Progress in the genetic analysis of mammalian development can never be as rapid as it was with Drosophila and Caenorhabditis. Not only is the mammalian genome size larger and the generation time longer, but the embryos also develop much more slowly. In addition, it is more difficult to manipulate mouse embryos experimentally compared with Xenopus, chick, or zebra fish embryos, since they are adapted to grow within the protective and nutritive environment of the mother. Despite these drawbacks, there is a unique challenge to understanding how genes control the growth and differentiation of the mammalian embryo. To a large extent, this challenge is an intellectual one and derives from our curiosity to know how human form is generated and how it has evolved from that of simpler organisms. But at a practical level, we also need to know how mutations and chemicals produce human malformations, congenital defects, and childhood cancers, and whether the productivity of agricultural animals can be improved. This knowledge, and the ability we now have to change the genetic program, must inevitably make a great impact on society and have far-reaching effects on the way in which we think about ourselves.

The roots of our knowledge about how genes control mammalian development can be traced back to experiments carried out in the early 1900s on the inheritance of coat colors in a variety of domestic animals. Since then, the mouse has become firmly established as the primary experimental mammal, and more information has accumulated on its genetics than on that of any other vertebrate, including humans. More than 1000 gene-based loci and 4000 anonymous simple-sequence-length polymorphisms (SSLPs) or microsatellite markers have already been mapped onto its haploid set of 20 chromosomes, giving a marker every 0.35 cM on average (Copeland et al. 1993; Dietrich et al. 1994b). These numbers are rapidly increasing as a result of either large-scale collaborative mapping efforts (see Appendix 2) or smaller-scale insertional mutagenesis and gene-trap programs. In addition, extensive linkage conservation or synteny exists between the mouse and human genomes, so that progress with the Human Genome Project will contribute to knowledge of the mouse genetic map and vice versa (Copeland et al. 1993; O'Brien et al. 1994). A detailed analysis has also been made of large regions of the mouse genome that encompass many mutations, deletions, and chromosomal rearrangements, such as the H-2, t, dilute-short ear, and albino complexes. In addi-
tion, several yeast artificial chromosome (YAC) libraries have been constructed to facilitate analysis of large regions of DNA (for reviews, see Klein 1975; Frischauf 1985; Rinchik et al. 1985; Silver 1986, 1993a).

As well as genetic mapping strategies, new techniques of molecular biology, including whole-mount in situ hybridization and reverse transcriptase-polymerase chain reaction (RT-PCR), are being used to reveal the temporal and spatial patterns of expression of specific genes at different stages of development. Novel cell autonomous lineage markers have also been produced for following cell fate (see Table 5 in Section A). However, the most compelling reason for excitement and optimism about studying developmental genetics in the mouse, instead of another vertebrate such as the zebrafish (Brachydanio rerio) (Driever et al. 1994), is undoubtedly our ability to manipulate the genome of the mouse in a variety of different ways. The first edition of Manipulating the Mouse Embryo emphasized the potential importance of introducing new genetic information into transgenic mice by microinjecting DNA into the pronucleus of the fertilized egg or by infecting embryos with retroviral vectors. The targeting of mutations to specific genes by homologous recombination in pluripotent embryonic stem cells was still only a dream, tenaciously followed by a small group of scientists, who, like many others before them, persisted in the face of considerable skepticism from their contemporaries. It is one of the triumphs of mammalian developmental genetics that the idea of gene targeting proved to be correct. Indeed, the technique has today become almost routine, producing a wealth of often unexpected and therefore highly stimulating data about the in vivo function and interaction of genes in the context of the developing organism.

The aim of this manual, as with the first edition, is to provide a simple technical guide for scientists who want to learn some of the techniques for manipulating the mouse embryo and for introducing genes and mutations into transgenic mice. This revised manual also includes an expanded and more up-to-date summary of early mouse embryogenesis and information about accessing new databases of mouse genetics. As before, we sincerely hope that making this information available to a wide audience will help to continue the spirit of international cooperation established by the first mouse geneticists.

**Mendelian Inheritance and Linkage: The Beginnings of Mouse Genetics**

Historians of science on both sides of the Atlantic acknowledge the American scientist William E. Castle as one of the founding fathers of mammalian genetics. As first director of the new Bussey Institute of Experimental Biology at Harvard, from 1909 to 1937, he encouraged work on the inheritance of variable characteristics in a wide range of organisms, including birds, cats, dogs, guinea pigs, rabbits, rats, and even mice (Russell 1954; Keeler 1978; Morse 1978, 1981). He was also responsible for introducing Thomas Hunt Morgan to Drosophila (Shine and Wrobel 1976). Castle had a profound influence on the course of mammalian genetics through the many scientists who came to visit or study at the Bussey Institute.

Of all the mammals studied by these early geneticists, the mouse became the mammal of choice because of its small size, resistance to infection, large litter size, and relatively rapid generation time (see Table 1). Mice were also favored because
Table 1  Some Vital Statistics of the European House Mouse, *Mus musculus*, in the Laboratory

<table>
<thead>
<tr>
<th>Genome</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of chromosomes</td>
<td>40</td>
</tr>
<tr>
<td>Diploid DNA content</td>
<td>~6 pg (3 x 10^9 bp)</td>
</tr>
<tr>
<td>Recombination units</td>
<td>1600 cM (2000 kb/cM)</td>
</tr>
<tr>
<td>Approximate number of genes^a</td>
<td>0.5–1.0 x 10^5</td>
</tr>
<tr>
<td>Percent of genome as five families of highly repeated DNA sequences (B1, B2, R, MIF-1, and EC1)^b</td>
<td>8–10%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reproductive biology^c</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestation time</td>
<td>19–20 days</td>
</tr>
<tr>
<td>Age at weaning</td>
<td>3 weeks</td>
</tr>
<tr>
<td>Age at sexual maturity</td>
<td>~6 weeks</td>
</tr>
<tr>
<td>Approximate weight</td>
<td>birth 1 g</td>
</tr>
<tr>
<td></td>
<td>weaning 8–12 g</td>
</tr>
<tr>
<td></td>
<td>adult 30–40 g (male &gt; female)</td>
</tr>
<tr>
<td>Life span in laboratory</td>
<td>1.5–2.5 years</td>
</tr>
<tr>
<td>Average litter size^d</td>
<td>~6–8</td>
</tr>
<tr>
<td>Total number of litters per breeding female</td>
<td>4–8</td>
</tr>
</tbody>
</table>

^aMcKusick and Buddle (1977).
^bBennett et al. (1984).
^cParameters such as gestation time, weight, and life span vary between the different inbred strains. Details can be found in a number of books listed in Appendix 2, e.g., Altman and Katz (1979), Festing (1979), and Heiniger and Dorey (1980).
^dLitter size depends on the number of eggs liberated at ovulation and the rate of prenatal mortality, both of which vary with age of mother, parity, and environmental conditions (e.g., diet, stress, and presence of strange male) and with strain (reflecting genetic factors such as efficiency of placentation). Prenatal mortality in inbred strains can be approximately 10–20% (for references, see Boshier 1968).

of the interesting pool of mutations affecting coat color and behavior that was readily available from breeders and collectors of pet mice, or mouse “fanciers.” One of these mutants, *albino* (see Fig. 42 in Section A), was used by Bateson in England, Cuenot in France, and Castle in the United States for the first breeding experiments demonstrating Mendelian inheritance in the mouse (for references, see Castle and Allen 1903). A few years later, *albino* and another old mutation of the mouse fanciers, *pink-eyed dilute* (see Fig. 42 in Section A), were used by J.B.S. Haldane for the first demonstration of linkage in mice (Haldane et al. 1915). Sadly, this work was interrupted in 1914 when Haldane volunteered for service in the First World War, leaving his sister to continue their experiments for a while in the Department of Comparative Anatomy in Oxford (Clark 1984; N. Mitchison, pers. comm.). It was not until after the war that Haldane was able to turn his attention to the wider aspects of mammalian genetics and, along with others, begin developing mathematical models of inheritance and natural selection.

**Origins of the Laboratory Mouse**

If William E. Castle and J.B.S. Haldane are founding fathers of mouse genetics, then the mother is undoubtedly Abbie E.C. Lathrop. A self-made woman, Abbie Lathrop established around 1900 a small mouse “farm” in Granby, Massachusetts, to breed
Figure 1 Genealogy of the more commonly used inbred mouse strains. This figure is based, in part, on data provided by Michael Potter and Rose Lieberman in 1967; it was extended by Jan Klein in 1975 and revised by Potter in 1978. H-2 haplotypes are shown in parentheses. (Reproduced, with permission, from Altman and Katz 1979.)
Figure 1 (See facing page for part A and legend.)
mice as pets. However, her mice were soon in demand as a source of experimental animals for the Bussey Institute and other American laboratories, and she gradually expanded her work to include quite sophisticated and well-documented breeding programs. For example, in collaboration with Leo Loeb, she carried out experiments to study the effects of genetic background, inbreeding, and pregnancy on the incidence of spontaneous tumors in her mice (Shimkin 1975; Morse 1978). As source material for the farm, Abbie Lathrop used wild mice trapped in Vermont and Michigan, fancy mice obtained from various European and North American sources, and imported Japanese “waltzing” mice. Waltzing mice had been bred as pets in China and Japan for many generations and were probably homozygous for a recessive mutation that causes a defect in the inner ear and thus nervous, circling, behavior. The Granby mouse farm was, to a large extent, the “melting pot” of the laboratory mouse, and, as shown in Figure 1, many of the old inbred strains can be traced back to the relatively small pool of founding mice that Lathrop maintained there. At present, about 400 different inbred strains are available and their origins and characteristics are regularly listed in the journal *Mouse Genome* (see, e.g., Festing 1993)

The formal systematics of the laboratory mouse is far from simple and reflects the existence of several subspecies of the European mouse species, *Mus musculus*, from which it was ultimately derived. The nature of this complexity has been revealed by the application of restriction-fragment-length polymorphism (RFLP) studies to mouse DNA. Analysis of the RFLP of mitochondrial DNA (which is maternally inherited through the egg cytoplasm) has shown few differences among old established strains, compared with the wide variations seen among wild mice

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**Figure 2** Geographical distribution of the five biochemical groups of the house mouse species complex in Europe. (Redrawn from Bonhomme et al. 1984.)
and newer strains derived from them. In fact, on the basis of mitochondrial DNA RFLPs, it has been argued that at least five of the primary strains (DBA, BALB/c, SWR, PL, and C57–C58) were derived originally from a single female of the subspecies *Mus musculus domesticus* (Ferris et al. 1982). This taxonomic group is found in western and southern Europe and is the source from which all wild mice in the northern parts of the United States were derived by migration with humans across the north Atlantic shipping lanes. A second taxonomic group or subspecies, *Mus musculus musculus*, is found in central and eastern Europe, Russia, and China, and only interbreeds with *domesticus* over a narrow band from north to south through central Europe (Fig. 2) (Bonhomme et al. 1984). In addition to having distinct mitochondrial DNA RFLPs, the two groups also show different patterns using DNA probes specific for the Y chromosome, which is inherited only through the male. Unexpectedly, in view of the mitochondrial RFLP data, many old inbred mouse strains, including A/J, BALB/c, C57BL/6, CBA/HeJ, C3H, DBA/2, 129/Sv, and 163/H, have Y chromosome RFLPs of the *musculus* type. The most likely explanation is that the Y chromosome came from Japanese pet mice, for example, those bred on the Granby mouse farm. A list of the origin of the Y chromosome of different inbred strains has been published (Nishioka 1987). In view of the mixed origin of the laboratory mouse, it has been agreed to refer to standard inbred strains as *Mus musculus* only (Auffray et al. 1990).

**Origin of Inbred Strains and Other Resources of Mouse Genetics**

An inbred strain is defined as one that has been maintained for more than 20 generations of brother-to-sister mating and is essentially homozygous at all genetic loci, except for mutations arising spontaneously (Altman and Katz 1979; Morse 1981). The derivation of inbred strains represents one of the most important phases in the history of mouse genetics, and it revolutionized studies in cancer research, tissue transplantation, and immunology. One of the pioneers of the innovation was Clarence C. Little. He was originally a student of Castle’s at the Bussey Institute, where he studied the inheritance of mouse coat color, and he later went on to found the Roscoe B. Jackson Memorial Laboratory (usually known as the Jackson Laboratory) in Bar Harbor, Maine (Russell 1978; Morse 1981). Other pioneers were Lionel Strong, Leo Loeb, and Jacob Furth (Morse 1978; Strong 1978). Among the first inbred strains were DBA, which was named after the coat color mutations it carried—*dilute* (d), *brown* (b), and *nonagouti* (a), and C57 and C58, which were derived from females 57 and 58 from the Granby mouse farm. While carrying out these early inbreeding experiments, both Little and Strong worked between 1918 and 1922 at the Carnegie Institution of Washington at Cold Spring Harbor, thus establishing the laboratory (then known as the Station for Experimental Evolution) as one of the birthplaces of mouse genetics (Keeler 1978; Strong 1978).

In deriving inbred strains, great tenacity was required to maintain the strict brother-to-sister matings through times when the breeding stocks reached a very low ebb due to disease or accidents, and accounts of these difficult times make fascinating reading (Morse 1978). It also required intellectual courage to challenge the widely held belief that inbreeding to virtual homozygosity would be impossible due to recessive lethal mutations in the founding pairs. Today, more than 400 inbred mouse strains are available. Each has a standardized nomenclature, to indicate strain and substrain, and details of their history, characteristics, and availability are
regularly listed in the journal *Mouse Genome* (see, e.g., Festing 1993). Standard methods for maintaining breeding colonies and testing mice for genetic purity have been described previously (see, e.g., Nomura et al. 1985), and computerized databases for tracking breeding colonies are available (Silver 1993b). Unfortunately, newcomers to the field should be aware that examples of cross-contamination of stocks from commercial sources are by no means rare, even in the present day.

One of the driving forces behind the initial establishment of inbred strains was the need to rationalize studies on the genetics of cancer susceptibility. Inbred strains were also essential for solving the problem of why spontaneous tumors could be transplanted into some mice and not others. Although many groups studied this problem, a major contribution was made by Peter Gorer, working in Haldane's department at University College, London. Using A, C57BL, and DBA strains of mice and a transplantable A strain tumor, he showed for the first time that mice resistant to tumor growth produced antibodies against antigens present not only on the tumor cells, but also on blood cells of strain A mice. One particularly strong antigen was called Antigen II. In 1948, Gorer and the American geneticist George Snell together showed that the gene specifying Antigen II was closely linked to the fused (*Pu*) locus (now known to be on chromosome 17), and they called the gene *Histocompatibility-2*, or *H-2* (Gorer et al. 1948). In a series of outstanding experiments, for which he was awarded the Nobel prize in 1980, Snell went on to identify and map many of the minor histocompatibility loci as well. All of this work was carried out at the Jackson Laboratory and owes much to the unique environment built up there by C.C. Little and his colleagues. It was the first laboratory in which many inbred strains were maintained under conditions of strict breeding and health monitoring, and from the time of its foundation, a spirit of cooperation prevailed (Morse 1978; Russell 1978; Snell 1978).

To identify the histocompatibility genes, Snell developed the concept of congenic inbred strains, in which a short segment of the chromosome around a marker gene is transferred from one strain into an inbred genetic background by repeated backcrossing and selection. Like the inbred strains, congenic strains have a strict nomenclature (Snell 1978; Altman and Katz 1979; Morse 1981). For example, B10.129-H-12b is a strain in which the allele (H-12b) derived from the strain 129/J has been transferred onto the C57BL/10 inbred background. Congenic strains carrying X-linked genes from wild mice have also been developed for studies on X chromosome inactivation (Nielsen and Chapman 1977; Chapman et al. 1983). Many of the congenic strains originally developed by Snell and subsequently by others are widely available from commercial sources, including the Jackson Laboratory. Appendix 2 describes how lists of available mice can be accessed via Internet.

Another important innovation in mouse genetics was the development of recombinant inbred strains by Donald W. Bailey and Benjamin A. Taylor (Morse 1981). These strains were derived by crossing two different highly inbred progenitor strains and then inbreeding random pairs of the F₂ generation to produce a series of recombinant inbred or RI strains (Table 2). Their usefulness is in localizing within chromosomes any new locus that shows a polymorphism between the two progenitor strains. This is done by comparing the strain distribution pattern (SDP) of the new polymorphism with the many SDPs already established for enzyme, protein, or DNA RFLPs associated with known loci. One advantage of the system is that the data are cumulative; the patterns already published (Lyon and Searle 1989), or stored on computer at the Jackson Laboratory (see Appendix
Table 2  Schematized Construction of Eight RI Strains

Progenitor inbred strains  
AABBCC x aabbcc

F₁  
AaBbCc

F₂  
AaBbCc x AaBbCc

Inbreeding for more than  
20 generations

<table>
<thead>
<tr>
<th>RI Strain</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>B</td>
<td>B</td>
<td>B</td>
<td>b</td>
<td>b</td>
<td>B</td>
<td>b</td>
<td>B</td>
<td>b</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>c</td>
<td>c</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>c</td>
</tr>
</tbody>
</table>

Construction starts from two progenitor strains that have alternate alleles at three unlinked loci. The three alleles segregate and assort independently during the inbreeding process and eventually become genetically fixed. Each allele then has a unique strain distribution pattern (SDP).

2), provide a unique and expanding database for mapping the mouse genome. Another advantage is that since living animals are available, differences in behavior or neurological responses can be studied (Takahashi et al. 1994). Thus, RI strains have been used to map loci affecting susceptibility of mouse strains to drug and alcohol addiction (Berrettini et al. 1994; Crabbe et al. 1994). One disadvantage of RI strains is that they are expensive to maintain, but purified DNA is available from the Jackson Laboratory for mapping cloned genes (see Appendix 2). Another disadvantage is the difficulty often encountered in finding polymorphisms among the progenitor strains. This is due in part to the rather restricted origin of laboratory mice, as discussed in the previous section. Fortunately, an alternative mapping technique is now readily available based on backcrossing F₁ hybrids between an inbred mouse strain and M. spretus, a wild mouse species found in Spain. Because M. m. domesticus and M. spretus are different species, the chances of finding an RFLP for any given DNA probe are much higher (Robert et al. 1985; Avner et al. 1988). A disadvantage of the system is that the F₁ males are sterile, so that once a cross has been made, the offspring cannot be bred to produce lines, and the amount of DNA is finite. Backcrosses between other inbred strains derived from wild mice (e.g., M. castaneous) and M. domesticus have also been established. Details of collaborative interspecific backcross mapping panels from which DNA is freely available can be found in Appendix 2. For a progress report of the use of backcross panels and other methods for generating a linkage map of the mouse, see Copeland et al. (1993).

Wild mice have contributed to laboratory studies in other ways. For example, as shown originally by the German geneticist Alfred Gropp, they can be used to introduce cytogenetic variations into the karyotype of M. m. domesticus, which otherwise consists of 40 acrocentric chromosomes that are very difficult to distinguish (see Section G, Karyotyping Mouse Cells). Gropp discovered in high Swiss valleys inbred groups of mice that have seven pairs of bi-armed (or Robertsonian fusion) chromosomes produced by the centric fusion of pairs of normal chromosomes (Gropp and Winking 1981). Individual Robertsonian chromosomes have been crossed into inbred laboratory strains where they can be used to generate embryos...
that are monosomic or trisomic for particular chromosomes (Epstein 1985) or have inherited two copies of a chromosome from one parent (Cattanach and Kirk 1985). They also provide markers for cytogenetic experiments, including the mapping of genes by in situ hybridization (see, e.g., Münke et al. 1985). Since their discovery in mice of the Valle di Poschiavo, centric fusions have been found in mice in other localities and in laboratory strains. Like inbred strains, they have a strict nomenclature; for example, Rb (11.16)H is a Robertsonian fusion involving chromosomes 11 and 16 and was the second of a series identified at the MRC Radiobiology Laboratory at Harwell (H) (Lyon and Searle 1989). For more information on the genetics and natural history of M. m. domesticus and its relatives, see the excellent symposium volume Biology of the House Mouse (Berry 1981) and the excellent and very recent book by Lee Silver (1994).

Origins of Developmental Genetics of the Mouse

Because of their availability from the mouse fancy, many of the first mutants used in breeding experiments sported visible differences in coat color, hair morphology, and pigmentation patterns (see Fig. 42 in Section A). In fact, these old mutations have proved to be an extremely valuable resource for studying a whole range of interesting biological problems, and many of the genes involved have now been cloned. For example, the Dominant white spotting (W) (see Fig. 42 in Section A) and Steel (Sl) pigmentation mutants have defects in the genes encoding, respectively, a transmembrane tyrosine kinase receptor and its ligand required for the growth and survival of melanocytes, primordial germ cells, and hematopoietic cells. The waved-1 and waved-2 hair mutations turned out to involve the genes encoding the transforming growth factor-α (TGF-α) and the epidermal growth factor (EGF) receptors, respectively (Luetke et al. 1993, 1994; Mann et al. 1993). The dilute (d) mutation, which causes a dilution of pigmentation because the melanocytes cannot extend their dendritic cellular processes, is now known to have originated in a retroviral insertion into a gene encoding a novel myosin heavy chain expressed in the cytoplasm of melanocytes (Mercer et al. 1991), and the agouti gene that is expressed in hair follicles and regulates pigment production by melanocytes (see Fig. 42 in Section A) has also been cloned (Bultman et al. 1992).

Over the years, a large number of mouse mutants affecting other complex neurological, physiological, and morphogenetic processes have been identified. Some were uncovered during the early days of inbreeding as recessive mutations in wild or fancy mice. Others have arisen as spontaneous mutations in laboratory stocks of already inbred mice. Another important source has been the offspring of mice exposed to X-rays or chemical mutagens. Much of this work has been carried out in two laboratories established shortly after the Second World War in response to the need for research into the biological effects of radiation: the Oak Ridge National Laboratory, Tennessee, and the MRC Radiobiology Unit in Harwell, near Oxford. As well as generating a whole range of important radiation-induced mutants and chromosomal rearrangements, these laboratories have done outstanding work on basic mouse genetics. For example, at Oak Ridge, Liane Russell mapped a series of overlapping deletions covering the dilute-short ear region on chromosome 9. This region encompasses several genes involved in pre- and postnatal development and is flanked by the dilute locus, which affects pigmentation, and the short-ear locus, which regulates the differentiation of the skeleton (Russell 1971; Rinchik et
The retroviral insertion into the *dilute* gene described earlier provided the first molecular handle into the detailed analysis of the complex (Mercer et al. 1991), and subsequent positional cloning led to the identification of the *short-ear* locus as the gene encoding the growth factor known as BMP-5 (bone morphogenetic protein-5) (Kingsley et al. 1992). Work at Oak Ridge also generated an overlapping set of deletion mutants around the *albino* locus, and at least one of the genes that results in postnatal lethality as a homozygous deletion has now been definitively identified (Kesley et al. 1993). On the other side of the Atlantic, Mary Lyon, working first at the Department of Genetics in Edinburgh and then at the MRC Radiobiology Laboratory at Harwell, was the first to describe the phenomenon of random-X chromosome inactivation in somatic tissues of female mice (Lyon 1961). At Harwell, she also generated many new, and for a long time unappreciated, ideas about the genetic organization of the *t* complex, which is described below (Lyon et al. 1979). By whatever route mouse mutants and chromosomal variants are derived, they are very expensive and time-consuming to isolate and maintain, and those that have been conserved and are catalogued in the sources listed in Appendix 2 are testament to an enormous amount of hard work, dedication, and foresight by many mouse geneticists.

Looking back, it is also easy to underestimate the painstaking work that went into describing the pathology and etiology of many of the early morphological mutants. It soon became apparent that to understand how a whole range of defects in the adult could be caused by mutation in a single gene, it was necessary to trace the mutant phenotype back to the early embryo. One geneticist who made a speciality of this approach was Hans Grüneberg, a refugee from Germany, who in 1938 was invited by Haldane to work at University College, London. Originally a physician, Grüneberg was motivated by a belief that mouse mutants could be used as models for understanding human congenital defects. For more than 40 years, he described a whole variety of mutants, in particular those with skeletal abnormalities (see, e.g., *pudgy*, Fig. 42 in Section A). He traced many of them back to early postimplantation stages when they first showed signs of defects in the process of somite formation and differentiation. His books, *Animal Genetics and Medicine* (1947), *The Genetics of the Mouse* (1952), and *The Pathology of Development* (1963), are classics of their kind and were as influential as Ernst Hadorn's *Developmental Genetics and Lethal Factors*, which was written in 1955 and translated into English in 1961.

Foremost among the pioneers of mouse developmental genetics in the United States were L.C. Dunn, a contemporary of Thomas Hunt Morgan at Columbia University, and his colleagues Dorothea Bennett and Salome Gluecksohn-Waelsch, originally a student of the renowned German embryologist, Hans Spemann. Dunn and his disciples can be credited with describing many homozygous lethal mouse mutants, but their most significant contribution has been to promote the genetic analysis of the *t* complex on chromosome 17. The first mutant forms of this complex were discovered by a Russian cancer research scientist, Nelly Dobrovolskaia-Zavadskaya, working at the Pasteur Institute in Paris on the effects of radiation. She found that one of the offspring of an X-irradiated mouse had a short tail and defined it as having a dominant mutation, *T* or *Brachyury*. Later, Dobrovolskaia-Zavadskaya crossed one of her *T* mice with a wild mouse she had caught and was surprised to find that the offspring had no tails at all. In fact, we now know that the wild mouse carried a variant form of the *t* complex which is called a *t* haplotype.
and which interacts with $T$ to produce taillessness. In 1932, realizing the complexity of the system she had uncovered and the limitations of her own resources, Dobrovolskaia-Zavadskaia passed her mice on to Dunn.

Soon a number of different $t$ haplotypes were discovered in wild mouse populations, and it was shown that embryos homozygous for different $t$ haplotypes die at different stages of development (Bennett 1975). The recessive lethal mutations were maintained at high levels in the wild because males heterozygous for wild-type and $t$-haplotype forms of chromosome 17 transmit the latter to more than 90% of their offspring, a phenomenon known as transmission ratio distortion. Fifty years on, the $t$ complex has been extensively analyzed by molecular techniques and has been shown to cover more than 16 cM of DNA, or about one third of chromosome 17 (equivalent to 1% of the entire genome), and to contain four large inversions, one of which includes the H-2 complex (Herrman et al. 1986). It seems very likely that these inversions trap recessive embryonic lethal mutations in the many unrelated genes contained within the region by inhibiting recombination between wild-type and mutant chromosomes, and the complex is further held intact by transmission distortion (for reviews, see Silver 1988, 1993a). Making use of a number of different $T$ mutations and rearrangements within the $t$ complex, Bernhard Herrmann, working in collaboration with Hans Lehrach and his colleagues at the EMBL in Germany, cloned the $T$ gene in 1990. This was the first example of the positional cloning of a mouse developmental mutation and it had a great impact on the field at the time. The Brachyury gene product turned out to be a DNA-binding protein and putative transcription factor expressed initially in the primitive streak of the embryo and then in the notochord and tailbud. In homozygous mutant embryos, the absence of $T$ leads to lethal developmental defects at approximately 10 days p.c. (Herrmann et al. 1990; Herrmann 1991; Kispert and Herrmann 1993; Stott et al. 1993; Wilson et al. 1993). Moreover, the $T$ gene product and pattern of expression have been highly conserved during vertebrate evolution, and homologs have been found in Xenopus (Xbra) (Smith et al. 1991), zebra fish (no tail) (Schulte-Merker et al. 1992, 1994), and ascidians (Yasuo and Satoh 1993). Recent work has shown that a gene related to Brachyury is present in Drosophila and that $T$ is a member of a quite large gene family encoding transcription factors with a so-called T-box DNA-binding domain (Bollag et al. 1994).

