

Understanding the Human Condition: Experimental Strategies in Mammalian Genetics

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Abstract

Mice have become the mammalian model of choice for the application of genetics in biomedical research due to the evolutionary conservation of physiological systems and their attendant pathologies among all mammals as well as the exceptional power of genetic research technologies in the species. Beginning from aberrant phenotypes, a large number of mouse mutants and natural polymorphisms have been cloned, providing much information about the molecular basis of physiological processes. Additionally, the variable expression of these mutations in different inbred strain backgrounds has demonstrated the importance of modifier genes, which are also susceptible to cloning. Research efforts are keeping pace with these developments. In the area of gene discovery, large, government-funded mutagenesis programs now exist, and as a matter of great practical importance, recent evidence suggests that the same genes may be involved in the natural polymorphisms affecting disease in mice and humans. In parallel, dramatic advances are also being made in our ability to measure physiological processes in mice, and the advent of expression profiling promises revolutionary advances in understanding phenotype at the molecular level. Gene-driven approaches have relied on engineering the mouse genome, including adding, subtracting, and replacing genes and, most recently, the ability to control gene activity reversibly. Together, these multiple advances in our technical abilities have created extraordinary opportunities for future discovery.

Key Words: cloning; genetics; genetic engineering; genotype; mice; mutation; phenotype; polymorphism

Introduction

Genetically defined and engineered mice have become the mammalian models of choice in biomedical research for many reasons. Their biology and genome, about which we have accumulated a vast amount of information in the last century, is similar enough to humans' to be highly informative about our own species. Although yeasts, worms, and flies are excellent models for studying the cell cycle, cell fate, and numerous developmental processes in lower eukaryotes, mice are far more

suitable for studying both the normal function and pathologies of the immune, endocrine, nervous, cardiovascular, skeletal, and other complex physiological systems of mammals. All mammals share these basic systems and, as far as we know, develop the common diseases associated with them: cancer, atherosclerosis, hypertension, both types of diabetes, osteoporosis, glaucoma, and many others. All of these pathologies are found naturally in mice; and those that are not, such as cystic fibrosis and Alzheimer's, can be induced by manipulating the mouse genome and environment. Each of these diseases, whether spontaneous or induced, varies greatly in its expression among different mouse strains, reflecting the importance of modifier genes as major determinants of disease severity in mice as well as humans.

However, the most compelling reasons for choosing mice over other mammalian models derive from the extensive, sophisticated technologies we have for analyzing and manipulating the mouse genome. With mice, it is possible to replace existing genes with altered versions or to add entirely new genes, including functional human genes. Immunodeficient animals can be used as hosts for both normal and diseased human tissue. In addition, the murine genetic resources available for research purposes are exceptional: hundreds of inbred strains, thousands of single gene mutations, marker strains for analyzing chimeras at the cellular level, genetic mapping resources such as recombinant inbred and congenic lines, and chromosomal rearrangements and fusions. Moreover, all of this is available in a creature that is relatively inexpensive to maintain and reproduces several times a year.

We are beginning to see revolutionary advances in our understanding of mammalian biology in general and human biology in particular. Just as public health measures and antibiotics sharply reduced the incidence of infectious diseases in the 20th century, so these new understandings will dramatically alter the impact of physiological diseases in the 21st century. We can expect new therapeutics, early intervention, and ultimately prevention, all based on a molecular understanding of pathobiology and increasingly focused on our individual differences (both environmental and genetic).

Genetic Research Strategies Using the Laboratory Mouse

Genetic research in the laboratory mouse integrates two complementary approaches: phenotype-driven and geno-

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type-driven approaches. The former begins with phenotypes and attempts to discover the genes responsible for them. The latter begins with specific genes, alters them by genetic engineering, and examines the phenotypic consequences. Each approach has generated significant research findings on its own, and each is likely to benefit from continuing improvements in technologies and data management systems. Together, the two approaches comprise a gene discovery/analysis cycle so powerful that it is causing a revolution in scientific thinking as great as any in history.

