. Once activated, they maintain their repeating pattern through a positive-feedback loop between the cells on either side of each boundary, defined by activity of the transcription factor genes *engrailed* and *cubitus interruptus (ci)*. The *engrailed* cells emit an inducing factor called hedgehog, which maintains activity of *ci* in the neighboring cells, and the *ci* cells emit a factor called wingless which maintains activity of *engrailed* in the neighboring cells (Fig. 11.16).

After cellularization of the blastoderm, it is no longer possible for one nucleus to influence another simply by producing a transcription factor and allowing it to diffuse into the surroundings. In a multicellular embryo communication necessarily involves the secretion of inducing factors and the activation of cell-surface receptors. Whereas all the gap and pair-rule genes code for transcription factors, the segment polarity genes, which start to function after cellularization, may code either for transcription factors or for components of the signaling machinery. Most of the segment polarity genes have mutant phenotypes in which the segmental pattern of denticle bands is replaced by a continuous lawn of denticles. This spiky appearance is underlined by some of the gene names, such as *hedgehog*, *armadillo*, or *pangolin*.

engrailed codes for a homeodomain transcription factor and comes on at the cellular blastoderm stage, forming a 14-stripe pattern by the extended germ band stage. Each band characterizes the anterior quarter of a parasegment. Expression of *engrailed* is initially activated by the pair-rule genes as described above (Fig. 11.15). *ci* encodes a Gli-type zinc-finger transcription factor and is repressed by engrailed such that its expression pattern consists of 14 stripes in between the *engrailed* stripes.

The maintenance of the pattern depends on mutual interactions. This may be shown by the fact that if a gene required for one of the states is absent, the pattern is initially set up correctly, but it then rapidly decays. The key genes for maintenance make up the components of two intercellular signaling systems. In the *ci* cells, a factor called wingless is produced, homologous to the vertebrate Wnt factors. This stimulates a wingless receptor coded by two *frizzled* genes. The signal represses a kinase coded by the *zeste-white-3 (= gsk3)* gene, which in turn represses a protein coded by the *armadillo (= β-catenin)* gene. As two repressions equal one activation this means that Wingless activates armadillo and causes it to move into the nucleus, together with

the product of the *pangolin* gene (= *Lef/Tcf*), to activate target genes. Among the targets is *hedgehog*, so hedgehog protein is secreted by the *engrailed* cells and binds to the receptor encoded by *patched*. Patched is a constitutively active repressor of smoothened, another cell-surface protein, which activates the Ci protein. Hedgehog inhibits patched, thus lifts the repression of smoothened and thereby enables Ci protein to enter the nucleus and to activate the *wingless* gene. The system is shown in Fig. 11.16.

There is an essential polarity to this system, because the signals hedgehog and wingless only activate their targets on one side and not on both. This polarity arises from the action of other pair-rule genes that restrict the competence to express *wingless* to the posterior half of the parasegment. The way the system works is well illustrated by examining the mutant phenotype of *patched*. Unlike many of the other segment polarity genes, mutants of *patched* do not show a lawn of denticles. Instead they show a remarkable pattern in which there are extra segment boundaries in between the normal ones, so the pattern approaches a 32-segment one. The reason is that patched is a repressor, so inactivation causes activation of the patched targets, including *wingless*. But *wingless* can only be turned on in its competence domain, roughly the posterior half of the parasegment, so its domain is enlarged but it does not become ubiquitous. Now, the anterior edge of the enlarged *wingless* domain abuts the competence domain of *engrailed*, leading to activation of an ectopic stripe of *engrailed*. Since a parasegment border appears at junctions between *engrailed* and *ci* regions, this causes the appearance of an ectopic border (Fig. 11.17). Exactly the same phenotype is produced by overexpression of *hedgehog*, driven by a ubiquitous promoter. This represses the activity of patched all over and hence also causes the enlargement of the *wingless* domain and the formation of an ectopic *engrailed* stripe.

The segment polarity genes all have vertebrate homologs that are important in development and have been mentioned in previous chapters (Table 11.1). Although these homologs exist and the biochemical pathways are substantially the same, this does not mean that the developmental functions are necessarily the same. The *engrailed–cubitus* loop is the motor of segmentation in insects and other arthropods, but it is probably not involved in vertebrate segmentation. Likewise, the Wnt pathway is an essential feature of dorsoventral polarity determination in *Xenopus*, but has no comparable function in *Drosophila*.

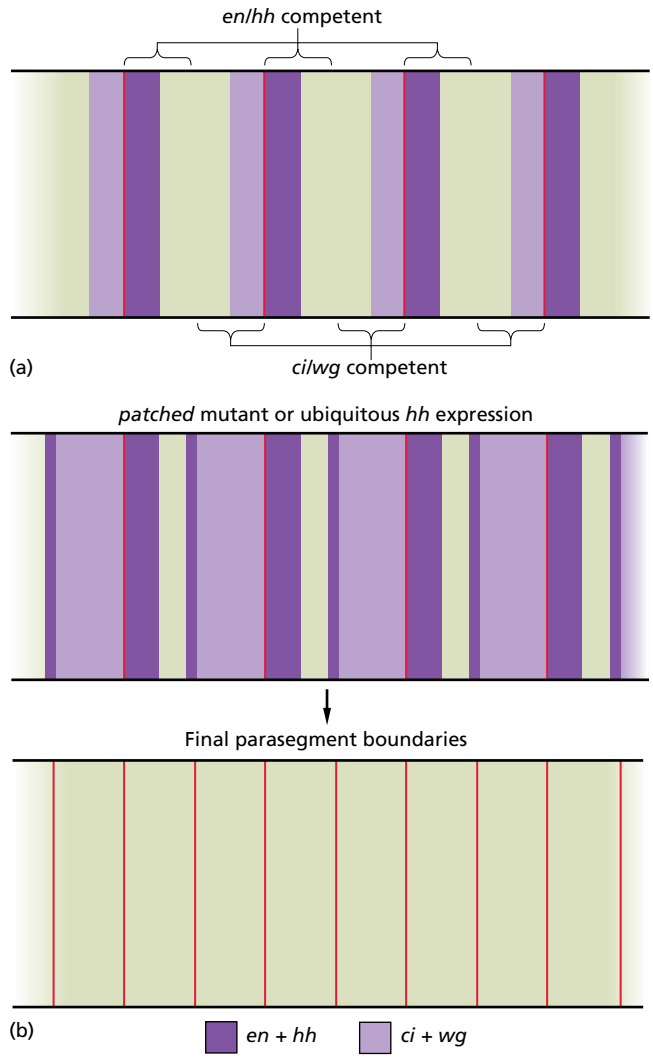

Fig. 11.17 (a) Normal segmentation domains. (b) The effect of ubiquitous expression of *hedgehog* is the same as that of removal of *patched*. *cubitus* becomes activated over its entire region of competence and the resulting Wingless signal induces a second file of *engrailed*-expressing cells from the region of *engrailed* competence. A parasegment border forms wherever *cubitus* and *engrailed* cells are juxtaposed, so the end result is to double the number of segments.

Table 11.1 Vertebrate homologs of segment polarity genes.

Drosophila	Vertebrate
engrailed	*en1* and *en2*
wingless	*wnt1-wnt12*
hedgehog	*sonic, Indian, desert, banded hedgehog*
cubitus interruptus	*gli-3*
frizzled 1,2	*Many frizzleds*
zeste-white-3	*glycogen synthase kinase 3*
armadillo	*beta-catenin*
pangolin	*lef/tcf*
patched	*patched*

Hox genes

The *Drosophila* Hox complex is split into two regions called the Antennapedia and the Bithorax complexes. The Antennapedia complex corresponds to vertebrate paralog groups 1–6 and contains the genes *labial*, *proboscipedia*, *Deformed*, *Sex combs reduced*, and *Antennapedia*. The Bithorax complex corresponds to vertebrate paralog groups 7–10 and contains the genes *Ultrabithorax (Ubx)*, *abdominal-A*, and *Abdominal-B*. The

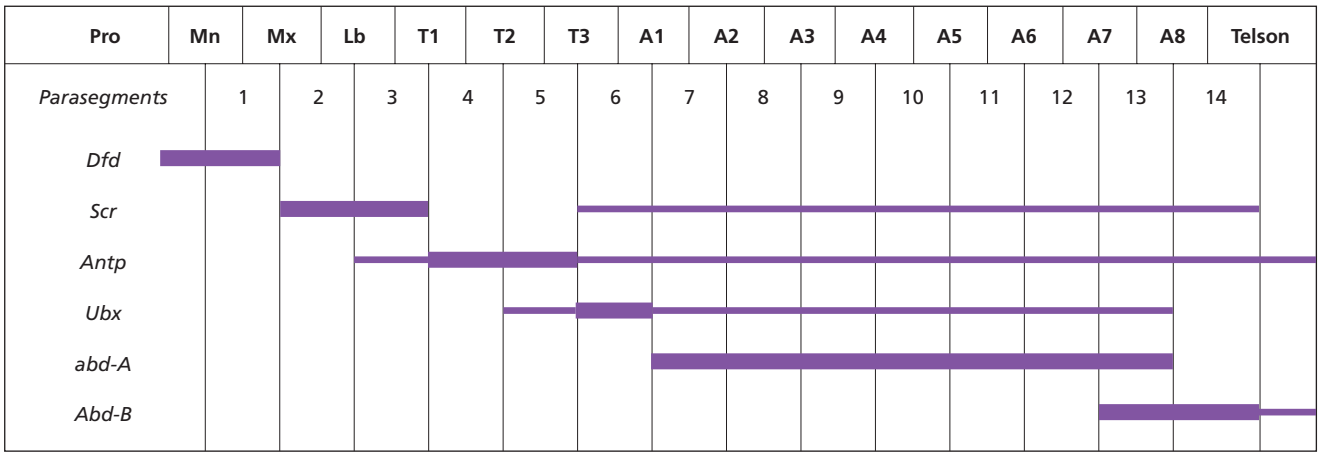

Fig. 11.18 Schematic expression of Hox genes at the extended germ-band stage.

