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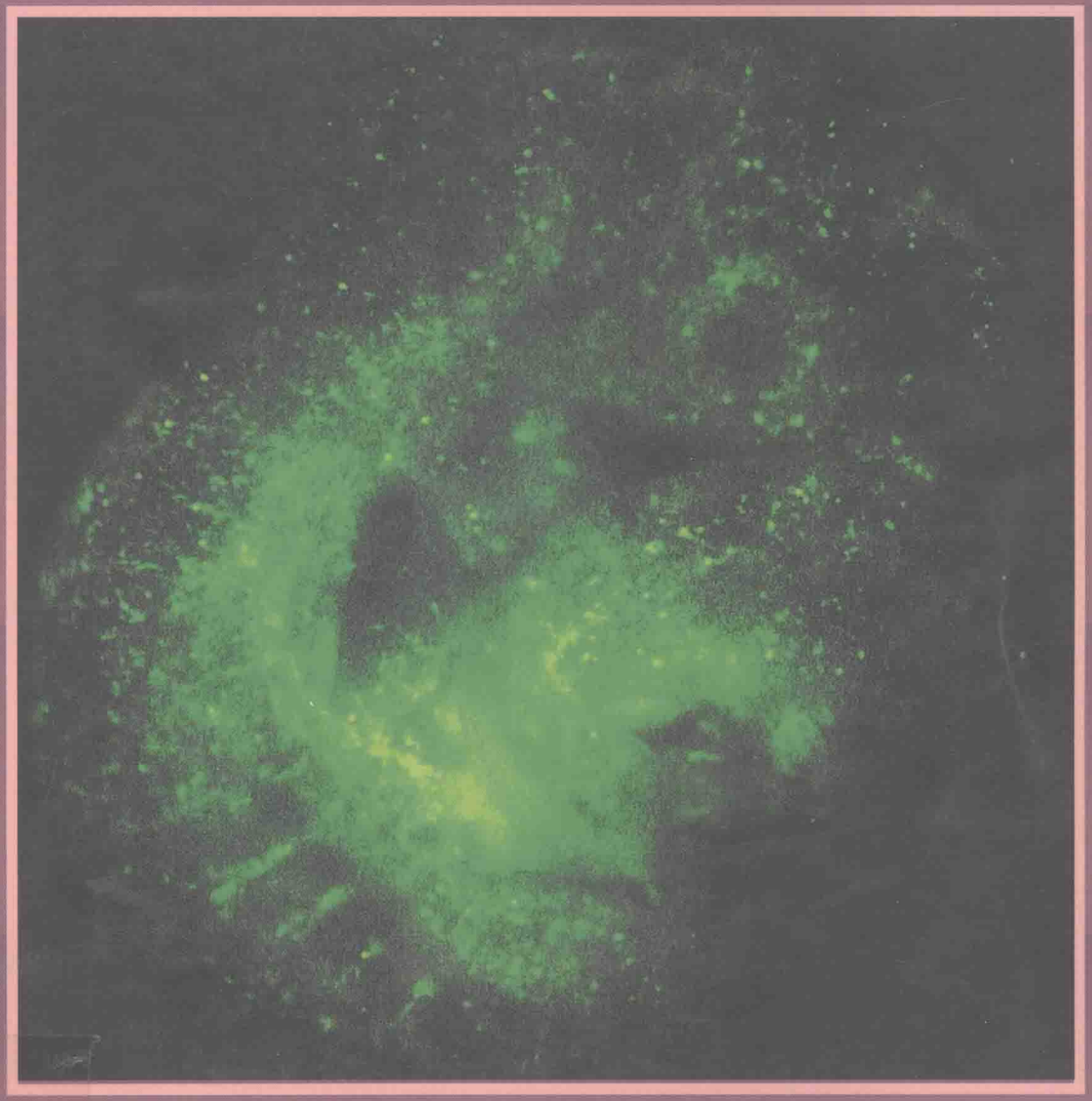
小鼠胚胎操作实验指南

