Manipulating the Mouse Embryo

A LABORATORY MANUAL
Second Edition

Brigid Hogan
Howard Hughes Medical Institute and Vanderbilt University Medical School

Rosa Beddington
National Institute for Medical Research, London

Frank Costantini
Columbia University

Elizabeth Lacy
Memorial Sloan-Kettering Cancer Center

Cold Spring Harbor Laboratory Press
1994

Developmental Genetics and Embryology of the Mouse: Past, Present, and Future

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 zebra fish (*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 pluripotential 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

Genome	
Number of chromosomes	40
Diploid DNA content	~6 pg (3×10^9 bp)
Recombination units	1600 cM (2000 kb/cM)
Approximate number of genes[a]	$0.5–1.0 \times 10^5$
Percent of genome as five families of highly repeated DNA sequences (B1, B2, R, MIF-1, and EC1)[b]	8–10%
Reproductive biology[c]	
Gestation time	19–20 days
Age at weaning	3 weeks
Age at sexual maturity	~6 weeks
Approximate weight	birth 1 g weaning 8–12 g adult 30–40 g (male >female)
Life span in laboratory	1.5–2.5 years
Average litter size[d]	~6–8
Total number of litters per breeding female	4–8

[a]McKusick and Ruddle (1977).

[b]Bennett et al. (1984).

[c]Parameters 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).