Antennapedia complex also contains *bicoid* and *zerknüllt*, which do not function as Hox genes in *Drosophila*, but have Hox-like homologs in other insects. Except for *Deformed* where a substantial protein concentration is achieved by the cellular blastoderm stage, the Hox genes are turned on slightly later than the gap and pair-rule classes, and their protein products have generally built up to an effective level by the extended germ-band stage. As in other animals, the order in which the genes are expressed in the anteroposterior axis is also the order of their arrangement on the chromosome.

The approximate expression domains of the Hox genes at the extended germ-band stage are shown in Fig. 11.18 (*proboscipedia* is expressed only in the larva). The function of these genes is to impart different characters to the different segments. In general if the expression domains are altered by mutation or by overexpression experiments, then the identity of the segments is altered in a predictable way. Loss-of-function mutations generally produce anteriorizations and gain-of-function mutations generally produce posteriorizations. However, their action does not depend simply on the combination of gene products present in a region, because individual structures within a segment may also be specified by a peak of expression of one of these genes. For example, expression of *Antennapedia* in parasegment 5 excludes the tracheal pits, which are the only cells of this parasegment to express *Ubx*.

The properties of the system are illustrated by a consideration of the *Ubx* gene, which is the homolog of the Hox7 paralog group in vertebrates. The expression of *Ubx* starts around cellularization and shows an initial peak in parasegment 6 and subsequent lesser expression from parasegments 5 to 13. It is also expressed in the mesoderm. Later expression is prominent in the ventral ganglia of these segments and in the metathoracic (T3) imaginal discs of the larva. Null mutants of *Ubx* show a transformation of parasegments 5 and 6 to parasegment 4.

Initial control of *Ubx* expression is by hunchback which acts as a repressor. There is an activation by fushi tarazu, which gives a transient pair-rule character to the *Ubx* pattern. This pair-rule control is important for aligning the parasegmental register of segment polarity and homeotic genes. Once it is established, the posterior boundary of *Ubx* expression is maintained by repression from abdominal-A. Ubx itself represses *Antp*, so maintaining its posterior boundary. Maintenance, at least in the visceral mesoderm, also depends on a positive feedback loop involving wingless and dpp. To ensure more permanent regulation there are groups of genes that are responsible for Hox maintenance in the long term, such as *Polycomb* and *extra sex combs*. How they work is poorly understood, but they are concerned with chromatin structure, and the demarcation of active and inactive domains within the genome. These genes tend to be ubiquitously active and repressive, so loss of function mutants give ectopic Hox activity (e.g. the *extra sex combs* mutant derepresses *Sex combs reduced* in T2–3, leading to the formation of male sex combs on the second and third legs as well as on the first leg where they are normally found). Homologs of the *Polycomb* group are found in all eukaryotes and are called **chromobox genes.**

The anteroposterior body pattern

The long and complex series of interactions described above leads to a fully specified body plan by the extended germ band stage. This specification has two essential components. There is the repeating pattern composed of parasegments whose boundaries are defined by the juxtaposition of bands of cells in which the *engrailed* (anterior) and *ci* (posterior) systems are active. There is also the nonrepeating sequence of Hox gene expression zones. Although the Hox domains overlap, there is a clear sequence from anterior to posterior in which a single gene predominates.

Each element of this pattern appears to be initiated locally by combinations of concentrations of the products of the maternal systems, the gap genes, and the pair-rule genes. The pair-rule genes have a particularly important role in that they must control

the register between the segment polarity and the homeotic selector genes so that each segment acquires the correct identity. By the extended germ-band stage the products of the controlling systems have decayed or are decaying and so the maintenance of the pattern is ensured by separate means, such as: positive-feedback loops, for example of *Ubx*; mutual reinforcement of neighboring states, for example of *engrailed* and *ci*; or inhibition between neighboring states, for example of *Antp* by *Ubx*.

Up to the formation of the general body plan the regional specification of *Drosophila* is quite well understood. But the ultimate role of the developmental genes is to activate appropriate combinations of genes to carry out the differentiated functions of the relevant cells. How this is done is still poorly understood, and this is partly because the cell biology, histology, and general biochemistry of *Drosophila* is not nearly as well studied as that of vertebrates.

Key Points to Remember

- The mutagenesis screens for genes affecting early *Drosophila* development led to the discovery of most of the classes of gene that control development not just in *Drosophila* but in all animals.
- *Drosophila* and other insect embryos initially develop as a syncytium. This means that during the first 3 hours transcription factors can diffuse from one nucleus to another to control gene expression.
- The dorsoventral and anteroposterior patterns are specified largely independently. The dorsoventral pattern is set up by a series of interactions between oocyte and follicle cells, starting with the eccentric position of the oocyte nucleus and culminating in a gradient of dorsal protein in the nuclei of the syncytial blastoderm.
- The cytoplasmic determinants that control anteroposterior pattern are also laid down in the oocyte before fertilization. They are: *bicoid* mRNA in the anterior; *nanos* and *oskar* mRNA in the posterior; and activation of torso at the termini.
- Development proceeds in a stepwise fashion with each domain of cells being defined by the expression of a combination of transcription factors encoded by the gap and pair-rule genes. These regulate the expression of the segmentation genes, defining the 14 repeating units of the embryo; and the Hox genes that define the anteroposterior character of each parasegment.
- Segmentation is controlled by a cross-activation between stripes of engrailed-expressing cells that secrete hedgehog, and cubitus interruptus-expressing cells that secrete wingless.
- Anteroposterior pattern is controlled by transcription factors encoded by Hox genes which are activated in a nested pattern such that all are on at the posterior end with each gene having a specific anterior expression limit. Loss-of-function mutants generally cause anteriorization, while gain-of-function mutants generally cause posteriorization.

Further reading

See also the general textbooks referenced in Chapter 2.

Website for data on *Drosophila* genes

The Interactive Fly: http://www.sdbonline.org/fly/aimain/1aahome.htm

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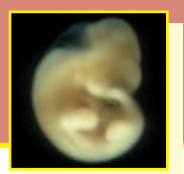
Chapter 12



Caenorhabditis elegans

Caenorhabditis elegans is a small, free-living soil nematode and has been used for developmental biology research since the 1960s. Among developmental biologists it is usually known as "the worm." In one sense it is the best known animal on Earth since the location and lineage of every cell in embryo, larva, and adult is known. Also its genome was the first of any animal to be completely sequenced. The genome contains 19,099 genes, of which about 2000 are mutatable to lethality.

Caenorhabditis elegans is kept on petri plates and feeds on bacteria. Genetic screening is easy because it is possible to examine large numbers of worms, and the generation time is only 3 days. The worms are self-fertilized **hermaphrodites**, so recessive mutations will automatically segregate as homozygotes in two generations without the need to set up any crosses (Fig. 12.1). Genetic stocks can be preserved in liquid nitrogen. This ease of genetic analysis means that large numbers of mutants are available, and, as in *Drosophila*, investigation of a biological problem often starts with a mutant affecting the process in question. As an alternative to the isolation of mutants it is now also easy to inhibit gene action by **RNA interference** (see Chapter 3). If double-stranded RNA complementary to an endogenous message is introduced this results in the production of embryos resembling the corresponding maternal effect mutation (**phenocopies**). The dsRNA can be administered by injection into the somatic tissues of the worm, or by feeding. A convenient method is to express the required dsRNA from a plasmid in *E. coli* and then use these bacteria as the food for the worms: sufficient dsRNA is absorbed intact to exert its biological effect.

It is also easy to make **transgenics** by injection of the required DNA into the gonad, where it is incorporated as an extra-chromosomal element into the germ cells. Such transgenics are not stable because the element can be lost at meiosis or mitosis. However, transient transgenesis is often sufficient for experimental purposes.

It is possible to make **genetic mosaics** for some parts of the genome. Mosaics arise by the spontaneous loss of small free

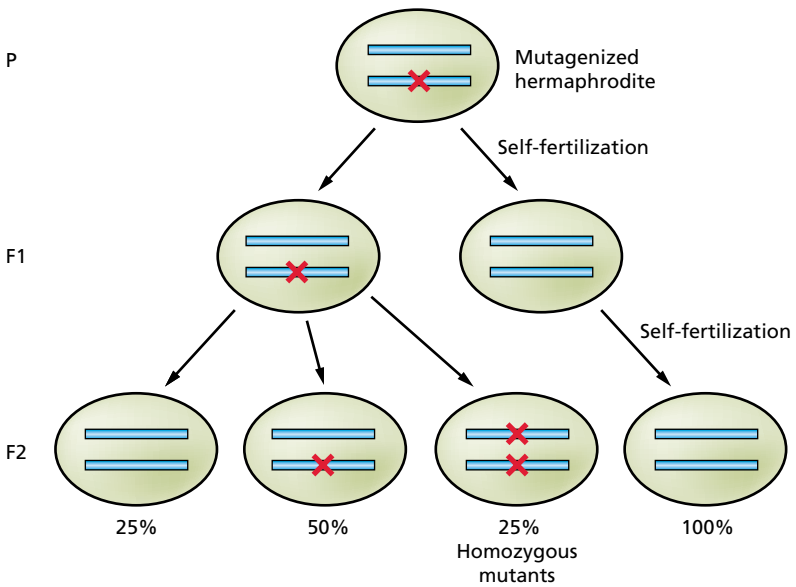


Fig. 12.1 Segregation of homozygotes after mutagenesis and two generations of self-fertilization. In the P generation a particular mutation will be found in just a few gametes, having occurred in a single germ cell.

chromosome fragments which are duplications of normal chromosomal regions. If the main chromosome carries a mutant allele and the free chromosome fragment the wild-type allele, then when this is lost from a single cell its descendant cells will all be mutant but the rest of the animal will be wild type. Mosaics can be very useful for establishing in which region of the embryo the function of a gene is required.

The negative features of *C. elegans* are the small size of the eggs and the tough egg case, both of which make microsurgical experiments difficult.

In *C. elegans*, it is conventional to capitalize the names of proteins. So for example the *glp-1* gene encodes the GLP-1 protein. As usual, many mutations affecting early development are maternal-effect and in such cases it is the genotype of the mother and not the zygote that determines the embryo phenotype. In *C. elegans* a gene that is lacking maternally will also usually be lacking in the embryo. This is because the normal mode of propagation from a self-fertilized hermaphrodite means that a –/– parent will produce –/– zygotes, so both will lack the gene. But it is still necessary to remember that the gene products that control early development are deposited in the egg during oogenesis.