Manipulating the Mouse Embryo

A LABORATORY MANUAL

（影印版）

〔加〕A. 纳吉　M. 格斯滕斯坦
〔德〕K. 文特斯滕　〔美〕R. 贝林杰　著



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内 容 简 介

小鼠是遗传学、发育学研究的重要模式生物之一，在功能基因组和后基因组时代，在生物医学科研和生物技术药物开发过程中，小鼠胚胎操作具有不可替代的作用，应用领域日趋扩大。本书是冷泉港实验室出版社 *Manipulating the mouse embryo: a laboratory manual* 的影印本，对小鼠的发育遗传学、胚胎学研究，小鼠发育，转基因小鼠、嵌合小鼠制备的普遍问题，移植后胚胎的复原、体外培养等方面的理论基础进行了翔实的阐述，并且对小鼠胚胎的一系列操作技术和方法进行了详细的说明，具有较大的实验指导意义。每一个方案都经过专家的精心挑选和雕琢，实验设计严谨、准确、简洁、规范，可操作性强，值得称道。本书的版式设计侧重于方便读者使用，正文中穿插了丰富的图表作为实验设计的辅助说明，附录中还列出基本溶液、缓冲液的配方和配制方法，以及实验注意事项等。

本书适合于从事发育生物学、细胞生物学、基因组学、遗传学、分子生物学、药物设计和开发以及功能基因组学研究的相关教学科研人员以及相关学科的本科生研究生参考使用。

正文内彩图集中列于书末彩色图版，请读者对应参考。
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Manipulating the Mouse Embryo Companion Web Site

Manipulating the Mouse Embryo: A Laboratory Manual, Third Edition, has a companion Web Site (www.mousemanual.org) that provides supplemental information about this field of research. The site will include:

- Suppliers' links and information
- References linked to Medline
- Links to other databases of value to working scientists

Additional information will be added after the book is published.

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Preface

IT HAS BEEN 20 YEARS SINCE THE FIRST Cold Spring Harbor Laboratory Molecular Embryology of the Mouse course that led to the initial publication of this manual. In 1983, the generation of transgenic mice by pronuclear injection of zygotes had only recently been accomplished and was far from being routine. Embryonic stem cell lines had only recently been produced and their germ-line potential and usefulness had yet to be demonstrated. Homologous recombination was still a dream. Large-scale mutagenesis in the mouse existed only in the minds of a few pioneering individuals. In addition, the molecular embryology of the mouse was still in its infancy. As we reflect back in time, it is astonishing how much progress has been made in the fields of mouse developmental genetics and molecular embryology. We now have a publicly assembled and annotated mouse genome available to all researchers. Genomic and cDNA clones can be easily identified in the databases, ordered, and received within days. Any gene can be added to the mouse germ line or altered by design. Chromosomes can be engineered. Living embryonic tissues can fluoresce brightly with a myriad of designer colors. Embryos and gametes can be cryopreserved and archived. Mice can be cloned. There seem to be no limits for utilizing the mouse to address fundamental biological questions and provide novel biomedical insights for human biology and disease.

As the mouse developmental genetics and molecular embryology fields have progressed and evolved, so has the "Mouse Manual." The current edition is built upon the solid foundation of the previous editions and the efforts of the original editors, Brigid Hogan, Frank Costantini, Liz Lacy, and Rosa Beddington. This new edition has been significantly reorganized, incorporating many innovations since the publication of the second edition in 1994. New chapters and protocols have been added, including mouse cloning, intracytoplasmic sperm injection, artificial insemination, cryopreservation of embryos and gametes, and guidance for current vector designs. New techniques such as the introduction of foreign DNA into mouse embryos by electroporation have been added. In addition, chapters on generating and analyzing transgenic mice and chimeras have been considerably expanded. All of the surgical techniques have been moved to a single chapter. Methods to visualize living embryos and new reporter genes such as fluorescent proteins have also been added.

We are very grateful to the many people who generously helped us produce the present edition. They provided new and updated protocols, figures, and images, and served as an incredibly helpful source of expert information. We thank (in alphabetical order) Kathryn Anderson, Gusztav Belteki, Sally Camper, Chris Cretekos, S. K. Dey, Mary Dickinson, Hao Ding,

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A.N., M.G., K.V., R.B.