The nature of most of the $t$-associated genes, including those that regulate transmission distortion, has yet to be determined (Silver 1993a). Likewise, there are many other interesting “classical” mutations affecting embryonic pattern formation, organogenesis, and early neural development mapping elsewhere in the genome that must still be cloned. There are several ways in which this challenge may be met in the future (for reviews, see Reit and Bernstein 1991; Magnuson and Faust 1993). As more and more cloned genes and anonymous microsatellite markers are mapped, some may by chance fall near developmental loci and provide a starting point for a short stint of genomic “walking.” In addition, a DNA fragment or a retrovirus used to generate transgenic mice may by chance insert into a gene controlling a specific morphogenetic process. The foreign DNA will then provide a unique handle for isolating the endogenous gene (for review, see Meisler 1992). One of the first examples of such chance insertional mutagenesis into a developmental gene was the cloning of limb deformity by Rick Woychik and his colleagues in the laboratory of Phil Leder (Woychik et al. 1985, 1990; Maas et al. 1990). Finally, studies in fields such as hematopoiesis, immunology, tumor biology, and
developmental neurobiology are constantly leading to the identification of new growth factors, receptors, and cell-cell adhesion molecules. It is very likely that at least some of these proteins will be encoded by genes already identified by classical genetics. More systematic strategies for identifying new genes and cloning developmental mutants in the mouse are discussed at the end of this Introduction.

**Origins of Experimental Mouse Embryology**

Mammalian genetics had a clear beginning with the rediscovery of Mendel’s laws in 1900 and was initially championed by a relatively small number of enthusiasts, centered around leaders such as Castle, Little, and Haldane. In contrast, mammalian embryology is a much older science, and it would be beyond the scope of this Introduction to trace the complex lineage of modern ideas back through many communities and continents to the classical experimental embryologists like Boveri, Roux, Spemann, Hadorn, Nieuwkoop, and Waddington. From the beginning, mammalian embryology was closely associated with studies into human and veterinary reproductive physiology, and it is through these links that social pressures have had their influence on academic research. For example, the accelerated pace of research into mammalian reproduction and embryology in the late 1950s and 1960s was due, in part, to the realization of the need for new methods of human population control and increased food production. Many laboratories in the United States and Europe were funded by the Population Council, Inc., and by the Ford Foundation. Other support came from bodies like the Agricultural Research Council in Great Britain that were anxious to see improvements in the fertility and yields of farm animals. For a history of mammalian embryology, see references in Needham (1959), Oppenheimer (1967), Mayr (1982), and Austin (1961).

Much of the early experimental work in mammalian embryology was done using rabbit embryos. This included accurate descriptions of preimplantation stages (Van Beneden 1875), oviduct transfer (Heape 1890), filming of morulae dividing in culture (Lewis and Gregory 1929), and other in vitro observations (for reviews, see Pincus 1936; Austin 1961). Rabbits were used initially because the eggs are relatively large and easy to handle, being surrounded by a thick mucin coat, and the female ovulates only after mating, so that the age of the embryos could be timed quite precisely. However, these advantages were soon outweighed as more became known about the reproductive physiology and genetics of mice.

The first report of attempts to culture mouse embryos in vitro to the blastocyst stage was by John Hammond, Jr., son of his namesake, the great animal husbandry scientist who introduced the technique of artificial insemination for cattle. Working at the Strangeways Laboratory in Cambridge, England, Hammond Jr. succeeded in culturing eight-cell morulae to blastocysts, but embryos removed at two-cell stage soon died (Hammond 1949). It was not until 1956 that a breakthrough was made by an Australian veterinary scientist Wesley Whitten working at the Australian National University in Canberra after training in Oxford. The motivation behind these experiments was to obtain a defined medium in which the possible requirement of steroid hormones for embryo development could be tested. Using Krebs-Ringer’s bicarbonate solution supplemented with bovine serum albumin, Whitten succeeded in culturing one-cell mouse eggs to the blastocyst stage (Whitten 1956).
Whitten later emigrated to the United States and continued his work in the Jackson Laboratory, but he also collaborated closely with John Biggers of the University of Pennsylvania in Philadelphia. It was in Biggers' laboratory that another veterinarian, Ralph Brinster, began his research career by defining the precise nutritional requirements of the preimplantation mouse embryo and, in the process, established the microdrop culture technique (for review of early work, see Brinster 1965; Whitten and Biggers 1968; Biggers et al. 1971).

These culture conditions, although in the end simple enough, opened up a whole new range of experiments. At the same time, the work of Anne McLaren in the United Kingdom on optimizing conditions for oviduct and uterine transfer (McLaren and Michie 1956) made it possible to overcome the final hurdle and routinely turn cultured eggs into live mice (McLaren and Biggers 1958). Together, these technical improvements meant that it was at last feasible to test the end result of experimental manipulations on large numbers of embryos. For example, Kristof Tarkowski in Warsaw was able to start analyzing the developmental potential of single mouse blastomeres, using the classical embryological approach of killing one blastomere and seeing how the other would develop. He was also able to make the first aggregation chimeras, an idea conceived and accomplished during a visit to the University of Bangor in north Wales (I. Wilson, pers. comm.). Tarkowski's original method involved breaking the zona pellucida mechanically and pushing the embryos together in a small drop of medium, which was technically extremely difficult. The whole process was made much easier by Beatrice Mintz in Philadelphia, who discovered that the zona could be gently digested by Pronase. So here again, a relatively simple procedure, once established, opened up a wealth of biological problems that could be tackled experimentally.

Chimeras derived from embryos of two or more genotypes have been used to study such diverse topics as melanocyte migration and pigment patterns, sex determination, germ cell differentiation, immunology, tumor clonality, size regulation, and cell lineage (for review, see McLaren 1976). The use of culture systems also led to the development of routine methods for both in vitro fertilization and the parthenogenetic activation of mouse oocytes. In addition, Ralph Brinster was able to carry out the first experiments on the injection of purified globin mRNA into mouse eggs (Brinster et al. 1980) which, as described below, set the stage for the production of transgenic mice. With similar vision and persistence, Davor Solter, at the Wistar Institute in Philadelphia, succeeded in transferring nuclei between fertilized mouse eggs. This technology was crucial in revealing the importance of parental gene imprinting in mammalian development (McGrath and Solter 1984b; Surani et al. 1990; Forejt and Gregorova 1992; Barlow 1994).

As far as studies on the postimplantation mouse embryo are concerned, there was considerable debate and confusion about the lineage of the different embryonic and extraembryonic tissues. The various conflicting theories have been summarized by Rossant and Papaioannou (1977). To resolve these problems, and to ask when early embryonic cells become committed to their developmental fate, Richard Gardner in Cambridge, England, developed the technique of generating chimeras by injecting isolated cells into host blastocysts (Gardner 1968). To test the developmental potential of different parts of the postimplantation embryo, several laboratories also developed methods for culturing isolated pieces of tissue in vitro and in ectopic sites. In this way, Nikola Skreb and his colleagues in Zagreb showed that the early embryonic ectoderm contains cells capable of contributing to all three germ layers of the fetus.
These studies on the pluripotentiality of cells from the normal embryo were complemented by the use of teratocarcinomas as a model system for studying early embryonic development, an approach pioneered by Leroy Stevens at the Jackson Laboratory and by Barry Pierce at the University of Colorado. Teratocarcinomas are gonadal tumors that contain a chaotic mixture of different tissue types, all derived from a population of undifferentiated stem cells known as embryonal carcinoma cells. Stevens first observed that male mice of the inbred 129 strain have a low incidence of testicular teratoma arising from primordial germ cells (Stevens and Little 1954). Stevens also identified modifier genes such as ter that increase the frequency of teratomas in the testis and eventually developed a strain (129/Sv) in which the incidence is as high as 30%. It is likely that the nature of the ter gene will soon be known (Asada et al. 1994). Stevens also developed the LT strain in which about 50% of females develop ovarian teratocarcinomas.

The availability of transplantable teratocarcinomas inspired many new experiments, and it was not long before it was shown by Boris Ephrussi in France and Gordon Sato in the United States that cells from the tumors could be grown in vitro as cultures that consisted of both differentiated derivatives and undifferentiated embryonal carcinoma stem cells. The potential of this culture system for studying the biochemistry and molecular biology of early mammalian embryonic cells was also recognized by François Jacob, and because of his influence and the work of his research group in the Pasteur Institute, many cell biologists and biochemists were attracted to the teratocarcinoma system and the study of mouse developmental genetics (Jacob 1983).

Finally, the availability of teratocarcinomas led to the demonstration by Brinster (1974), Mintz and Ilmensee (1975), and Papaioannou et al. (1975) that embryonal carcinoma stem cells could be reintegrated into blastocysts and contribute to many normal adult tissues. However, it was not until the development of blastocyst-derived embryonic stem cell lines, independently by Gail Martin in San Francisco (Martin 1981) and Martin Evans and Matt Kaufman in Cambridge, England (Evans and Kaufman 1981), that integration of cultured cells into the germ line could be achieved with high efficiency and reproducibility. As described below, the availability of embryonic stem cells, now known as ES cells, revolutionized mammalian developmental genetics. In conjunction with the technique of homologous recombination, ES cells have opened up exciting new approaches to studying gene function and interaction during embryonic development, and they are also widely used to engineer mouse models for human diseases and congenital abnormalities.

**Manipulating the Mouse Genome**

The first report of the direct introduction of new genetic material into the mouse embryo actually predates the widespread use of recombinant DNA techniques. In 1974, Rudolf Jaenisch and Beatrice Mintz found that when purified SV40 DNA was injected into the blastocoel cavity of mouse blastocysts, viral DNA sequences could be detected in somatic tissues of many of the resulting animals, suggesting that they had integrated into the genome of embryonic cells. In addition, Jaenisch (1976) discovered that Moloney murine leukemia virus could be stably introduced into the germ line by viral infection of preimplantation mouse embryos. However, these studies did not immediately lead to attempts to introduce cloned eukaryotic genes into the germ line.
In 1980, it was reported that the microinjection of the cloned herpes simplex virus (HSV) thymidine kinase (tk) gene into the nuclei of cultured fibroblasts led to the stable incorporation and expression of the tk gene in 5–20% of the recipient cells (Anderson et al. 1980; Capecchi 1980). This finding suggested that the microinjection of DNA into the one-cell mouse embryo might allow the efficient introduction of cloned genes into the developing mouse and led a number of investigators to test this possibility. The first successful introduction of a cloned gene into mouse somatic tissues by pronuclear injection was reported by Gordon et al. (1980). Shortly thereafter, several groups were successful in introducing cloned genes into somatic tissues as well as into the germ line by this technique (Brinster et al. 1981b; Costantini and Lacy 1981; Gordon and Ruddle 1981; Harbers et al. 1981; E. Wagner et al. 1981; T. Wagner et al. 1981). The structure, inheritance, and expression of foreign genes in transgenic mice and the applications of this technique for the study of mouse development are discussed in Section E.

ES cells were first derived from blastocysts in culture by Evans and Kaufman (1981) and Martin (1981). Shortly thereafter, they were shown to be capable of contributing to many different tissues in chimeras, including the germ line, when injected into host blastocysts and returned to a foster mother (Bradley et al. 1984). The first reports of genetic manipulation of ES cells were by Robertson et al. (1986), who demonstrated that cells containing integrated retroviruses could be transmitted through the germ line, and Gossler et al. (1986), who showed that the neo gene could be similarly transmitted. Manipulation of a specific gene was shown by Hooper et al. (1987) and Kuehn et al. (1987), who selected mutant ES cells defective in the X-linked gene, hypoxanthine phosphoribosyl transferase (HPRT), and used them to derive HPRT-deficient male mice. Two methods used were selection of pre-existing "spontaneous" mutant cells (Hooper et al. 1987) and disruption of a normal gene with a retrovirus (Kuehn et al. 1987). Unfortunately, differences in the metabolism of purines by mouse and humans meant that the HPRT-deficient male mice did not show the symptoms of Lesch-Nyhan disease. Nevertheless, an important breakthrough had been made with the demonstration that it was possible to genetically manipulate ES cells in a controlled way in the culture dish and introduce the mutation into the germ line. The stage was then set for experiments designed to target mutations to specific genes by homologous recombination in ES cells. This was initially achieved using HPRT (Doetschmann et al. 1987; Thomas and Capecchi 1987; Koller et al. 1990). Germ-line transmission of a targeted mutation in a gene other than HPRT was first obtained with c-abl (Schwartzberg et al. 1989) and β2-microglobulin (Zijlstra et al. 1989). To date, more than 100 genes have been disrupted by homologous recombination and transmitted through the germ line, and a partial listing of the mouse lines with targeted mutations can be found in the TBASE database now held at Johns Hopkins University (see Appendix 2). The isolation of ES cells and their manipulation in culture are described in Section F.

The Systematic Search for New Genes and Developmental Mutants in the Mouse

During the past decade, a number of different strategies have been used to search systematically for novel genes regulating development in the mouse. One of the most fruitful strategies has been so called "homology searching," which involves
screening cDNA or genomic libraries for mouse genes related to those that have been found to regulate growth and development in other organisms, in particular Drosophila, C. elegans, and Xenopus. It also includes searching for genes related to proto-oncogenes, growth factors, and receptors discovered in research with mammalian cells. This approach has led to the identification of highly conserved gene families encoding proteins with related functional motifs. One such example includes transcription factors with related DNA-binding domains such as the homeo, paired, zinc finger, winged-helix, and helix-loop-helix domains. Other examples are polypeptide-signaling molecules, including those related to growth factors and oncogene products (e.g., proteins related to FGFs, TGF-βs, EGFs, hedgehogs, and Wnts). To these can be added transmembrane receptors for different classes of signaling molecules, and proteins involved in axonal guidance, cell migration, and cell adhesion. The extraordinary success of this approach has led to the growing realization that not only have individual elements of developmental pathways such as polypeptide signaling molecules and transcription factors been conserved during evolution, but probably whole regulatory circuits have been conserved as well. This implies that some simple ancestral organism established basic mechanisms for determining anteroposterior, dorsoventral, and proximodistal axes and for determining the fate and proliferation of cells according to their position along these axes. Once these mechanisms were set up, it would be easier for future organisms to elaborate upon them by gene duplication and divergence to produce more complex morphologies than to completely re-invent alternative pathways. It remains to be seen to what extent this principle of conservation pervades embryonic development and whether screens based on alternative hypotheses lead to the discovery of whole new classes of developmental genes unique to vertebrates in general, and mammals in particular. Meanwhile, it is obviously best to rely on several different experimental approaches to the problem, particularly since it is likely that some conserved genes will have diverged too far in nucleotide sequence to be detected by conventional methods for homology searching.

One alternative strategy to identify developmental genes is to carry out large-scale mutagenesis screens. Until ES cells were available, such mutagenesis experiments in the mouse could be performed only by investigators with access to relatively large breeding colonies. The most popular protocol used by mouse geneticists is to treat male mice with ethylnitrosourea (ENU) or with chlorambucil (Russell et al. 1989) and to then screen offspring for dominant or recessive mutations. Examples of interesting mutants recently generated by ENU mutagenesis are Min (multiple intestinal neoplasia), which has a mutation in the mouse homolog of the human APC (adenomatous polyposis colon) gene (Su et al. 1992), and Clock, which has a defect in establishing circadian rhythm (Vitaterna et al. 1994).

In the future, as more and more microsatellite markers and genes are mapped, chemical mutagenesis programs may become more cost-effective in the mouse, since the positional cloning of disrupted genes will be easier. Meanwhile, the availability of ES cells has opened up an entirely different and very powerful way of carrying out large-scale insertional mutagenesis screens. This potential was quickly recognized by Martin Evans and his group at Cambridge, who infected ES cells with a defective retrovirus carrying the neoR gene, which allowed cells with an insertion to be selected in culture (Robertson et al. 1986). These cells were then used to generate mouse lines from which homozygous mutants could be bred. One of these lines, 413.d, has been shown to have an insertion in nodal, which en-
codes a TGF-β-related gene expressed around the embryonic node during gastrulation (see Fig. 22 in Section A) (Conlon et al. 1991; Zhou et al. 1993).

More recently, insertional mutagenesis screens have involved vectors that tag the insertion site with a reporter sequence such as lacZ, which both facilitates cloning and allows the expression pattern of the disrupted gene to be visualized. Different "trap" vectors have been designed for identifying coding sequences, enhancers, and promoters (Gossler et al. 1989; Friedrich and Soriano 1991; Skarnes et al. 1992; for a list of currently available gene, promoter, and enhancer-trap vectors, see Gossler and Zachgo 1993). Other innovations include the use of retroviral vectors designed to allow rapid sequencing of flanking DNA (von Melchner et al. 1992; Chen et al. 1994) and vectors designed to select for insertion into genes encoding secreted or membrane-associated proteins (W.C. Skarnes and R Beddington, unpubl.). Screens can also be set up to select for insertions into genes that are specifically expressed when ES cells differentiate or that are activated or repressed in response to growth factors.

As the mouse genome project accelerates, it will become easier to generate a range of compound mutants by interbreeding different mouse mutant lines and to map multiple modifier genes using microsatellite markers (see, e.g., Dietrich et al. 1993). In the future, high priority will also be placed on devising techniques for conditionally activating, disrupting, or tagging a particular gene in a specific tissue or cell type during development. Methods for conditionally activating genes are discussed in Section E (Production of Transgenic Mice). Strategies for selectively disrupting genes will be particularly important in cases where homozygous null mutants of a gene expressed at several different stages of embryogenesis die early in development, precluding analysis of gene function at later stages. Two methods for tissue-specific DNA recombination are under study at the present time. The first is to cross a transgenic mouse line carrying the P1 bacteriophage Cre recombinase under the control of a tissue-specific regulatory element with another transgenic line in which loxP recombination sites have been inserted on either side of the targeted gene. The second method involves a similar strategy but using yeast FLP recombinase and recombinase-specific flanking sequences (O’Gorman et al. 1991; Lasko et al. 1992; Orban et al. 1992; Gu et al. 1993, 1994). Although still rather speculative, ideas like these will continue to vitalize the study of mouse genetics and to open up exciting new opportunities for the future.
Section A

SUMMARY OF MOUSE DEVELOPMENT
This section provides a brief survey of mouse development for those researchers who are completely new to mammalian embryology. For detailed accounts, some excellent textbooks are listed in Appendix 2. We also provide a very short section on the genetics of coat color for researchers who become interested in this topic after handling mice for the first time. Finally, a few normal and mutant mice are illustrated to provide at least some idea of the genetic variation that exists in the laboratory mouse.
Embryonic development of the mouse begins with fertilization of the egg by the sperm. One important feature of mouse embryogenesis is that early development is much slower than in organisms such as the sea urchin, Drosophila, and Xenopus. By 24 hours after fertilization, embryos of these species are well on their way to becoming free-living, feeding larvae, and they contain more than 60,000 cells, organized into many different tissue layers. In contrast, the mouse embryo is still at the two-cell stage and will continue to divide slowly without any increase in mass as it moves along the oviduct into the uterus for implantation 4.5 days after fertilization. This slow development allows the uterine tissue time to prepare for receiving the embryo. The embryo, in its turn, generates the first two tissue lineages (the trophectoderm and the primitive endoderm) that form a major part of the placenta and the extraembryonic yolk sacs required for successful interaction with the mother. Once implantation has been achieved, a dramatic increase in the growth rate of the embryo occurs, particularly in the small group of pluripotential cells known as the epiblast, or primitive ectoderm, from which the fetus will develop. The epiblast is in many ways the equivalent of the cellular blastoderm of Drosophila, or the blastodisc of the chick. Between the fifth and tenth day after fertilization, the three primary germ layers—the ectoderm, mesoderm, and definitive endoderm—are formed as a result of gastrulation, and the basic body plan and organ primordia of the future mouse are established. To summarize briefly, the notochord is laid down along the midline of the anteroposterior axis, and the paraxial mesoderm on either side of the notochord is divided up into reiterated pairs of somite blocks, generating an obvious segmented pattern, while the lateral plate mesoderm remains unsegmented. The neural plate is induced and folds up into the neural tube, which is subdivided into forebrain, midbrain, hindbrain, and spinal cord. The placodes of the nose, ear, and lens are formed from the surface of the ectoderm. The neural crest cells start their migration, and the heart and circulatory system and limb buds are established. It is during this early postimplantation period that many of the genes controlling the differentiation and morphogenesis of the adult organs are gradually brought into play.

The gestation period for the mouse embryo is 19–20 days, depending on the strain. Figure 1 and Table 1 show the timing of the different stages, based on the development of F₁ hybrids between C57BL/6 females and CBA males; for some inbred strains, such as C3H, the process is somewhat slower. Figure 2 is a summary of our current knowledge of tissue lineages in the mouse embryo.

Although embryonic development starts with fertilization, both the egg and the sperm are themselves products of complex maturational processes initiated when the primordial germ cells enter the genital ridges. This summary of mouse development thus begins with a description of the origin and growth of the germ cells, which are among the most fascinating cells in the whole organism.

*Figure 1* Time course of development. (0–5 days) Cleavage and blastulation; (5–10 days) implantation, gastrulation, and early organogenesis; (10–14 days) organogenesis; (14–19 days) fetal growth and development. For more details, see Table 1.
Figure 1 (See previous page for legend.)
Table 1  Development of the Mouse Embryo

<table>
<thead>
<tr>
<th>Stage</th>
<th>Age (days p.c.)</th>
<th>Features</th>
<th>System</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>extraembryonic</td>
</tr>
<tr>
<td>1</td>
<td>0–1</td>
<td>one-cell egg</td>
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<tr>
<td>2</td>
<td>1</td>
<td>two-cell egg</td>
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<tr>
<td>3</td>
<td>2</td>
<td>morula, 4–16</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>cells</td>
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<tr>
<td>4</td>
<td>3</td>
<td>morula-blastocyst</td>
<td>trophectoderm</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>free blastocyst without zona</td>
<td>formed</td>
</tr>
<tr>
<td>6</td>
<td>4.5</td>
<td>implanting blastocyst</td>
<td>primitive</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>egg cylinder</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>proamniotic cavity in primitive ectoderm</td>
<td>Reichert's membrane forming</td>
</tr>
<tr>
<td>9</td>
<td>6.5</td>
<td>embryonic axis determined</td>
<td></td>
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<tr>
<td>10</td>
<td>7</td>
<td>early–mid-primitive streak</td>
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<table>
<thead>
<tr>
<th>Stage</th>
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<th>Features</th>
<th>Extraembryonic</th>
<th>Circulation</th>
<th>Intestinal tract</th>
<th>Nervous/sensory</th>
<th>Urogenital</th>
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<tr>
<td>11</td>
<td>7.5</td>
<td>late primitive streak</td>
<td>allantois appearing</td>
<td>blood islands in foregut pocket visceral yolk sac</td>
<td>neural plate</td>
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<tr>
<td>12</td>
<td>8</td>
<td>1–7 somites</td>
<td>allantois contacts chorion</td>
<td>first aortic arch hindgut pocket</td>
<td>neural folds, otic placode</td>
<td>germ cells near base of allantois</td>
<td></td>
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<tr>
<td>13</td>
<td>8.5</td>
<td>8–12 somites; turning of embryo</td>
<td>paired heart primordia fusing anteriorly</td>
<td>thyroid rudiment, second pharyngeal pouch, hepatic diverticulum</td>
<td>neural folds close at level of somites 4–5</td>
<td>pronephros</td>
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<tr>
<td>14</td>
<td>9</td>
<td>13–20 somites</td>
<td>blood circulates in visceral yolk sac</td>
<td>heart begins to beat, three paired aortic arches</td>
<td>oral plate ruptures</td>
<td>anterior neuropore closes, olfactory placode</td>
<td>pronephric duct still solid</td>
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<td>15</td>
<td>9.5</td>
<td>21–29 somites; forelimb bud at level of somites 8–12</td>
<td>common ventricle and atrium, dorsal aortae fused</td>
<td>lung primordia, pancreas evagination, vitelline duct closed</td>
<td>posterior neuropore closes, otic vesicle</td>
<td></td>
<td></td>
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<tr>
<td>16</td>
<td>10</td>
<td>30–34 somites; hindlimb bud at level of somites 23–28</td>
<td>primary bronchi</td>
<td>lens placode</td>
<td>Wolffian ducts contact cloaca in older specimens</td>
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<tr>
<td>Week</td>
<td>Stage</td>
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<td>17</td>
<td>10.5</td>
<td>35–39 somites; tail rudiment</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>sixth aortic arch umbilical loop, cloacal membrane</td>
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<td></td>
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<td>deep lens pit mesonephric tubules</td>
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<td>18</td>
<td>11</td>
<td>40–44 somites</td>
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<td>spleen primordium</td>
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<td></td>
<td></td>
<td>lens vesicle closing, rims of olfactory placode fusing</td>
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<td>19</td>
<td>11.5</td>
<td>6–7 mm, forefoot plate</td>
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<td>partitioned atrium, unpaired ventricle</td>
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<td>bucconasal membrane</td>
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<td></td>
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<td>ureteric buds</td>
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<td>20</td>
<td>12</td>
<td>7–9 mm, hindfoot plate</td>
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<td>partition of arterial trunk begins</td>
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<td></td>
<td></td>
<td>tongue, thymus, and parathyroid primordium</td>
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<td></td>
<td></td>
<td>pineal body evaginates</td>
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<td>sexual differentiation gonads in older specimens</td>
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<td>21</td>
<td>13</td>
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<td></td>
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<td>palatine processes vertical, dental laminae</td>
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*a*Adapted from Thieler (1972, 1983) based on the development of C57BL/6 x CBA F₁ hybrids. Embryos of some inbred lines of mice may develop more slowly.

*b*In this manual we use the following convention for timing pregnancy and the age of embryos. Assuming that fertilization takes place around midnight on a 7 p.m.–5 a.m. dark cycle, then at noon on the following day (i.e., the day on which the vaginal plug is found), the embryos are aged "half-day postcoitum" or "0.5 day p.c. According to this convention, the day on which the plug is found is day 0 of pregnancy. At noon on the next day, the embryos are 1.5 days p.c., and so on. Note, however, that the embryos of one litter are not synchronized in their development.
Figure 2  Summary of the lineages of tissues constituting the mouse embryo. (Hatched areas) All tissues that will give rise to the embryo proper and extraembryonic cells; (closed areas) extraembryonic tissues; (open areas) tissues of the embryo proper. (Adapted from Gardner 1983.)
Origin of Germ Cells and Their Migration to the Genital Ridges

The primordial germ cells (PGCs) are derived from the epiblast (Falconer and Avery 1978; Gardner et al. 1985). There is no evidence for a segregated germ line in the inner cell mass of blastocysts up to 4.5 days postcoitum (p.c.) because single epiblast cells at this stage can give rise to both somatic tissue and gametes (Gardner et al. 1985). PGCs are first detected during gastrulation at 7.0 days p.c. as a distinct cell population located in the extraembryonic mesoderm of the posterior amniotic fold (Fig. 3B) (Ginsburg et al. 1990). Subsequently, they come to underlie the posterior part of the primitive streak (Ozdenski 1967) and become incorporated into the base of the allantoid, forming a coherent cluster of approximately 75 cells at the headfold (presomite) stage (Fig. 3A). PGCs are distinguished by their large round shape and high expression levels of tissue-nonspecific alkaline phosphatase (Hahnel et al. 1990) and high levels of Oct-3/4 mRNA (Rosner et al. 1990; Scholer et al. 1990a,b). Evidence that germ cells may have segregated as a separate lineage by mid gastrulation comes from experiments in which small fractions of the 7.0- and 7.5-day p.c. embryonic region of the egg cylinder, composed of all three germ layers, were isolated in culture. The ability of these fractions to generate large, alkaline-phosphatase-positive cells was assessed. These studies showed that the potential to give rise to PGCs is restricted to the posterior aspect of the embryo (Snow 1981).