Phenotype-driven Approaches

Spontaneous Single Gene Mutations

Single gene, point mutations are the classic substrates of genetics. Indeed, by 1980, when ethylnitrosourea was reported to be a very high potency mutagen for mice, there were already hundreds of spontaneous mutations that had arisen in various mouse colonies around the world (Green 1981; Lyon and Searle 1989; Lyon et al. 1996). Initially these mutations were important for establishing Mendelian inheritance in mice, exploring mechanisms of heredity, and creating genetic maps. Later, they were applied to studying biological processes. One early success was the discovery of the major histocompatibility complex (H2 in mice, HLA in humans), and another was the discovery of the importance of receptor-ligand interactions in cellular differentiation. The former discovery resulted from immunological studies related to tumor transplantation (Gorer 1948; Klein 1975). The latter came from bone marrow transplant studies between the *Steel* and *W* mutants and normal mice showing that the *Steel* and *W* genes determine the ability to send and receive signals essential for stem cell differentiation (McCulloch et al. 1964; Russell 1979; Russell and Bernstein 1968; Russell et al. 1968). This concept of extracellular signals and receptors was confirmed when molecular studies revealed that *W* encodes the c-kit tyrosine kinase receptor (Bernstein et al. 1990; Chabot et al. 1988; Geissler et al. 1988) and that *Steel* encodes its ligand, the Steel growth factor (SLF) (Brannan et al. 1991; Flanagan et al. 1991).

Important insights into modifier genes were provided by the *obese*, *ob*, and *diabetes*, *db*, mutations, which originally arose in the C57BL/6J (B/6) and C57BLKS/J (BKS) mouse strains, respectively. As the names indicate, these mutations originally caused distinct phenotypes, *ob* causing obesity, and *db* causing both noninsulin-dependent diabetes and obesity. However, this difference in phenotype actually reflected differences in the genetic backgrounds of the inbred strains and not the mutations themselves (Coleman 1978; Coleman and Hummel 1973): On a B/6 background, *ob* and *db* mutations caused only obesity; on a BKS background, they caused both obesity and diabetes. These results provided the first clear evidence of physiological modifier genes segregating among inbred strains, and researchers now routinely place mutations on different genetic back-

grounds to search for modifiers. Thus, polymorphic modifiers have provided many valuable biological insights, one of which is that they are probably responsible for the wide range of phenotypes that human mutations commonly express. Nadeau (2001) has provided a review of natural polymorphisms acting as genetic modifiers in mice.

When Coleman (1973, 1978) joined *ob* and *db* mutants as parabiotic pairs so that each shared the other's circulation, the *ob* mutant behaved as if it lacked a satiety factor that controlled food intake, and the *db* mutant behaved as if it lacked the receptor for that factor. This insight was confirmed when Friedman's group cloned the *ob* gene and showed that it encodes leptin, a diffusible peptide controlling food intake (Zhang et al. 1994). Later, the *db* gene was cloned and shown to encode the leptin receptor (Barinaga 1996; Chen et al. 1996; Chua et al. 1996; Tartaglia et al. 1995).

During the mid-1990s, researchers cloned other obesity-causing mutations, including the *Ay* (yellow) allele of *agouti* (Miller et al. 1993), *Cpe^{fat}* (Naggert et al. 1995), and *tubby* (Kleyn et al. 1996). As a result, some types of obesity are now understood to be a consequence of defects in the neural signaling pathways that regulate food intake and metabolism.

It was not long before cloning single gene mutations became a virtual cottage industry, and many scores of mutants have now been successfully identified at the molecular level.

Induced Mutations

Mutational analyses of physiological pathways are now being substantially accelerated with the use of powerful mutagens (e.g., ethylnitrosourea), which enhance natural mutation rates by a thousand-fold or more. As a result, it is now feasible to use sophisticated phenotyping techniques that were previously too expensive to apply on a large scale.