Normal development

Adult anatomy

The adult is highly elongated ("worm shaped"; Fig. 12.2). The outer layer is the **hypodermis** which is one cell thick, largely syncytial, and secretes a thick cuticle. Beneath the hypodermis are four longitudinal bands of mononucleate muscle cells. There is a through gut with a muscular pharynx and an intestine. There is a nerve ring surrounding the pharynx, a ventral nerve cord, and tail ganglia. The main body cavity of nematodes is described as a **pseudocoelom** rather than a coelom because it is not lined all round with mesoderm. The **gonad** opens into a mid-ventral vulva. In the hermaphrodite this has two arms which are both bent back on themselves. Within the gonad, the cells nearest the vulva mature as sperm, while the more distant ones divide continuously as a syncytium and then become cellularized as **oocytes**. These mature into eggs, become fertilized as they encounter the sperm on their way out, and are laid as cleavage stage embryos.

Although most worms are hermaphrodite, there are also occasional males whose gonad has just one arm and opens posteriorly at the cloaca. Hermaphrodites have an XX-chromosome constitution while males are XO. They arise when an X-chromosome is lost by disjunction during meiosis. If a male and hermaphrodite mate, then the male sperm outcompete those of the hermaphrodite, resulting in an outcross.

Even in the adult stage nematodes have rather few cells, and during embryonic and larval development *C. elegans* shows almost complete invariance of cell lineage, meaning that every individual embryo shows exactly the same sequence and orientation for every cell division. Embryos are laid at about 30 cells, hatch at about 14 hours with 558 cells The larva feeds and grows, undergoing four molts before reaching the adult stage with 959 somatic cells plus about 2000 germ cells. The first stage larva also has the option of entering a dormant **dauer larva** phase if nutrients are in short supply (see Chapter 18). After the last molt the adult worm shows no further cell division of somatic tissues and can grow only by cell enlargement.

C. elegans does possess a HOX cluster containing six Hox genes, although as there are some intervening genes it is not a true cluster. The genes obey the rule of colinearity of chromosomal position and anterior expression limit. They are called: *lin39*, *ceh13*, [gap], *mab5*, *egl5*,[gap], *php3*, *nob1*. The last three belong to the Abdominal B, or posterior class. Only *ceh13*, *php3*, and *nob1* have embryonic phenotypes in loss-of-function mutations.

Embryonic development

Fertilization in *C. elegans* is somewhat unusual. The sperm are amoeboid, with no flagellum or acrosome. Oocytes are fertilized before the first meiotic division. The sperm can enter at any position and the point of sperm entry defines the future **posterior** of the zygote. Following fertilization and the completion of meiosis, there is a cytoplasmic rearrangement associated with a "pseudocleavage" or formation of a furrow which does not progress to a full cleavage. The early cleavages are asymmetrical (Fig. 12.3). The first forms an anterior AB and posterior P cell. AB then forms ABa and ABp while P behaves in a **stem cell**-like manner, keeping a P daughter while successively cutting off EMS, C and D blastomeres. The residual P cell (P4) is the **germ**

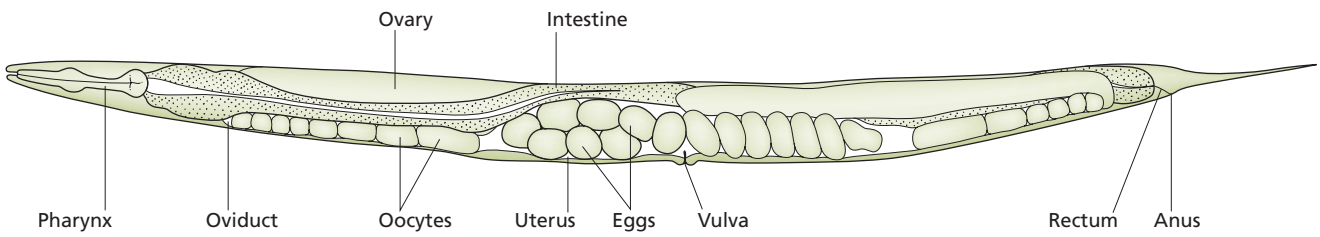


Fig. 12.2 Adult anatomy of *C. elegans*. (After Sulston. In: Bard 1994. *Embryos*. Wolfe, figure 4.4, p. 56.)

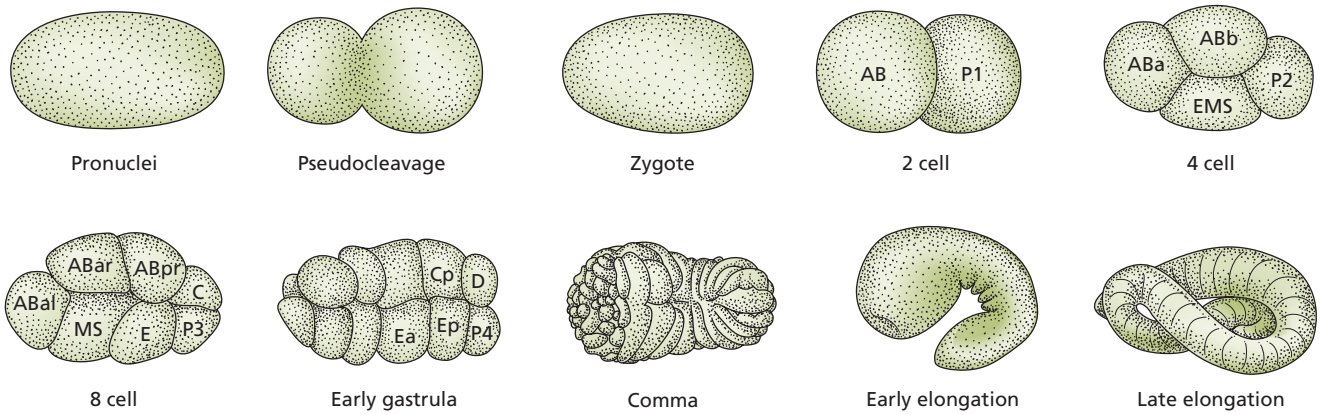


Fig. 12.3 Embryonic development of *C. elegans*.

cell precursor, dividing only once more in embryonic life. Maternal components are sufficient to direct development up to about 26 cells, as this is the earliest stage that defects are apparent if embryos are raised in α-amanitin, an inhibitor of RNA polymerase II. In the germ line zygotic transcription of RNA polymerase II genes remains repressed until about the 100-cell stage.

The egg contains RNA-rich **P-granules** which are initially randomly dispersed but which concentrate in the posterior during the cytoplasmic rearrangement period. During each successive division these granules concentrate in the region that will become the new P cell. Of the original founder cells, AB, MS, and C all produce a variety of cell types while the others generate a single cell type: P4 becoming the germ line, E becoming the gut, and D becoming muscle. "**Gastrulation**" in *C. elegans* is rather prolonged but can be considered as starting at the 26-cell stage when the two E cells move into the interior. These are followed by the myoblasts derived from C and D, and the pharyngeal cells derived from ABa. The ventral cleft, which resembles a blastopore, closes at about the 300-cell stage.

Because of the relatively small cell number and the invariance such that all individuals undergo exactly the same sequence of cell divisions, the complete **cell lineage** of embryo, larva, and adult has been determined by direct observation, the first part of which is shown in Fig. 12.4. Although in one respect setting a high standard of precision, the lineage falls short of a complete fate map as it shows only the "family tree" of the cells but not their spatial relationships at the different stages. Development was originally thought to be entirely mosaic in character, because in almost all cases when a cell is removed by laser microbeam irradiation all of its descendants are lost and there is no consequence for the development of neighboring cells. However, a number of inductive interactions are now known, so *C. elegans* does not really differ greatly from the other model species in this regard.

The precision of the cell lineage makes it less useful to define which parts of the embryo belong to the different germ layers than it is for the other animal types. The "official" germ layers are:
ectoderm: AB, Caa, Cpa;
mesoderm: MS, Cap, Cpp, D;
endoderm: E.

However, AB produces the pharyngeal muscles which would normally be considered a mesodermal type and MS produces some pharyngeal neurons which would normally be considered ectodermal.

Classic Experiments

Mechanism of unequal cell division

The breakthrough depended on isolation of mutants of maternal-effect genes in which the normal polarization of the zygote was lost. This showed that PAR protein complexes became positioned within the cell by mutual repulsion and controlled degradation. Homologs of the *par* genes are now known to be involved in asymmetrical cell divisions in many other animals.

Kemphues, K.J., Priess, J.R., Morton, D.G. & Cheng, N. (1988) Identification of genes required for cytoplasmic localization in early *C. elegans* embryos. *Cell* **52**, 311–320.

Guo, S. & Kemphues, K.J. (1995) *Par-1*, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. *Cell* **81**, 611–620.