Alkaline phosphatase staining has also been used to follow the complex migration of PGCs from the base of the allantoid to the genital ridges (Figs. 3 and 4) (Clark and Eddy 1975; Eddy et al. 1981; Eddy and Hahnel 1983). At 8.0 days p.c., the small nest of PGCs begins to disperse, and by the early somite stage, the majority of alkaline-phosphatase-positive cells (~200 in all) are found intercalated into the epithelium of the hindgut (Ginsburg et al. 1990). The PGCs leave the gut endoderm and traverse the dorsal mesentery toward the coelomic angles, the first cells reaching the genital ridges by 11.0–11.5 days p.c. (Figs. 3A and 4). By 13 days p.c., approximately 25,000 PGCs have colonized each gonad primordium. The genital ridges arise from intermediate mesoderm adjacent to the mesonephros and are first visible as a distinct urogenital ridge at approximately 10.0 days p.c. (see Lateral Plate and Intermediate Mesoderm, p. 81). However, the first signs of overt sexual dimorphism in the gonadal primordia are not apparent until 12.5 days p.c. During their migration from the allantoid, PGCs divide approximately once every 16 hours (Tam and Snow 1981). Their movement from the gut to the genital ridges involves active migratory movements, probably dependent on a suitable substratum and possibly involving chemotactic signals to ensure that the majority of cells converge on the genital ridge (Fig. 5). Female germ cells that migrate into prospective ovaries cease proliferation and enter meiosis, becoming arrested at prophase of the first meiotic division. In contrast, male germ cells entering prospective testes continue to proliferate until mitotic arrest occurs at about 14.0 days p.c. It is not uncommon for some PGCs to lodge in mesonephric tissue adjacent to the genital ridges and thus fail to enter the developing gonads (McLaren 1984). Such ectopic PGCs, regardless of their genetic sex, tend to enter meiotic arrest, characteristic of the female developmental pathway (McLaren 1984). As discussed below, this highlights the importance of the gonadal somatic tissue in influencing the meiotic or mitotic behavior of PGCs.

Several mutations are known to affect germ cell proliferation and cause sterility in homozygotes by severely reducing the number of germ cells reaching
Figure 3 Primordial germ cells and their migration pathway. (A) Schematic representation of the localization of PGCs at the base of the allantois around the hindgut pocket in an 8.5-day embryo, and the migration of PGCs along the hindgut and into the genital ridges in a 10.5-day embryo. (B) 7.5-day embryo (neural-fold-stage) stained in whole mount for alkaline phosphatase activity. Note the cluster of PGCs around the base of the allantois. (Reprinted, with permission, from Ginsberg et al. 1990.)
**Figure 4** Germ cells in the 10.5-day mouse embryo. (A) Germ cells stained with alkaline phosphatase lying in the hindgut mesentery of a 10.5-day mouse embryo (bright-field illumination). (B) Immunofluorescence microscopy of an adjacent section stained to reveal laminin in the basement membranes of the mesentery and genital ridge. Bar, 20 μm. (Photographs provided by Dr. D. Stott, St. George's Hospital Medical School, London.)

**Figure 5** Timing of female and male germ cell development in the genital ridge and early gonad of the mouse. (Adapted from Monk and McLaren 1981.)
the genital ridges. Recently, the molecular nature of two of these mutations has been defined. White spotting (W) and viable white spotting (Wv) reduce both the proliferation and migration of primordial germ cells (Mintz and Russell 1957). In contrast, in mice homozygous for mutations in Steel (Sl), the germ cells migrate toward the genital ridges, but once there, this already depleted population fails to proliferate and all PGCs degenerate (McCoshen and McCallion 1975). Both mutations also affect neural crest cells and progenitors of the hematopoietic system, two other populations of cells that must move as individual cells over long distances in the embryo. The pigment cell descendants of the neural crest from Sl/Sl, but not W/W, embryos can be rescued by providing them with a wild-type environment (Mayer 1973), suggesting that the Sl product acts non-cell-autonomously. Cloning of the Sl gene has confirmed this by showing that the gene product is a peptide-signaling molecule, Steel factor (SF) (also known as stem cell factor [SCF], kit-ligand [KL], and mast cell growth factor; see Witte 1990). Moreover, the various W mutations have been shown to be disruptions of the proto-oncogene c-kit (Chabot et al. 1988; Nocka et al. 1989), which is the transmembrane tyrosine kinase receptor for SF and expressed in PGCs. Certainly, SF is required for both survival and proliferation of PGCs in vitro, and it has recently been shown that primordial germ cells can give rise to permanent pluripotent cell lines in vitro if cultured in the presence of a variety of peptide growth factors, including SF (Matsui et al. 1992; Resnick et al. 1992). These cells, known as embryonic germ (EG) cells (see Fig. 33), appear to be equivalent to embryonic stem (ES) cells, at least with respect to their developmental potential, capacity to form chimeras, and ability to give rise to functional sperm (Labosky et al. 1994; Stewart et al. 1994).

**Sex Determination and Germ Cells**

The local gonadal environment has a profound influence on germ cell differentiation because in general the genetic sex of the gonad determines the type of gamete produced. In XX→XY chimeras, formed by aggregating morulae (see Section C) or by injecting cells into the blastocyst (see Section D), XX germ cells in a predominantly XX gonadal environment will enter meiotic arrest, typical of the female pathway, whereas in XY gonadal soma, they become mitotically arrested, the characteristic prelude to spermatogenesis (see McLaren 1984). Such experiments, in combination with the study of W mutants where the number of primordial germ cells is severely reduced in the genital ridge, have identified the somatic tissue of the gonad as the primary determinant of sexual differentiation. Three principal cell types make up the somatic element of the male gonad: Sertoli cells, Leydig cells, and connective tissue. However, it has not yet been established which of these cells is primarily responsible for initiating testis determination. Sertoli cells have been favored as the cell type responsible because they are the first to differentiate. Moreover, in XX→XY chimeras that are phenotypically male, the vast majority of Sertoli cells are XY, whereas Leydig cells and connective tissue demonstrate more variation in genotypic sex (Burgoyne et al. 1988).

It would therefore seem that a gene carried on the Y chromosome (Tdy) acts earlier in development than any ovarian determining counterpart to direct the indifferent gonad into testis differentiation. Thus, testis determination is essentially synonymous with sex determination, and Tdy has been rechristened Sry (Sex-determining region Y gene). This gene has been cloned and shown to encode a
DNA-binding protein with an HMG motif (Gubbay et al. 1990; Sinclair et al. 1990). It is expressed at 10.5 days p.c., when the urogenital ridge first appears, and during the critical phase (11.5–12.5 days p.c.) when the indifferent gonad acquires sexually dimorphic characteristics. In addition, its expression is limited to somatic cells since normal levels of Sry are found in W/W embryos (Koopman et al. 1990). Furthermore, in situ hybridization has shown that transcription at 11.5 days p.c. is restricted to the genital ridge and does not extend into the adjacent mesonephros. This candidate transcription factor is thus likely to initiate testis differentiation, thereby inhibiting ovary formation. Support for this idea came from the observation that transgenic XX mice which express Sry develop a testis (Koopman et al. 1991).

Normal testis differentiation, and in particular the development of testis cords, may also require a contribution of cells derived from the adjacent mesonephric region. Cells from this region can migrate into developing testes and contribute to peritubular myoid cells and other interstitial populations (Buehr et al. 1993). If these cells are prevented from entering the gonad, the testis fails to develop normal cords.

Prior to sex determination of the gonads, both primordia for the male and female reproductive tracts (the Wolffian and Mullerian ducts) are present. In the female, the Wolffian duct will degenerate, whereas in the male, the Mullerian duct regresses. In the absence of testis formation, the Wolffian duct does not survive, but this degeneration is not dependent on the presence of an ovary, indicating that regression is the default pathway (Austin and Edwards 1981). In the male, testosterone produced by the Leydig cells of the testis ensures survival and differentiation of the Wolffian duct into the vas deferens, seminal vesicles, and epididymis. In addition, anti-Mullerian hormone (AMH, also known as Mullerian inhibiting factor, MIF), a very early product of the Sertoli cells and a member of the transforming growth factor-β (TGF-β) superfamily, effects the degeneration of the Mullerian duct (Tran and Josso 1982; Munsterberg and Lovell-Badge 1991). In transgenic mice chronically expressing AMH, the Mullerian ducts are absent and the ovaries show signs of masculinization. This suggests that AMH may also have a role in testis differentiation, although whether this is due to a direct influence on somatic testicular cells or simply the elimination of germ cells entering meiosis in females is not known (Behringer et al. 1990; McLaren 1990).

**Spermatogenesis**

Spermatogenesis is probably the most exquisite example of a continuously synchronized and spatially organized sequence of differentiation (for review, see Hecht 1986). Since the time taken for spermatozoa to differentiate from a stem cell is more or less constant (~5 weeks in the mouse), any transverse section of a seminiferous tubule will contain a stereotyped array of cells from the basement lamina to the luminal surface, reflecting succeeding waves of spermatogenesis passing along the tubule. Unlike oogenesis, spermatogenesis relies on a population of true stem cells, which are capable of self-renewal as well as producing progeny for differentiation into spermatozoa (Fig. 6). These stem cells, which are the direct descendants of primordial germ cells, are large cells known as type-A spermatogonia. They first appear about 3–7 days after birth and lie on the basement membrane surrounding the seminiferous tubules. Following division, some daughters of type-
A spermatogonia differentiate into intermediate spermatogonia, and these in turn develop into type-B spermatogonia. Type-B spermatogonia, which are also located adjacent to the basement membrane, are smaller cells and behave like a transition population in that they can divide to produce more type-B spermatogonia. Consequently, type-B spermatogonia are more numerous than type A. Type-B spermatogonia enlarge and move away from the basement membrane, toward the lumen of the seminiferous tubule, thereby transforming into primary spermatocytes. It is at this stage that meiosis commences. During the first meiotic prophase, homologous chromosomes, including the X and Y chromosomes, pair and crossing over takes place. The products of the first meiotic division are known as secondary spermatocytes, each nucleus containing 20 chromosomes composed of two sister chromatids. The second meiotic division, in which the sister chromatids separate, produces spermatids containing a haploid genome. No further division occurs, and the subsequent differentiation into mature spermatozoa, which occurs at the luminal surface, involves extrusion of cytoplasm together with extensive differentiation. Finally, mature spermatozoa are released into the lumen, leaving superfluous cytoplasm, known as residual bodies, on the luminal surface.

The synchrony of spermatogenesis is due in part to incomplete cytokinesis so that all descendants of a type-B spermatagonium remain attached by cytoplasmic bridges, which are only lost when mature spermatozoa are released into the lumen of the seminiferous tubule. This means that although meiosis has already occurred, the complex differentiation required for spermatozoan development occurs within a shared cytoplasm and in the presence of most products of a diploid genome.

The testis is composed of an array of seminiferous tubules that originate from the testis cords apparent in developing male gonads at 12.5 days p.c. Germ cells that do not have a Y chromosome are at a selective disadvantage during spermatogenesis. They can, albeit rarely, enter meiosis but degenerate before the first meiotic metaphase. Therefore, the Y chromosome, in addition to carrying the sex determining gene, is thought also to contain a gene or genes essential for normal spermatogenesis (Burgoyne 1987).

Oogenesis

By 5 days after birth, all oocytes are in the diplotene stage of the prophase of the first meiotic division. They are therefore diploid but contain four times the haploid amount of DNA (4C). During the prolonged resting or dictyate stage, the paired homologous chromosomes are fully extended and transcription of oocyte (maternal) mRNA takes place. Studies on X chromosome activity have shown that only one X is active in XX primordial germ cells and 11.5-day p.c. oogonia, but that by 12.5 days p.c., both X chromosomes become active (Monk and McLaren 1981; McLaren 1983). In addition, it has been shown that the egg genome is globally undermethylated as compared with the sperm genome (Monk et al. 1987; Kafri et al. 1992).

Each oocyte is contained within a follicle composed of multiple layers of follicle or granulosa cells, which are of the same embryonic origin as Sertoli cells of the testis and have various roles in oocyte growth and differentiation (Figs. 7 and 8) (Richards et al. 1987). The follicle cells immediately surrounding the oocyte have numerous projections that form specialized junctions with the egg (Fig. 8). These
Figure 6 (See facing page for legend.)
Figure 7 Growth of the ovarian follicle.

Figure 6 Schematic representation of spermatogenesis and oogenesis in the mouse showing the difference in phases of mitosis and meiosis in males and females. Primordial germ cells (PGCs) first reach the gonads at approximately 11.0 days p.c. In the male, germ cells enter mitotic arrest at 14.0 days p.c., whereas female germ cells at this time become arrested in the first meiotic prophase. In the adult female, ovulation and resumption of meiosis are initiated by hormonal stimuli. In the male, type-A spermatagonia are first identified at 3–7 days postpartum, and thereafter, spermatogenesis is maintained by stem cell renewal and differentiation. Two homologous chromosomes (one from the father and one from the mother) are shown in the nucleus of the PGC. Sometime between the origin of the PGCs and its maturation in the gonad, parental imprinting of the chromosomes is erased and a new imprint is imposed.
Figure 8  Relationship between oocyte and follicle cells. (O) Oocyte; (JC) junctional complex; (ZP) zona pellucida; (FC) follicle cell. Bars: (A) 5 \( \mu \text{m} \); (B) 0.5 \( \mu \text{m} \).
junctional complexes involve gap junctions and allow metabolite transfer. They are maintained even when the follicle cells and oocyte are gradually separated by the deposition of the zona pellucida, a layer of extracellular material synthesized and deposited by the growing oocyte (Bleil and Wassarman 1980a,b; Greve and Wassarman 1985). The zona is composed of three major acidic sulfated glycoproteins (ZP1, Mr 200,000; ZP2, Mr 120,000; ZP3 Mr 83,000) and reaches a thickness of about 7 µm. It has been shown that ZP3 functions as a sperm receptor and initiates the acrosome reaction, which must occur if a sperm is to fertilize the egg. Molecular analysis has shown that the mouse ZP3 gene encodes a polypeptide of 402 amino acids, which is extensively glycosylated with both N-linked and O-linked complex oligosaccharides (for review, see Wassarman 1990). Sperm binding is localized not to the polypeptide backbone of ZP3 but to oligosaccharides associated with a glycopeptide fragment from the carboxy-terminal region of the intact protein (Rosiere and Wassarman 1992).

Apart from studies on the synthesis and processing of the zona glycoproteins, which together constitute about 10% of the total protein synthesis, relatively little is known about the gene activity of growing oocytes. Several groups have carried out two-dimensional gel electrophoretic analysis of total [35S]methionine-labeled proteins synthesized by maturing oocytes and unfertilized eggs (Van Blerkom 1981; Howlett and Bolton 1985). In addition, the synthesis of a number of specific proteins has been reported (for review, see Schultz 1986). For example, about 1.3% of the total protein synthesis of oocytes is devoted to tubulin (Schultz et al. 1979) and about 0.9% is devoted to actin in the mid growth phase (Bachvarova et al. 1989). Transcripts for a variety of genes have been localized in mouse oocytes by in situ hybridization or reverse transcriptase polymerase chain reaction (RT-PCR) (e.g., genes encoding the TGF-β-related proteins, DVR-6 [also known as Vgr-1 and BMP-6] and activin, and the transcription factor, Oct-4), but the biological significance of these RNAs, and whether they are translated into functional protein, is not always clear (Lyons et al. 1989; Scholer et al. 1990a,b; Albano et al. 1993). In some cases, it is known that RNAs accumulated in the growing oocyte are not polyadenylated or translated until meiotic maturation (e.g., transcripts for c-mos and tissue plasminogen activator; see below). In other cases, strong evidence exists that genes expressed in oocytes have a role in their growth and development. For example, it has been shown that growing oocytes (as well as PGCs) express the transmembrane tyrosine kinase receptor, c-kit, the product of the W locus. In contrast, somatic cells of the ovary, including the follicle cells, express Steel factor (SF), the product of the Steel (Sl) locus and the ligand for c-kit. Mutations in the Sl gene which decrease the expression of SF in the ovary, for example, Steel panda (SlPAN), inhibit the growth of oocytes and result in a reduced number of follicles and their arrest at the one-layered cuboidal stage (Manova et al. 1990, 1993; Manova and Bachvarova 1991; Huang et al. 1993).

Surprisingly, more than half of the primordial follicles present in the mouse ovary at birth degenerate before 3–5 weeks of age, but little is known about the hormonal and local factors controlling this loss (Faddy et al. 1983). The female mouse reaches sexual maturity at approximately 6 weeks of age, depending on the strain and environmental conditions. By this time, each ovary contains approximately 10⁴ oocytes at different stages of maturity. Techniques have been established for isolating and culturing both immature oocytes from preantral follicles and mature oocytes from antral follicles (see Section C; Eppig and Telfer 1993; O’Brien et al. 1993). Maturation of oocytes from antral follicles occurs spontaneous-
ly under these conditions; they can be fertilized in vitro and develop normally thereafter. Oocytes from preantral follicles, on the other hand, must be cultured for several days with their surrounding follicle cells before they can be fertilized in vitro.

**Ovulation**

As the oocyte increases in size, it gradually acquires the competence to enter the final stages of meiosis in response to either the correct hormonal stimulus (in vivo) or release from the follicle (in vitro). Ovulation requires the coordinated response of both the follicle cells and the oocyte, and under optimal conditions, it occurs spontaneously once every 4 days. However, cycle length can be influenced by many environmental factors and can be induced artificially by hormone injection (see Section C, Inducing Superovulation). In any one natural cycle, only a few follicles respond to an increase in the level of follicle-stimulating hormone (FSH), which is produced by the pituitary. The stimulated follicle cells break contact with the oocyte and increase their synthesis and secretion of high-molecular-weight proteoglycans and tissue plasminogen activator. At the same time, the follicle accumulates fluid, swells, and moves toward the periphery of the ovary, ready for the final maturation and release of the oocyte. The mature, fluid-filled follicle units are known as antral or Graafian follicles, after the scientist Regnier de Graaf who first described them in 1672. For an extensive review of the biosynthetic activity of follicle cells in vivo and in culture, see Hsueh et al. (1984) and Richards et al. (1987).

Ovulation occurs in response to a surge in the level of luteinizing hormone (LH), also produced by the pituitary. After LH stimulation, the oocyte undergoes nuclear maturation (Fig. 9). The nucleus (which is also known as the germinal vesicle) loses its membrane (a process known as germinal vesicle breakdown), and the chromosomes assemble on the spindle and move toward the periphery of the cell where the first meiotic division takes place. One set of homologous chromosomes, surrounded by a small amount of cytoplasm, is extruded as the first polar body, whereas the other set remains in metaphase II. It is in this state of arrest after the first meiotic division that the oocyte is finally released from the follicle, and meiosis does not resume after fertilization.

Studies in a number of laboratories have implicated the mouse proto-oncogene, c-mos, encoding a cytoplasmic serine/threonine protein kinase, in the process of meiosis. Transcripts for c-mos accumulate in the growing oocyte but are not polyadenylated or translated until resumption of meiosis and are then degraded by the two-cell stage. Injection of antisense oligonucleotides to c-mos into an oocyte that has initiated meiosis results in failure to proceed beyond metaphase II. It has been suggested that the absence of c-Mos protein results in a destabilization of a protein required to maintain meiosis (Mutter et al. 1988; O'Keefe et al. 1989, 1991; Paules et al. 1989). However, in mice lacking functionally active c-mos, oocytes undergo parthenogenetic activation, indicating that c-mos is required for metaphase arrest at meiosis II (Colledge et al. 1994; Hashimoto et al. 1994).

Meiotic maturation of the oocytes also triggers the synthesis and secretion of the protease, tissue-type plasminogen activator (tPA). The protein is synthesized on mRNA already present in the oocyte but is not translated until meiotic maturation. Activation of tPA mRNA involves addition of a poly(A) tail, and this is controlled by an AU-rich cytoplasmic polyadenylation element (CPE) in the 3'-noncoding region.
Figure 9 Ovulation and fertilization.

This CPE has been identified in several maternal mRNAs, in addition to that for tPA (Huybrechts et al. 1988a,b; Strickland et al. 1988; Salés et al. 1992).

Each ovulated oocyte is surrounded by its zona and a mass of follicle cells (cumulus cells) with their associated proteoglycan. The eggs are swept into the open end, or infundibulum, of the oviduct by the action of the numerous cilia on the surface of the oviduct epithelium. Other cells in the epithelium are secretory,
and at the time of ovulation, the section of the tube adjacent to the infundibulum becomes engorged and enlarged to form an ampulla where fertilization takes place. In a natural ovulation, 8–12 eggs are released (depending on the mouse strain), but the process is not synchronous and occurs over a period of 2–3 hours. After ovulation, the follicle cells remaining in the ovary differentiate into steroid-secreting cells (luteinized granulosa cells), which help to maintain pregnancy. Counting the number of bright yellow corpora lutea near the surface of the ovary is a way of determining how many eggs were, in fact, released.

**Fertilization**

Approximately 58 x 10^6 sperm are released into the female reproductive tract per ejaculation. Some sperm reach the ampulla within 5 minutes, but they are not competent for fertilization for about 1 hour. This process of maturation is known as capacitation, but its mechanism is unknown. To reach the surface of the egg, the sperm must penetrate first the cumulus mass and then the zona pellucida. As described earlier, the glycoprotein ZP3 has been identified as the sperm-binding protein in the zona. In many mammals, this binding is highly (but not absolutely) species-specific, and this prevents penetration by sperm from other species. ZP3 also triggers the acrosomal reaction, a process in which the acrosome (a secretory vacuole-like structure in the sperm head) fuses with the plasma membrane of the sperm head, releasing various hydrolytic enzymes. Unless the acrosomal reaction takes place, the sperm cannot fertilize the egg.

Fusion of the posterior part of the sperm head with the egg membrane triggers the cascade of reactions known as fertilization. One very early event is a change in the egg surface, inhibiting the fusion of additional sperm. Another event is the Ca^{++}-dependent release (exocytosis) of the cortical granules positioned beneath the plasma membrane. This event initiates the "zona reaction," which involves both cross-linking of the glycoproteins of the zona and modification of the ZP3 glycoprotein so that it no longer binds sperm or elicits the acrosomal reaction. These events also help to prevent polyspermy. During fertilization, the head, mid-

| **Table 2** Some Properties of the Ovulated, Unfertilized Oocyte |
|---------------------|-------------------|
| **Diameter**        | 85 μm             |
| **Volume**          | 270 pl (volume of pronucleus 1 pl) |
| **Protein**         | 23 ng             |
| **Total DNA**       | 8 pg              |
| **Mitochondrial DNA** | 2–3 pg (note that much of the DNA of the unfertilized egg is mitochondrial) |
| **Number of mitochondria** | 10^5                   |
| **Genomic DNA (haploid number of chromosomes but diploid (2C) amount of DNA)** | 6 pg |
| **Ribosomal RNA**   | 0.2–0.4 ng        |
| **Poly(A)**         | 0.7 pg (120–200 nucleotides long) |
| **Poly(A)^+ RNA**   | exact amount not determined |
| **Transfer RNA**    | 0.14 ng           |
Table 3  Timing of Events during the First Cell Cycle of Embryos Derived from (C57BL ♀ × CBA ♂) F₁ ♀ × CFLP (Outbred) ♂ following In Vitro Fertilization

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<tr>
<td>Formation of ♂ pronucleus</td>
<td>4–7</td>
</tr>
<tr>
<td>Formation of ♀ pronucleus</td>
<td>6–9</td>
</tr>
<tr>
<td>DNA replication</td>
<td>11–18</td>
</tr>
<tr>
<td>Cleavage</td>
<td>17–20</td>
</tr>
</tbody>
</table>

Information supplied by S. Howlett, Department of Anatomy, University of Cambridge, U.K.

For morphology, see Fig. 4.

piece, and a large part of the tail of the sperm are all incorporated into the egg cytoplasm. The midpiece of the sperm contributes paternal centrioles and mitochondria to the zygote, but the latter are enormously diluted out by the mitochondria of the oocyte.

Fertilization triggers the second meiotic division and extrusion of the second polar body. Nuclear membranes, including nuclear lamin proteins, then form around the maternal and paternal chromosomes, forming separate haploid male and female pronuclei that move toward the center of the egg. DNA replication takes place during this migration. The pronuclei do not fuse, but the membranes break down and the chromosomes assemble on the spindle; the first cleavage occurs soon after. Because of asynchrony in ovulation and fertilization, the first cleavage occurs over a number of hours in a population of naturally fertilized zygotes. More synchronous development can be obtained by in vitro fertilization (see Section C). Unfertilized eggs remain viable for about 12 hours and sperm for about 6 hours.

Some properties of the ovulated unfertilized mouse oocyte are given in Table 2, and the sequence of events from onset of nuclear maturation to blastocyst formation is illustrated in Table 3 and Figures 9, 10, and 11.

Cytoskeletal Organization of the Egg before and after Fertilization

Throughout the cytoplasm of the oocyte, there is a complex matrix of cytoskeletal elements, including actin, tubulin, and certain cytokeratins (Fig. 12) (Lehtonen et al. 1983a; Maro et al. 1984; Schatten et al. 1985). The different systems presumably help to coordinate events at the cell surface with changes in the pronuclei as they migrate toward the center of the egg. This migration is inhibited by both cytochalasin B (which inhibits actin polymerization) and colcemid (which inhibits tubulin polymerization), and both inhibitors are required together in nuclear transfer experiments to enable the nuclei to be withdrawn into a karyoplast (see Section D, Nuclear Transplantation in the Mouse Embryo). The earliest developmental changes in actin organization in the egg are seen at fertilization (Maro et al. 1984). In the ovulated oocyte, the plasma membrane above the meiotic spindle is devoid of concanavalin A (Con A)-binding sites and microvilli and is underlaid by an actin-rich subcortical layer. Fertilization results in the formation of a second Con-A-free zone, the fertilization cone, which is around the site of sperm entry. The plasma membrane in this region is also underlaid by an actin-rich layer. As the pronuclei move toward the center of the egg, the distribution of actin filaments becomes more uniform and the Con-A-free regions disappear.
Figure 10  Morphology of preimplantation mouse development. (1) Preovulatory oocyte with germinal vesicle intact. (2) Preovulatory oocyte showing breakdown of germinal vesicle (GVBD) (2.5–4.5 hr post-hCG). (3 and 4) Extrusion of first polar body (~10 hr post-hCG) followed by ovulation (~11–13.5 hr post-hCG) and fertilization. (5) Resumption of meiosis by female set of chromosomes and extrusion of second polar body (occurs over the period 17–33 hr post-hCG). (5 and 6) Decondensation of sperm nucleus and formation of male pronucleus. (7) Formation of nuclear membrane around haploid set of female chromosomes to form female pronucleus which is subcortical, near the second polar body, and smaller than the male pronucleus (process complete in the majority [75%] of the embryos by ~26 hr post-hCG). (8) Migration of pronuclei to center of egg. (9 and 10) Formation of visible nucleoli within both pronuclei. DNA replication (complete in the majority of embryos by ~28 hr post-hCG). (11 and 12) Breakdown of pronuclear membranes and disappearance of visible nucleoli. Ruffling of embryo surface indicating reorganization of the cytoskeleton preparatory to cleavage (observed from ~27 hr post-hCG until cleavage is completed). (13) Elongation of embryo. (14 and 15) Formation of "waist." (16) Newly formed two-cell embryos with visible nucleoli (the majority of embryos have cleaved by ~32 hr post-hCG). (17) Later-stage two-cell embryo with nuclei visible. (18–25) Later stages of preimplantation development. (18) Four-cell embryo. (19–20) Six- to eight-cell embryo. (21) Compacting eight-cell embryo. (22) Compacted 8- to 16-cell embryo. (23 and 24) Early blastocysts. (25) Fully expanded blastocyst. (All timings are given in hours after injection of hCG. Information and figure provided by Dr. H. Pratt, Department of Anatomy, University of Cambridge.)
Parthenogenesis

Parthenogenetic activation of unfertilized eggs can be elicited by exposing them to a variety of agents, including alcohol (see Section C), hyaluronidase, the Ca++ ionophore A23187, Ca++/Mg++-free medium, heat/cold shock, and anesthetics. In addition, about 10% of the oocytes of the LT/Sv strain of mice undergo spontaneous activation with high frequency, either in the oviduct or in the ovary. Those LT parthenogenetic embryos that implant develop to the egg cylinder stage (7 days p.c.) and then become disorganized and die, whereas those remaining in the ovary give rise to teratomas. A similar phenomenon of high-frequency parthenogenetic activation of oocytes resulting in the formation of ovarian teratomas has been reported in female mice deficient in c-mos (Colledge et al. 1994; Hashimoto et al. 1994).