[d]Litter 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

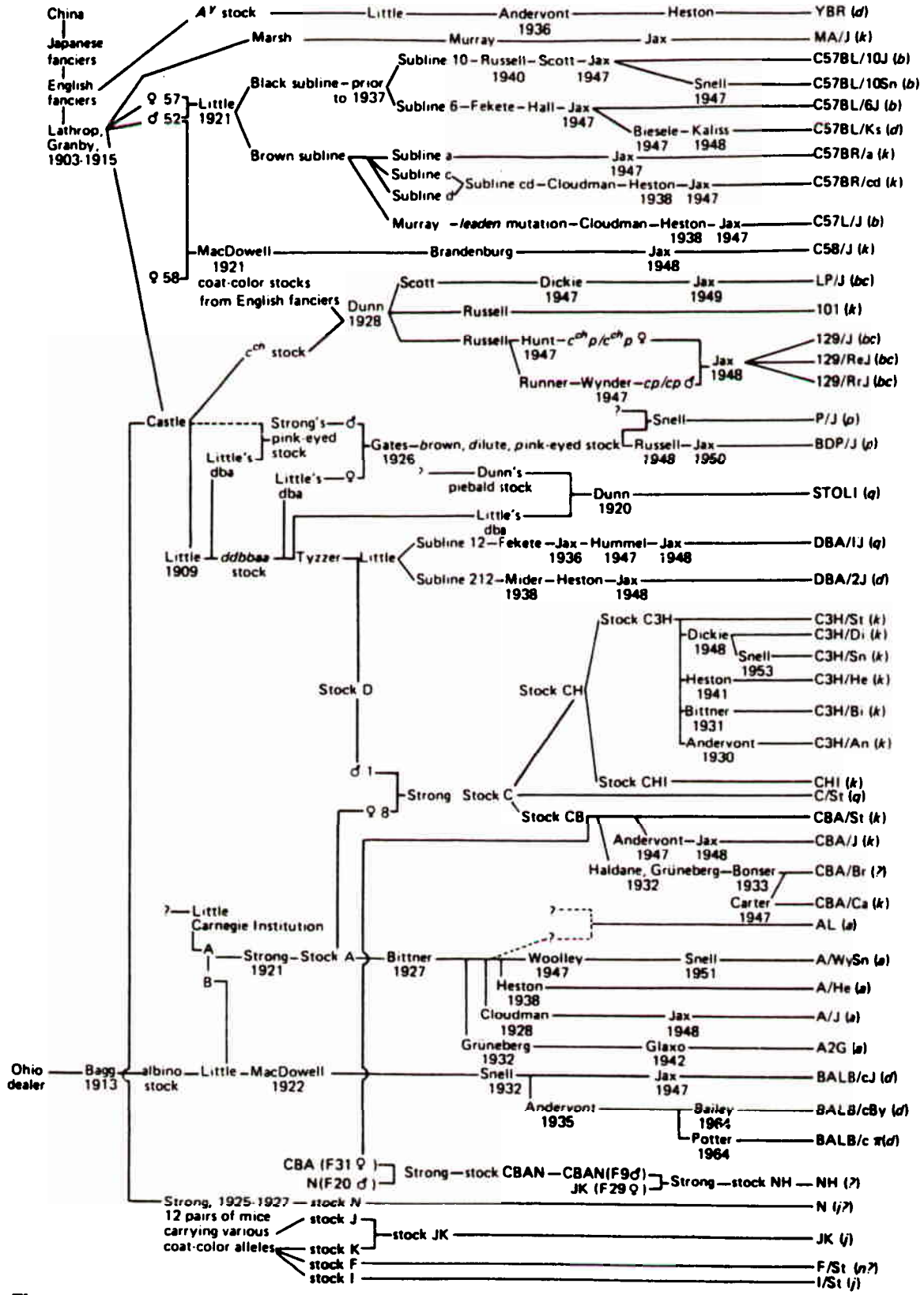

Figure 1 Genealogy of the more commonly used inbred mouse strains. This figure is based, in part, on data provided by Michael Potter and Rose Lieberman in 1967; it was extended by Jan Klein in 1975 and revised by Potter in 1978. *H-2* haplotypes are shown in parentheses. (Reproduced, with permission, from Altman and Katz 1979.)