Regional specification in the embryo

Asymmetrical cleavages

Asymmetric division is important in numerous cases of tissue differentiation and stem cell behavior in higher animals, and some of the basic mechanism were discovered by studying *C. elegans*. Asymmetrical division involves two processes, the establishment of cytoplasmic

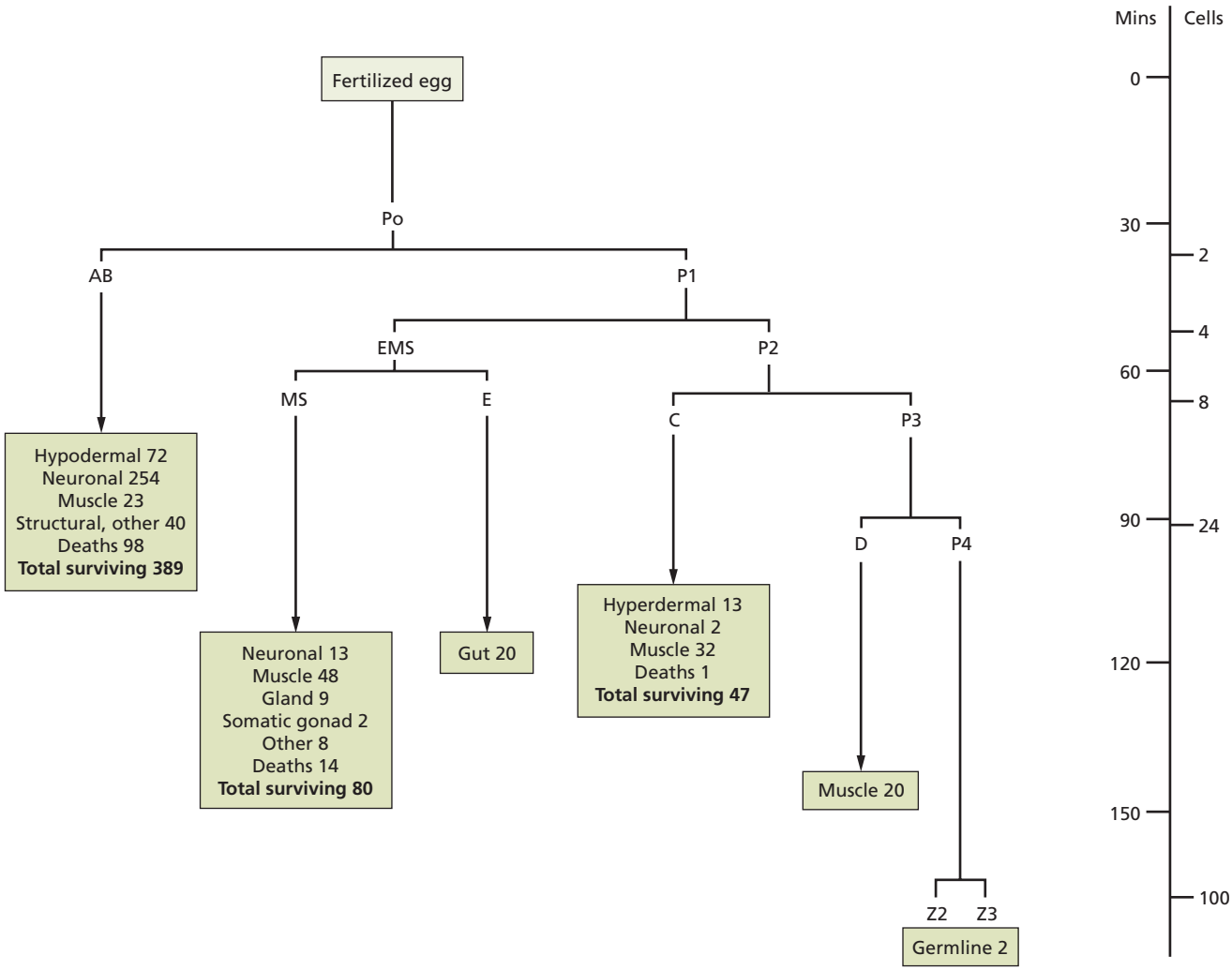


Fig. 12.4 Early cell lineage of *C. elegans*.

polarity and the correct orientation of the mitotic apparatus (Fig. 12.5).

Normally the early blastomeres will divide in a direction at right angles to their last cleavage. The AB cell follows this rule as it divides orthogonal to the first cleavage but the P1 cell does not do so, instead dividing parallel to the first cleavage. It does this because of rotational alignment, which is a 90 degrees rotation of the centrosomes and nucleus, driven by the positioning of microtubule attachments on the cell cortex.

A series of maternal-effect genes affecting the asymmetry of cell divisions have been isolated by mutagenizing worms that are themselves unable to lay eggs. Such worms can still reproduce, because the larvae arising from self-fertilization simply eat their way out of the body of the hermaphrodite. To do the screen, one F1 larva is put in each dish. If it carries a mutation on one chromosome, then 25% of its (F2) offspring will be homozygous for that mutation. If the mutation is zygotic lethal, then the affected embryos will simply fail to develop. However, if the mutation is a maternal-effect lethal, then the F2 generation will develop into worms but they will then fill up with inviable F3 embryos that cannot develop and so do not eat their way out (Fig. 12.6).

Some of these maternal-effect lethals affect the cleavage planes and character of early blastomeres and are called *par* genes ("partitioning defective"). Embryos produced by homozygous mothers have symmetrical early cleavages and arrest as amorphous cell masses:

par-1 codes for a SerThr kinase which binds nonmuscle myosin. After the cytoplasmic rearrangement it is found in the posterior cortex of the zygote.

par-2 codes for a cytoplasmic protein with adenosine triphosphate (ATP)-binding and zinc-binding (RING) domains. It is also localized to the posterior of the zygote.

par-3 codes for a cytoplasmic protein containing a PDZ (protein-protein recognition) domain. It forms a complex with PAR-6 (another PDZ domain protein) and an atypical

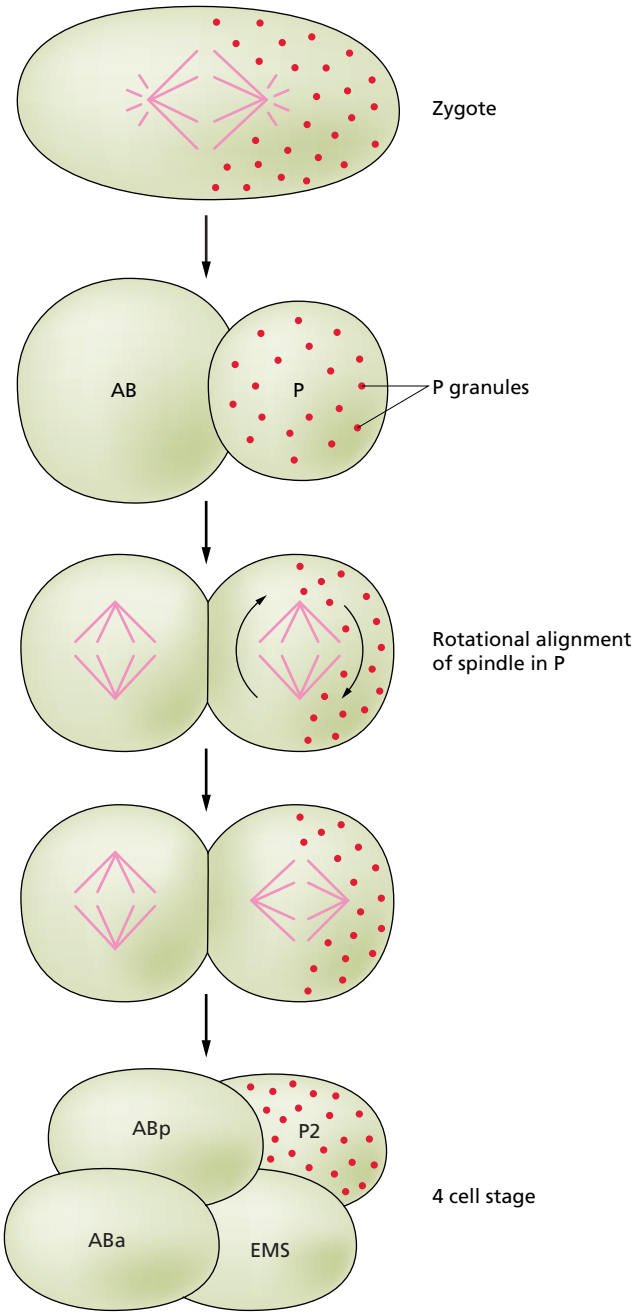


Fig. 12.5 Asymmetrical division involving segregation of P-granules into P cells and rotational alignment of the P1 spindle.

protein kinase C (aPKC3), and the complex becomes associated with the plasma membrane in the anterior of the zygote.

In the unfertilized egg the PAR proteins are uniformly distributed. Following fertilization the sperm aster repels the PAR-3 complex from the posterior. The PAR-3 complex then repels PAR-1 and -2 such that they become concentrated in the posterior cortex (Fig. 12.7). This mutual repulsion seems to be the key element of the cell polarization and effectively serves to amplify the small change brought about by sperm entry into a big change affecting the overall structure of the zygote. Evidence for the process comes from observing the distribution of one PAR protein in the absence of another. In the absence of PAR-2 there is no movement of the PAR-3 complex to the anterior, and in the absence of PAR-3 there is no movement of PAR-1 and -2 to the posterior. It remains uncertain to what extent the localizations are achieved by actual movement and to what extend by differential degradation, but the mechanism is known to involve the phosphorylation of PAR-3 by PAR-1, which allows binding of other cytoplasmic proteins of the 14-3-3 class and resulting destablization of the PAR-3 complex.

In terms of specification of commitment the function of the PAR proteins is to control the disposition of cytoplasmic determinants in the zygote and early blastomeres as described below. As far as the orientation of mitotic spindles is concerned, embryos lacking PAR-2 show rotational alignment in neither AB nor P. Embryos lacking PAR-3 show rotational alignment of both AB and P cells. The double mutant, *par-2*$^{-}$ *par-3*$^{-}$, produces embryos showing rotational alignment in both cells, like *par-3*$^{-}$. This means that something other than the *par* genes must be causing the rotational alignment and that the PAR-3 complex normally suppresses it in the AB blastomere and its absence allows it in P1.

There are mammalian and *Drosophila* homologs of the *par* genes, and these are thought also to be involved in the acquisition of cell polarity and in the control of asymmetrical cell division. In mammalian epithelia the PAR-3 complex is found in junctional complexes (see Chapter 13). It has been shown that overexpression of a PAR-1 homolog can alter cell polarity, for example converting a normally columnar epithelial cell type to a liver type with intercellular lumens resembling bile canaliculi. In *Drosophila* neuroblasts the PAR-1 homolog is found in the cortical crescent (see Chapter 14).

Determinants

Several cytoplasmic determinants responsible for regional specification have been identified (Figs 12.7, 12.8). Their mode of action has been deduced from the maternal-effect mutant phenotype, and from the effect on their localization of mutating other genes, including the *par* genes. Localization may be studied by immunostaining for the protein, or by observing the intracellular position of a transgenic GFP fusion protein by fluorescence microscopy.