The genotype of the parthenogenetic embryo (also known as a parthenogenone or parthenogenote) can vary depending on the experimental conditions and, in particular, on the postovulatory age of the activated oocyte. The most important factor may be the state and orientation of the cytoskeletal elements in the egg when activation takes place. The following are among the possible genotypes
Figure 12 Distribution of tubulin in early mouse embryos. (A) Schematic representation of tubulin distribution from unfertilized egg through to eight-cell stage. (B) Unfertilized egg showing cytoplasmic asters. (C) Pronucleus-stage fertilized egg. (Photograph provided by Dr. Gerald Schatten, Department of Biological Science, The Florida State University.)

arising from the parthenogenetic activation of an oocyte from an F₁ (heterozygous) female (Fig. 13):

1. Uniform haploid (second polar body successfully extruded).
2. Mosaic haploid (second polar body behaves as a normal blastomere).
3. Heterozygous diploid (results from suppression of second polar body formation or from fusion of the pronucleus and the second polar body). The heterozygosity in these parthenogenones is the result of recombination during meiosis.
4. Homozygous diploid (results from diploidization of the haploid female genome).

Note also that up to 20% of eggs activated by alcohol may be aneuploid as a result of nondisjunction (Kaufman 1982, 1983b). Most parthenogenones, and particularly the uniform haploids, die before the blastocyst stage. A minority continue to develop past implantation, for example, up to the egg cylinder stage (LT/Sv and mosaic haploid parthenogenones) and the early limb bud stage (heterozygous diploids; Kaufman et al. 1977). However, normal development to term has not been obtained from any class. The reason for this lies in the inactivation of specific genes by imprinting during oogenesis (see Imprinting, p. 95). ES cells can be derived from diploid parthenogenetic blastocysts, and these will differentiate into a wide range of cell types in culture and contribute to the formation of many tissues in chimeras (see Section F).
Summary of Mouse Development

**Figure 13** Possible products of parthenogenetic activation of mouse oocytes. Embryos of different genotype are produced depending on whether or not a second polar body is extruded and on the timing and nature of the first cell division.

**Early Cleavage: One-cell Embryo to Eight-cell Uncompacted Morula**

Despite the small size of cleavage-stage mouse embryos, a considerable amount of information is now available about changes in the pattern of RNA and protein synthesis during preimplantation development (for reviews, see Schultz 1986; Kidder 1992). In summary, up to the mid two-cell stage (27 hours postfertilization), the embryo appears to rely largely on protein and RNA synthesized during oogenesis. By the mid two-cell stage, many embryonic genes are switched on. Coincidentally, much of the maternally inherited mRNA appears to be degraded rapidly, but maternally coded proteins can persist beyond this time. Three principal methods have been used to study protein synthesis and gene expression in the preimplantation embryo: (1) two-dimensional SDS-polyacrylamide gel electrophoresis of polypeptides produced in vivo, or in vitro from embryonic RNA added to the reho-
ulocYTE lysate protein synthesis system, (2) amplification of specific embryonic RNAs using techniques based on RT-PCR, and (3) construction and screening of cDNA libraries.

Two-dimensional gel electrophoresis has revealed a number of changes in the pattern of $^{35}$S-methionine-labeled proteins synthesized by early-cleavage embryos (see, e.g., Latham et al. 1991). It has been difficult to time these changes precisely, particularly in relation to other cellular events (e.g., DNA synthesis and cell division) because of asynchrony both within a population of normally fertilized embryos and between blastomeres of individual embryos. This asynchrony can be reduced by in vitro fertilization, by picking out embryos after cleavage to two cells, and by dissociating and recombining groups of blastomeres at specific stages of the cell cycle before metabolic labeling with radioactive precursors. Several processes may be responsible for the following changes in the pattern of protein synthesis seen after fertilization.

1. Increased turnover rates of some proteins made on stable maternal mRNAs. Evidence for such mechanisms has been found in mammalian eggs (Howlett and Bolton 1985).

2. Posttranslational modification of proteins synthesized on either maternal or embryonic RNA. Some evidence exists for modification of proteins by phosphorylation, glycosylation, or proteolytic cleavage (Van Blerkom 1981; Cascio and Wassarman 1982; Pratt et al. 1983).

3. Selective use of subspecies of maternal mRNAs. There is now clear evidence from the study of carefully timed embryos and from comparison of in vitro and in vivo translation products that some mRNA species are utilized or suppressed selectively.

4. Specific degradation of maternal mRNAs carried over from the oocyte. At the two-cell stage, there is a sharp fall in the level of total and poly(A)$^+$ RNAs (Clegg and Piko 1983), in the translation of globin mRNA injected into the zygote (Brinster et al. 1980), and in the translation of proteins thought to be encoded by maternal RNA (for review, see Johnson 1981; Pratt et al. 1983). In a particularly clear series of experiments using Northern hybridization to embryonic RNA, Giebelhaus et al. (1983) showed a marked loss at the two-cell stage of the pools of actin and histone H3 mRNAs present in the egg. Subsequently, the level of these mRNAs in the embryo began to rise after the four-cell stage, when transcription from the embryonic genome is under way.

5. Synthesis of proteins on mRNAs transcribed de novo from the embryonic genome. Experiments in which eggs are incubated in the presence of the RNA polymerase inhibitor α-amanitin have shown that new RNA synthesis is required for development beyond the two-cell stage and for the synthesis of many new proteins (Flach et al. 1982). Transcription of both ribosomal and poly(A)$^+$ RNAs, which is apparently down-regulated at the time of germinal vesicle breakdown, is thought to resume at a low level around the mid two-cell stage. Timing of the onset of synthesis of paternally coded proteins has come from studies on the expression of genetic enzyme variants or antigens, and the synthesis of a few specific proteins has been followed by metabolic labeling and immunoprecipitation.
Analysis of Embryonic Gene Expression Using RT-PCR Techniques and cDNA Libraries

The RT-PCR technique has opened up new horizons for the analysis of gene expression in preimplantation mouse embryos. Total RNA isolated from small numbers of eggs or cleavage-stage embryos, or from single blastocysts, is first used as a template for reverse transcription, primed by oligo(dT). Specific sequences in the transcribed cDNA are then amplified by PCR. One of the first applications of this technique to preimplantation mouse embryos was the identification of RNAs for a number of growth factors (Rappolee et al. 1988). Maternal transcripts for platelet-derived growth factor A (PDGF-A) and transforming growth factor-α (TGF-α) were shown to be present in unfertilized, ovulated eggs and to decline during the two-cell stage, only to reappear as zygotic transcripts in late cleavage–early blastocyst-stage embryos. TGF-β1 transcripts were not present in the egg but increased in amount during cleavage. Other studies have used RT-PCR to identify transcripts for insulin-like growth factor II (IGFII), IGFII receptor, IGFI receptor, insulin receptor, and epidermal growth factor (EGF) receptor during preimplantation development (Kidder 1992; Rappolee et al. 1992; Wiley et al. 1992). In many cases, specific antiserum have revealed the presence of protein corresponding to the mRNAs found by RT-PCR or by in situ hybridization (Rappolee et al. 1988; Palmieri et al. 1992; Pari et al. 1992; Wiley et al. 1992; Albano et al. 1993). These findings have raised the possibility that growth factors produced by the preimplantation embryo may act in an autocrine loop to promote cell proliferation and/or survival. Although fertilized eggs can develop into blastocysts in defined medium lacking serum or growth factors, it is now known that certain purified factors added to the medium (e.g., IGFII, TGF-α, and insulin) can significantly enhance the growth rate and final cell number of preimplantation embryos (Harvey and Kaye 1990, 1992; Rappolee et al. 1992).

Another approach to the identification of genes expressed in preimplantation mouse embryos has been the construction and screening of large and representative cDNA libraries from different stages (Weng et al. 1989; Rothstein et al. 1992). Libraries have been screened with probes for known mouse genes or evolutionarily conserved genes from other species, or used for subtractive hybridization to yield cDNAs for genes differentially expressed at two different stages.

Restriction in the Developmental Potential of Embryonic Nuclei

Nuclear transplantation experiments in Xenopus have shown that blastula-stage nuclei can support the development of an enucleated egg to the tadpole stage. In contrast, nuclear transplantation experiments in the mouse by McGrath and Solter (1984a,b,c) present a different picture. These authors found that even nuclei transplanted from cleavage-stage embryos into enucleated eggs are unable to support development beyond the blastocyst stage. To account for these results, they argue for a close synchrony between nuclear events, cell division, and the redistribution of cytoplasmic molecules, which is disrupted by transplantation between stages. It is as though fertilization sets in motion clocks in both the nucleus and cytoplasm, and these clocks must be synchronized for correct development; the greater the time difference (e.g., eight-cell nucleus into one-cell cytoplasm), the poorer the development observed.
Compaction and the Formation of the Blastocyst: The First Differentiation Events

Details of the timing of early events in preimplantation mouse development and the experimental evidence for changes in cell potency and fate have been reviewed by Pederson (1986). Up to the early eight-cell stage, there is good evidence that the blastomeres of the mouse embryo are equipotent. Single blastomeres from two-cell and four-cell morulae can each give rise to a mouse. Early eight-cell-stage blastomeres cannot generate a mouse by themselves, but when recombined with genetically marked morulae, they can give rise to a wide range of different tissues in chimeric offspring (Kelly 1977). As cleavage proceeds to the 16-cell stage, however, there is a gradual restriction in the developmental potency of the cells, eventually resulting in the generation of two distinct lineages: the trophoderm (TE) and the inner cell mass (ICM). This differentiation process starts with compaction, when the blastomeres flatten and increase their contact with each other and develop distinct apical and basal membrane and cytoplasmic domains (polarization). Cells that end up on the inside of the compacted embryo give rise to the ICM, whereas the outer cells give rise to the trophoderm. However, even at late morula stages, normal blastocysts can be formed from exclusively inside or exclusively outside cells. Eventually, though, the process becomes irreversible, with the formation of a fully expanded blastocyst consisting of a hollow vesicle of trophoderm surrounding a fluid-filled cavity (the blastocoele) and a small group of ICM cells. The trophoderm has all the features of a true epithelium, with apical junctional complexes forming a complete permeability seal against the outside environment. The trophoderm overlying the ICM is known as the polar TE, and the cells surrounding the blastocoele cavity constitute the mural TE.

Some of the cellular changes associated with compaction are listed in Table 4. It should be stressed that they do not occur synchronously within all the cells of one embryo. Likewise, the cell cycles are not synchronized (Graham and Deussen 1978). The changes associated with compaction clearly point to alterations in both the surface properties of the cells and the organization of the cytoskeleton. The molecular basis of these changes, the signal(s) eliciting them, and their relationship to each other and to the cell cycle are areas of active research.

Changes in Cell Adhesiveness with Compaction

From the point of view of cell adhesiveness, there is good evidence that compacting embryos do not synthesize the extracellular matrix protein fibronectin or collagens (I through IV) (Wartiovaara et al. 1979; Leivo et al. 1980). Laminin is synthesized by morulae (Table 4) (Cooper and MacQueen 1983) and can be localized histochemically between the cells of the compacted morula (Fig. 14) (Leivo et al. 1980), but there is no evidence that it has a role in cell-cell adhesion. In contrast, compaction is completely inhibited by polyvalent rabbit antibodies (either whole serum or IgG Fab fragments) against a surface glycoprotein originally identified as uvomorulin (Hyafil et al. 1980, 1981; Peyrieras et al. 1983). It is now known that uvomorulin is a member of a diverse family of Ca\(^{++}\)-dependent transmembrane cell adhesion molecules known as cadherins and is identical to E-cadherin (for reviews, see Takeichi 1991; Kemler 1993). Synthesis of uvomorulin/E-cadherin is therefore not unique to compacting morulae, but antibody-blocking experiments point to its fun-
Table 4  Changes Occurring in Blastomeres during Compaction

1. Increased Ca\(^{++}\)-dependent adhesiveness, both to each other and to lectin-coated beads.

2. Increased spreading on adhesive surfaces, using lamellipodia-like cell processes.

3. Ability to express contact-induced cell polarization as shown by regionalization of membrane and cytoplasmic domains (microvilli, lectin-binding sites, and intracellular organelles).

4. Establishment of gap-junction-mediated intercellular communication (ionic coupling and dye transfer) between all cells of the morula unit.

5. Gradual development of apical, zonular tight junctions between outside cells, generating an impermeable outer epithelial layer.

Agents that inhibit features of compaction include cytochalasin B, tunicamycin, low Ca\(^{++}\), rabbit anti-F9 embryonal carcinoma serum or Fab fragments, and some monoclonal antibodies against uvomorulin all tend to prevent or reverse the cell-spreading effects but not polarization.

Dramatic importance at this stage of development. Uvomorulin/E-cadherin RNA and protein synthesis is initiated in the morula around the four-cell stage prior to compaction, and a key event appears to be its trafficking to the basolateral domains of the cell surface, a process that may be associated with posttranslational modifications of the protein (for review, see Kidder 1992). E-cadherin is a transmembrane protein and its cytoplasmic region is specifically complexed with a number of molecules, including \(\beta\)-catenin, \(\gamma\)-catenin or plakoglobin, and \(\alpha\)-catenin. These may mediate both interaction of E-cadherin with the cytoskeletal system and with other proteins of adherens junctions and transduce signals generated by cell-cell interactions from the cell surface to the nucleus (Ozawa et al. 1990; for reviews, see Kemler 1993; Peifer 1993).

Cell Polarization with Compaction

One of the essential features of compaction is the polarization of the blastomeres, so that they show distinct apical and basolateral membrane domains reminiscent
of many epithelial tissues. These domains are clearly seen by scanning electron microscopy of compacted embryos that have been dissociated by incubation in the absence of calcium (Reeve and Ziomek 1981); the outer poles of the cells have numerous microvilli, whereas the inner surfaces are smooth. Transmembrane receptors such as the EGF receptor may become preferentially localized to one domain, for example, the basolateral surface of the TE cells (Dardik et al. 1992). Conversely, the Na⁺ glucose transport protein has been localized to the apical domain of polarized blastomeres (Wiley et al. 1991). Cytoplasm organelles also appear to be polarized after compaction, with nuclei taking up a basal position. The onset of polarization can be followed in vitro by incubating pairs of isolated precompaction blastomeres. During culture, the microvillous surfaces and Con-A-
binding sites always develop at the poles opposite the points of cell-cell contact (Ziomek and Johnson 1980; Johnson and Ziomek 1981). An important question under investigation is whether this redistribution of plasma membrane domains precedes, or results from, a reorganization of cytoskeletal elements. The rules that emerge about polarization of mouse embryos at the morula stage may apply more generally to the differentiation of epithelial tissues from nonpolarized precursor cells at later stages of development.

**Segregation of the Trophoderm and Inner Cell Mass Cell Lineages**

As outlined above and in Table 4, compaction is associated with cellular polarization. This property forms the basis of a polarization hypothesis to account for the differentiation of the two distinct cell lineages of the blastocyst, the trophoderm and the ICM. Cleavage planes through compacted morula cells horizontal to the polarized axis will generate basal or inside cells, and apical or outside cells, each inheriting different membrane and cytoplasmic molecules (e.g., plasma membrane glycoprotein receptors and cytoskeletal organizing centers). These inherited molecules are thought to be responsible for initiating differences in the developmental potentials of the inner and outer cells. According to this hypothesis, differentiation is the result of cellular polarization elicited early in compaction. According to an alternative inside/outside microenvironment hypothesis, differentiation does not occur until after a network of tight junctions between the outer cells has formed. This generates distinct inside and outside microenvironments to which the cells respond, inside cells by becoming ICM and outside cells by becoming trophoderm. For a full description of compaction and a discussion of the polarization and microenvironment theories, see Johnson and Ziomek (1981), Pratt et al. (1981), Gardner (1983), Johnson et al. (1986), and Pratt (1989).

**Implantation**

During the fifth day of development, the blastocyst hatches from the zona and is ready for implantation. Hatching may be effected by a trypsin-like enzyme that digests the glycoprotein matrix of the zona pellucida and is synthesized by cells in the mural trophoblast (Wassarman et al. 1984), but uterine enzymes probably have a major role in vivo. Escape from the zona may also be facilitated by rhythmic expansion and contraction of the blastocyst. Hatching is independent of the uterine environment and will occur normally in vitro. At the time of implantation, the walls of the uterus become tightly apposed so that the uterine lumen is more or less occluded, and changes in the surface of the uterine epithelium make it a conducive surface for blastocyst attachment. The mouse blastocyst first adheres by its abembryonic pole (the mural trophoderm furthest from the ICM) to the anti-mesometrial uterine wall (Fig. 15). No preformed attachment sites exist within the uterus, and the more or less even spacing of implantation sites is thought to result from the peristaltic movements of the uterus. Blastocyst attachment induces the formation of a uterine crypt (Fig. 15) and also stimulates the uterine stroma to form a spongy mass of cells known as decidual tissue. The process is known as the decidual reaction, and the mass of decidual cells around a single embryo can be referred to as the "deciduum," meaning "the thing that falls off." Alternatively, many
Figure 15 (See facing page for legend.)
texts refer to "the decidua," although this is, strictly speaking, the plural of deciduum. The decidual reaction only occurs in a uterus appropriately primed by progesterone and estrogen, but it will occur in response to stimuli other than the embryo, such as mechanical trauma or oil droplets. Thus, the decidual reaction is dependent on high levels of estrogen during estrous, followed by a few days when progesterone predominates, and finally a small surge in estrogen on the fourth day of gestation. This final surge of estrogen coincides with a surge in uterine expression of leukemia-inhibiting factor (DIA-LIF) (Bhatt et al. 1992; Smith et al. 1992), and mutant female mice with nonfunctional DIA-LIF cannot support implantation (Stewart et al. 1992). Therefore, the production of this cytokine by the uterine endometrial glands seems to be an essential ingredient in the maternal initiation of implantation.

The decidual reaction involves a rapid increase in the permeability of local capillaries causing the uterine stroma to become swollen and edematous. The stromal cells in the decidual tissue proliferate, increase in size, and establish numerous tight junctional complexes with their neighbors (for review, see Finn 1971). In due course, the epithelium separating the blastocyst from the stroma is eroded, and again this is not embryo-dependent because it also occurs in artificially induced deciduoma. However, degeneration of the epithelium allows the trophoblast cells, which phagocytose the moribund epithelial cells, to invade the deciduum. The invasive nature of trophoblast cells may be associated with their synthesis of the protease urokinase-type plasminogen activator, as well as various metalloproteinases and their inhibitors (Strickland and Richards 1992). It is unknown what limits trophoblast invasion to the uterus, but it is thought that the deciduum must have a role in restricting the distribution of these cells, whose behavior otherwise much resembles that of metastatic tumor cells. Blastocysts in vitro will simulate implantation, in that they will attach to plastic or extracellular matrix substrata and spread out over the dish (Enders et al. 1981).

If estrogen is absent on the fourth day of gestation, which is the case in lactating females or in females that have been ovariectomized after fertilization (see Section F), the blastocysts will not implant but instead enter a quiescent phase known as "delay" (diapause) (Mantelenakis and Ketchel 1966; Yoshinaga and Adams 1966). This state can be maintained for up to 10 days, but it can be reversed at any time by the removal of suckling young or the administration of estrogen. Cell proliferation and DNA synthesis cease within a few days of the onset of delay (McLaren 1968), but the differentiation of primitive endoderm occurs at the same time that it would in normal blastocysts (Gardner et al. 1988). The mechanism by which small quantities of estrogen influence the progression of development is not known, but it is possible that the production and availability of DIA-LIF might mediate the effect, since

**Figure 15** Schematic representation of implantation and the orientation of the embryo with respect to the uterus in vivo. (A) The blastocyst implants such that the ICM is located toward the mesometrial aspect of the uterus. The longest side of the asymmetrical blastocyst attaches either to the right or left wall of the uterus. (B) This asymmetry is evident as a slightly tilted ectoplacental cone at 5.5 days p.c. (C) The primitive streak (defining the posterior aspect of the embryo) can form at either pole of the plane of tilt, such that the posterior axis of the embryo coincides with either the right wall or the left wall of the uterus.
this cytokine supports the continued proliferation of ES cells and prevents their differentiation in vitro (Smith et al. 1992).

The orientation of implantation has been studied to investigate whether there is any relationship between asymmetries in implantation and the future definitive axes of the embryo (Smith 1985; N.A. Brown et al. 1992). Histological sections of peri-implantation embryos have shown that the three primary axes of the embryo (anteroposterior, dorsoventral, right-left) correlate with the three axes of the uterine horn (oviduct-cervix [long] axis, mesometrial–antisomesometrial axis, and right-left axis; Fig. 15). The anteroposterior axis of the egg cylinder is perpendicular to the long axis of the uterus, and the primitive streak lies toward either the right or left side of the uterine horn. The dorsal side of the embryo at the egg cylinder stage lies toward the mesometrium, but once the embryo has turned (see Turning, p. 74), it is the right-left axis that is parallel to the mesometrial–antisomesometrial axis of the uterus, the right side of the embryo facing the placenta. The orientation of C-shaped 9.5-day p.c. embryos in the uterus is always that either the head is toward the oviduct and the dorsal surface is toward the left wall or the head is toward the cervix and the dorsal surface is toward the right wall. The dorsoventral relationship derives from the asymmetric implantation of embryos in mice, whereby the abembryonic pole of the blastocyst (that furthest away from the ICM) always attaches to the antisomesometrial wall of the uterus. The anteroposterior relationship has been ascribed to early asymmetry in the blastocyst, which is preserved during implantation such that the longest side of the blastocyst comes to abut either the left wall or right wall of the uterus (Fig. 15), with no intermediate positions observed. However, it has yet to be proved experimentally whether this asymmetry in the blastocyst has any causal role in determining the anteroposterior axis. Recent examination of the correlation between asymmetry in the ectoplacental cone, visualized as a distinct tilt presumed to derive from the original asymmetry of the blastocyst, and the location of the primitive streak indicates that the anteroposterior axis of the embryo is not random with respect to this ectoplacental cone asymmetry but that its polarity is, in that the primitive streak may form either on the same side as the tilt or on the opposite side (Gardner et al. 1992).

Trophoblast and Its Derivatives

As described above, an essential feature of the differentiation of the trophoderm of the early blastocyst is the organization of the cells into a typical epithelium. The cells have apical junctional complexes and distinct apical and basal membrane domains. The junctional complexes involve extensive desmosomes (Fig. 16), and associated with these are large numbers of intermediate filament bundles (Jackson et al. 1980).

During postimplantation development, the trophoderm does not remain as a simple epithelium but becomes regionally specialized with respect to morphology and growth potential (for review, see Gardner 1983). One subpopulation, the mural trophoderm, is derived from the cells that surround the blastocoel cavity but are not in contact with the ICM. These cells cease division and become large and can contain up to 1000 times the haploid amount of DNA due to chromosomes becoming polytenic (Varmuza et al. 1988). These are the so-called primary trophoblastic giant cells. In contrast, the trophoderm cells in close
Figure 16  Mouse blastocyst at ~4.5 days of development. (A) Section showing outer epithelial vesicle of trophoderm (TE) surrounding the blastocoele cavity and the epiblast and primitive endoderm (PrEnd). (B) Junction between two trophoderm cells showing desmosomal junction (DJ) and interdigititation of the plasma membranes. (C) Trophodermal cells showing abundant cytoplasmic glycogen, desmosomal junction, and thin basal lamina (BL) on the internal surface.
proximity to the ICM and its derivatives remain diploid and continue to proliferate rapidly.

After implantation, this population of so-called polar trophectoderm spreads in several directions. First, some cells migrate around the embryo, replacing the primary mural trophoblastic giant cells and themselves becoming polyploid. Second, a finger-like projection of polar trophectoderm penetrates down into the blastocoel cavity, forming the extraembryonic ectoderm of the egg cylinder and pushing the ICM derivatives ahead of itself. This projection develops a central cavity and becomes epithelial. After the formation of the extraembryonic mesoderm, the extraembryonic ectoderm retracts toward the placenta where it forms the chorion. Finally, some trophectoderm cells continue to penetrate into the endometrium forming the bulk of the placenta. Some of these cells, and cells of the chorion, also become polyploid (secondary giant cells).

Proliferation of the early trophectoderm appears to be controlled by its proximity to ICM derivatives; in the absence of ICM derivatives, TE cells do not proliferate but instead become giant, which has obvious advantages in preventing continued growth of trophectoderm if the embryo dies in utero.

Studies have been initiated to characterize genes specifically expressed in trophoblast cells. For example, differential screening of a cDNA library made from 13.5-day p.c. mouse placenta yielded a TE-specific cDNA encoding a novel, secreted protein expressed in differentiated cells (Lescisin et al. 1988). In situ hybridization studies have shown that the proto-oncogene, c-fms, which encodes the receptor for the cytokine, CSF-1, is expressed at high levels in the trophoblast from about 9.5 days p.c. (Regenstreif and Rossant 1989). At the same time, the ligand, CSF-1, is expressed in the uterus, specifically in the epithelium. This finding raised the possibility that the c-fms/CSF-1 system has a paracrine role in promoting the growth of the embryo and its interaction with the uterus. Subsequent studies have shown that the CSF-1 gene is inactive in osteopetrotic (op/op) mutant mice (Yoshida et al. 1990). Homozygous op/op females have a markedly reduced fertility when mated with op/+ or +/+ males, but placental and fetal weights of the implanted embryos are normal. These and other results suggest that maternal CSF-1 is not absolutely required for embryo development but nevertheless may contribute to reproductive success at several different stages, for example, during ovulation, implantation, and colonization of the uterus with macrophages (Pollard et al. 1991). Recent experiments have shown that the Mash-2 gene, which encodes a basic helix-loop-helix transcription factor of the achaete-scute family, is specifically required for normal development of the trophoblast lineage. Embryos lacking Mash-2 die from placental failure around 10 days p.c. (Guillemot et al. 1994).

The Second Round of Differentiation: Formation of the Primitive Endoderm and Ectoderm

Like the formation of the trophoderm lineage, the second differentiation event in mammalian embryogenesis is also characterized by the appearance of an epithelial layer—in this case, the primitive endoderm—on the free surface of a group of nonpolarized cells, the ICM (see Fig. 17, below). The remaining core of ICM cells then becomes organized into a layer known as the primitive ectoderm. (The primitive endoderm is also known as the hypoblast and the primitive ectoderm is known as the epiblast or embryonic ectoderm.) The differentiation of the primitive endoderm begins around 4.0 days p.c., shortly before implantation, when
there are only 20–40 cells in the ICM. Because of the small number of cells involved, it has so far been very difficult to make precise statements about the sequence of cellular and molecular changes involved in this differentiation and how they are related to the cell cycle and to intercellular communication and organization (for reviews of primitive endoderm and ectoderm differentiation, see Gardner 1983; Hogan et al. 1983).

Good evidence from injection chimera experiments, using glucose phosphate isomerase (GPI) as a lineage marker, indicates that primitive endoderm cells do not colonize the endodermal tissues of the fetus, but only the extraembryonic parietal and visceral endoderm in the yolk sacs surrounding the developing embryo (Gardner 1982, 1983). Similar experiments have shown that the primitive ectoderm lineage gives rise to the ectodermal, mesodermal, and endodermal tissues of the fetus, to the germ cells, and to the mesodermal components of the extraembryonic membranes and placenta (Gardner and Rossant 1979). These lineages are summarized in Figure 2.

**Lineage Markers Used with Mouse Embryos**

Until recently, the only genetically determined lineage markers available for use with mouse embryos have been the GPI allozymes GPI-1AA and GPI-1BB. These differ in electrophoretic mobility and can be assayed in tissue homogenates (see, e.g., Gardner and Rossant 1979). Their sensitivity and precision have therefore been limited. However, a variety of markers are now available that can be used at the cellular level on tissue sections (see Table 5).

**The Primitive Ectoderm Lineage**

At the time of implantation (~4.5 days p.c.), the blastocyst is composed of three distinct tissue lineages: trophoderm, primitive endoderm, and epiblast (Fig. 17). The epiblast comprises the smallest population, consisting of only 20–25 cells, situated between the polar trophoderm and the primitive endoderm. The cells are interconnected by gap junctions, but they are apolar. Shortly after implantation, the epiblast cells organize into a simple epithelium surrounding a small central cavity—the proamniotic cavity (Fig. 17). This process is associated with visible cell death and probably a high degree of cell intermixing. Like all epithelia, the epiblast cells are now polarized, attached at their apices by junctional complexes and contain a subapical concentration of cytokeratin polypeptides (Jackson et al. 1981). Their basal surface lies on a continuous basal lamina, which separates them from the primitive endoderm (Leivo et al. 1980). At the interface with the extraembryonic ectoderm, it appears that epithelial continuity is lost, although the two populations are tightly apposed, and they form discrete populations with respect to gap junctional communication (Lo and Gilula 1979).