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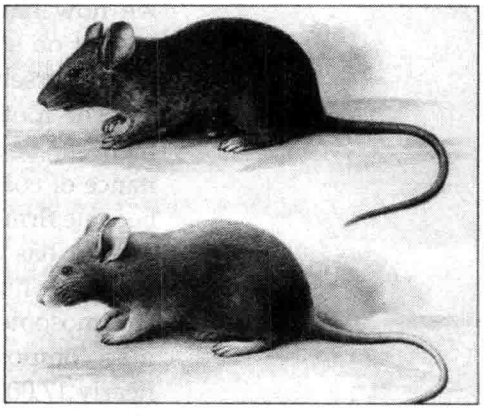
CHAPTER 1

Developmental Genetics and Embryology of the Mouse

Past, Present, and Future

THESE ARE PROBABLY THE BEST OF TIMES to be studying mouse genetics and embryology. Currently, the mouse germ line can be experimentally manipulated in almost every conceivable way either through direct injection of cloned DNA into zygotes or through the genetic modification of embryonic stem (ES) cells. Large-scale mutagenesis projects are yielding thousands of new mouse mutants. Fortunately, all of these mice do not have to be maintained "on the shelf" because mice can be archived by cryopreservation of embryos and gametes. Mice can now be routinely cloned by somatic nuclear transfer, creating new questions about genome programming. Finally, perhaps the most significant recent advance is the availability of the first public annotated assembly of the mouse genome sequence (MGSC Version 3), which will greatly facilitate biomedical research using mice (Lindblad-Toh et al. 2001; http://www.ncbi.nlm.nih.gov).



Courtesy of Ian Jackson

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INTRODUCTION

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. 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. The mouse genome, which is contained on a haploid set of 20 chromosomes, has been assembled and predicts 46,370 gene models. A physical map composed of nearly 300 bacterial artificial chromosome (BAC) contigs with nearly 17,000 markers is also now available (Gregory et al. 2002). There is extensive linkage conservation or synteny between the mouse and human genomes, so that progress with the Human Genome Project has contributed to knowledge of the mouse genetic map and vice versa (Copeland et al. 1993; O'Brien et al. 1994, http://www.ncbi.nlm.nih.gov/Homology/).

The techniques of molecular biology, including whole-mount in situ hybridization, reverse transcriptase-polymerase chain reaction (RT-PCR), DNA microarrays and sophisticated imaging methods, 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 2.5). However, the most compelling reason for excitement and optimism about studying developmental genetics in the mouse, instead of another vertebrate, 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* (1986) emphasized the potential importance of introducing new genetic information into transgenic mice by microinjecting DNA into the pronucleus of the zygote or by infecting embryos with retroviral vectors. The targeting of mutations to specific genes by

homologous recombination in pluripotential ES 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. Today, the technique has become 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 second edition of this manual (1994), like the first edition, provided a simple technical guide for scientists who wanted to learn some of the techniques for manipulating the mouse embryo and for introducing genes and mutations into mice. The current edition of this manual includes new and expanded chapters on ES cell genetic manipulations, mouse chimeras, mouse cloning, assisted reproduction strategies, and embryo and gamete cryopreservation. 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 1.1). Mice were also favored because 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. 2.42), 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 dilution* (see Fig. 2.42), 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 Castle and Haldane are the founding fathers of mouse genetics, then the mother is undoubtedly Abbie E.C. Lathrop. A self-made woman, Lathrop established around 1900 a small mouse "farm" in Granby, Massachusetts, to breed mice as

TABLE 1.1. Some vital statistics of the European house mouse, *Mus musculus*, in the laboratory

Genome	
Number of chromosomes	40
Diploid DNA content	~6 pg (2.6×10^9 bp)
Recombination units	1600 cM (2000 kb/cM)
Approximate number of genes[a]	46,370
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]Gene models, MGSC Version 3 (http://www.ncbi.nlm.nih.gov).

[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 Chapter 17; e.g., Altman and Katz (1979), Festing (1979), and Heiniger and Dorey (1980).

[d]Litter size depends on the number of oocytes 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 ~10–20% (for references, see Boshier 1968).

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 1.1, many of the old inbred strains can be traced back to the relatively small pool of founding mice that Lathrop maintained there. At present, more than 400 different inbred strains are available, and their origins and characteristics are listed in the *Mouse Genome Informatics* (MGI) database (http://www.informatics.jax.org/mgihome/genealogy).