SKN-1 (pronounced "skin-1") is a transcription factor of the bZIP type and confers an EMS type of development on its nuclei. The mRNA is present maternally and is not localized, but the protein accumulates only in the P1 nucleus, and later in the descendant P2 and EMS nuclei, then becomes lost after the 12-cell stage. Embryos without SKN-1 lack pharynx and intestine because the E and MS blastomeres develop like the C blastomere (hence too much "skin"). Although in the normal embryo

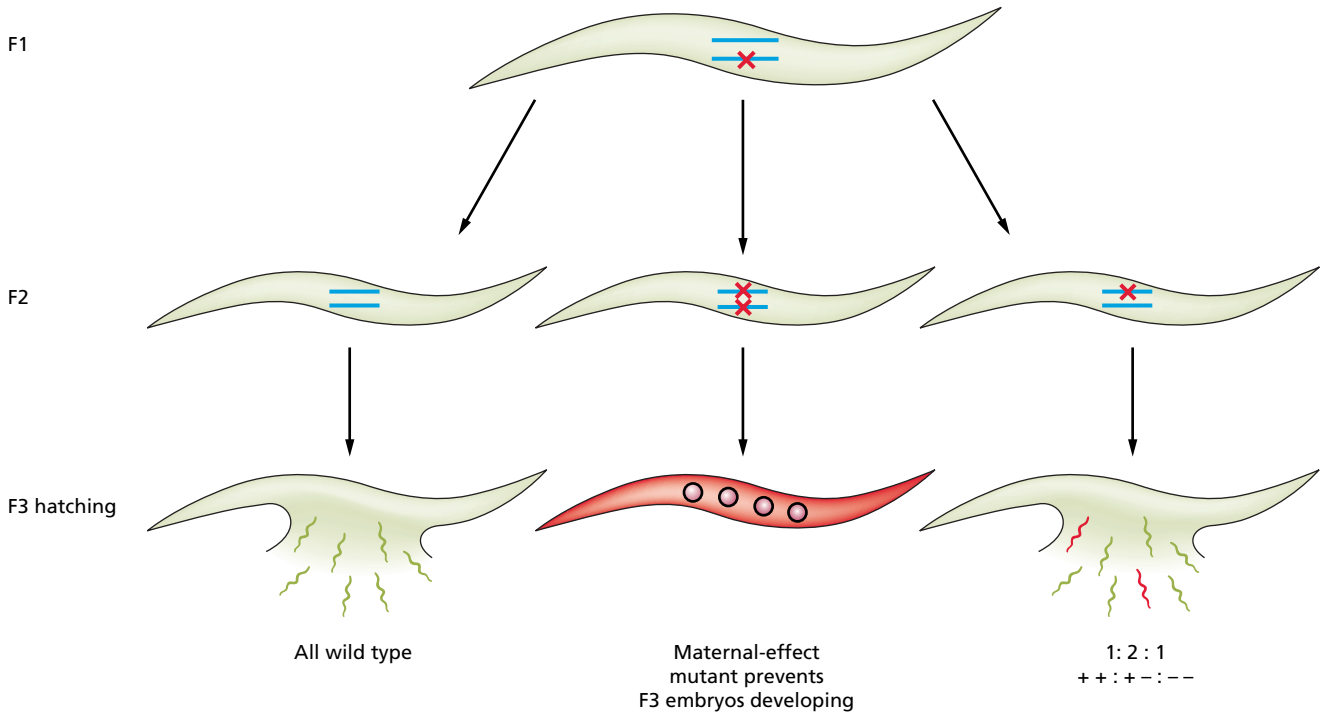


Fig. 12.6 Maternal screen. The hermaphrodites are vulvaless and cannot lay eggs, so the larvae eat their way out, destroying the parents. But those F2 worms carrying arrested embryos due to a maternal-effect mutation will persist.

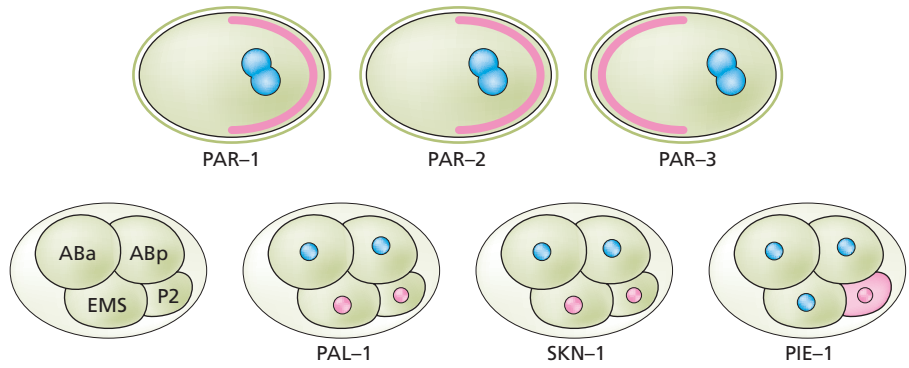


Fig. 12.7 Cytoplasmic determinants in *C. elegans*. The PAR-1 and PAR-2 proteins become localized to the posterior and the PAR-3/PAR-6/aPKC3 complex to the anterior following fertilization. Localization of SKN-1, PIE-1, and PAL-1 proteins occurs as shown by the four-cell stage.

SKN-1 is present both in EMS and in P2, its transcription factor activity is repressed in P2 by the PIE-1 protein. PIE-1 is responsible for repression of all RNA polymerase II-mediated transcription in the early germ line. Embryos lacking PIE-1 still have a normal distribution of SKN-1 protein, but the P2 cell now develops like EMS, because SKN-1 is active in both cells.

Mex-1 mRNA is initially ubiquitous but becomes lost from cells other than the P lineage. The protein also concentrates in the posterior of the zygote. MEX-1 appears to prevent SKN-1 from entering AB. Embryos lacking MEX-1 have SKN-1 in the nuclei of the two AB cells as well as in P2 and EMS. As a consequence they have AB descendants developing like the normal MS descendants, leading to too much muscle. Embryos lacking both MEX-1 and SKN-1 have a similar phenotype to that caused by lack of SKN-1 alone, with AB normal but EMS developing like C. These results confirm that normal AB behavior depends on the absence of SKN-1.

PAL-1 is a homolog of the *Drosophila caudal* gene and the vertebrate cdx family. Like these genes, it is needed for posterior development. The mRNA is present all over the early embryo, but is normally only translated in EMS and P2. Translation is repressed during the early stages, and in AB cells, by MEX-3, which acts on the 3′UTR of the *pal-3* mRNA. The *mex-3* mRNA and MEX-3 protein are initially uniform then become more abundant in AB cells and are lost after the four-cell stage. Embryos lacking MEX-3 express PAL-1 protein all over and are posteriorized in morphology with the AB descendants resembling the normal descendants of blastomere C.

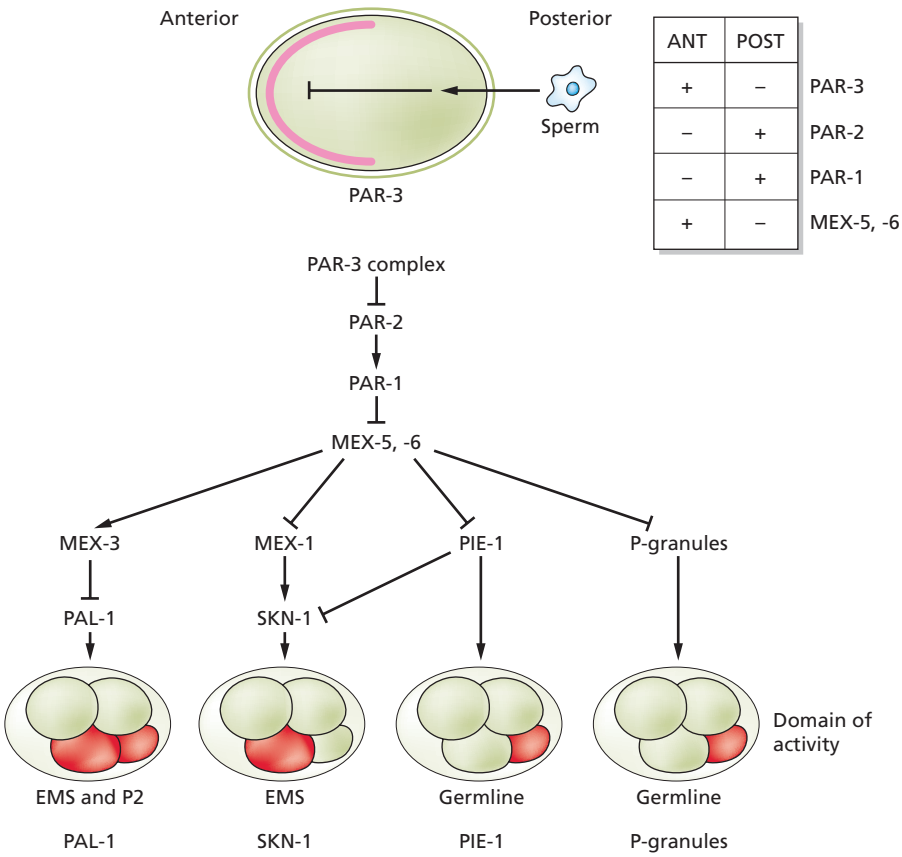


Fig. 12.8 Mechanistic connections between the sperm entry point and the segregation of cytoplasmic determinants. The sperm aster repels the PAR-3 complex and this unleashes a cascade of localization events depending on protein movement, differential degradation, and translational control. Note that SKN-1 is present in P2 but not active because of the presence of PIE-1.

These results show how the character of each of the early blastomeres is specified by the particular combination of determinants which it inherits. The spatial disposition of the determinants is controlled by the PAR system and PAR-1 seems to be the main effector, acting on the cytoplasmic proteins MEX-5 and MEX-6 such that they become localized to the anterior (Fig. 12.8). These in turn act on the MEX-1 and PIE-1 proteins, and the P-granules, to localize them all to the posterior where they direct the formation of the P lineage of blastomeres. The evidence for this is that in the absence of PAR-1, all of MEX-5, -6 and -1 proteins, PIE-1 protein, and the P-granules are uniformly distributed. In the absence of the MEX-5 and -6 proteins, PAR-1 distribution is normal, but MEX-1, PIE-1, and P-granules are uniform, showing that PAR-1 normally regulates the localization of MEX-5 and -6 and that they in turn control the disposition of the other components. These effects are exerted mostly through differential protein degradation.