During the early postimplantation period, epiblast cells stain positively for alkaline phosphatase, produced from the embryonic and tissue-nonspecific alkaline phosphatase genes (Hahnel et al. 1990). They also react with anti-SSEA-1 and anti-uvomorulin antibodies and express high levels of Oct-3/4 (see Beddington and Lawson 1990). These markers distinguish epiblast during the early stages of gastrulation but disappear as it differentiates into the definitive germ layers. However, some of them persist as markers of primordial germ cells (see Section 1). From implantation to the onset of gastrulation, the epiblast population undergoes
**Figure 17** Schematic representation of mouse development from implantation up to the neurula stage (5 somites). (A) The embryo is composed of three distinct tissue lineages at the time of implantation: trophoderm (gray), primitive endoderm (beige), and epiblast (blue). The primitive endoderm subsequently differentiates into parietal (B) and visceral (C) endoderm. The epiblast becomes organized into an epithelium surrounding the proamniotic cavity (C). The primitive streak forms at approximately 6.5 days p.c. (D) and mesoderm (red) emerges as two wings of tissue underlying the epiblast and extending into the extraembryonic region. Lacunae appear in the extraembryonic mesoderm (E) and coalesce to form the cavity of the visceral yolk sac (exocoelom) bounded by the chorion mesometria and the amnion antimesometria (F).
Figure 17 (Continued) The streak extends toward the distal tip of the egg cylinder (E), and by 7.5 days p.c. (F), the node is clearly visible at the anterior end of the primitive streak. The head process and subsequently the notochord (brown) arise from the node and underlie the midline of the neurectoderm (G). Definitive gut endoderm (yellow) is also laid down from the anterior primitive streak and is initially contiguous with the notochord. At the posterior end of the streak, the allantois forms (F). By 8.5 days p.c. (G), the neurectoderm (purple) has become organized into distinctive neural folds. The heart develops rapidly and the more anterior paraxial mesoderm is arranged into paired somite blocks. Caudally, the primitive streak continues to supply additional mesoderm for future trunk structures.
Table 5 Cell Autonomous Markers Used to Follow Cell Lineages during Mouse Development

<table>
<thead>
<tr>
<th>Marker</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Dyes or enzymes for microinjection</td>
<td></td>
</tr>
<tr>
<td>1. Dii, DiO</td>
<td>Serbedzija et al. (1989);</td>
</tr>
<tr>
<td></td>
<td>Beddington (1994)</td>
</tr>
<tr>
<td>2. Lysinated dextran rhodamine and rhodamine-conjugated dextran</td>
<td>Gimlich and Braun (1985);</td>
</tr>
<tr>
<td></td>
<td>Stern et al. (1988);</td>
</tr>
<tr>
<td></td>
<td>Lawson et al. (1991)</td>
</tr>
<tr>
<td>3. Horseradish peroxidase</td>
<td>Lawson et al. (1991)</td>
</tr>
<tr>
<td>B. Transgenic mouse lines</td>
<td></td>
</tr>
<tr>
<td>1. Line carrying about 1000 copies of the β-globin genes; ES lines are</td>
<td>Lo (1986); Lo et al. (1987)</td>
</tr>
<tr>
<td>available; for staining technique, see Section H, DNA-DNA In Situ</td>
<td></td>
</tr>
<tr>
<td>Hybridization</td>
<td></td>
</tr>
<tr>
<td>2. LacZ driven by a constitutive promoter</td>
<td>Beddington et al. (1989);</td>
</tr>
<tr>
<td></td>
<td>Tan et al. (1993)</td>
</tr>
<tr>
<td>HPRT</td>
<td></td>
</tr>
<tr>
<td>5. Replication-defective retrovirus containing lacZ reporter</td>
<td>Sanes et al. (1986); Price et al.</td>
</tr>
<tr>
<td></td>
<td>(1987); DeGregori et al. (1994)</td>
</tr>
<tr>
<td>C. Genetic differences between inbred mouse strains</td>
<td></td>
</tr>
<tr>
<td>1. Monoclonal antibodies specific for H-2b and H-2k</td>
<td>Ponder et al. (1983)</td>
</tr>
<tr>
<td>2. Satellite DNA sequence distribution between M. musculus and M.</td>
<td>Rossant (1985)</td>
</tr>
<tr>
<td>caroli</td>
<td></td>
</tr>
<tr>
<td>3. Null mutation in cytoplasmic malic enzyme (Mod-1&lt;sup&gt;b&lt;/sup&gt; vs.</td>
<td>Gardner (1984)</td>
</tr>
<tr>
<td>Mod-1&lt;sup&gt;b&lt;/sup&gt;)</td>
<td></td>
</tr>
<tr>
<td>4. Carbohydrate polymorphism recognized by Dolichos biflorus</td>
<td>Schmidt et al. (1985)</td>
</tr>
<tr>
<td>agglutinin</td>
<td></td>
</tr>
<tr>
<td>5. Y-specific probe in XX-XY chimeras</td>
<td>Jones and Singh (1981); Bishop</td>
</tr>
<tr>
<td></td>
<td>et al. (1985)</td>
</tr>
<tr>
<td>6. Monoclonal antibody (OX7) specific for the Thy-1.1 allele of Thy-1</td>
<td>John et al. (1972); Morris and</td>
</tr>
<tr>
<td>a surface glycoprotein on T lymphocytes and certain other tissues,</td>
<td>Barber (1983)</td>
</tr>
<tr>
<td>including fibroblasts and embryonic brain cells; has been used to</td>
<td></td>
</tr>
<tr>
<td>follow grafts of embryonic tissue into adult brain; congenic pairs of</td>
<td></td>
</tr>
<tr>
<td>Thy-1.1 and Thy-1.2 mice are available</td>
<td></td>
</tr>
<tr>
<td>7. High- and low-activity alleles of β-glucuronidase; use limited to</td>
<td>Mullen (1977)</td>
</tr>
<tr>
<td>central nervous system</td>
<td></td>
</tr>
</tbody>
</table>
subtle changes. The cells lose their capacity to colonize the blastocyst, surface polysaccharides are modified, changes in polypeptide synthesis can be detected, X-inactivation occurs (see below), and the length of the cell cycle decreases (see Gardner and Beddington 1988). In addition, during this time, the overall methylation of the genome increases. Studies have shown that CpG islands of specific genes undergo programmed methylation changes that are stage-specific. In the early embryo, a general wave of demethylation occurs, so that by the 16-cell and blastocyst stages, most CpG sites are demethylated. The DNA gradually becomes remethylated, and by 6.5 days p.c., almost all genes studied show the methylation pattern characteristic of adult somatic tissues (Monk et al. 1987; Kafri et al. 1992).

**Primitive Ectoderm Cells Divide Rapidly**

Unlike most lower vertebrates, gastrulation in the mouse is associated with rapid cell proliferation. As shown in Tables 6–8, analysis of cell number and mitotic index in a random bred strain of mouse has shown that the cells of the primitive ectoderm (epiblast) divide extremely rapidly between 5.5 and 7.5 days p.c. (Snow 1977). At 5.5 days p.c., the epiblast consists of about 120 cells and this increases to 660 cells by 6.5 days p.c. when gastrulation begins. To account for the number of ectoderm and mesoderm cells present at 7.0 days p.c., given that mesoderm cells

---

**Table 6** Total Cell Numbers in the Embryonic Germ Layers

<table>
<thead>
<tr>
<th>Age (days p.c.)</th>
<th>Number of embryos (no. of litters)</th>
<th>Endoderm *</th>
<th>Mesoderm *</th>
<th>Epiblast/Ectoderm *</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>14(5)</td>
<td>95</td>
<td>-</td>
<td>120</td>
</tr>
<tr>
<td>6</td>
<td>6(3)</td>
<td>130</td>
<td>-</td>
<td>250</td>
</tr>
<tr>
<td>6.5</td>
<td>13(5)</td>
<td>250</td>
<td>-</td>
<td>660</td>
</tr>
<tr>
<td>7</td>
<td>7(2)</td>
<td>430</td>
<td>1220</td>
<td>3290</td>
</tr>
<tr>
<td>7.5</td>
<td>16(5)</td>
<td>680</td>
<td>6230</td>
<td>8060</td>
</tr>
</tbody>
</table>

Reprinted, with permission, from Snow (1977).

*Number of cells.

**Table 7** Mean Cell Cycle Times Required to Account for the Growth of the Epiblast

<table>
<thead>
<tr>
<th>Age (days p.c.)</th>
<th>5.5</th>
<th>6</th>
<th>6.5</th>
<th>7</th>
<th>7.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cells</td>
<td>120</td>
<td>250</td>
<td>660</td>
<td>4510</td>
<td>14,290</td>
</tr>
<tr>
<td>Number of divisions</td>
<td>1.04</td>
<td>1.32</td>
<td>2.71</td>
<td>1.58</td>
<td></td>
</tr>
<tr>
<td>Mean cell cycle</td>
<td>11.5</td>
<td>9.1</td>
<td>4.4</td>
<td>6.7</td>
<td></td>
</tr>
</tbody>
</table>

Reprinted, with permission, from Snow (1977).

**Table 8** Estimated Cell Cycle Times (Hours) for Various Regions of the Embryo

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Epiblast/ectoderm</th>
<th>Mesoderm</th>
<th>Proliferative zone</th>
<th>Other epiblast</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.4</td>
<td>4.8</td>
<td>22.2</td>
<td>2.2</td>
<td>5.1</td>
</tr>
<tr>
<td>7</td>
<td>7.2</td>
<td>3.2</td>
<td>3.2</td>
<td>7.5</td>
</tr>
<tr>
<td>7.5</td>
<td>8.1</td>
<td>13.9</td>
<td>3.6</td>
<td>8.5</td>
</tr>
</tbody>
</table>

Reprinted, with permission, from Snow (1977).
are derived from the epiblast following ingestion through the primitive streak, the
cell cycle of the epiblast at 6.5 days p.c. is estimated to be as short as 4.4 hours.
Since the epiblast also gives rise to definitive endoderm during this period, this
may be an underestimate. Studies using a variety of techniques have indicated that
the division rate is not uniform throughout the gastrulation-stage embryo but is
significantly faster in a subpopulation of cells. In rat embryos, this population has
been localized to the primitive streak (MacAuley et al. 1993), whereas in the mouse,
using different techniques, a more limited distribution at the anterior of the primi-
tive streak has been proposed by Snow (1977). It is unclear whether a real species
difference exists or whether the apparent difference is due to the techniques used.
The mesoderm emerging from the primitive streak proliferates more slowly, with a
cell cycle time of approximately 8–10 hours.

**The Epiblast Is a Pluripotent Tissue**

Several lines of evidence show that epiblast cells, or at least some of them, are
pluripotent up until the later stages of gastrulation. It is these experiments that
have identified the epiblast as the sole founder tissue of the fetus, including both
the somatic tissues and the germ line.

1. When single epiblast cells from 4.5-day p.c. blastocysts are injected into 3.5-day
p.c. blastocysts of a different genotype, their progeny in chimeras populate all
somatic tissues of the fetus or liveborn mouse as well as germ cells (Gardner
and Rossant 1979; Gardner et al. 1985). They also contribute to extraembryonic
mesodermal components of the chorioallantoic placenta (chorionic mesoderm
and allantois) and the amnion. This ability to colonize the blastocyst is lost by
5.5 days p.c.

2. Blastocysts can give rise to cell lines in vitro (ES cells) that are totipotent (Evans
syngeneic mice give rise to teratocarcinomas (see Teratocarcinoma Cells and
Embryonic Stem Cells, p. 92, and Fig. 33), which are tumors composed of a vari-
ety of both differentiated cell types and undifferentiated stem cells known as
embryonal carcinoma (EC) cells; EC cells are responsible for the progressive
growth and transplantability of the tumor. Furthermore, ES cells injected into
3.5-day p.c. blastocysts contribute to the soma and germ line of adult chimeras
(Bradley et al. 1984), and single ES cells have been shown to give rise to trophec-
toderm, primitive endoderm, and fetal somatic tissues in midgestation
chimeras (Beddington and Robertson 1989). ES cells can also be derived from
blastocysts arrested in delay (see Section F), when the epiblast lineage has prob-
able already segregated (Gardner et al. 1988), further emphasizing the
pluripotency of epiblast tissue.

3. Isolated epiblast tissue from 6.5- or 7.5-day p.c. embryos of all mouse strains
tested can give rise to teratocarcinomas, containing both pluripotent EC cells
and a large repertoire of differentiated tissues, at a very high frequency when
transplanted into an ectopic site in adult syngeneic mice (see Fig. 33) (Diwan
and Stevens 1976). Very small pieces of epiblast (~100 cells) from the anterior
region of the 7.5-day p.c. embryo also generate teratocarcinomas (Beddington
1983). Because primordial germ cells are located at the posterior aspect of the
embryo at this stage (see Origin of Germ Cells and Their Migration to the Genital Ridge, p. 28), the potential to give rise to pluripotent EC cells must be a property of epiblast tissue and not just of a subpopulation of PGCs within it. This potential to generate teratocarcinomas is lost by 8.5 days p.c. (Damjanov et al. 1971).

4. When preimplantation embryos are transferred to an ectopic site, they develop more or less normally to the early egg cylinder stage (equivalent to 6.0 days p.c. of normal development) before becoming disorganized and forming either a teratoma (differentiated tissues but no EC cell population) or a teratocarcinoma. Similarly, in the LT strain of mice, which shows a high incidence of ovarian teratomas, spontaneous parthenogenetic activation of oocytes is followed initially by apparently normal development to the egg cylinder and even early gastrulation stages, before embryogenesis degenerates into teratocarcinogenesis (for review, see Martin 1980; Stevens 1983).

5. Single 7.5-day p.c. epiblast cells injected with lineage tracer into the intact embryo (see Table 5) can give rise to derivatives in a variety of embryonic tissues (Lawson et al. 1991).

**General Description of Gastrulation and the Formation of Mesoderm and Definitive Endoderm**

Gastrulation is the process by which the bilaminar egg cylinder of the 6.0-day p.c. embryo is transformed into a multilayered, three-chambered conceptus and the embryo itself acquires the full array of fetal primordia arranged according to the characteristic vertebrate body plan. All of these definitive tissues of the embryo and the extraembryonic mesoderm components are derived from the simple epithelium of the epiblast, which contains about 800 cells at the onset of gastrulation (Snow 1977). Not surprisingly, such reorganization and production of new tissues require an extremely complex and coordinated combination of morphogenesis, proliferation, cytodifferentiation, and pattern formation. Conceptualizing the morphogenetic movements associated with gastrulation is particularly difficult with respect to the mouse embryo, since the epiblast is folded into a cup. However, the movements are thought to be essentially the same as those seen in the chick embryo, where the blastoderm is a flattened sheet (Fig. 18).

Gastrulation begins at about 6.5 days p.c. when the primitive streak forms in a localized region of the epiblast adjacent to the embryonic/extraembryonic junction (Fig. 17), but the signals that lead to the initiation of gastrulation at a particular point along the embryonic/extraembryonic junction remain unknown. This region marks the future posterior of the embryo. The epithelial continuity of the epiblast is lost in the streak region (~10–15 cell diameters in width), and cells move or delaminate through the streak to emerge between the epiblast and visceral endoderm as a new intermediate layer of mesoderm (Fig. 19). Some cells also intercalate into the outer visceral endoderm layer to provide the first cohort of definitive or gut endoderm. The origin of the streak marks the posterior aspect of the embryo and provides the first unequivocal definition of the anteroposterior axis. The onset of expression of several genes, for example, goosecoid (Blum et al. 1992), T (Wilkinson et al. 1990), Evx-2 (Dush and Martin 1992), and follistatin (Albano et al. 1994), coincides with both the appearance and location of the streak, although the
Figure 18  Schematic representation of gastrulation in the chick and mouse embryo.  (A)  Chick blastoderm before and after formation of the primitive streak and Hensen's node.  Arrows mark the direction of migration of cells within the epithelial sheet of the epiblast.  Mesoderm cells are delaminating from the epiblast and accumulating between the upper and lower epithelial sheets.  (B)  Primitive-streak-stage mouse embryo in section.  If flattened out, it would resemble the chick blastoderm above.
Figure 19 (Top) Transverse cross-fracture through the primitive streak of an early primitive-streak-stage embryo showing the ingress of epiblast cells and the lateral spread of mesoderm cells from the midline. (Bottom) Stereo pair showing the cellular organization of newly formed mesoderm. Arrow marks anterior. (PS) Primitive streak. (Photograph provided by Dr. Patrick Tam, The Chinese University of Hong Kong.)
role of these genes in mouse gastrulation has yet to be established. However, it is clear that once the streak is fully extended, the epiblast in its immediate vicinity shows differential transcriptional activity according to its anteroposterior position relative to the streak.

As gastrulation progresses, the streak elongates from its origin at the embryonic/extraembryonic junction toward the distal tip of the egg cylinder, eventually extending down the entire side of the cylinder. In reality, this elongation is probably due to extension of the streak proximally by the addition of new delaminating tissue behind the anterior aspect of the early streak. The location of epiblast cells that will pass through the streak (all those with the exception of prospective neur ectoderm and surface ectoderm) can be surmised from the fate maps shown in Figure 20. In addition to providing an avenue through which epiblast cells can pass to form the new mesoderm and endoderm layers, the primitive streak is an actively proliferating cell population (Hashimoto and Nakatsuji 1989; MacAuley et al. 1993). However, it is not clear whether the streak also contains a resident population of proliferating stem cells in addition to the transitory population of epiblast cells. Clonal fate map studies (see below) indicate that only in the anterior third of the streak is there a population of cells that has some of the properties of a stem cell pool: Such cells give rise both to progeny in the new germ layers and to descendants that remain within the streak. More caudally, the streak seems only to be a thoroughfare for delaminating epiblast traffic (Lawson and Pedersen 1992).

During the first 24 hours of gastrulation, nascent mesoderm moves in two directions. The most posterior mesoderm pushes its way proximally into the extraembryonic region, displacing the extraembryonic ectoderm toward the ectoplacental cone (Fig. 17E). It also moves laterally around the circumference of the egg cylinder in both the embryonic and extraembryonic regions (Fig. 17E). Definitive endoderm emerges from the anterior aspect of the streak and moves predominantly anteriorward. This means that the posterior regions of the streak may remain associated with primitive endoderm for most of the gastrulation phase (Tam and Beddington 1987). It should be emphasized that much of this apparent rostral or lateral movement of new tissue is actually due to rapid expansion of the egg cylinder by proliferation of tissues anterior and lateral to the primitive streak, rather than to extensive migratory movements.

In the posterior extraembryonic region, and to a lesser extent anteriorly and laterally in the extraembryonic region, mesoderm accumulates and increases in volume due to the acquisition of intercellular lacunae (Fig. 17E). These lacunae will eventually coalesce to form a new, mesoderm-lined cavity, the exocoelom (Fig. 17F). The expanding extraembryonic mesoderm population pushes the proximal rim of epiblast and the distal rim of extraembryonic ectoderm toward the center of the proamniotic cavity; this results in the formation of distinct bulges, most pronounced posteriorly, which are known as the amniotic folds (Fig. 17E). These folds meet and fuse so that a new chamber is formed in the egg cylinder, separated from the embryonic region by the amnion and from the ectoplacental cone by the chorion (Fig. 17F). The mesodermal and visceral endoderm walls of this chamber will expand to form the visceral yolk sac. Following the formation of the exocoelom, some, if not all, of the nascent extraembryonic mesoderm still emerging from the posterior streak gives rise to a distinct structure, the allantois (Fig. 17F), which grows mesometrially across the exocoelom to fuse with the chorion. This will form a major component of the chorioallantoic placenta and provide a direct link for nutrient and waste exchange between fetus and placenta.
Figure 20 Fate maps of epiblast before and during gastrulation. (A) The epiblast origin of prospective tissues is plotted onto the left half of the embryo. Considerable overlap occurs between the boundaries of different prospective tissues. (B) The overall topography of the mouse fate map is comparable to that of other vertebrates during gastrulation. The embryos are depicted flattened out and viewed from the dorsal aspect.
The Node

At the most anterior aspect of the streak, there is a specialized structure about 20 cells in diameter, known as the node (Figs. 17F and 21), which is equivalent to Hensen's node in the chick and the dorsal blastopore lip of Xenopus; this structure has a crucial role in organizing and patterning the midline axis of the embryo. The morphology and developmental fate of the mouse node has been reviewed recently (Sulik et al. 1994). It is first clearly recognizable at the late primitive streak stage when the streak extends to the distal tip of the egg cylinder (for conventions used in staging gastrulating mouse embryos, see Downs and Davies 1993). However, fate maps (Fig. 20) indicate that a region with the developmental fate characteristics of the later node exists at mid-streak stages, although at this stage, it does not appear as a discrete morphological structure. At later stages, the node region is recognized by a slight indentation at the tip of the egg cylinder, and there is no visceral endoderm overlying this region. Consequently, the node is a bilaminar structure, with dorsal and ventral layers, in contrast to the trilaminar composition of the rest of the embryonic region of the egg cylinder. The two layers of the node may be more intimately associated than the definitive germ layers (which are separated from each other by basal lamina) since the node cannot be separated into constituent tissue layers by conventional enzyme digestion (see Section C).

The ventral layer of the node is derived from the overlying epiblast and is initially intercalated laterally with the epithelial layer of definitive endoderm, which is also derived from the epiblast. At this stage, the ventral cells of the node are known as the notochordal plate; each cell in this layer is distinguished by the presence of a single, motile, central cilium (Fig. 21C,D). Later, the notochord separates as a rod of cells from the endoderm, which becomes a continuous epithelial layer. By analogy with the chick embryo, the notochord precursors probably undergo extensive cellular rearrangements during gastrulation and become extended and intercalated along the anteroposterior axis (Fig. 21C) (Jurand 1974; Poelman 1981; Sausedo and Schoenwolf 1994; Sulik et al. 1994).

DII (1-1-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate) labeling experiments (Beddington 1994) and injection of horseradish peroxidase (HRP) into individual cells in the node region (Lawson and Pederson 1992) indicate that the node contains a population of resident cells responsible for providing most of the axial mesoderm. Just anterior of the notochordal plate is a population of compact midline mesoderm cells known as the head process, first distinguishable at the late primitive streak stage (~7.5 days; Jurand 1962; Poelman 1981; Tam et al. 1983). The ultimate fate of these cells is presently unknown. Unlike the cells of the notochordal plate, head process cells are not intercalated with the endoderm but emerge between the ectoderm and primitive endoderm. They underlie the future forebrain and possibly also the midbrain neurectoderm and are thought to be equivalent to the prechordal plate of the avian embryo. Indeed, given the confusion over the term "head process," prechordal plate is probably a more precise term (Sulik et al. 1994). The generally accepted view is that in the mouse, the head process or prechordal plate mesoderm originates from the epiblast at the node and migrates anteriorly. However, some of the axial mesoderm underlying the future fore/midbrain may derive from the leading edges of the lateral wings of mesoderm, which emerge from the early primitive streak and meet in the anterior midline (Fig. 17E; Tam et al. 1993 and pers. comm.). The most anterior mesoderm derived from the primitive streak gives rise to cardiogenic mesoderm.
Definitive endoderm also emerges from the node region and flanks the developing notochord (Figs. 17F and 21B) (Bedington 1981; Lawson et al. 1991; Tam and Bedington 1992; Tam et al. 1993). There is also evidence from chick and mouse that floor plate cells of the neural tube are derived from cells situated in the node region (Selleck and Stern 1991; Sulik et al. 1994).

Recently, evidence has emerged that the mouse node has "organizing" properties analogous to those of the amphibian dorsal blastopore lip and Hensen's node of the chick. First, it appears that the distal half of the embryonic region of a mid-streak embryo can induce additional anterior structures in *Xenopus* embryos if transplanted into the ventral side of the blastocoelic cavity (Blum et al. 1992). Second, the mouse node can effect duplications of digits in the developing avian limb by simulating an ectopic zone of polarizing activity (see Limbs, p. 82), and this behavior may relate in part to its production of retinoids (Hogan et al. 1992). Finally, and conclusively, heterotopic grafting of the node to a posterolateral position in a late-streak-stage mouse embryo results in the induction of a second neural axis (Bedington 1994).

Figure 22 illustrates some examples of differential gene expression along the length of the streak and within and surrounding the node. Although future research will undoubtedly show regional expression of many more than the ten genes depicted, one can already see that the formation of different tissues from the streak and node during gastrulation has an explicit molecular foundation. Furthermore, if the expression patterns of the homologs of *T*, *goosecoid*, and *HNF3-ß* in zebra fish, *Xenopus*, and the chick are compared at an equivalent stage of gastrulation (Bedington and Smith 1993), and more recently the distribution of hedgehog transcripts in zebra fish and mouse (Echelard et al. 1993; Krauss et al. 1993), there is almost complete conservation of molecular pattern. This strongly suggests that even if we do not know whether the signals that initiate gastrulation are conserved in the mouse, there is a strong argument for supposing that, once under way, similar molecular mechanisms will operate during the execution of gastrulation in all vertebrates.

**Fate Maps of Gastrulation**

Two different methods have been used to construct fate maps of the gastrulating epiblast, and both have generated comparable results. Since labeling of specific cells requires direct access to the embryo, these studies have used whole embryo culture (see Section I, Culture of Primitive Streak/Early Somite Stage Embryos) in order to follow development during gastrulation and early organogenesis. The first method employed was to graft orthotopically 7.5-day p.c. [³H]thymidine-labeled epiblast tissue into embryos of the same stage (Bedington 1981, 1982). The second method is more precise in that it involves less perturbation to the embryo and follows the development of descendant clones derived from a single epiblast cell injected iontophoresically with HRP or HRP and rhodamine dextran (Lawson et al. 1991; Lawson and Pederson 1992). The fate maps generated for pre- and early-streak embryos by such a clonal analysis, together with maps derived from orthotopic grafting in the late-streak embryo (Bedington 1981, 1982; Tam 1989), are illustrated in Figure 20. Three important points emerge from this work. The fate map of the mouse epiblast during gastrulation is clearly similar to those charted in the chick, *Xenopus*, and teleosts at a similar stage; i.e., the topography of prospective tissues appears to be well conserved during vertebrate evolution (Bed-
Figure 21 Scanning electron micrograph of neural-fold-stage embryo showing the position of the node and notochord. (A) Midsagittal section showing the allantois (A), amnion (Am), embryonic ectoderm (E), extraembryonic endoderm (ExEn), extraembryonic mesoderm of the yolk sac (ExM), foregut diverticulum (FG), presumptive heart (H), node (N), notochord (NC), and neural folds (NF). (B) Cross section through a similar stage embryo immediately anterior of the node. Note that in the midline, there are only two cell layers; the ciliated notochord cells (NC) tightly apposed to the embryonic ectoderm (E) which will form the neural plate. (Continued on facing page.)
Figure 21 (Continued.) The paraxial mesoderm (M) lies on either side of the notochord, between the ectoderm and the definitive endoderm (En), which is still adjacent to or continuous with the notochord. (Photographs provided by Dr. R.E. Poelman, Rijksuniversiteit, Leiden, The Netherlands.) (C,D) Scanning electron micrograph of the ventral surface of 7.5-day p.c. embryo showing the morphology of the node and notochord cells. (C) Cells in the node (N) and notochord (NC) have microvilli and a large single cilium as well. This is shown at higher magnification in part D. In contrast, adjacent endoderm cells (En) have only a large number of microvilli. (Photographs provided by Dr. K. Sulik, University of North Carolina, Chapel Hill.)
**Figure 22** Some examples of localized gene expression in the primitive streak, node, and emerging notochord, adjacent mesoderm, and floor plate. Many more than the ten genes depicted show regional expression in this part of the embryo, but even this small subset of genes illustrates that the origin of different tissues from different levels of the streak and node results from differential gene expression.
The borders between different prospective tissues are not absolute but show a considerable degree of overlap, and a single epiblast cell can give rise to derivatives in all three germ layers. Therefore, the fate maps do not reflect a predetermined mosaic of cells already committed to forming specific tissues (Lawson et al. 1991). This interpretation is supported by the observation that heterotopically grafted epiblast tends to give rise to tissues characteristic of its new location, rather than adhering to its original developmental fate (Beddington 1982).