The first mouse mutagenesis projects to search for specific phenotypes were modest. Two early successes were the identification of the *min* mutation, the mouse equivalent of the adenomatous polyposis coli mutation in humans (Moser et al. 1990, 1993), and the *clock* mutation (King et al. 1997; Vitaterna et al. 1994), which alters circadian rhythms by encoding a transcriptional element whose regulation feeds back on itself. Various agencies then funded significantly larger programs, first in Europe (Hrabe de Angelis et al. 2000; Nolan et al. 2000) and Australia and later in the United States and Canada (Nadeau et al. 2001). One cluster of National Institutes of Health programs focuses on neurological and behavioral disorders and another on mutations affecting heart, lung, blood, and sleep disorders.

Ultimately, the utility of these efforts will depend greatly on our ability to clone the induced mutations efficiently. Although still a relatively laborious process, positional cloning is easier than it used to be because bacterial artificial chromosome contigs of human and mouse geno-

mic DNA are available in public databases. First, mutant and wild-type mice must be selectively crossed to produce hundreds, often thousands, of offspring to localize the mutation genetically to a chromosomal region comprising a few hundred kilobases of DNA. Then, markers flanking the region are used to identify the region's clones and candidate genes from the assembled human and mouse genome sequences. The candidate genes in the region are evaluated, and the most promising one is sequenced in detail in both mutant and wild-type mice to find the mutated site. The putative assignment must then be confirmed by identifying independent mutant alleles of the same gene or by reiterating the phenotype in appropriate transgenic or knockout mice. Positional cloning will become even easier as the publicly assembled sequence of the mouse genome becomes increasingly available.

From this description, it is apparent that positionally cloning a gene is easier when multiple mutant alleles exist and when the mouse genomic sequence is already available. It would be even easier if we could (1) increase recombination frequencies in mice and thereby reduce the very large cost and animal space required to construct high resolution genetic maps; (2) reclon the equivalent bacterial artificial chromosome from multiple DNA sources by using techniques similar to transformation-associated recombination cloning (Larionov 1999), thereby making it easier to compare homologous wild-type and mutant DNA sequences; and (3) reliably identify the site of single base pair differences between mutant and wild-type DNA in several hundred kilobase stretches without having to sequence them fully.

Natural Polymorphisms

Naturally occurring polymorphisms (NPs¹) complement the information obtained from single gene mutations by describing both natural variation and the genetic interactions important in most serious human diseases. They can identify critical control steps in complex physiological processes, thus defining a subset of genes whose products provide particularly attractive targets for therapeutic intervention. They also can predict the location of human quantitative trait loci (QTL¹), a matter of great practical consequence. The basic strategy for locating NPs is to intercross a pair of inbred mouse strains that differs substantially in the trait of interest and then, using sets of markers that cover as much of the entire genome as possible, identify chromosomal regions determining phenotypic differences among the progeny. When possible, it is advantageous to make the cross between a pair of strains that have been used previously as the progenitors of a set of recombinant inbred

lines. This cross makes it possible to study segregation of the trait among the recombinant inbred lines as well as the progeny of a conventional cross.

NPs were initially used in the early decades of the 20th century to prove that Mendelian factors determine tumor transplantation in mice, and they ultimately led to the discovery of the major histocompatibility complex. Snell's work analyzing the major histocompatibility complex by making H-2 congenic strains, for which he received the Nobel prize, also identified a series of minor histocompatibility genes as NPs that acted cumulatively. (For a historically interesting review, see Snell and Stimpfling 1966.) We now call such genes QTL.

The analysis of QTL was stimulated by a highly influential paper in which Lander and Botstein (1989) proposed a method of interval mapping across an entire genome that provided both a means of locating QTL and calculating the fraction of the total phenotypic variance that is determined by each QTL. (For a review of the statistical approaches used, see Broman 2001.)

The earliest efforts to apply QTL analysis in mammals examined insulin-dependent diabetes (type I) in crosses between susceptible nonobese diabetes (NOD) mice and other resistant strains (Prins et al. 1993; Rodrigues et al. 1994). Such experiments were feasible in mammals based on the development of microsatellite or simple sequence repeats (Dietrich et al. 1994, 1996; Rassmann et al. 1991; Tautz 1989) as marker systems that could be used on a genome-wide scale.