MEX-5 and -6 also concentrate MEX-3 in the anterior. In the absence of PAR-1, MEX-3 is present all over the embryo. Normally, MEX-3 in the anterior inhibits the production of PAL-1 by translational control, confining the activity of PAL-1 to the posterior. But in the absence of PAR-1, leading to uniform MEX-3, there is no expression of PAL-1 and posterior development is defective.

Inductive interactions in C. elegans

It was initially thought that *C. elegans* functioned entirely on the basis of cytoplasmic determinants, because of the invariant fate map and the **mosaic** behavior of most cells after laser ablation of their neighbors. However, it is now known that there are many inductive interactions as well.

A structure that depends on induction for its formation is the pharynx. There are two successive signals of which the first is repressive from the P2 cell, and the second is positive from the descendants of MS (Fig. 12.9). Both signals operate through the Notch pathway, but using different ligands of Notch.

Normally the anterior part of the muscular pharynx is produced by the ABa cell. If ABa and ABp are interchanged then ABp will form the anterior pharynx instead of ABa, showing that position of the cell rather than its lineage is important. However, if P2 is prevented from touching ABp then ABp forms pharynx as well as ABa, showing that there must normally be a signal from P2 to ABp that suppresses pharynx formation.

This repressive signal is encoded by the maternal-effect *apx-1* (anterior pharynx excess) gene. Embryos lacking APX-1 show formation of anterior pharynx from both AB blastomeres, instead of just ABa. *apx-1* codes for a Delta-like ligand. The receptor is encoded by another maternal-effect gene, *glp-1* (the

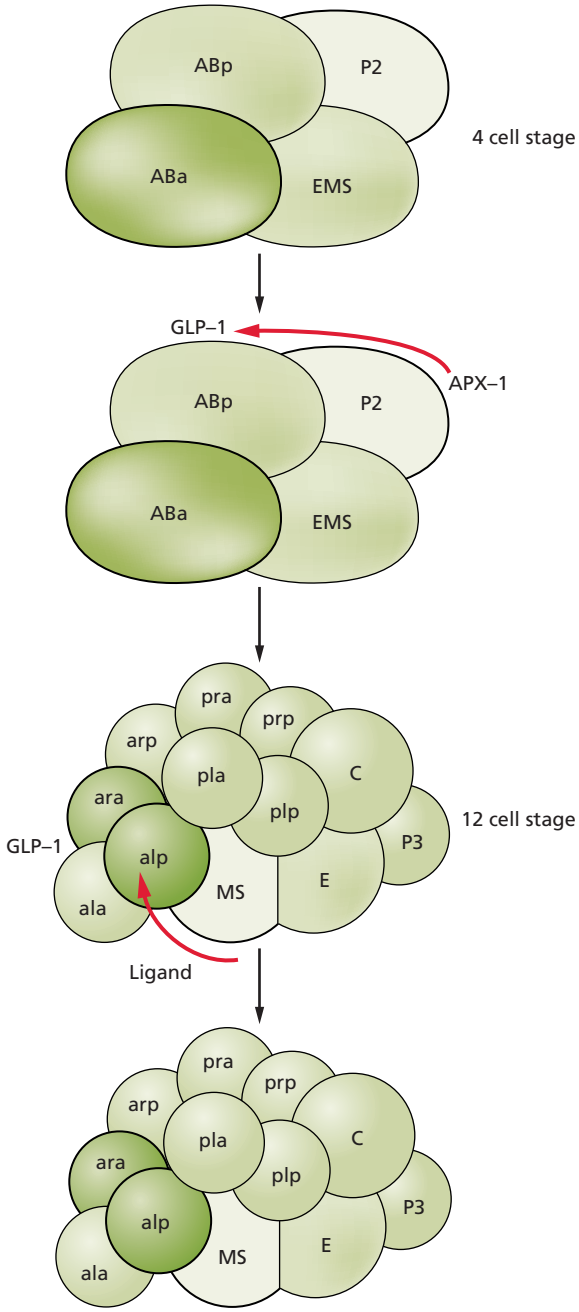


Fig. 12.9 Two inductive interactions leading to formation of the pharynx.

name refers to an effect on **g**erm-**l**ine **p**roliferation) whose product is a Notch-type receptor. Embryos lacking GLP-1 also have ABp developing as ABa in most respects, but unlike *apx-1*⁻, *glp-1*⁻ mutants do not actually go on to form pharynx from the two equivalent ABa-like blastomeres. This is because formation of pharynx is not, in fact, a default for ABa, but depends on a subsequent positive inductive interaction. This may be shown by the fact that an isolated AB cell does not produce any pharynx. Also, laser ablation of the MS cell between eight and 12 cells prevents ABa forming pharynx, showing that its presence must be necessary during this time interval. At the 12-cell stage the MS blastomere touches the two ABa grand-daughters (ABalp and ABara), and emits the second signal responsible for inducing the pharynx. Moreover in *apx-1*⁻ embryos the descendants of the ABp cell that produce pharynx are ABpra, ABprp, and ABplp, all of which also contact MS at the 12-cell stage. Remarkably, it seems that the receptor for this second signal is also GLP-1, since, as we have seen, the mutant embryos do not form a pharynx, even though in other regards the two AB lineages behave the same. The *glp-1* mRNA is uniformly distributed in the embryo up to the eight-cell stage but the protein is found only in the AB descendants. This is because of differential translation regulated by a sequence in the 3′-UTR of the message, with translation in the posterior being repressed by a cascade of factors ultimately controlled by PAR-1. The GLP-1 protein disappears at the 28-cell stage (when there are 16 AB descendants). The ligand for GLP-1 expressed by MS is distinct from APX-1.

Hence there is a double requirement for GLP-1, first as the receptor mediating repression of pharynx formation by the action of P1 on ABp, and then as the receptor mediating positive induction of pharynx formation by the action of MS on ABa. These two separate requirements are clearly shown by the phenotypes of temperature-sensitive mutants of *glp-1*, kept for different time periods at the nonpermissive temperature. If the nonpermissive temperature is given only around the four-cell stage then the phenotype is just like the maternal effect phenotype of *apx-1*⁻, with ABp as well as ABa forming pharynx. If the nonpermissive temperature is maintained until the 12-cell stage then the phenotype is like the maternal effect *glp-1* null mutant, with equivalent cell divisions of ABp and ABa but no subsequent pharynx formation.

Ultimately, formation of the entire pharynx is dependent on the zygotically expressed gene *pha-4*, encoding a winged helix transcription factor homologous to the FoxA genes important in vertebrate gut development (see Chapter 16). This is responsible for activating a "pharyngeal enhancer" controlling expression of pharyngeal genes in all the component cell types of the organ. The loss-of-function mutant lacks the entire pharynx, both the part formed from ABa and the part formed from MS. Use of a temperature-sensitive allele shows that there is a requirement for *pha-4* throughout development, for both early and late differentiation events.

The intestine is composed of 20 cells derived from the E blastomere. These cells polarize, intercalate with each other, and become arranged around a gut lumen and joined with junctional complexes. The developmental specification of the E blastomere depends on a signal from the P2 cell. This emits a Wnt-type signal that causes the nearer part of EMS to become E and the further part to adopt the default specification of MS. This may be shown by removing the P2 cell, which causes both progeny of EMS to resemble MS. A series of *mom* (more mesoderm) mutants have a similar effect to loss of P2. These turned

out to encode members of the Wnt pathway, and it was shown by mosaic analysis that the signaling components including the Wnt homolog itself (*mom2*) were required in the P2 cell while the receptor homolog (*mom5*) was required in EMS. Loss-of-function maternal-effect mutants of these Wnt pathway components will convert E into a second MS. However the reverse phenotype results from loss of function of *pop-1*, which is an HMG domain transcription factor comparable to the Tcf and Lef factors in vertebrates. This converts MS into a second E, suggesting that formation of E depends on inhibition of POP-1 activity by the Wnt signal, whereas in vertebrates the Wnt signal will normally activate the POP-1 homologs.

A zygotically active gene fulfilling the description of a master regulator for the intestine is *end-1*. This encodes a transcription factor of the GATA class and is expressed only in the E cell and its progeny. It activates a set of intestine-specific target genes. Transcription of *end-1* is activated by SKN-1 and repressed by POP-1, meaning that the formation of intestine requires both the correct early placement of SKN-1 by the PAR-1/MEX-1 system, and the later Wnt signal from the P2 blastomere.

In summary, the molecular basis for regional specification of the blastomeres in *C. elegans* is now fairly well understood. It depends both on the correct placement of cytoplasmic determinants, and on the occurrence of inductive signals between adjacent blastomeres. The asymmetrical localization of both the determinants and the components of the signaling systems depend on the operation of the PAR system.

Analysis of postembryonic development

The vulva

The vulva is the epidermal structure that is formed in larval life around the mid-ventral opening of the gonad (Fig. 12.10). Its formation is controlled by an EGF-like signal from an internal cell called the **anchor cell**. It arises from the cells called P5p, P6p, and P7p, which are the three posterior daughters of embryonically generated ectodermal cells P5, P6, and P7:

P5p makes seven vulval descendants;
P6p makes eight vulval descendants;
P7p makes seven vulval descendants.

In the fourth larval stage these 22 cells undergo various movements and fusions to make the vulva itself. In addition, the surrounding cells, called P3p, P4p, and P8p, are competent to make vulva, but in normal development they each divide just once to make two cells that later enter the syncytial hypoderm. In discussions of the vulva the following convention is used:

formation of eight cells = primary fate 1°; normally followed by P6p;
formation of seven cells = secondary fate 2°; normally followed by P5p and P7p;
formation of two cells = tertiary fate 3°; normally followed by P3p, P4p, P8p.