**Generation of Regional Diversity in the Mesoderm**

Little is understood about diversification within the mesoderm population. Indeed, it is not clear that "mesoderm," as a homogeneous progenitor tissue for the various different mesoderm derivatives, ever exists. Rather, it is possible that cells emerging from the primitive streak are already committed to specific mesodermal fates and express different genes. Clearly, the axial mesoderm of the head process and notochord shows a unique pattern of gene expression (Fig. 22). Similarly, other genes such as *Msx-1*, *Msx-2*, and *lim-1* are expressed in lateral mesoderm but not more medially (Davidson and Hill 1991; Barnes et al. 1994), whereas genes such as the *forkhead* domain gene, *MF-1*, follistatin, *sek*, and *Mox-1* and *Mox-2* (Candia et al. 1992; Nieto et al. 1992; Sasaki and Hogan 1993; Albano et al. 1994) appear to demarcate presomatic or paraxial mesoderm. Certainly, fate mapping studies have demonstrated that different mesodermal derivatives arise from different rostro-caudal regions of the primitive streak (Tam and Beddington 1987, 1992; Tam et al. 1993; Lawson and Pedersen 1992), and there is evidence that different genes are expressed within different subpopulations of the primitive streak (Fig. 22). However, the developmental lability of nascent mesoderm from different levels of the streak has not yet been tested directly.

By 8.5 days p.c., the diversity of mesodermal derivatives is already apparent. In the extraembryonic region, mesoderm has formed part of the chorion, part of the amnion, the mesothelium lining the exocoelom, capillary endothelium, fibroblasts, hematopoietic precursors (the blood islands), and the allantois. Within the embryo, at least seven different categories of mesoderm can be distinguished on the basis of morphology and anatomy: cranial mesoderm, cardiac mesoderm, somites and presomatic mesoderm, intermediate mesoderm, lateral plate mesoderm (splanchnopleure and somatopleure), blood vessels, and notochord. Undoubtedly, future molecular descriptions will demonstrate an even greater variety of mesodermal components.

**Tail Bud**

The tail bud replaces the primitive streak as the source of new caudal tissue on the tenth day of gestation. It is thought that this transition from a streak to a solely tail-bud supply of tissue coincides with closure of the posterior neuropore and corresponds to the lumbosacral region of the anteroposterior axis. The remnant of the primitive streak, which is located immediately behind the posterior neuropore, becomes or is replaced by a population of mesodermal cells at the distal tip of the growing tail. Tail-bud tissue divides rapidly (Tam and Beddington 1986) and has the
potential to give rise to somitic and lateral mesoderm, neural tube, notochord, and gut endoderm; however, if it is removed, both somitogenesis and axial elongation are arrested (Tam 1984; Tam and Tan 1992). The tail bud thus appears to behave like a pluripotent progenitor population for tail development. However, the mechanisms by which this tail-bud population is maintained and the cues that control the final size of the body axis have yet to be elucidated.

**Turning**

At the early somite stage, the mouse embryo is U-shaped, and because the germ layers are initially inverted in the mouse, the ectoderm (neural tube and surface ectoderm) lies on the inside of the conceptus and the endoderm (gut) lies on the outside. Turning effectively reverses this topography to restore the typical conformation of a vertebrate embryo (for a detailed description, see Kaufman 1992). Figure 23 illustrates the basic movements effected by turning, which involve both the caudal and rostral sections of the embryo rotating in opposite directions while the whole conceptus rotates 180° anticlockwise about the midpoint of the U. These movements are also responsible for the complete envelopment of the embryo by its embryonic and extraembryonic membranes.

**Somites and Their Derivatives**

The somites and their derivatives, in particular the vertebrae and ribs, represent the most obvious example of segmental pattern in vertebrate embryos. Their genesis and fate have been most extensively studied in the chick embryo. Somites arise from strips of mesoderm, the presomitic mesoderm, or segmental plate, which lie on either side of the neural tube immediately anterior to the primitive streak or, at later stages, the tail bud (Figs. 24 and 25). The presomitic mesoderm and its derivatives are known collectively as the paraxial mesoderm. In the mouse, somitogenesis commences at about 8.0 days p.c. and continues up to the 14th day of gestation (Tam and Tan 1992), with approximately 65 pairs of somites being generated altogether, in a rostral to caudal gradient of maturation. Discrete somites are not evident in the cranial region anterior to the otic vesicle, although some paraxial mesoderm is present, arranged into seven pairs of metameric condensations, known as somitomeres and recognized by scanning electron microscopy (Fig. 24) (Meier and Tam 1982). Most of the skull is derived from the neural crest (see below), and the principal derivatives of cranial paraxial mesoderm are the musculature of the eyes and the branchial arches. Somitomeres have also been observed in the presomitic mesoderm prior to overt somite segmentation. However, lineage studies make it unlikely that a presomitic somitomere constitutes the direct precursor of a somite (Tam and Beddington 1986). Cell mixing within each strip of presomitic mesoderm indicates that cells may be moving between somitomeres, and therefore, they do not represent a constant population of cells. Once somites have formed, little or no cell mixing occurs between them.

Periodically (approximately every 1.5 hours) and apparently synchronously, mesoderm at the rostral aspect of each strip of presomitic mesoderm becomes organized into an epithelial sphere and a new pair of somites is formed. Thus, the file of somites is extended by the addition of new somites caudally. At early somite
Figure 23 Schematic representation of turning of the mouse embryo (after Kaufman 1992). (A) Changes in the conformation of the embryo and arrangement of embryonic and extraembryonic membranes from 8.5 days p.c. to 9.5 days p.c. (B) Vertical sections through the mid-trunk region of the embryo during turning to illustrate how the amnion comes to envelop the embryo while contained within the visceral yolk sac.
Figure 24  Schematic representation of a neural-fold-stage chick or mouse embryo. (A) Viewed with ectoderm in position. (B) The ectoderm and neural tube have been removed to reveal the underlying mesoderm. (1-3) The first somite blocks to condense; (I-VIII) represent the cranial somitomeres detected only by stereo scanning electron microscopy. The fate of somitomeres and their contribution to craniofacial development are discussed in Trainer et al. (1994). In vivo, there are up to six somitomeres in the segmental plate of the mouse embryo.

Figure 25  Scanning electron micrograph of the neural-fold-stage mouse embryo (~8.5 days p.c.). (1) External morphology showing headfold with preotic sulcus (PS), and prosencephalon (Pro), mesencephalon (Mes), metencephalon (Met), and myelencephalon (My) regions of the prospective brain. The metencephalon/myelencephalon boundary is approximately between rhombomeres R1/R2 or R2/R3, just rostral to Krox 20 expression in R3. White arrowhead is foregut and H is heart. (2) Right half of the embryo from which the neuroepithelium has been removed. There are three somites (S) and seven somitomeres in the cranial region and six in the prosomite mesoderm (curved arrows). (Photos courtesy of Patrick Tam, Childrens Medical Research Institute, Wentworthville, NSW, Australia.)
stages, each nascent somite appears to be marked by high but transient expression of *Notch*, a homolog of the *Drosophila Notch* gene (Del Amo et al. 1992; Reaume et al. 1992). Each somite goes through a similar maturation sequence. The epithelial structure is maintained for about 10 hours, after which cells on the ventral margin of the block disperse and move toward the notochord; these cells constitute the sclerotome, the precursor of the vertebrae and ribs (Fig. 26). Dorsally, the epithelial organization of the somite is retained in the dermomyotome population. The medial myotome component will give rise to the muscles of the vertebrae and back, whereas the lateral myotome cells generate the muscles of the body wall and limbs. The dermatome will provide the dermis of the skin of the trunk and tail. A family of basic helix-loop-helix proteins, which include Myf-5, Myo-D, myogenin, and Myf-6, has been implicated in myogenesis in vitro and in vivo, and the genes are expressed in a defined sequence in the myotome region of developing somites from 8.0 days p.c.: *myf-5* is expressed first, then myogenin, then *myf-6*, and finally Myo-D (Sassoon et al. 1989; Bober et al. 1991; Ott et al. 1991). However, contrary to expectation, inactivation of Myo-D, by homologous recombination in ES cells, results in normal homozygous mice that exhibit elevated expression of *myf-5* (Rudnicki et al. 1992). Equally perplexing is the observation that mice lacking active *myf-5* suffer defective rib development, but apparently no skeletal muscle abnormalities, although the timetable of embryonic myogenesis may be somewhat retarded (Braun et al. 1992). However, animals mutant for both *myf-5* and *myo-D* do lack muscle (Rudnicki et al. 1993).

Experiments in the chick and mouse suggest that patterning of the somites extends beyond their visible separation into distinct epithelial blocks and that early in development, they acquire anteroposterior and dorsoventral positional cues. For example, the rostral and caudal halves of each somite appear to have different properties, and this is reflected in the fact that both neural crest cells and motor axons are excluded from the caudal half of somites (Serbedzija et al. 1990). Further-

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**Figure 26**  Schematic representation of the differentiation of the somites in the trunk region of the mouse embryo. The ectoderm has been pulled back on one side to reveal the somites adjacent to the neural tube. (A) The somite blocks have an epithelial organization. (B) Some cells migrate around the notochord to form the sclerotome cells of the vertebrae. The remainder differentiate into dermatome and myotome.
more, experiments in avian embryos indicate that each vertebrum is derived from
the caudal sclerotome of one somite and the rostral sclerotome of the adjacent
somite (Goldstein and Kalcheim 1992). Somites are not equivalent along the
anteroposterior axis. In the chick, transplantation of somites has demonstrated
that they develop autonomously with respect to sclerotomal derivatives. Thus, a
somite from the future thoracic region (Fig. 27) will give rise to an ectopic rib if
transplanted to the prospective cervical region. However, in such experiments, the
dermomyotome exhibits greater developmental lability and will give rise to skeletal
muscle derivatives appropriate to its new location. The expression of certain genes
(e.g., members of the HOX family; Fig. 27) provides visible evidence for anteropos-
terior patterning within the paraxial mesoderm, and the combination of Hox genes
expressed may define the axial character of the vertebrae. For example, a null
mutation in Hox C8 results in a homeotic transformation such that the vertebrae
derived from somites expressing the highest levels of Hox C8 acquire a more
anterior character (Le Mouellic et al. 1992). This is consistent with the notion that
as more 5′ Hox genes are activated in more caudal somites (see Fig. 28), a more
posterior identity to the vertebral derivatives is ascribed (Kessel and Gruss 1991).

There is good evidence from experiments in the chick and mouse that the
dorsoventral patterning of the somites, i.e., whether the cells will give rise to muscle
dorsal or cartilaginous sclerotome ventral, is controlled by diffusible factor(s)
from the notochord and neural tube. These signals act early in the development of
a somite and are of at least two kinds. In one case, the notochord and the floor
plate produce ventralizing signals that induce the adjacent somite to differentiate
into sclerotome. If a supernumerary notochord or floor plate is grafted in a chick
embryo dorsally between the somite and the neural tube, the nearby cells will
change their fate and develop not into muscle but into cartilage (Pourquie et al.
1993). On the other hand, it has been shown that local factors produced by the ad-
jacent neural tube are required for the dorsalization of the somite and for promo-
ting the differentiation of muscle precursors. If the neural tube is removed, axial
muscles (but not muscles derived from the lateral part of the somite) fail to develop
(Christ et al. 1992; Bong et al. 1992). It therefore seems that the dorsoventral pat-
terning of the somite is regulated by different factors produced in different regions
of the neural tube. Regionalization of the neural tube is in turn influenced by the
floor plate and, ultimately, by the notochord (see Generation of Regional Diversity
in the Early Brain and Neural Tube, p. 84). This hierarchy of pattern controls
appears to underlie the abnormal differentiation of the somites in mouse mutants
in which the growth and differentiation of the notochord are defective, e.g.,
Brachyury (Dietrich et al. 1994a), Danforth’s short tail (Sd) (Koseki et al. 1993),
pintail (Pt), and truncate (tc) (Lyon and Searle 1989). Moreover, the ventralizing ef-
efect of the notochord on the somite is mediated, at least in part, by the expression

Figure 27 Diagram showing the relationship between specific somites and their vertebral
derivatives and the anterior boundary of expression of different Hox genes in the paraxial
mesoderm. Due to natural degeneration of one of the occipital somites, the somite number
ascribed to each vertebra varies according to the method of counting used. Therefore,
correspondence of boundaries with respect to a specific somite number can only be consid-
ered accurate to within ±1 somite. Vertebrae serve as more reliable and standardized land-
marks.
Figure 28 Alignment of the vertebrate Hox complexes into 13 paralogous groups and comparison with Drosophila HOM-C (after McGinnis and Krumlauf 1992). The current nomenclature for each gene is given within the box, whereas the old name is given below the box. The gray areas indicate homology with specific genes of Drosophila HOM-C. (lab) labial; (pb) proboscipedia; (Dfd) Deformed; (Scr) Sex Combs Reduced; (Antp) Antennapedia; (Ubx) Ultrabithorax; (Abd) Abdominal.
of the paired-domain transcription factor, Pax-1, and mutations in Pax-1 affect
cerotome differentiation, as first reported in different mutations of the undulated
(uni) locus (Balling et al. 1988, 1992; Koseki et al. 1993).

**Lateral Plate and Intermediate Mesoderm: Kidney and Genital Ridges**

The mesoderm lateral to the somites is subdivided into intermediate mesoderm
(adjacent to the somites) and lateral mesoderm (on the lateral margins of the em-
byo). Lateral mesoderm is split in two to form the coelom. Dorsally, the somato-
pleure is associated with overlying ectoderm and continuous with the mesoderm of
the amnion; ventrally, the splanchnopleure is associated with endoderm and
continuous with the visceral yolk sac mesoderm (Fig. 17G). Lateral mesoderm will
provide much of the mesenchyme involved in development of the viscera and con-
nective tissue of the limbs, the mesothelial lining of the body cavity, and the
mesenteries.

In the caudal part of the trunk region and proximal tail of 9.0–9.5-day p.c. em-
byros, the intermediate mesoderm differentiates into the pronephric duct and
nephric primordium. Soon afterward (9.5 days p.c.), the urogenital ridge can be
discerned extending from the mid-trunk to the mid-tail region. The lateral aspect
of the urogenital ridge forms the mesonephros (visible at 10.0 days p.c.), and the
medial portion forms the gonads. Although vesicles and ducts differentiate within
the mesonephros, and show some segmental organization, they probably never
function as an excretory system, and the mesonephros is to all intents and pur-
poses a vestigial structure, although it can influence gonad differentiation. In addi-
tion, caudally, the mesonephric duct gives rise to an important offshoot, the
ureteric bud, which is required to induce the formation of the definitive
metanephric kidney. The mesoderm induced to form the kidney primordium
(metanephric blastema) is caudal intermediate mesoderm.

Kidney differentiation and morphogenesis are probably the best studied
organogenetic processes in mammals (Saxen 1987) and serve as an excellent ex-
ample of the complexities involved in establishing causal mechanisms responsible for
tissue interactions. Kidney development has been shown to depend on reciprocal
interactions between the epithelium of the ureter and the metanephric mesen-
chyme: The ureter induces the mesenchyme to become epithelial and form
tubules, whereas the mesenchyme induces the ureter to branch. Although the
metanephric mesenchyme can be induced by tissues other than the ureteric bud
(e.g., embryonic spinal cord), no other mesenchyme in the embryo will form
tubules in response to the inducing signal. This suggests that the caudal interme-
diate mesenchyme has already acquired special but, as yet, undefined properties
predisposing it to nephric development. The initial induction requires direct cell
contact between inducer and mesenchyme, and this appears to trigger a complex
cascade of interdependent events. During the past few years, various molecules
have been identified whose expression correlates with particular phases of tubule
induction (e.g., Wilms' tumor gene, WT-1 [Pritchard-Jones et al. 1990], syndecan
[Vainio et al. 1989], and nerve growth factor receptor [Sariola et al. 1991]). The fact
that kidney development can be analyzed in vitro, by employing organ culture
methods, bodes well for establishing the biological role of these molecules: More
sophisticated, controlled, and reliable tissue recombination experiments and more
precise application of specific stimulatory or inhibitory molecules can be carried
out in vitro than in vivo. These kinds of studies provide an important complementary approach to molecular genetic analysis.

**Limbs**

Limb development has been used as an experimental system for studying vertebrate pattern formation for many years. These classical embryological experiments were based on tissue grafting, and they identified three important regions of the limb bud responsible for establishing the characteristic pattern of the mature limb: the apical ectodermal ridge, the underlying progress zone, and the zone of polarizing activity. The theoretical models of pattern formation derived from these experiments have provided the foundation for interpreting the complex patterns of gene expression revealed by more molecular techniques. As with many other aspects of mouse development, the pioneering studies were done initially in the chick and only subsequently confirmed in the mouse (for reviews, see Tickle and Brickell 1991; Bryant and Gardiner 1992; Hinchcliffe et al. 1992; Mendelsohn et al. 1992).

The limb buds develop as outgrowths of lateral mesoderm enclosed in surface ectoderm. In the mouse, the forelimb buds first appear at approximately 9.0–9.5 days p.c. adjacent to somites 7–12 (for a useful staging regime, see Wanek et al. 1989). By 9.5–10 days p.c., the hindlimb buds are distinguishable at the level of somites 23–28. Lineage studies in chick/quail chimeras have shown that the bone and cartilaginous elements (including muscle tendons) are derived from the lateral mesoderm of the initial outgrowth, whereas the limb musculature originates separately from myotome cells migrating from the somite into the limb bud at a later stage. As the limb buds grow, the surface ectoderm overlying their distal tip thickens into a distinct structure known as the apical ectodermal ridge (AER). Grafting experiments have identified the cells of the AER as essential for maintaining the proliferation and patterning of the underlying progress zone (PZ). Removal of the AER or PZ during limb bud outgrowth results in a truncated limb lacking distal structures. Conversely, replacing the PZ of an older limb bud with that of a younger limb bud results in an elongated limb with duplicated proximodistal elements. For example, the usual forelimb proximodistal pattern of humerus/radius + ulnar/digits may be transformed into humerus/radius + ulnar/a second radius + ulnar/digits. This indicates that the PZ contains information for patterning the proximodistal axis of the limb, and it appears to be the age of the PZ rather than the age of the AER that is important. The expression of various polypeptide signaling molecules, e.g., members of the Wnt family (Gavin et al. 1990), BMP family (Lyons et al. 1992), and FGF-4 (Niswander and Martin 1992), has been detected in the AER; these factors may be responsible for inducing and maintaining the PZ. The patterning activity of the PZ itself is not understood, but two homologs of the *Drosophila msh* gene, known as *msx-1* and *msx-2* (previously called *Hox* 7 and *Hox* 8, although they are not related to the *Hox A,B,C,D* family), are expressed differentially along the proximodistal axis (for review, see Izpisua-Belmonte and Duboule 1992). *msx-1/Hox* 7 is particularly strongly expressed in the PZ, and its expression can be induced by ectopic grafting of the AER, whereas *msx-2* is expressed in the AER and anterior mesoderm (Robert et al. 1991).

The anteroposterior patterning of the limb requires a region of the limb bud mesenchyme situated at its posterior margin immediately proximal to the PZ. These cells, which are morphologically indistinguishable from the rest of the limb
mesoderm, constitute the zone of polarizing activity (ZPA). If an additional ZPA is grafted to the anterior margin of a limb bud, mirror image duplications of the AP axis result, the elements forming nearest to both the host and grafted ZPA always being posterior in character. These duplications occur quantally and stepwise, in that extra whole digits, as opposed to partial elements, are always formed and their anteroposterior character follows a predictable sequence. This all or none kind of differentiation indicates that the pattern is established by threshold responses within a continuous gradient. In both the chick and mouse, the node, at the anterior aspect of the primitive streak, can reproduce the effect of a transplanted ZPA when grafted to the anterior margin of a developing chick limb bud (Hogan et al. 1992). Identical duplications also result from anterior implantation of a bead soaked in retinoic acid (RA; Tickle et al. 1982). Evidence exists that retinol can be metabolized to RA within the limb, and in particular in the ZPA. This should generate a gradient of RA across the anteroposterior axis of the limb, the highest concentration being at the posterior margin (Thaller and Eichele 1978, 1990). However, it is not yet clear whether RA itself is the morphogen, whose concentration difference across the limb directly establishes anteroposterior pattern, or whether it locally induces ZPA activity (mediated by an unknown morphogen) in adjacent cells (see, e.g., Noji et al. 1991). Recent experiments have shown that in the mouse and chick, the ZPA (as well as the node) expresses the gene sonic hedgehog which encodes an extracellular protein likely to be a signaling molecule (Echelard et al. 1993; Krauss et al. 1993; Riddle et al. 1993). In chick embryos, ectopic anterior expression of sonic hedgehog in the limb bud produces anteroposterior duplications. Application of RA in an anterior bead induces the expression of sonic hedgehog, but it is not yet clear whether RA is involved in regulation of sonic hedgehog in vivo. What is clear is that members of the Hox A and Hox D family are expressed in a region-specific manner within the limb, consistent with a role in determining the final anteroposterior pattern (for review, see Izpisua-Belmonte and Duboule 1992; Izpisua-Belmonte et al. 1992). For example, posterior cells express all Hox D genes 5' to Hox D-9, whereas anterior cells express only Hox D-13. If anterior mesoderm from the mouse limb bud is transplanted into the posterior region of a chick limb bud, Hox D-11 expression is induced in the mouse cells. Thus, whatever the morphogen responsible for initiating the anteroposterior polarity of the limb, members of the Hox A and D families are likely to be involved in translating this information into the final pattern of cartilaginous elements. Intriguingly, it has been shown that ectopic expression of Hox B-8 in regions rostral to its normal anterior boundary (somes 10/11) results in mirror image duplication in the forelimbs. This coincides with ectopic sonic hedgehog expression in the anterior margin of the limb bud, marking the apparent induction of a second ZPA (Charité et al. 1994).

Development of the Nervous System: Neurulation, Timing of Neural Tube Closure

The brain and spinal cord (central nervous system [CNS]) are derived from a thickened, medial strip of ectoderm anterior (or rostral) to the primitive streak. This is the neural plate, which is first distinguishable soon after 7.5 days p.c. and elongates by the addition of newly differentiated neurectoderm at its posterior (or caudal) aspect. As with lower vertebrates, the medial epiblast is probably induced to form neurectoderm by vertical signals from the underlying mesoderm (prechordal and
notochordal) emerging from the front of the primitive streak, and patterning is also influenced by planar signals propagated within the ectoderm itself. After its initial formation, the neural plate changes shape dramatically, and its lateral edges elevate to form the neural folds. Consequently, in cross section, the neurectoderm comes to resemble a "V," with the "hinge point" being the midline overlying the notochord. The cells of this medial hinge point will give rise to the floor plate and probably originate in the node (Lawson and Pedersen 1992; Schoenwolf et al. 1992; Sulik et al. 1994). Beginning at the level of the fourth and fifth somites, at about 8.0–8.5 days p.c., the neural folds fuse along the dorsal midline and thus the neural tube is formed. Subsequently, fusion proceeds both rostrally and caudally in a zipper-like fashion from the occipital/cervical region. Closure of the anterior neuropore is complete by the 15–20-somite stage (9.0 days p.c.), whereas the posterior neuropore remains open until the 32-somite stage (10.0–10.5 days p.c.). Neurulation is one of the most widely studied morphogenetic processes in vertebrate development, and a variety of motive forces are implicated in folding the simple sheet of neurepithelium and causing it to roll up into a tube. These forces include cell autonomous factors influencing cell shape (such as microfilaments, microtubules, and cortical cytoplasmic flow), cell division, convergent extension cell movements, and expansion of the extracellular matrix underlying the neural plate (for review, see Schoenwolf and Smith 1990).

**Generation of Regional Diversity in the Early Brain and Neural Tube**

It is becoming increasingly evident that the developing nervous system is a highly patterned tissue from a very early stage. At a gross level, there is obvious regional differentiation along the anteroposterior axis, generating morphologically distinct forebrain (prosencephalon), midbrain (mesencephalon), hindbrain (rhombencephalon), and spinal cord domains. Like the paraxial mesoderm, this pattern is reflected in the restricted anteroposterior expression domains of certain genes. The various aspects of anteroposterior patterning and segmentation in the early CNS have been studied most extensively in the developing hindbrain. More than 175 years ago, von Baer (1828) described periodic swellings (neuromeres) along the neural tube reminiscent of some segmental organization (Fig. 29). More recently, the eight particularly pronounced neuromeres in the hindbrain region (called rhombomeres) have been reexamined in the chick in the context of their relationship with segmentally distributed nerves, such as the motor neurons or sensory ganglia, emanating from this region of the brain (Lumsden 1991). These studies have revealed a convincing correspondence between individual rhombomeres and specific neuronal pathways and have indicated that individual rhombomeres come to comprise segregated tissue lineages (i.e., cells from one rhombomere can no longer populate adjacent rhombomeres). Consequently, a rather precise relationship exists between particular rhombomeres, or pairs of rhombomeres, and cranial motor nerves, sensory ganglia, and neural crest migration into adjacent branchial arches (Fig. 30A). However, these lineage relationships in the chick may not be a hard and fast prototype for mice, and the correspondence between specific rhombomeres and the origin of specific cranial nerves may not follow the same precise periodicity. This anatomical and cell lineage work has been augmented by descriptions of gene expression patterns in the hindbrain.
region, which have revealed an intriguing correlation between the boundaries of gene expression and rhombomere boundaries (Fig. 30A). One of the earliest genes to be expressed in a restricted pattern encodes a zinc finger protein, called Krox 20. In situ hybridization shows two early and distinct stripes of Krox 20 expression corresponding to the position of rhombomeres 3 and 5 (Wilkinson et al. 1989). Subsequently, the mature anterior boundaries of the more 3' members of Hox gene clusters have been shown to coincide with the anterior boundaries of odd-numbered rhombomeres, thus revealing a more striking two-rhombomere periodicity. Recent evidence suggests that Krox 20 can directly trans-activate Hox B2, whose expression is highest in rhombomeres 3–5 (Sham et al. 1993). Furthermore, mutations in Hox genes with different anterior boundaries in the hindbrain (Hox A3, anterior boundary r5; Hox A1, anterior boundary r4) result in complementary phenotypes, indicating that Hox genes may have a determinative role in specifying derivatives of the hindbrain only in their most rostral domains of expression and
that different hindbrain derivatives may be specified by different Hox genes (Chisaka and Capeschi 1991; Lufkin et al. 1991).

The segmental organization of the hindbrain is now well established, but less is known about the patterning of the midbrain and forebrain. Recently, a neuromeric model of forebrain organization has been proposed, based on the temporal and spatial expression of a number of genes (Puelles and Rubenstein 1993). These include the mouse homologs of the Drosophila orthodenticule and empty spiracle genes (Otx1, Otx2, Emx1, and Emx2) (Holland et al. 1992; Simeone et al. 1992) and members of the Wnt, Pax, Pou, homeodomain, helix-loop-helix, and other gene families. A somewhat different model for the segmental organization of the chick embryonic diencephalon has been put forward by Figdor and Stern (1993).