Since the initial efforts on diabetes, QTL have been located for a wide variety of phenotypes (Rikke and Johnson 1998), including atherosclerosis (Hyman et al. 1994; Ko et al. 2001; Mu et al. 1999; Paigen et al. 1987a,b; Pitman et al. 1998; Purcell et al. 2001); epilepsy (Frankel et al. 1995a,b; Gershenfeld et al. 1999; Legare et al. 2000; Maihara et al. 2000; Miner and Marley 1995); obesity (Brockmann et al. 2000; Horvat et al. 2000; Schalling et al. 1999; Taylor et al. 1999, 2001); asthma (De Sanctis and Drazen 1997; De Sanctis et al. 1999; Dwyer-Nield et al. 2000; Ewart et al. 2000; Nicolaides et al. 1997; Symula et al. 1999); lupus (Gu et al. 1998; Santiago et al. 1998); osteoporosis (Benes et al. 2000; Hosoi 2001; Shimizu et al. 1999); hypertension (Sugiyama et al. 2001a,b); and a variety of cancers including lung (Festing et al. 1994, 1998; Lin et al. 1998), skin (Nagase et al. 1996, 1999, 2001), and colon (Gould and Dove 1998; MacPhee et al. 1995).

Because of the great interest in QTL analysis, much effort is currently being devoted to develop single nucleotide polymorphisms (SNPs¹) (Lindblad-Toh et al. 2000; Riley et al. 2000) as genetic marker systems in mice. SNPs are very abundant (occurring approximately 1/1000 base pairs in both mouse and human DNA) and, if successfully developed, may substantially reduce the present high cost of genotyping. Much of the technology development is coming from efforts to use SNPs in human genetic studies.

The major drawback of NPs in contrast to single gene mutations is the substantially greater difficulty of position-

¹Abbreviations used in this article: ES, embryonic stem; NP, naturally occurring polymorphism; QTL, quantitative trait loci; SNP, single nucleotide polymorphism.

ally cloning them. The first step of accurately mapping an NP depends on the magnitude of the difference between the phenotype of interest in two inbred mouse strains and the fraction of the phenotypic variance the NP contributes. The larger the absolute magnitude of an NP's individual effect, the easier it is to locate, which explains why positional cloning of NPs has proved much easier when only one or a few of them are operative in a genetic cross. The subsequent assignment of a phenotype difference to a particular NP is also difficult because there are many more nucleotide differences between the alleles of two inbred mouse strains than there are between a mutant allele and its wild-type (where there is presumably only a single difference).

Despite these difficulties, a number of NPs in mice and rats have been identified: hearing loss (Ikeda et al. 1999; Johnson et al. 1997, 2000; Noben-Trauth et al. 1997; Shastry 2000), two loci determining gallstone formation (Paigen et al. 2000), two for glaucoma (S. W. M. John, The Jackson Laboratory, unpublished results, 2001), hypertension in rats (Cover et al. 1995; St. Lezin et al. 1999), defective fatty acid and glucose metabolism in hypertensive rats (Aitman et al. 1999), and modifiers of intestinal neoplasia (Cormier et al. 1997).

Because QTL represent chromosome regions rather than single genes, and their associations with particular phenotypes are often subtle, their underlying genes have been difficult to identify. That situation is rapidly changing. Some notable examples in rodents are the identification of *Pla2g2a* polymorphisms as associated with differential susceptibility to intestinal tumorigenesis in mice (Cormier et al. 1997), of *Il4* and *Il13* polymorphisms as associated with asthma phenotypes in mice (Symula et al. 1999), of *Pctrl* polymorphisms as associated with differential susceptibility to plasmacytomas in mice (Zhang et al. 2001), of *Cd36* (*Fat*) as associated with defective fatty acid and glucose metabolism in rats (Aitman et al. 1999), and the implication of GABAergic genes as associated with alcohol and barbiturate withdrawal in mice (Buck and Finn 2001).