Hermaphrodites are able to reproduce without a vulva, because the larvae just chew their way out of the body. Therefore it is possible to screen worms for viable mutations with various vulval defects. These viable mutations are often hypomorphic, with the corresponding null alleles of the same genes being lethal. The main classes of mutant are *vulvaless* and *multivulva*, the latter forming supernumerary vulvas from the same P3p–P8p cell group.

The six P3p–P8p cells are said to make up an **equivalence group**, because they are all competent to form vulva and they can replace each other in various experimental situations. This is clearly shown by their relations with the gonadal **anchor cell**, which lies internally adjacent to P6p. If the anchor cell is ablated by laser microbeam radiation, then all the P3p–P8p cells follow the tertiary fate 3° and no vulva is produced. If one of the P3p–P8p cells is removed by laser microbeam, then its neighbor will take its place and a normal vulva will result. If the anchor cell is moved relative to the P3p–P8p cells, as in various *displaced gonad* mutants, then whichever three of the P3p–P8p cells are nearest will produce the vulva.

There are several *vulvaless* mutants, giving a similar phenotype to anchor cell ablation. The anchor cell ligand is encoded by *lin-3*, and is a homolog of EGF. The receptor is an EGF receptor homolog encoded by *let-23*. *let-60* is a gene with several different alleles. Loss-of-function mutants give a vulvaless phenotype while gain-of-function mutants give a multivulva phenotype. *let-60* in fact encodes a homolog of the Ras protein, familiar as an intermediate in the ERK signal transduction pathway activated by EGF or FGF signaling (see Appendix). Constitutively active Ras will produce multivulva, while inactive Ras will produce vulvaless. Double mutant combinations work in a predictable way, for example the combination of *let-23*$^{-}$ and *let-60 gof* gives a multivulva phenotype, confirming that the Ras requirement lies downstream of the receptor. It is possible to visualize a gradient of EGF response centered on P6p in worms transgenic for an EGF reporter, which is a *lacZ* gene whose transcription is activated by EGF signaling. The EGF signal also works partly by lifting of a continuous inhibition from the syncytial hypoderm, which is in contact with the P3p–P8p cells. In the *lin-15* mutation a multiple vulva is formed from all six cells with or without the anchor cell. Genetic mosaic experiments show that the P3p–P8p cells themselves need not be *lin-15*$^{-}$, and so it is thought that the mutation must prevent the formation of an inhibitor by the syncytial hypoderm which normally represses vulva formation, and which is overcome by the anchor cell signal.

Although the gradient of EGF signaling should theoretically be enough to generate the three cell fates, there is also a secondary signal emitted by P6p that activates the Notch pathway in P5p and P7p. In normal development the combination of both these signals serves to control the formation of the vulva.

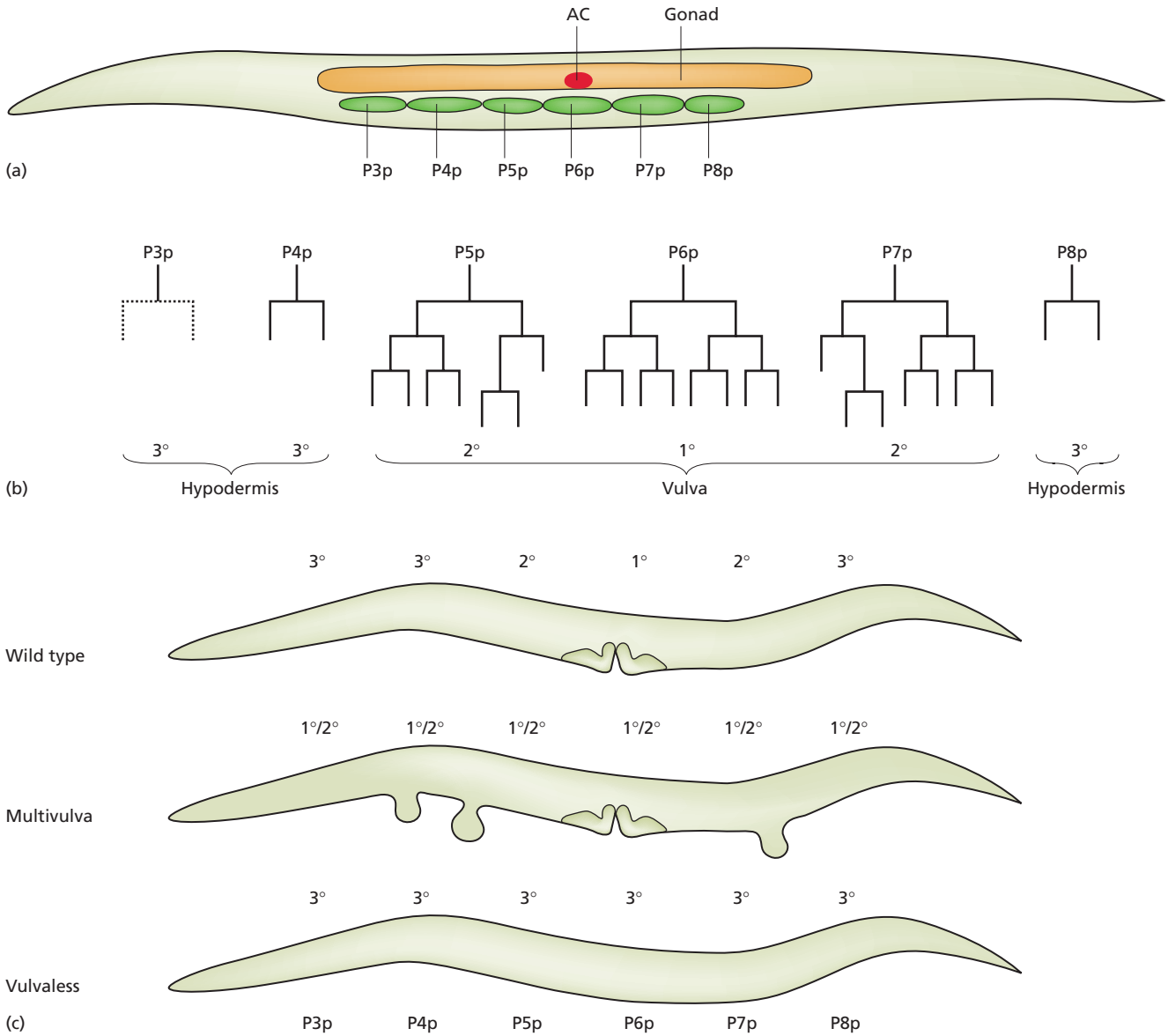


Fig. 12.10 Development of the *C. elegans* vulva. (a) Relationship of the equivalence group cells to the gonad and anchor cell. (b) Normal lineage of each cell. (c) Mutant phenotypes. The fate of each of the cells is indicated for each phenotype.

The germ line

An important aspect of postembryonic development is the maturation of the **germ line**. This derives from the P lineage which inherits various determinants, some associated with the P granules. As mentioned above, the PIE-1 protein causes a general repression of genes transcribed by RNA polymerase II during the early stages. In the newly hatched larva, the germ line consists of just two cells descended from the P lineage: Z2 and Z3. These express the *cgh-1* gene, which encodes an RNA helicase homologous to *vasa* in *Drosophila*. The *cgh-1* gene is active in the germ line thereafter, and the CGH-1 protein is one of the components of the P-granules found in the egg and segregated to the germ-line lineage in early development. Treatment of worms with RNAi directed against *cgh-1* causes death of the oocytes and formation of nonfunctional sperm, suggesting a key function in the later stages of germ cell development.

In the larval and adult worm the germ cells lie within the gonad. The most mature cells lie near the vulva and the most immature cells, which are still mitotic, at the blind ends of the gonad. These mitotic germ cells form a syncytium and become cellularized as they enter meiosis. In the hermaphrodite, the

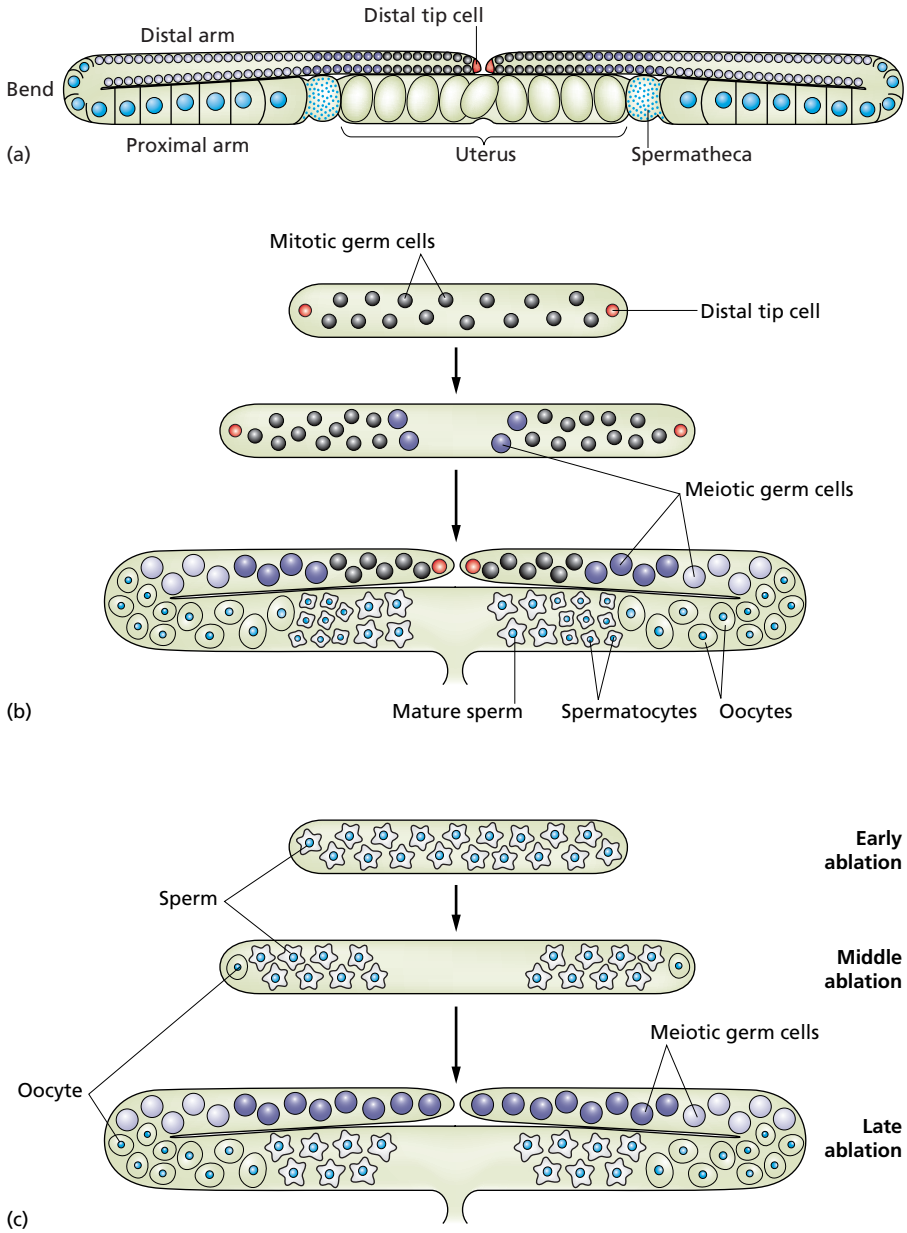


Fig. 12.11 Development of the *C. elegans* gonad. (a) Adult hermaphrodite gonad. (b) Normal development. (c) Effect of removing the distal tip cell at different stages.