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

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DNA (which is maternally inherited through the oocyte cytoplasm) has shown few differences among old established strains, compared with the wide variations seen among wild mice 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. 1.2) (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).

CREATION 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 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

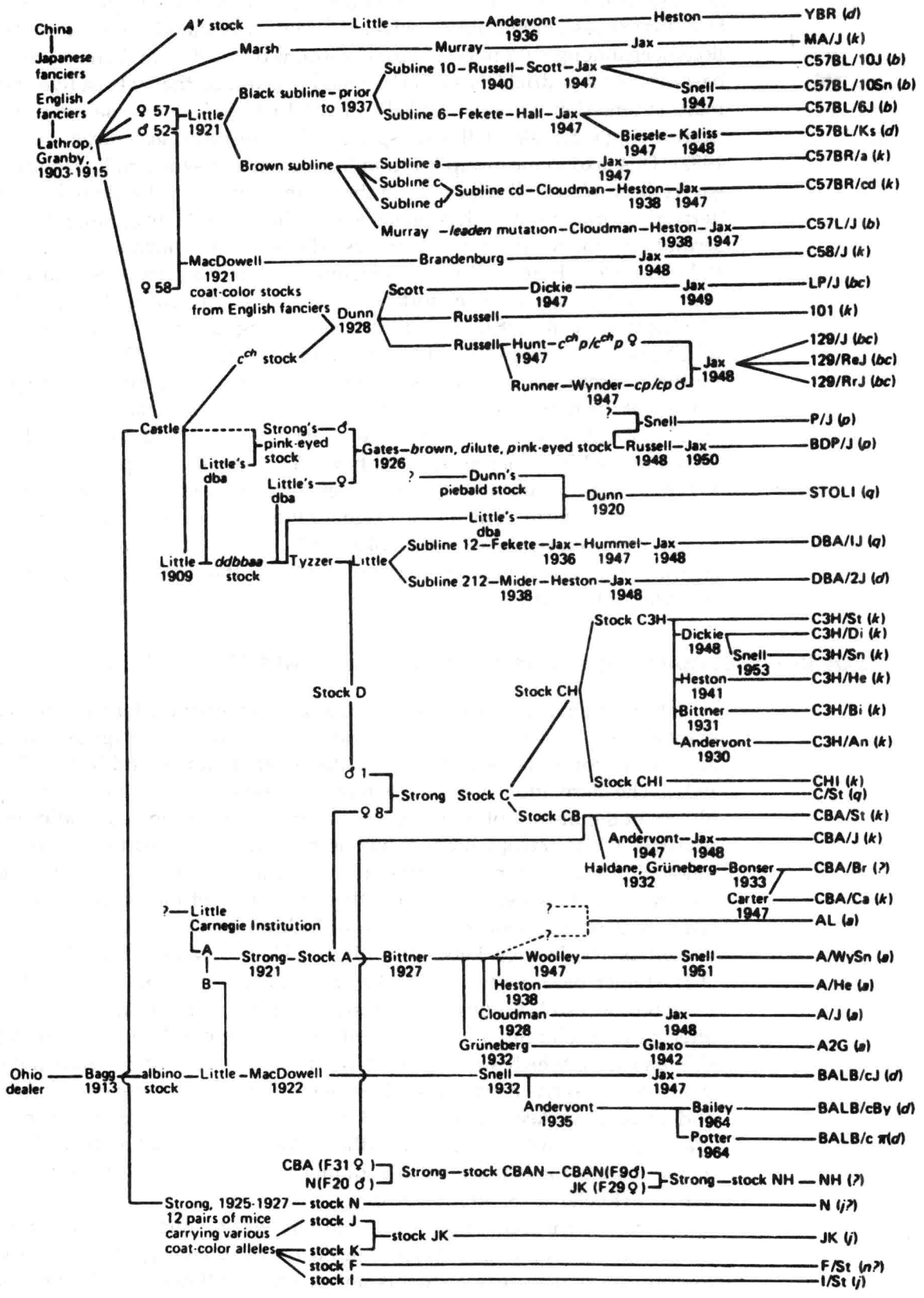

FIGURE 1.1. (*See facing page for legend.*)