At the cellular level, the initially columnar epithelium of the neural plate acquires a more complex stratified arrangement, and once the neural tube has formed, proliferating cells become restricted to the ventricular layer, adjacent to its lumen. In due course, the differentiation of specific neurons follows a stereotyped dorsoventral pattern: Motor neurons are located ventrally in the spinal cord, whereas commissural neurons and the neural crest (see below) arise in dorsal positions. This dorsoventral pattern is also presaged by the differential expression of genes, such as the Pax and Lim genes, in restricted domains (Fig. 30B) (Ericson et al. 1992; for review, see Deutsch and Gruss 1991). It is thought that the dorsoventral polarity of the neural plate is established early in development before closure of the neural tube, at least in part by interaction with the notochord, which underlies the ventral midline. The neur ectoderm cells lying immediately above the notochord are induced to differentiate into the floor plate, a small group of wedge-shaped cells extending along the ventral midline. In avian embryos, the addition of an ectopic notochord or floor plate next to the neural tube, or their deletion, results in marked but predictable changes in the dorsoventral pattern of the neural tube. In particular, the immediate proximity of a notochord induces a new floor plate and ventralizes adjacent neural tissue (Yamada et al. 1991, 1993; Goulding et al. 1993; Placzek et al. 1993). Recent experiments support a model in which the forkhead domain gene, HNF-3β, is an important regulator of floor plate development in the mouse. It is hypothesized that HNF-3β expression in the notochord induces the expression of sonic hedgehog, which encodes an extracellular signaling molecule (Echelard et al. 1993; Sasaki and Hogan 1994). This, in turn, induces the expression of HNF-3β in the ventral midline cells of the neural tube, and this activates the transcription of other floor plate marker genes. Thus, misexpression of HNF-3β in the

**Figure 30** (A) Anatomical anteroposterior pattern in the hindbrain and branchial arches corresponding to different patterns of Hox and Krox 20 gene expression. Asterisk indicates that Hox A1 subsequently regresses caudally from this anterior boundary, whereas Hox B1 persists as a single stripe of expression in r4. (r1-8) Rhombomeres; (gV-XI) cranial ganglia; (B1-4) branchial arches. The anatomical relationships between specific rhombomeres and the origin of cranial nerves and neural crest are based on studies in the chick and should not be taken as a definite map of the mouse hindbrain. (B) Transverse section through the spinal cord. Dorsoventral and mediolateral patternings are evident from the different domains of expression of members of the Pax gene family.
dorsal midbrain leads to the ectopic expression of floor plate genes such as HNF-3β, HNF-3α, BMP-1, and SP (Sasaki and Hogan 1994).

In addition to ventral signals, there may also be dorsal influences emanating from the roof plate (Basler et al. 1993) and/or the dorsal ectoderm, and the correct dorsoventral patterning of the neural tube probably requires an interplay between such dorsal and ventral signaling systems. A late function of the floor plate is guidance of axonal projections by contact-mediated and diffusible signals that promote directed outgrowth of commissural axons (for review, see Dodd and Jessell 1993).

**Neural Crest**

The neural crest is a transient population of cells that originates in the dorsal part of the neural tube at the junction between neurepithelium and surface ectoderm. Crest cells migrate extensively as single cells and move away from the neural tube to ventral and dorsolateral locations where they differentiate into a wide variety of cell types. Their derivatives include adrenomedullary cells, bone and cartilage, melanocytes, glial and Schwann cells, and several different kinds of neurons (sensory cranial nerves, parasympathetic, sympathetic, and sensory ganglia). Their developmental potential is influenced by their axial location, since cranial neural crest can give rise to bone, cartilage, and odontoblasts (one of the principal precursors in tooth formation), whereas the trunk crest cannot differentiate into these different cell types. In the head, the neural crest gives rise to most of the skull bones, with only minor components of the posterior skull being derived from cephalic mesoderm or from the first few somites (Coulby et al. 1993).

Neural crest cells in the mouse first appear on the dorsal surface of the neural tube at a level corresponding to the third or fourth somite rostral to the most recently formed somite (Erickson and Weston 1983). Thus, they both emerge and migrate in a rostral-to-caudal sequence. Migration of neural crest cells in the mouse has been studied most thoroughly by labeling the plasma membrane of cells with the lipid-soluble, hydrophobic fluorescent dye DiI (Serbedzija et al. 1990, 1991, 1992). Dye injected into the neural tube of embryos subsequently cultured in vitro shows that trunk neural crest cells emerge between 8.5 and 10.5 days p.c. (Serbedzija et al. 1990). Two pathways of migration are evident: (1) a ventral pathway through the rostral half of somites and (2) a dorsolateral pathway between the dermamyotome and the epidermis (Fig. 31). The ventral migration is composed of two phases, an early phase between 8.5 and 9.5 days p.c., in which neural crest cells reach more ventral destinations such as the sympathetic ganglia and dorsal aorta, and a later phase between 9.5 and 10.5 days p.c., which contributes to the dorsal root ganglia and Schwann cells of the motor axons. In the sacral region, neural crest cells emigrating from the neural tube also populate the enteric nervous system of the postumbilical gut (Serbedzija et al. 1991). In contrast to the situation for the trunk, cranial neural crest migration follows a caudal-to-rostral sequence, beginning first in the midbrain region at about the 5-somite stage and in the forebrain region at about the 10-somite stage. At all levels, cell emigration lasts approximately 9–12 hours. Neural crest cells emerging from the hindbrain region follow segmental pathways in that substantial streams of crest are only observed lateral to r2, r4, and r6 and populate the adjacent first, second, and third branchial
Figure 31 Schematic representation of neural crest migration. (Closed arrows) Neural crest cells arising from the dorsal neural tube migrate either ventrally through the rostral half of the somites or dorsolaterally between the dermamyotome and the epidermis.

arches, respectively. However, Dil labeling of r3 and r5 shows that at least some neural crest differentiates in these rhombomeres and joins the segmental pathways from adjacent rhombomeres. This argues that the segmental distribution of crest in the hindbrain region is not due to any segment-specific failure to form neural crest cells (Serbedzija et al. 1992), although the relatively small contribution from r3 and r5 may result from rhombomere-specific cell death of neural crest cells (Graham et al. 1993).

It remains somewhat controversial whether the crest migration pathways are truly specific, paved, as it were, with particular extracellular molecules that direct crest cells to their final destination. Certainly, a variety of extracellular matrix molecules, such as hyaluronic acid, collagen types I and III, laminin, and fibronectin, have been described along the neural crest migration routes. In the chick, antibodies against the integrin fibronectin receptor perturb cranial neural crest migration (Bronner-Fraser 1985). However, it is possible that the so-called pathways are simply the only navigable routes available to migrating cells, other regions being impenetrable due to preexisting embryonic structures or an extracellular matrix incompatible with cell movement. As yet, it is not understood what prompts the neural crest cells to delaminate in the first place or what makes them stop and differentiate at one of their potential destinations. Certain genes, such as W (Dominant white spotting) and Sl (Steel), affect neural crest migration and differentiation. The demonstration that Sl/Sl neural crest melanoblasts could be rescued by provision of wild-type skin, whereas W/W melanoblasts could not, was the first indication that W and Sl encoded interacting receptor and ligand molecules (Mayer 1973).
Formation of the Branchial Arches and the Pharyngeal Region

The six pairs of branchial (or visceral) arches, which arise in the pharyngeal region of the embryo in cranial caudal sequence starting at 9.0 days p.c., constitute major building blocks of the head and neck (with the exception of the fifth arch, which is rudimentary). Each arch, which grows from its distal tip, is an ectoderm-covered bar of mesenchyme (much of it neural crest in origin) that curves laterally and ventrally around the future oral cavity (Sulik and Schoenwolf 1985). The most rostral arch (b1) forms first, and bifurcates distally so that the maxillary prominence grows out from its dorsal surface, while the remainder of the arch (the mandibular prominence) continues to grow more ventrally. The maxillary and mandibular prominences become more overtly separated at 11 days p.c. and contribute to the upper and lower jaw, respectively. The second arch makes a major contribution to the neck region, effectively overgrowing the smaller third and fourth arches. The relationship between the more rostral branchial arches (b1–b4) and the hindbrain, with respect to Hox gene expression, neural crest migration, and innervation, is shown in Figure 30A.

The branchial arches are separated from each other externally by deep ectodermal grooves (visceral grooves) and internally by similar endodermal invaginations (pharyngeal pouches; Fig. 32), and each arch receives its own blood supply from a specific offshoot of the aorta (aortic arches). The first branchial or pharyngeal pouch, possibly in association with the second pouch, will give rise to the Eustachian tube and contribute to the middle ear cavity, whereas the third

![Diagram](image)

**Figure 32** Schematic representation of the relationship between the branchial arches (1, 2, 3), pharyngeal pouches (I, II, III), and aortic arches (i, ii, iii).
pouch will give rise to the thymus and parathyroid glands, and the fourth to ultimobranchial bodies. The ultimobranchial bodies fuse with the thyroid and are thought to provide its complement of parafollicular cells. The thyroid itself differentiates from an endodermal thickening in the floor of the pharynx, which enlarges to form a ventral diverticulum.

The roof of the primitive mouth cavity, which is lined with epithelium continuous with surface ectoderm, gives rise to Rathke's pouch. This enlarges dorsally and abuts the infundibulum, a ventral outgrowth from the floor of the diencephalon. Together, the two will form the pituitary gland, located immediately ventroposterior to the optic chiasma. Rathke's pouch gives rise to the anterior lobe (or adenohypophysis) of the pituitary, and the infundibulum gives rise to the posterior part (or neurohypophysis). The outgrowth of Rathke's pouch marks the anterior limit of the notochord. The parotid salivary glands also originate from the ectodermal lining of the roof of the stomodeum, whereas the submandibular and sublingual salivary glands arise from endoderm in the floor of the oral cavity.

**Gut**

The early development of the gut is poorly understood. Cell lineage studies in the blastocyst and during gastrulation (see above) have demonstrated that the epiblast and not the primitive endoderm is the precursor of the fetal gut. However, it is not clear exactly how definitive endoderm intercalates into the preexisting visceral embryonic endoderm layer nor how long residual primitive endoderm descendants may persist in the developing gut. The majority of epiblast cells are recruited into the gut primordium through the anterior part of the primitive streak; since they emerge on the ventral surface of the egg cylinder, the future roof of the gut is located medially, whereas the prospective ventral gut resides more laterally (Beddington 1981; Poelmann 1981; Lawson et al. 1991, 1987). Before the gut becomes a separate tube, the lateral margins of the definitive endoderm are continuous with the visceral yolk sac endoderm, and medially, the notochord is intercalated into the roof of the gut. The notochord will detach itself from the endoderm to form a discrete midline rod. Rostrally, the blind ending of the gut tube in the pharynx abuts the ectodermal lining of the primitive oral cavity (the stomodeum), but this ectoendodermal membrane (the buccopharyngeal membrane) ruptures at about 9.0 days p.c. due to a combination of cell death and cell rearrangements, so that the gut opens into the amniotic cavity. Later, a similar process occurs in the hindgut to generate the cloacal membrane (10–10.5 days p.c.), which ruptures to provide the anal opening. The gut endoderm gives rise to a number of derivatives, such as those originating from the pharyngeal pouches (see section above), most of which are derived from its ventral aspect (e.g., thyroid, ventral pharyngeal endoderm [first evident at 8–10 somite stage]; lung buds, ventral endoderm of the caudal pharyngeal region [first evident at 22–28 somite stage]; liver, ventral endoderm at the foregut-midgut junction [first evident at 8–10 somite stage]; and pancreas, rostral midgut [first evident at 25–30 somite stage]). The signals prompting the outgrowth of endoderm epithelium at the appropriate position for the various organ primordia are not well understood, although it is believed that the endoderm acquires its own rostrocaudal polarity early during development. Recent studies have shown that genes encoding DNA-binding transcription factors of the forkhead domain family are expressed in an anteroposterior sequence along the definitive
gut from the gastrulation stage (Monaghan et al. 1993; Sasaki and Hogan 1993). The subsequent differentiation and morphogenesis of each gut derivative invariably require intimate cooperation and interaction with adjacent lateral plate mesenchyme (for review, see Saxen et al. 1976).

**Teratocarcinoma Cells and Embryonic Stem Cells**

Transplantation of epiblast dissected from postimplantation embryos up to mid gastrulation to a well-vascularized site in an immunologically compatible host results in the formation of tumors. These tumors usually contain a chaotic array of differentiated tissues, such as skin, bone, striated muscle, and nervous tissue, and represent a caricature of normal development (for reviews, see Stevens 1967; Damjanov and Solter 1974). They contain many of the normal derivatives of epiblast (the fetal tissues) and even structures (such as hair follicles) requiring local tissue interactions for their formation. However, highly sophisticated organotypic differentiation dependent on complex tissue interactions, such as liver or kidney formation, does not occur, nor is there any indication of the organized pattern of tissues characteristic of the embryo. In addition to these benign, differentiated tissues, a high percentage of these tumors contain a population of undifferentiated proliferating cells known as EC cells, which closely resemble ICM or epiblast cells in their morphology and biochemical characteristics (Diwan and Stevens 1976). EC cells behave as true malignant stem cells in that even a single EC cell is capable of giving rise to a new teratocarcinoma, complete with its own repertoire of mature tissues and dividing stem-cell population (Kleinsmith and Pierce 1964). Therefore, it is the EC cells that are responsible for generating not only more EC cells, but also the plethora of differentiated tissues. Early embryos from other mammalian species transplanted to an ectopic site can generate benign teratomas with a characteristic array of chaotic differentiated derivatives. However, the production of EC cells in such transplants appears to be a peculiarity of the mouse, although EC cells are found in human tumors, also called teratocarcinomas, derived from germ cells in the gonads. Postimplantation embryos from any strain of mouse can be used to generate teratocarcinomas containing EC cells, but strain differences, presumably reflecting differences in the immune system, have been observed in the ability of host animals to support progressive growth of teratocarcinomas (Solter et al. 1975). For example, C57BL/6 and AKR strains are nonpermissive for EC cell growth, although embryos of these strains will give rise to a normal percentage of teratocarcinomas if transplanted to F₁ hosts.

As with most malignant tumor stem cells, EC cells can be isolated in culture and propagated as permanent cell lines. Under appropriate conditions (Section I, Culture of Teratocarcinoma Cells), these cells can be induced to differentiate into a number of different cell types in vitro, thereby providing a convenient differentiation system in culture (for reviews, see Graham 1977; Martin 1980). One of the most remarkable features of EC cells is that when injected back into a normal blastocyst, they are able to resume embryonic development and participate in the formation of a normal chimeric mouse, derived partly from the host blastocyst and partly from the tumor stem cells (Brinster 1974; Mintz and Illmensee 1975; Papaioannou et al. 1975). EC cells also occasionally give rise to normal gametes in these chimeras and therefore could transmit their genotype to subsequent generations (Stewart and Mintz 1982). This astonishing triangular relationship among em-
bryo, tumor, and tissue culture cell line promised a completely unexpected route for undertaking sophisticated molecular genetics in the mouse. Here was a population of "embryonic cells" that could be grown in culture and subjected to molecular genetic manipulation and selection and then be returned to an embryo where they could form a chimera and transmit their modified genome to future generations of mice. However, the realization of such an experimental genetic program required that EC cells colonize the germ line at a high frequency and this was not the case. Their chequered history, including proliferation as a malignant stem cell in vivo, may select for many genomic changes incompatible with gametogenesis (for review, see Papaioannou and Rossant 1983).

If cell lines could be isolated directly from the embryo, without resorting to a tumor phase, colonization of the germ line might be improved. This has now been achieved, and ES cells can be recovered routinely from both normal and delayed blastocysts of at least two inbred mouse strains (129 and C57BL/6). If due care is taken in their maintenance, they can be grown for many generations in culture and will still contribute to the germ line of chimeras at a high frequency (Evans and Kaufman 1981; Martin 1981; Bradley et al. 1984). Consequently, ES cell lines provide one of the most powerful tools in mammalian genetics. Not only can they serve as a substrate for random mutagenesis, but they can also be used to select for extremely rare events such as homologous recombination (see Section F). This allows the production of specific mutations in known genes and, following the establishment of new lines of mice carrying the mutation, a detailed examination of the mutant phenotype (for reviews, see Capecci 1988; Reith and Bernstein 1991).

In addition to their use as a genetic tool, ES cells are at present the only normal stem-cell population that can be maintained indefinitely in vitro without losing stem-cell properties. Thus, they provide an invaluable resource for investigating the parameters of stem-cell growth and maintenance and identifying factors involved in sustaining their proliferation at the expense of differentiation (e.g., DIALIF; Smith and Rathjen 1991). Furthermore, some progress has been made toward developing culture conditions that select for the differentiation of ES cells into populations of tissue-specific stem cells, such as hematopoietic and neuronal stem cells that can be isolated in vitro (see, e.g., Lindenbaum and Grosfeld 1991; Wiles and Keller 1991). Like EC cells, ES cells are tumorigenic if introduced into adult mice, where they form teratocarcinomas. They therefore also provide an interesting population for studying factors involved in the suppression of malignancy. Section F describes the isolation, maintenance, and manipulation of ES cells in vitro and available methods for making chimeras. The interrelationship of tumor cells, embryonic stem cells, and the normal embryo is schematically represented in Figure 33.

Size Regulation

The mouse embryo has a remarkable capacity to compensate for either a substantial increase or decrease in cell number (for review, see Snow et al. 1981). If two (Tarkowski 1961), or even nine (Petters and Markert 1980), morulae are aggregated together (Section D, Making Aggregation Chimeras), and the resulting giant blastocysts are transferred to pseudopregnant recipients, the ensuing offspring are normal in size. In quadruple-size embryos, some regulation of cell number occurs before implantation, although the ICM:trophectoderm ratio remains dispropr-
Figure 33 Interrelationship between the early embryo, embryonic stem (ES) cells, and embryonic carcinoma (EC) cells. ES cells can be derived in vitro from the ICM of blastocysts. Like the epiblast of the egg cylinder, ES cells form transplantable teratocarcinomas if inoculated into syngeneic adult hosts. The stem cells of these tumors (EC cells), like ES cells, can participate in normal development if introduced into a blastocyst. Both can contribute to the somatic tissues of liveborn chimeras, but, unlike EC cells, ES cells colonize the germ line at a high frequency, allowing their genotype to be transmitted to subsequent generations. EG cell lines, showing all the characteristics of ES cells, can be isolated directly in vitro from the posterior portion of 8.5-day p.c. embryos, the region that contains the primordial germ cell population.

The rate of cell replication is high (Rands 1987). However, the most dramatic phase of regulation occurs between 5.5 and 6.5 days p.c. and appears to involve an increase in cell cycle time simultaneously in all tissues of the conceptus without a significant increase in cell death (Lewis and Rossant 1982). By the onset of gastrulation, cell numbers are more or less normal. Furthermore, the timetable of development is not severely perturbed in giant embryos, although there is some evidence that proamnion formation may be advanced, indicating that this process is dependent on total cell number, rather than the number of cell divisions that have occurred during preceding development (Lewis and Rossant 1982).

Likewise, if cell number in the preimplantation embryo is reduced by either destroying cells or separating blastomeres, the final size of liveborn offspring is not affected. However, compensatory growth occurs quite late in development. Halvesized embryos, produced from development of a single blastomere isolated at the two-cell stage, appear morphologically normal, but they are half the size of control embryos up to 10 days p.c. (Tarkowski 1959). By 11.5 days p.c., they have regained normal size; although it is not known how this is achieved, it may be significant that it coincides with the maturation of a fully functional placenta. A similar timetable of "catch up" growth is seen in XO embryos that are small and retarded
in growth up to 10.5 days p.c. but close to normal at 12.5 days p.c. However, XO embryos are invariably underweight at birth (Burgoyne et al. 1983). A substantial decrease in cell number can also be tolerated by gastrulating embryos. Administration of mitomycin C, which randomly kills cells, to 6.5–7.5-day p.c. embryos in utero can rapidly reduce total cell number to 15% of that found in untreated embryos. However, these diminutive embryos complete gastrulation on schedule and regain normal fetal size and weight by 13.5 days p.c. Elevated proliferation in all tissues is first evident at the neurula stage. Remarkably, only subtle developmental abnormalities can be detected in these conceptuses, and the defects are more consistent with some degree of asynchronous development between different tissues during organogenesis than with destruction of specific progenitor populations. This implies that the epiblast must remain a developmentally labile tissue during gastrulation if the embryo can recover from such a drastic teratogenic insult. However, the primordial germ cell population, although showing some compensatory expansion between 9.5 and 10.5 days p.c., remains about half the size of the same population in normal embryos (Snow et al. 1981), and defects are found in specific vertebrae (Gregg and Snow 1983).

Imprinting

Imprinting is the phenomenon whereby the activity of some genes is influenced by their parental origin. Its occurrence in mammals has only recently been recognized and was deduced from a number of different lines of research, including classical genetic studies and studies on the pattern of X-inactivation and the development of diploid parthenogenetic, gynogenetic, and androgenetic embryos (for reviews, see Solter 1988; Cattanach and Beechey 1990; Surani et al. 1990). If mice heterozygous for reciprocal or Robertsonian translocations are crossed, some offspring will inherit regions of a particular autosomal chromosome only from one parent. Despite diploidy being maintained, it emerged that anomalous phenotypes are seen if certain chromosomes or regions of chromosomes are inherited from only one parent.

Parthenogenetic embryos derived from activated eggs (see Parthenogenesis, p. 43) can develop apparently normally up to the blastocyst stage. A minority form egg cylinders following implantation, and occasional development to early limb bud (25 somites) stage has been observed. However, none of the embryos progress to term and all have very poorly developed extraembryonic tissues. More recently, nuclear transplantation (Section D) has been used to reconstitute fertilized eggs with pronuclei either only of paternal origin (androgenotes) or only of maternal origin (gynogenotes). In both cases, diploidy and even heterozygosity are maintained, but neither class of embryo develops normally. Gynogenotes can develop up to about 10 days p.c. (similar to parthenogenotes) but subsequently die with extremely poor development of extraembryonic tissues (Surani and Barton 1983). Androgenotes fair even worse, exhibiting poor preimplantation development and seldom forming embryos with recognizable somites, although the extraembryonic tissues in this case are relatively well developed. Control embryos, on the other hand, subjected to the same manipulations but reconstituted with a pronucleus from each parent develop normally. When chimeras are made between parthenogenotes and normal embryos, development can continue to term, although the chimeric offspring tend to be small, and parthenogenetic cells are
selected against in the latter part of gestation and are particularly underrepresented in skeletal muscle and liver. In addition, the parthenogenetic cells contribute poorly, if at all, to the trophoblast, probably because polar trophoeoterm lacking a paternal genome does not proliferate in response to a signal from the underlying ICM. In contrast, similar chimeras made with androgenotes tend to be larger than control embryos and show a disproportionate androgenetic contribution to mesodermal derivatives compared with ectodermal tissues. Androgenetic ES cells, derived from blastocysts developed from reconstituted eggs, retain their imprint (at least in part) despite several passages in vitro. Chimeras made between these cells and wild-type embryos do not survive if the ES cell contribution is high, and even when the contribution is low, the offspring consistently show dramatic skeletal defects and die at an early age (Mann et al. 1990).

Evidence that development to term requires the presence of both a maternal and a paternal genome has been augmented by the identification of specific genes whose expression is influenced by their parental origin. The first example was identified when examining the phenotype of offspring derived from mice containing a targeted null mutation in the insulin-like growth factor II (Igf2) gene (DeChiara et al. 1991). When the Igf2 mutation was inherited from the father, heterozygous offspring were approximately 60% the size of normal littermates. In contrast, heterozygotes inheriting the mutation from the mother were normal in size. This led to the conclusion that in normal individuals, only the paternal Igf2 allele is transcriptionally active, and this was subsequently confirmed by molecular analysis. Interestingly, this imprinting is tissue-specific in that expression of maternal Igf2 is seen in the choroid plexus late in gestation. It has now been demonstrated that the IGFII receptor (thought to be important in reducing levels of circulating IGFII) is imprinted in a reciprocal manner, with only the maternal allele being active (Barlow et al. 1991). This gene is located on chromosome 17, and its imprint may explain the lethality of a deletion called Thp when inherited from the mother but not from the father. H19, a gene that does not appear to code for a protein and whose function is unknown and that is closely linked to Igf2, has also been shown to be imprinted: Only the maternal allele is active (Bartolomei et al. 1991). It is possible that H19 may be the gene primarily imprinted, its activity precluding transcription of the Igf2 gene on the same chromosome. Neither the number of primary imprinted genes in the genome nor the mechanism of imprinting is known. Methylation has been invoked as one means of achieving a heritable, epigenetic, and reversible (in the germ line) state of gene activity, but it may be more important in maintaining the imprinted state rather than setting it up (for review, see Monk and Grant 1990). At present, it is not known when the imprint in PGCs is changed, such that the parental imprint apparent in somatic cells of the embryo is erased and the new imprint that will be inherited by the next generation is established. Erasure and setting up of a new imprint could be a simultaneous or a multistep process.

**X-inactivation**

In female mammals, one of the two X chromosomes is inactivated in all somatic cells, which results in dosage compensation for X-linked genes (Lyon 1961). This inactivation is random with respect to which X is silenced, except in the trophoblast and primitive endoderm lineages, where the paternally derived X chromosome is
preferentially inactivated (Takagi and Sasaki 1975; West et al. 1977). The timing of X-inactivation during development is illustrated in Figure 34 (for review, see Chapman 1986). Cytogenetic and biochemical studies have indicated that inactivation occurs first in the trophoblast and primitive endoderm of the blastocyst and subsequently in the early postimplantation epiblast. X-inactivation also occurs in female primordial germ cells, probably at the same time inactivation occurs in the epiblast, although both X chromosomes appear to be active during oogenesis. The activity of X chromosomes in EC and ES cell cultures in vitro has also been studied, and there is some evidence from certain cell lines that both X chromosomes are active before differentiation (implying that the cell lines were derived from embryonic cells prior to X-inactivation). However, once differentiation is induced, X-inactivation occurs. In ES cells known to contain two active X chromosomes, complete loss or partial deletion of one X chromosome occurs, which suggests that inadequate dosage compensation is deleterious to stem-cell growth (Rastan and Robertson 1985).

In general, X-inactivation once initiated is stably inherited by all clonal descendants. Unlike the situation in humans, where some X-linked genes coding for proteins escape inactivation, all such X-linked genes appear to be transcriptionally silent on the inactivated mouse X chromosome (Ashworth et al. 1990). Recently, an X-linked gene (Xist) has been described in humans and the mouse which maps to the region of the X thought to be responsible for inactivation (X-inactivation center, Xce), and more importantly, it has been shown to be actively transcribed only from the inactive X chromosome (Borsani et al. 1991; C.J. Brown et al. 1991). Interestingly,

\[ \text{Figure 34} \quad \text{Timing of X-inactivation during development. Shown are the changes in X chromosome activity in the female mouse embryo. (m) Maternal; (p) paternal; (+) active; (−) inactive. (After Monk and Grant 1990.)} \]
ly, this candidate RNA transcript for initiating and maintaining X-inactivation does not appear to code for a protein product and is localized predominantly to the nucleus (Brockdorff et al. 1992; C.J. Brown et al. 1992). Ironically, the onset of expression of Xist in the preimplantation mouse embryo and the prediction that it will be transcribed in trophectoderm but not the ICM of the blastocyst (Kay et al. 1993) make it one of the best candidates for a gene that will be differentially expressed in the inner and outer cells of the morula.

**Extraembryonic Tissues**

The extraembryonic tissues form an integral part of the life support system essential for the maintenance, nourishment, and protection of the fetus within the uterus. These tissues constitute the placenta, the parietal yolk sac (parietal endoderm and trophoblast), the visceral yolk sac (visceral endoderm and mesoderm), and the amnion (mesoderm and ectoderm) (Fig. 35). Studies on gene expression in extraembryonic tissues have focused on proteins synthesized at high levels in order to fulfill certain specialized functions. Examples cited below are the synthesis of fetal serum proteins such as β-fetoprotein (AFP), transferrin, and apolipoproteins by the visceral endoderm and the production of extracellular matrix glycoproteins such as laminin and type IV collagen, which form part of the basement membrane laid down by the parietal endoderm. Other studies have revealed more esoteric properties of these tissues. For example, in the trophoblast and the parietal and visceral endoderm (but not in the visceral mesoderm or amnion), the paternal X chromosome is specifically inactivated (Takagi and Sasaki 1975; Kratzer et al. 1983; Lyon and Rastan 1984), and both repetitive and single-copy

![Figure 35 Placenta and extraembryonic membranes of the 13.5-day embryo.](image-url)
DNA sequences are undermethylated (Chapman et al. 1984). In addition, in all extraembryonic tissues, and in particular the amnion, there is a high level of expression of the proto-oncogene, c-fos (Muller et al. 1983; Curran et al. 1984). The physiological significance of many of these observations is not yet known.