early cohorts of germ cells differentiate as sperm and the later ones as oocytes. The tip of each branch of the gonad contains an important somatic cell called the **distal tip cell** whose function is to maintain the neighboring germ-cell nuclei in mitosis. As the gonad grows during larval life, the germ-cell syncytium elongates and cells progressively leave the range of influence of the distal tip cell, whereupon the cells stop mitotic division and enter meiosis. Ablation of the distal tip cell at any stage by laser irradiation causes all the remaining mitotic nuclei to enter meiosis. The composition of the gonad following this is then appropriate to the stage of maturation reached, so an early ablation would create all sperm while a late ablation would result in a nearly normal arrangement of oocytes and sperm (Fig. 12.11). The distal tip cell acts by expression of *lag-2*, which is another homolog of *Delta*. The receptor, encoded by *glp-1* (the same gene required for pharynx induction) is present on the germ-cell syncytial membrane. Zygotic loss-of-function mutants of *glp-1* or of *lag-2* have the same effect as ablation of the distal tip cell.

The GLP-1 signal inhibits the activity of a pair of proteins GLD-1 and -2, respectively an RNA binding protein and a polyA polymerase, that are present in the germ line and are needed for progression to meiosis. It also activates expression of *daz-1*, encoding another RNA binding protein required for oocyte

maturation. *Daz-1* is a homolog of the human gene *daz* (**d**eleted in **az**oospermia), although this is required for spermatogenesis rather than oogenesis. The importance in *C. elegans* of genes such as *cgh-1* and *daz-1* that encode homologs of proteins required for germ cell development in higher animals is another example of the remarkable similarity of developmental mechanisms across the animal kingdom.

Programmed cell death

Cell death, or **apoptosis**, is important in many developmental contexts, and is now known to depend on the action of proteases called caspases. As with asymmetrical cell division, the discovery of the mechanism of programmed cell death is an area in which *C. elegans* genetics has made an important contribution to general cell biology.

During normal development of *C. elegans* about 1 in 8 cells die. They are mainly small cells and collectively represent only 1% of the biomass. Most deaths are autonomous and occur shortly after the cell was born. A few depend on signals from neighboring cells, shown by the fact that the cell will survive when its neighbor has been ablated by laser radiation. The sequence of events is the same as mammalian apoptosis, with a condensation of the nucleus, a shrinkage of the cell to a membrane-bound body, and engulfment by neighboring cells.

Cell-death-defective (*ced*) mutants affect all the cell deaths in the organism, while some other mutants affect the decisions of particular cells to die. Most of the *ced* mutants interfere with the engulfment of the dead cells, but three of them are components of the actual death program itself. In loss-of-function mutants of *ced-3* and *ced-4* , all of the cells normally destined to die now survive. The *ced-9* gene has both loss-of-function and gain-of-function alleles. In loss-of-function mutants there is excessive cell death, while in gain-of-function mutants there is some survival of cells that normally die. Excess cell survival is also shown on overexpression of wild-type *ced-9*. Double mutants of the type *ced-9*$^-$/*ced-3*$^-$ or *ced-9*$^-$/*ced-4*$^-$ also show target cell survival, so it follows that the normal function of *ced-9* must be to repress *ced-3/4*, which are themselves downstream of *ced-9* and necessary to execute the death program (Fig. 12.12). Genetic mosaic experiments show that the *ced-3/4* wild-type gene has to be present in the target cell itself in order for it to die.

ced-9 codes for the homolog of the mammalian protein BCL2. Discovered originally as an **oncogene** product, it is a cytoplasmic protein which is an inhibitor of apoptosis. CED-9 and BCL2 are interchangeable, therefore worms transgenic for mammalian BCL2 show inhibition of cell death, and *ced-9*$^-$ mutants can be rescued by BCL2. *ced-3* codes for a homolog of the interleukin 1β-converting enzyme (ICE). This is a cysteine protease that cleaves at Asp-X sequences, and is the prototype member of the family of caspases, of which many are now known. The caspases are the enzymes that actually bring about cell death, and the targets for the CED-3 protease include polyADPR polymerase

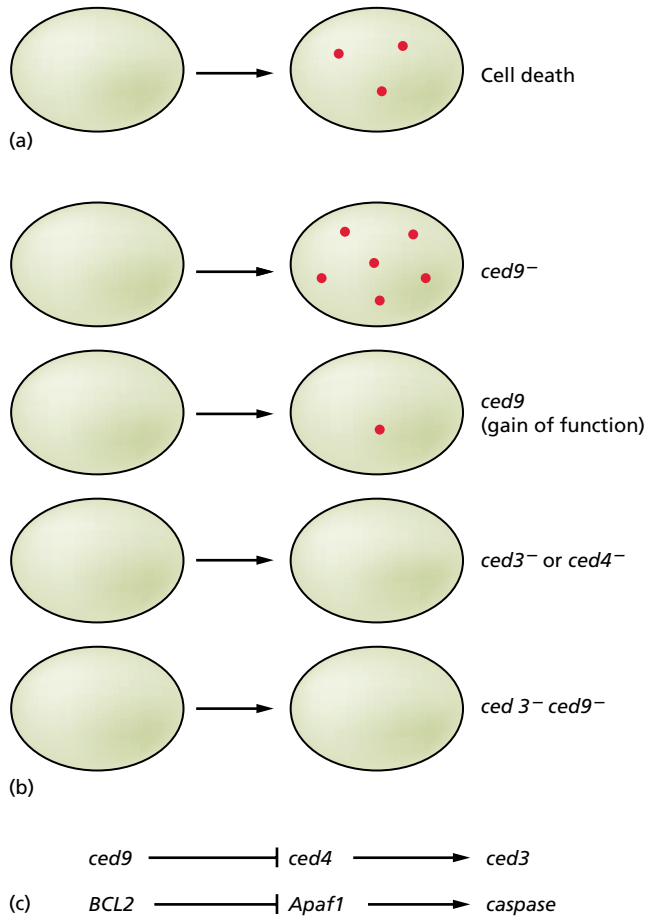


Fig. 12.12 The cell-death pathway. (a) Normal cell death. (b) Phenotypes of mutants. (c) The pathway in *C. elegans* and in mammals.

(involved in DNA repair), lamins (nuclear membrane proteins) and other nuclear proteins. CED-3 itself will cause apoptosis if introduced into mammalian cells. *ced-4* codes for a protein whose mammalian homolog is called Apaf1 and which activates procaspase 9. CED-4 itself similarly activates CED-3 and is inhibited by CED-9.

The final stage of the cell death process is the engulfment of the apoptotic cells by neighboring cells. *C. elegans* does not possess specialized phagocytes and the engulfment is carried out by nonspecialized cells. But as in mammalian phagocytes a critical step is the recognition of a particular phospholipid, phosphatidyl serine, on the surface of the cell to be engulfed. A number of the *ced* genes are defective in engulfment and their homologs are thought also to be important for the properties of mammalian phagocytes. Examples are *ced-1*, encoding a homolog of the mammalian SREC (scavenger receptor from endothelial cells), and *ced-7*, encoding an ABC (ATP binding cassette) transporter protein, one of a large class of proteins responsible for transport of small molecules and ions across cell membranes.

Classic Experiments

Cell lineage and cell death

The first two references are the papers describing the complete cell lineage of *C. elegans*, derived from painstaking observation by interference microscopy. The third is a detailed anatomical study. In the course of this work a number of programmed cell deaths were described. Later analysis of the cell death (*ced*) mutants showed that the biochemistry of the process was common to higher animals and allowed the elucidation of the pathway.

Sydney Brenner, Robert Horvitz, and John Sulson were awarded the Nobel Prize for Physiology for this work in 2002.

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Key Points to Remember

- *C. elegans* is very favorable for genetic experimentation. It is a self-fertilized hermaphrodite with a short generation time.
- The precise cell lineage of all cells in the embryo and adult has been described. This serves as a resource for all kinds of experimental work.
- The early regional pattern of the embryo arises through the action of the PAR proteins, which become segregated along the anteroposterior axis after fertilization and control the distribution of cytoplasmic determinants between the early blastomeres.
- Like other types of embryo, various steps of *C. elegans* development depend on inductive interactions. These utilize the same molecular pathways as other animals. For example, the formation of the pharynx from the AB cells depends on a Delta-like signal from the P2 and MS cells. The formation of the E lineage depends on a Wnt signal from the MS cell.
- Postembryonic development also involves inductive interactions. The EGF/Ras pathway is important for vulval development and the Notch pathway for the maintenance of the mitotic germ line.
- *C. elegans* has made important contributions to cell biology by helping to elucidate the mechanisms of cell polarization and of apoptotic cell death.

Further reading

Websites

Caenorhabditis elegans server: http://elegans.swmed.edu/
Introduction:
http://www.biotech.missouri.edu/Dauer-World/Wormintro.html
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