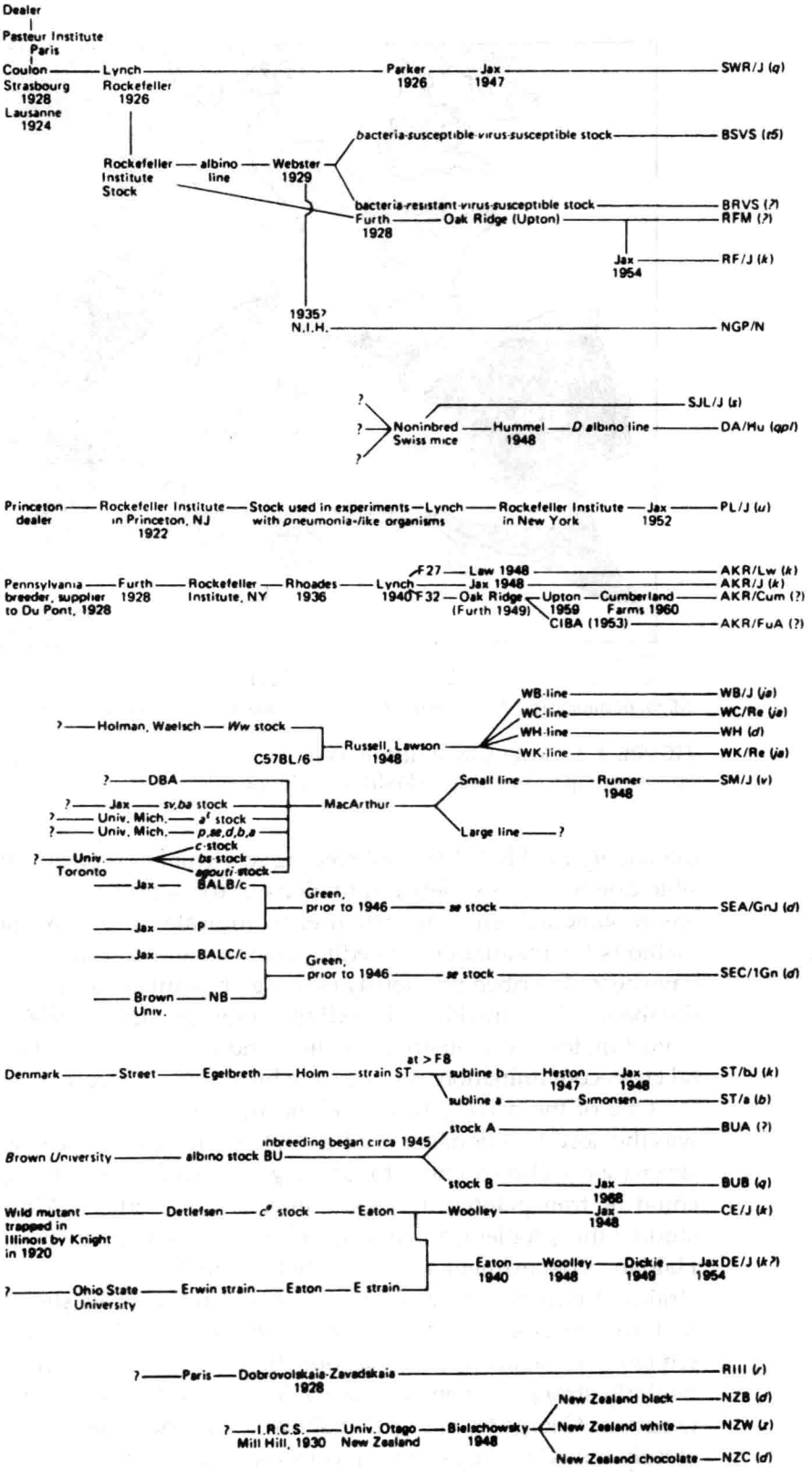

FIGURE 1.1. Genealogy of the more commonly used inbred mouse strains. This figure is based, in part, on data provided by Michael Potter and Rose Lieberman in 1967; it was extended by Jan Klein in 1975 and revised by Potter in 1978. H-2 haplotypes are shown in parentheses. (Reproduced, with permission, from Altman and Katz 1979.)