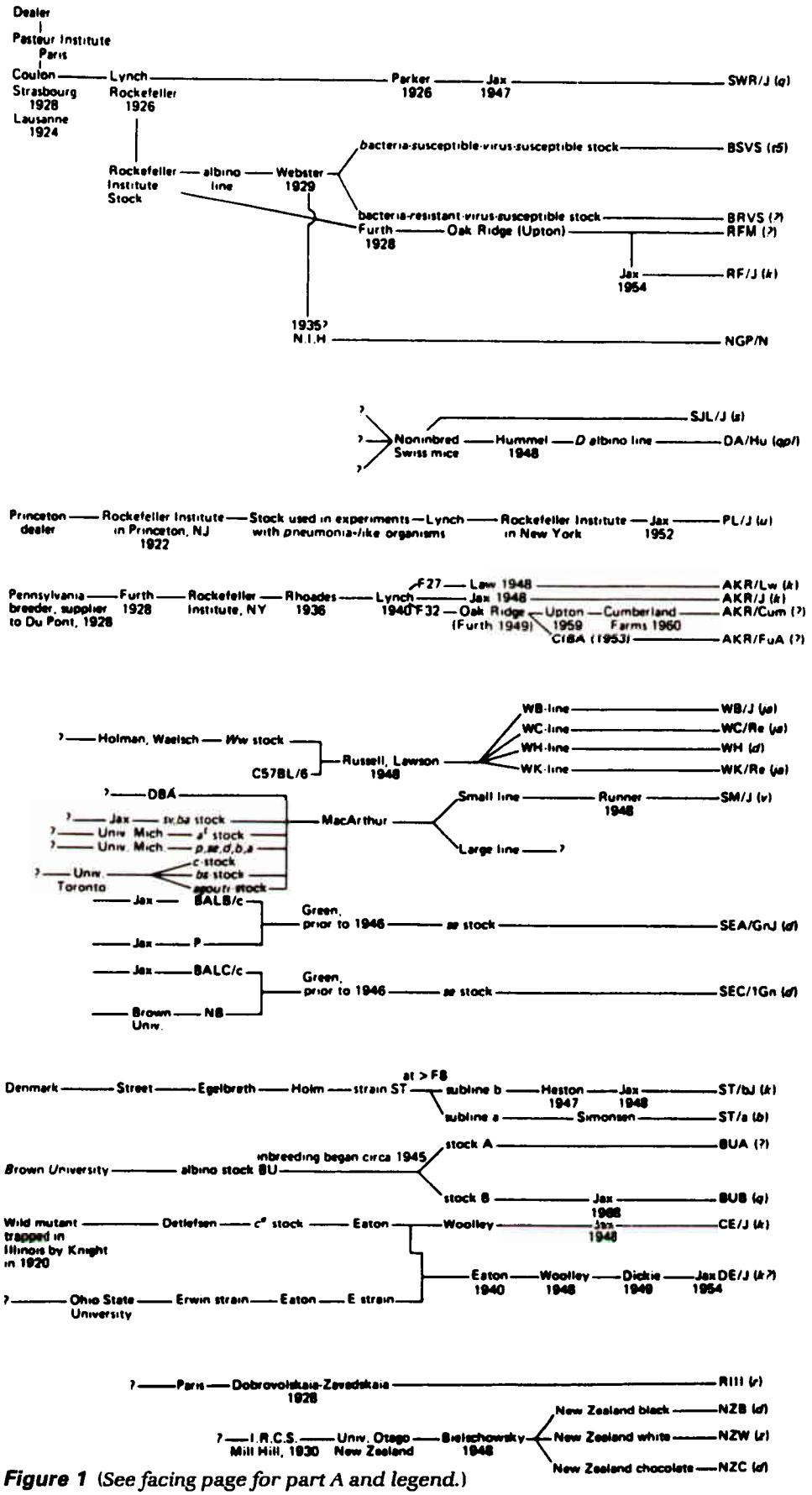

Figure 1 *(See facing page for part A and legend.)*

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

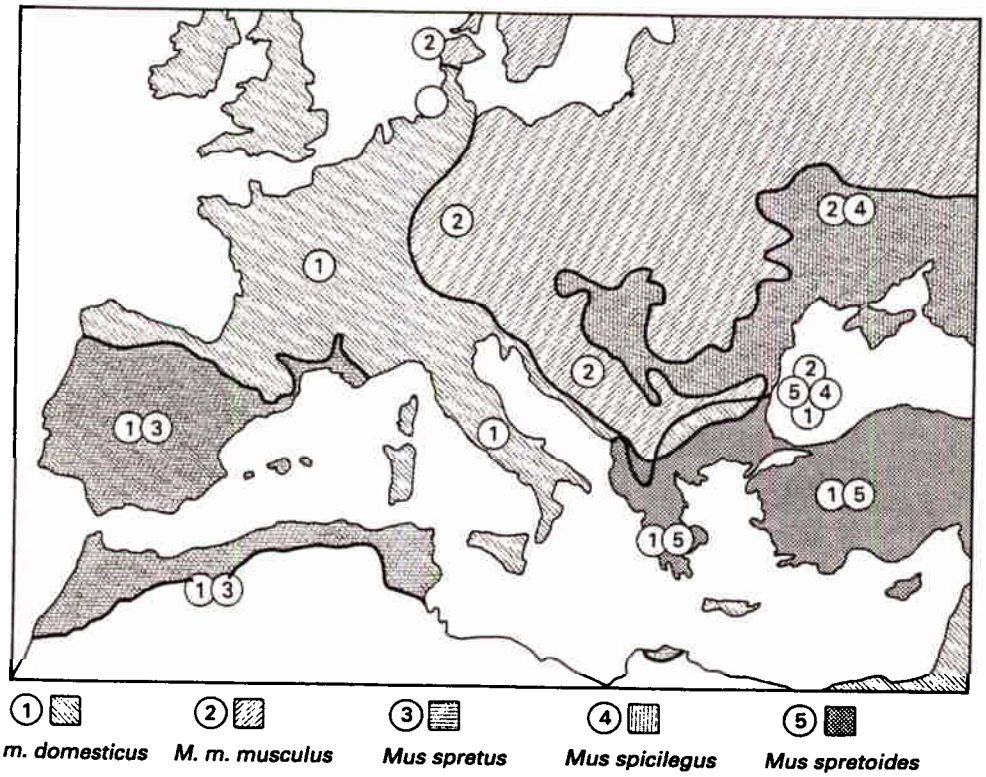

Figure 2 Geographical distribution of the five biochemical groups of the house mouse species complex in Europe. (Redrawn from Bonhomme et al. 1984.)

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 Lionelle 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* (*Fu*) 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-12^b is a strain in which the allele (H-12^b) 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 F_2 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
↓	
F_1	AaBbCc
↓	
F_2	AaBbCc x AaBbCc
↓	
Inbreeding for more than 20 generations	

RI Strain	1	2	3	4	5	6	7	8
	AABBCC	AABBcc	AAbbCC	AAbbcc	aaBBCC	aaBBcc	aabbCC	aabbcc
A	A	A	A	A	a	a	a	a
B	B	B	b	b	B	B	b	b
C	C	c	C	c	C	c	C	c

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 F_1 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 F_1 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)2H 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 (Luetteke 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

al. 1985). 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 (*b*one *m*orphogenetic *p*rotein-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-Zavadskaia, 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-Zavadskaia 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 Reith 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 Illmensee (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*^{r} 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 β_2-*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 neo^r 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.