**Extraembryonic Endoderm: The Primitive Endoderm Gives Rise Only to Visceral and Parietal Endoderm**

The endoderm cells of the yolk sacs surrounding the mouse embryo are derived from a precursor pool of about 20 bipotential primitive endoderm (PrE) cells (Fig. 17A,B). Lineage studies with Gpi alloenzyme-marked cells have shown that the primitive endoderm does not contribute to the definitive endoderm of the adult mouse (Gardner and Rossant 1979; Gardner 1982, 1983) but only to visceral and parietal extraembryonic endoderm. This lineage study has been confirmed using the Mod-1⁺/Mod-1¹ marker system (Gardner 1984).

PrE cells first differentiate on the blastocoelic surface of the ICM at 4–4.5 days p.c. and can be distinguished from the 20 or so primitive ectoderm cells by a number of morphological features (Nadijcka and Hillman 1974), in particular a more extensive endoplasmic reticulum swollen with secretory material (presumably including type IV collagen, laminin, and fibronectin; see below). The PrE cells do not, however, form a well-defined polarized epithelium at this stage (see, e.g., Enders et al. 1978). As the primitive and extraembryonic ectoderm layers grow and elongate to form the core of the egg cylinder (see Fig. 17), the outer endoderm cells differentiate into two morphologically and biochemically distinguishable subpopulations, the visceral endoderm (VE) and the parietal endoderm (PE). Endoderm cells that remain in contact with the egg cylinder become organized into a distinct epithelium of VE cells, with apical desmosomal junctions and microvilli, and many small and larger vacuoles and organelles distributed in the cytoplasm in a polarized manner. VE cells surrounding the primitive ectoderm are known as visceral embryonic endoderm and tend to be flatter or squamous, whereas those surrounding the extraembryonic ectoderm (visceral extraembryonic endoderm) are columnar and have a very vacuolated cytoplasm and numerous microvilli (Fig. 36) (Hogan and Tilly 1981). A completely different morphology is seen in the parietal PE cells (Figs. 36 and 37). These first appear at the time of implantation (Enders et al. 1978) when primitive endoderm cells migrate on to the inner surface of the trophectoderm that is covered by a thin basal lamina containing fibronectin and laminin (Wartiovaara et al. 1979; Leivo et al. 1980). In contrast to the VE cells, PE cells are individual and migratory, do not form specialized intracellular junctions, do not have an obvious polarity in the distribution of their intracellular organelles, and coexpress vimentin and cytokeratins (Lane et al. 1983; Lehtonen et al. 1983b). The most significant feature of PE cells is their enormously enlarged endoplasmic reticulum filled with secretory material, including components of the thick basement membrane (Reichert's membrane), which is laid down by the PE cells (see below). In vivo, the apical surfaces of PE and VE cells are closely apposed (Fig. 35), and the limited space between the two is filled with secretions of both the PE and VE cells and substances from the maternal circulation that have been filtered through Reichert's membrane.
Gene Expression in Visceral Endoderm

The most important functions of the visceral endoderm are (1) absorption, i.e., uptake of substances from the maternal circulation that have filtered through Reichert’s membrane into the cavity of the parietal yolk sac, and (2) secretion, i.e., production and secretion of serum components and other proteins such as AFP, transferrin, high- and low-density apolipoproteins, and α1-antitrypsin. The visceral endoderm therefore performs some of the same functions as both the fetal large intestine and liver (Meehan et al. 1984), even though these tissues are derived from a completely different lineage (see Fig. 2).

The morphology of visceral endoderm cells is highly specialized for absorption since the cells have numerous apically located microvilli and coated pits, as well as lysosomes, etc. (Figs. 36 and 38). Because the cells are polarized, it is likely

Figure 36 Visceral and parietal endoderm of the 7.5-day mouse embryo.
that the absorptive functions are located at the apical surface, whereas secretion of AFP, transferrin, and other serum components may take place via the basal surface, but this question has not been resolved. In the visceral yolk sac, the basal surface is adjacent to the mesoderm layer containing fetal blood vessels. The direction of secretion of other VE products such as plasminogen activator is a matter of speculation.

The most specific "marker" so far recognized for visceral endoderm is AFP, a glycoprotein ($M_r 68,000$) made only by the visceral endoderm, the fetal or regenerating liver, and localized regions of the embryonic intestine. AFP represents approximately 25% of the total protein synthesis of the visceral endoderm in 15.5-day mouse embryos, and its mRNA represents 15% of total poly(A)$^+$ RNA (Andrews et al. 1982a,b; Janzen et al. 1982). The precise function of AFP is unknown, but since it is the major $\gamma$-globulin in fetal blood, it may fulfill the same role as serum albumin in adult blood. The AFP gene is closely related to the albumin gene, probably by duplication and divergence of a common ancestral sequence (Gorin and Tilghman 1980; Eiferman et al. 1981).

Immunoperoxidase staining of sections of mouse embryos of different stages demonstrated that AFP is first detected in the visceral embryonic endoderm at 7 days p.c. (Dziadek and Adamson 1978; Dziadek and Andrews 1983). It is absent from visceral endoderm around the extraembryonic ectoderm as a result of the inhibitory influence of this tissue. If visceral extraembryonic endoderm is separated from the underlying extraembryonic ectoderm, the cells will start to synthesize AFP within 12 hours (Dziadek 1978). In situ hybridization studies have shown that AFP mRNA is present in all VE cells of the 14-day visceral yolk sac but is absent from the mesodermal cells (Dziadek and Andrews 1983).
Figure 38 Junction region between parietal and visceral endoderm in the 7.5-day mouse embryo. The parietal endoderm on Reichert's membrane has been folded back to reveal the underlying visceral endoderm layer around the egg cylinder. Magnification, 440X.

Gene Expression in Parietal Endoderm

The most obvious feature of parietal endoderm cells is the fact that they are specialized for synthesizing and secreting a thick basement membrane known as Reichert's membrane between themselves and the trophectoderm (Fig. 36). Until about 16 days of gestation (when it breaks down), Reichert's membrane is one of the major barriers between the maternal and fetal environments in the mouse. This is because in the rodent, the endothelial cells of the maternal blood vessels break down to give large sinuses, and the trophectoderm cells lying between these blood sinuses and the embryo do not remain as a continuous shell below the placenta but gradually die away. Although it is assumed that Reichert's membrane acts as a passive filter, keeping out maternal cells and large molecules, there is in fact little hard information about its permeability properties and even less is known about the relationship between the function of Reichert's membrane and its structure and composition. PE cells synthesize large amounts of basement membrane
components, including laminin, entactin, type IV procollagen, and heparan sulfate proteoglycan. The mature cells do not synthesize fibronectin (Hogan 1980; Smith and Strickland 1981; Amenta et al. 1983), and this extracellular protein is not considered to be a structural component of Reichert’s membrane (Semoff et al. 1982). However, it is likely that primitive endoderm cells do make fibronectin and possibly use it in migration on the trophectoderm (Hogan et al. 1983). RNA from PE cells has been used to isolate cDNA clones for both type IV procollagen and laminin (Kurkinen et al. 1983a,b; Barlow et al. 1984), and Reichert’s membrane is an excellent model system for studying basement membrane synthesis, assembly, and remodeling (Hogan et al. 1984). PE cells also synthesize large amounts of tissue plasminogen activator, which has a molecular weight, antigenicity, and inhibitor sensitivity different from that of the urokinase-type plasminogen activator made by VE (Marotti et al. 1982).

**Differentiation of the Extraembryonic Mesoderm**

Some of the mesoderm cells generated by the early primitive streak contribute toward the different extraembryonic tissues listed below (see Fig. 17 and Gardner 1983).

1. **Amnion.** The amnion is generated from both embryonic ectoderm and mesoderm. It appears first as a fold and then as a continuous roof over the top of the cup-shaped primitive ectoderm (Fig. 17). It then expands rapidly, and with the turning of the embryo, it forms a thin membrane surrounding the fetus. The ectodermal and mesodermal cells of the amnion have very different morphologies and are separated by a basement membrane (Fig. 39).

2. **Allantois.** The allantois starts as a finger-like projection of mesodermal cells from the posterior margin of the embryonic ectoderm where the primitive streak first arises. It expands upward, fuses with the chorion, is a major component of the labyrinth of the placenta, and gives rise to the blood vessels of the umbilical cord.

3. **Mesoderm of the visceral yolk sac.** Mesodermal cells generated from the posterior primitive streak migrate onto the inner surface of the visceral endoderm and give rise to the first hematopoietic tissue of the fetus in the form of blood islands in the visceral yolk sac. Mesodermal cells that cover the chorion also contribute to the placenta.

**The Structure and Function of the Placenta**

By midgestation, the placenta has become a very complex organ consisting of both fetal and maternal tissues and blood cells. The development of the placenta is described in detail by Theiler (1972), and only a schematic representation of two stages is given in Figure 40. An important feature distinguishing the mouse placenta from that of humans is that the maternal blood vessels break down, so that the blood cells come into direct contact with the fetal trophoblast. Listed below are the major fetal tissues of the mouse placenta:
1. **Trophoblast.** In the outer spongiotrophoblast layer closest to the uterine decidual tissue, most of the trophoblast cells are polyploid and giant, whereas in the inner labyrinth layer, many of the cells are diploid. In both layers are maternal blood sinuses containing maternal blood cells, and the outer layer, even after dissection from the uterus, is contaminated with maternal decidual cells (Rossant and Croy 1983). Trophoblast gene expression is discussed in Trophectoderm and Its Derivatives (p. 54).

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**Figure 39** (Top) Section through the amnion of a 10.5-day embryo. (Meso) Cell derived from mesoderm; (Ect) cell derived from ectoderm; (BM) basement membrane; (DJ) desmosomal junction. The mesodermal cells are not joined by specialized junctions. (Bottom) Higher magnification of an ectodermal cell (Ect) in top panel.
2. Mesoderm. After the allantois fuses with the chorion, it gives rise to fetal capillaries and blood vessels. These mingle with the trophoblast cells and with maternal blood sinuses in the labyrinth.


Apart from having an important function in the transfer of nutrients and metabolites into and out of the fetal circulation, the placenta synthesizes many steroid, polypeptide, and prostaglandin-type hormones that are required for the coordination of maternal and embryo physiology during pregnancy (see, e.g., Soares et al. 1985).

![Diagram of placenta](image)

**Figure 40** Schematic representation of the mouse placenta at 8.5 days p.c. (A) and 14.5 days p.c. (B). Note that in the older embryo, both the outer and inner placenta have a significant contribution of maternal cells. The best source of trophoblast tissue free of maternal contamination is therefore the 7.5- and 8.5-day p.c. ectoplacental cone (see Rossant and Croy 1985).
Mouse Coat Color and Its Genetics

As discussed above, the genes affecting coat color were among the first to be tested for Mendelian inheritance in mice. Since these early studies, more than 50 genes have been identified that affect hair growth and pigmentation. Here, only the briefest outline of the subject will be given to stimulate the curiosity of researchers who will be handling mice for the first time. For a full and scholarly account of the genetics of coat color, see Silvers (1979) and Grüneberg (1952). Various aspects of hair follicle development and coat color genetics have been reviewed by Hardy (1992) and by Jackson (1993).

During embryogenesis, each hair follicle develops from an epidermal thickening or placode that penetrates into the dermis and surrounds a condensation of mesodermal cells known as the dermal papilla (Fig. 41). In the mouse, there are ap-
approximately 50 hair follicles/mm², or approximately 500,000 over the entire skin (Potten 1985), and they are formed between 14 days p.c. and 3 days after birth. Melanocyte precursor cells (melanoblasts) migrate from the neural crest into the hair follicles and take up a position above the dermal papilla (Fig. 41). There are about 20–30 dendritic melanocytes in each follicle, and evidence suggests that they are derived by division from one founder melanoblast (Potten 1983). Each melanocyte has the capacity to synthesize two kinds of pigments, pheomelanin (yellow) and eumelanin (black or brown). Both pigments are derived from tyrosine and involve an initial conversion with the copper-containing enzyme tyrosinase. After this initial step, different enzyme systems generate the alternate chromatophores, which are then linked to proteins and incorporated into pigment granules of different size and shape. During active phases of the hair growth cycle, the melanocytes secrete these granules, which are taken up by the cortical and medullary epidermal cells of the hair shaft. Several different kinds of hair are found on the body, besides the whiskers (vibrissae); the three kinds of larger overhairs are the monotrich, awl, and achene, and the more abundant, smaller underhairs are known as zigzags. Their morphology is determined by the mesodermal component of the follicle.

Studies using a variety of techniques have identified a number of growth factors, signaling molecules, and transcription factors expressed in different regions of developing hair follicles and vibrissae, e.g., BMP-2 (Lyons et al. 1990), Notch (Franco del Amo et al. 1992), and Skn-1a and Skn-1b (Anderson et al. 1993). Moreover, adult mice homozygous for both targeted and spontaneous null mutations in the TGF-α gene have defects in hair morphology (Luetke et al. 1993; Mann et al. 1993).

Since the genes affecting hair color were among the first to be studied, the loci were named alphabetically. Here, only the first four are discussed: A (agouti), b (brown), c (albino), and d (dilute). The wild-type alleles in the European house mouse are probably A⁺, B⁺, C⁺, D⁺ (Grüneberg 1952; Bultman et al. 1994). All of these genes have now been cloned and their analysis has shed light on a number of important problems in cell biology and development. This rich harvest is likely to continue as more genes are characterized.

**A (agouti)**

More than 17 alleles of this gene located on chromosome 2 have been isolated. Hair from an agouti mouse is black with a subapical band of yellow. This pattern is generated by the influence of the hair follicle which transiently inhibits the production of black pigment by the melanocytes during an early phase of the growth cycle. Nonagouti (a/a) mice are therefore almost completely plain black (e.g., C57BL) or brown (C57BR), apart from a few yellow hairs on the ears and around the genitals. In newborn mice, the switch from eumelanin to pheomelanin production occurs between 3 and 6 days after birth so that chimeric pups made up of cells derived from agouti and nonagouti strains of mice cannot be distinguished before this time.

The agouti gene was isolated by a positional cloning strategy based on a radiation-induced chromosomal rearrangement with a breakpoint in the A gene (Bultman et al. 1992). It encodes a small, secreted protein and is expressed in the hair follicles and not in the melanocytes (Miller et al. 1993). As a result of studies on another coat color gene, known as the extension locus, which was shown to code
for the melanocyte-stimulating hormone (MSH) receptor (Robbins et al. 1993), a model was proposed for the mechanism of action of the A protein (for review, see Jackson 1993). MSH normally stimulates the melanocyte to produce black rather than yellow pigment. However, the function of the A protein is to antagonize the action of MSH with its receptor so that the melanocyte transiently produces yellow pigment during the period when A is expressed. This explains why the overall yellow coat color resulting from the constitutive expression of A in AV mutant mice (see below) can be overcome by injection of MSH, whereas similar treatment cannot reverse the effect of point mutations in the receptor encoded by the extension gene (see Silvers 1979 for references).

Molecular studies strongly suggest that the recessive allele a (nonagouti) is generated by insertion of a VL30 retrovirus-like transposable element in the first intron of the gene (Bultman et al. 1994). Reversion of a to A and AVW (white bellied agouti, in which the ventral part of the coat is cream and the dorsal part is agouti) occurs at a high frequency due to recombination involving the VL30 sequences. It is likely that the true wild-type allele of the gene, present in most wild mice, is AVW.

The mutation lethal yellow (AV) has been particularly well studied since it is a homozygous lethal, with AV/AV embryos dying around implantation. Heterozygous AVA/AV mice are completely yellow and show a variety of abnormalities, including increased susceptibility to spontaneous and induced tumors, obesity, and insulin-resistant diabetes. Studies have shown that the AV mutation results from a deletion that brings the A gene under the control of an adjacent gene, RaLy, which is constitutively expressed in all tissues (Michaud et al. 1993, 1994). The recessive lethality is unrelated to the A gene but is the result of the disruption in RaLy, a ubiquitously expressed gene that encodes an RNA-binding protein implicated in premessenger RNA processing. The dominant pleiotropic effects of AV, on the other hand, appear to be the result of ectopic expression of A.

b (brown)

The wild-type allele at this locus, B, produces black eumelanin, whereas the most recessive allele, b, produces brown. AVA/b/b mice are known as cinnamon, a color produced by their yellow-banded brown hairs. A tyrosinase-related gene has been mapped to the brown locus (Jackson 1988).

c (albino)

The wild-type (C) allele is dominant over all mutations at this locus, which encodes the tyrosinase enzyme (Kwon et al. 1987). These mutants result in a deletion or alteration in the structure of tyrosinase and do not affect the number or distribution of the melanocytes. Albino mice (c/c) have no pigment at all either in the coat or in the eyes (Fig. 42), whereas other mutants at the c locus have altered pigmentation, e.g., chinchilla ch and chinchilla mottled cm (See Fig. 42). It is important to realize that not all "albino" mice have the same coat color genes; in the presence of c/c, the effects of changes at the A, b, and d loci are masked.

A cluster of radiation-induced mutations around the c locus (the "albino deletion complex") encompass at least six genes involved in embryonic and early postnatal development. Recent studies have shown that the neonatal lethality caused by one class of deletions is due to the absence of the enzyme fumarate hydratase, which causes pleiotropic changes in the morphology, metabolism, and
gene expression of liver and kidney cells (Grompe et al. 1993; Kesley et al. 1993). The molecular basis of other mutations in the complex causing embryos to die at specific stages of prenatal development (see, e.g., Niswander et al. 1989) is not yet known.

*d* (dilute)

The dilute locus is one of a class of genes affecting coat color through an alteration in the morphology of the melanocytes. Hundreds of different dilute mutations have been identified. In d/d mice, the pigment granules are clumped, and the melanocytes are less dendritic than those in wild-type mice; other alleles of dilute are also associated with neurological defects. It is now known that the d mutation in DBA/2J mice is caused by integration of an ectropic murine leukemia virus (MLV) retrovirus genome into the gene, which encodes a protein related to the myosin heavy chain (Mercer et al. 1991). The gene is normally expressed in melanocytes and in neurons of the central and peripheral nervous systems, but precisely how the protein functions is still a matter of active investigation. Another mutation that causes abnormal coat color through an effect on melanocyte morphology is bg (beige). The bg/bg mouse is a model of the Chediak-Higashi syndrome in humans and is associated with a defective morphology of membrane-bound intracellular organelles such as lysosomes and melanosomes (see Fig. 42).

**Spotting Mutations**

A particularly interesting class of pigmentation mutants are those known as "spotting" (e.g., Dominant white spotting [W], piebald [s], splotch [Sp], and belted [bt]). Examples of W and bt are shown in Figure 42. Among other factors, spotting genes affect the migration, viability, or differentiation of melanoblasts, the precursors of the melanocytes that arise from neural crest cells (see Development of the Nervous System, p. 83). Between 8.5 and 9 days of gestation, neural crest cells are still located near the neural tube; by 11 days p.c., they have reached the skin of the trunk, and by 12 days, they have reached the limbs. Genes affecting melanocyte migration and/or survival are therefore more likely to result in the lack of pigment in the hair follicles on the ventral surface, forehead, and extremities, since the cells must migrate further to reach these locations.

The W gene is now known to encode the transmembrane tyrosine kinase, c-kit, which is expressed in melanoblasts, as well as germ cells and hematopoietic cells (Keshet et al. 1991) (see Origin of Germ Cells and Their Migration to the Genital Ridges, p. 28). Homozygous null mutations of c-kit result in the complete absence of melanocytes in the skin. In heterozygous animals, some mutations cause a less severe "piebald" pigmentation trait both in mice and in humans (Fleischman et al. 1991; Giebel and Spritz 1991). Other mutations of W result in characteristic patterns of white (e.g., Wsaah; Duttlinger et al. 1993).

The spotting mutation Splotch (Sp) involves the paired-box gene, Pax-3. Heterozygotes have white belly spots and occasionally spots on the back and feet. Homozygotes die about 14 days p.c. with massive neurological defects, including exencephaly and spina bifida (Epstein et al. 1991), which can be related to the early expression of Pax-3 in the developing nervous system. Mutations in the human homolog of Sp/Pax-3 cause Waardenburg's syndrome in heterozygotes, which is associated with deafness and pigmentation defects (Tassabehji et al. 1992, 1993).
Figure 42 (a) CBA X C57BL/10 F$_1$ hybrid. This mouse is heterozygous agouti/nonagouti (A/a) and homozygous wild type at the brown locus (B/B) and therefore shows the dominant agouti phenotype of black hairs with a subapical yellow band (see text). The F$_2$ will segregate both black, nonagouti (a/a), and agouti (A/a, A/A) offspring in the ratio 1:3. The ears have been clipped as part of a numbering system (see Section B). An agouti, black mouse is generally described as "brownish-gray," and this is the basic color of wild M. musculus domesticus mice.

(b) Nonagouti C57BL/6 (a/a; +/+ ) (left) and a coisogenic mouse homozygous for the beige mutation which arose in the Jackson Laboratory (a/a; bg$^b$bg$^b$) (right). Coisogenic strains differ only at one locus, as a result of a mutation arising in an inbred strain. Beige is a recessive mutation that causes clumping and fusion of intracellular melanosomes in both the melanocytes of the skin and the choroid of the eye (which are derived from the neural crest) and the retinal epithelium (which is derived from the neurectoderell). The overall result is lightening of coat and eye color. Another effect of beige is the fusion of lysosomes in a wide variety of cell types, including liver, visceral yolk sac endoderm, proximal kidney tubules, and leukocytes. One consequence is that bg/bg mice have defective natural killer cells and abnormal secretion of lysosomal enzymes. The mutation has been widely used as a model for the Chediak-Higashi syndrome in humans (Silvers 1979; Brandt et al. 1981).

(c) Homozygous nude mouse (nu/nu) (outbred background). Nude is a recessive mutation affecting the ectodermal component of the thymus, producing a small, cystic gland which does not provide an environment for T-cell maturation. The skin has the normal number of hair follicles, but the hairs are abnormally keratinized and break off at the skin surface. Homozygous nu/nu mice are widely used in immunological research and for the establishment of transplantable human tumors (Nomura et al. 1977).

(d) Albino (c/c). The albino locus encompasses the structural gene for tyrosinase. The recessive mutation c at this locus alters the structure of the tyrosinase enzyme, rendering it inactive. Note that the mutation is expressed in both melanocytes and retina. Albino mice were recorded in Greek and Roman times, and the c locus was the first to be studied for Mendelian inheritance in mice.

(e) Chinchilla mottled, an example of an unstable gene and position-effect variegation. Chinchilla (c$^{ch}$) is a recessive mutation at the albino locus (c) which reduces tyrosinase activity and the deposition of yellow and, to a lesser extent, black pigment. The chinchilla mottled mutation (c$^{m}$) arose in the progeny of a neutron-irradiated wild-type male. The mouse shown here is homozygous (c$^{m}$/c$^{m}$) and has a fine-grained variegated pattern, the darker patches corresponding to melanocyte clones phenotypically chinchilla (c$^{ch}$) and the lighter patches to clones phenotypically extreme chinchilla (c$^{e}$) or albino (c). The switch must occur late in development because the patches are small and finely intermingled. Lightly pigmented cells also occur in the retina, but only comprise 1% of the total, hence the dark eyes. There may be an environmental or positional effect on the expression of c$^{m}$, as the belly hairs of c$^{m}$/c$^{m}$ are invariably lighter than dorsal hairs, and the unpigmented cells of the retina are clustered around the optic nerve. One suggestion is that the c$^{m}$ mutation resulted in the gene being shifted near to heterochromatin, which in some cells extends into the gene and partially inactivates it (Deol and Truslove 1981). Other explanations are possible, however.

(f) Dominant spotting. A large series of mutations has been recorded at the dominant white spotting locus (W), which encodes the c-kit transmembrane tyrosine kinase receptor for Steel factor (stem-cell factor, mast-cell factor). All of them affect the proliferation and migration of three different cell populations; the neural-crest-derived melanocytes, the hematopoietic stem cells, and the primordial germ cells. Mutations can therefore cause defects in epidermal but not retinal pigmentation, as well as anemia and infertility. The mutations are expressed in the migratory cells themselves, rather than in the environment into which they move, and the phenotype can vary depending on genetic background. The mouse illustrated here is heterozygous for the allele extreme spotting (W$^{e}$/+) and is on an
DISAGGREGATION OF CLEAVAGE-STAGE EMBRYOS INTO INDIVIDUAL CELLS

EQUIPMENT
Embryos that have been allowed to recover for about 1 hour after removing the zonae
Calcium-free M16 containing 6 mg/ml BSA rather than the usual 4 mg/ml M16 with 4 mg/ml BSA (Section G)
Bacteriological plastic culture dishes

This procedure has been described by Ziomek and Johnson (1980).

PROCEDURE

1. Incubate the embryos without zonae in calcium-free medium for 10–15 minutes in drops under oil at 37°C in a humidified incubator with 5% CO₂. Disaggregate the blastomeres by pipeting through a flame-polished glass pipet.

2. Remove blastomeres from the calcium-free medium as soon as possible. The isolated blastomeres are very sticky, so place them individually or in small groups in single drops of conventional M16 medium in petri dishes to which they will not adhere (e.g., bacteriological dishes made by Sterilin). Cell death may be reduced by transferring the embryos into calcium-containing medium before disaggregation by pipeting (information provided by C.F. Graham).
EQUIPMENT

Precompaction cleavage stage embryos (e.g., 4–8 cell)
Pronase
M2
Microdrops of M16 in tissue culture dishes

1. Remove the zonae with Pronase. Observe the embryos throughout the incubation and take them out as soon as the zona distorts and swells (3–10 min). Wash in M2 and transfer to M16.

2. Incubate at 37°C until the zonae have completely gone (5–30 min). (If the zona is still present, the embryos will not mingle.)

3. Put the embryos together in pairs or triplets in microdrops in M16. Then push the embryos together with a glass pipet or needle and handle the dish gently so that contact is not broken. After 1 hour of incubation at 37°C the blastomeres should have mingled into a single embryo. If not, nudge them together again. Continue incubation until the embryo has compacted (1 day of culture), or until it has developed into a blastocyst (2 days of culture).

4. At either stage, the chimera is transferred to the uterus of a 2.5-day p.c. pseudopregnant female.

Blastocysts have a better chance of implantation, but balanced against this is the risk of poor development in vitro if culture conditions are not ideal. If only a small number of chimeras are to be transferred, use a genetically different 2.5-day p.c. pregnant female so that the litter size is large enough (five to seven).
agouti (A/A)(C3H/HeX101/H)F1 background (Loutit and Cattanach 1983). Note the extensive nonpigmented (and therefore melanocyte-free) patches on the flank and back, and the white blaze on the forehead. Homozygous Wv/Wv mice die soon after birth from anemia. They are completely white with black eyes.

(G) Belted (bt) is another spotting mutation. The white patches are areas that the melanocytes fail to reach by migration, or in which they cannot survive or proliferate (Silvers 1979). The homozygote (bt/bt) here is on a nonagouti (a/a) background that has been selected for increased expression of the belted phenotype, so that the mouse has white spots on the head and shoulders in addition to the classical "belt."

(H) This mouse is homozygous for pudgy, pink-eyed dilution, chinchilla, and agouti (pu/pu; p/p; cbc; A/A). Homozygous pudgy mice have extensive defects in the axial skeleton and ribs. In particular, there is a reduction in the number of caudal vertebrae, which are also very disorganized. The limbs, however, are normal. Pudgy embryos can be recognized as early as 9 days p.c. In the caudal region, the paraxial mesoderm condenses but does not become divided up into discrete somite blocks (Grüneberg 1961). Pink-eye dilution (p) is, like albino, one of the old mutations of the mouse fancy, and 17 alleles are known at the p locus (Silvers 1979). In p/p mutations, the structure of the melanosome is altered, and it is now known that the gene encodes a putative transmembrane transporter protein that may be responsible for transporting tyrosine into the melanosome. The human homolog of p, known as D15S12, is deleted in patients with Prader-Willi and Angelman syndromes (Gardner et al. 1992; Rinchick et al. 1993). Unstable (pum) alleles exist in which the effect of the p mutation is variegated, the rate of reversion varying with age and genetic background. It has recently been shown that the pum mutation involves the interruption of coding sequences by a head-to-tail tandem duplication of genomic DNA, one copy of which appears to be lost in revertant mice (Gondo et al. 1993).