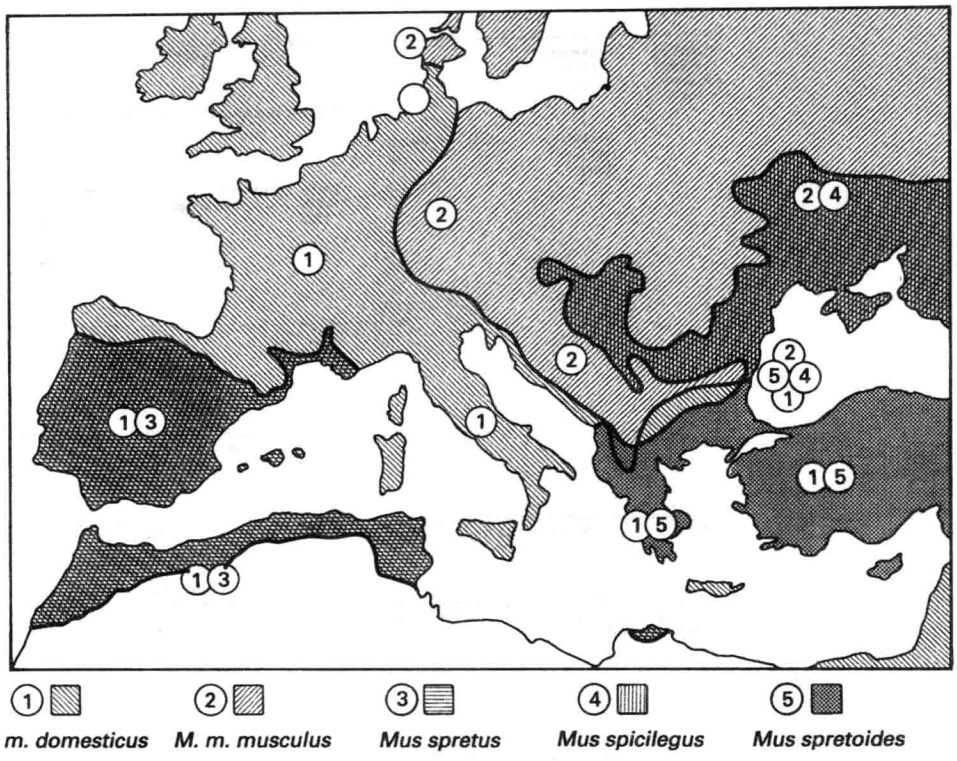

FIGURE 1.2. Geographical distribution of the five biochemical groups of the house mouse species complex in Europe. (Redrawn, with permission, from Bonhomme et al. 1984.)

the widely held belief that inbreeding to virtual homozygosity would be impossible due to recessive lethal mutations in the founding pairs. Each inbred strain has a standardized nomenclature, to indicate strain and substrain. 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 accidental cross-contamination of strains 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 *H2* (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, B6.C-*H1*^{b} *Tyr*^{c} *Hbb*^{d}/By is a congenic strain in which the *H1*^{b} allele derived from the BALB/cBy strain has been transferred onto the C57BL/6 inbred background. Amusingly, these mice are albino rather than black because the *Tyr*^{c} allele is tightly linked to the *H1*^{b} allele derived from the albino BALB/cBy strain. 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.

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 1.2). 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, provide a unique and expanding database for functional mapping of the mouse

TABLE 1.2. 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 AABBCC	2 AABBcc	3 AAbbCC	4 AAbbcc	5 aaBBCC	6 aaBBcc	7 aabbCC	8 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 segregrate and assort independently during the inbreeding process and eventually become genetically fixed. Each allele then has a unique strain distribution pattern (SDP).

genome. Another advantage is that because living animals are available, phenotypic differences in, for example, 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; however, purified DNA is available from The Jackson Laboratory. Initially, it was often difficult to find polymorphisms among the progenitor strains. This is due in part to the rather restricted origin of laboratory mice, as discussed in the previous section. An alternative mapping technique was developed 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. castaneus*) and *M. domesticus* have also been established.

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. 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. 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 book by Lee Silver (1994; publicly accessible through MGI).

ORIGINS OF DEVELOPMENTAL GENETICS OF THE MOUSE

Because of their availability from the mouse fanciers, many of the first mutants used in breeding experiments sported visible differences in coat color, hair morphology, and pigmentation patterns (see Fig. 2.42). 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*) 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

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