The drive to clone oligogenic NPs and locate chromosome regions for polygenic NPs will continue for several reasons: The genes underlying them are likely targets for therapeutic intervention; they represent epistatic gene interactions important in disease processes; and, most importantly, they are likely to predict the location of homologous QTL in humans (see below).

Concordance

As a matter of great practical consequence, recent evidence suggests that the same genes may be involved in the polymorphisms affecting disease susceptibility in mice and humans. Sugiyama and colleagues (2001 a,b) showed that seven of eight hypertension QTL identified in mice are concordant with homologous chromosomal locations associated with human hypertension, and that six of the eight are concordant in mice, rats, and humans. Fragmentary data suggest

the same may be true for several autoimmune diseases (Becker et al. 1998; Morel et al. 2001) and asthma (Zhang et al. 1999). The extent of genetic concordance will be revealed as additional human and mouse mapping data are compared.

Given the very large number of genes at which variation can theoretically affect a phenotype, the existence of substantial concordance between species suggests that phenotypic variation is particularly sensitive to quantitative variation in a small subset of genes—those coding for proteins that act at especially critical steps. These proteins obviously offer attractive targets for therapeutic or preventive intervention. We can detect concordance of mouse and human QTL not only because the vast majority of mammalian genes are held in common, but also because the genomes of various orders of mammals are arranged in a very similar genetic order—in large conserved blocks of chromosomal material. Nadeau and Taylor's (1984) original estimates suggested that the mouse and human genomes consist of 125 to 150 conserved chromosome segments, with the order of the segments differing between mouse and man. As our knowledge of the detailed structure of mammalian genomes has progressed, these preliminary estimates have been borne out. Although there are small internal rearrangements and gene duplications/deletions within these conserved blocks, to a great extent the location of a human gene predicts the location of its homologous mouse counterpart and vice versa (Copeland et al. 1993; Eppig and Nadeau 1995).

Cross-species concordance for major disease QTL has very large economic implications. Identifying the human chromosomal locations associated with hypertension took 5 yr and cost about \$35 million. In contrast, identifying hypertension QTL in the mouse study of Sugiyama and co-workers (2001a,b) required less than 1 yr and cost less than \$0.25 million. Moreover, recent estimates of the cost of identifying QTL in human populations by marker association (linkage disequilibrium), the most optimal approach at present, are dismaying. The average human DNA sequence that has not been disrupted by genetic recombination in the course of human history may average only 3 kilobases long (Kruglyak 1999). Given the nearly 3 billion base pairs in the human genome, fully genotyping one person at \$1.00/marker would cost at least \$500,000 with perfectly spaced markers, and probably much more. Even when genotyping costs are considerably reduced, a single study will likely cost tens to hundreds of millions of dollars. Recent evidence that even smaller lengths of DNA are not in linkage disequilibrium only complicates matters (Ardlie et al. 2001). At least for some time, a strategy of locating QTL in mice and then confirming their presence in humans has considerable attraction.

Expression Profiling

Expression profiling, the most recent revolutionary advance in phenotype-driven strategies, provides a quantitative de-

scription of the expression of thousands (even tens of thousands) of genes at one time (Eisen and Brown 1999; Harrington et al. 2000; Lipshutz et al. 1995; Ramsay 1998; Shatky et al. 2000). Profiling offers as radical an advance in technology over previous manual methods for assaying mRNA, which measured one or a few sequences at a time, as did automated DNA sequencing over hand sequencing. It will undoubtedly revolutionize our approaches to functional genomics.

Two expression profiling systems are available: Affymetrix chips and deposited arrays. In the Affymetrix system, 20 permuted DNA sequences for each of thousands of genes are deposited on chips in separate spots by a process similar to photolithography. In the deposited array system, thousands of microdroplets, each containing a different DNA sequence (either cDNA clones or synthesized oligonucleotides), are deposited on glass slides. In both systems, fluorescence-labeled cDNA prepared from mRNA samples is then hybridized to the arrays and the fluorescence is quantitated. Although Affymetrix chips were available first, deposited arrays are gaining popularity because they are substantially cheaper and more easily reconfigured.

Understandably, expression arrays have generated great interest. In one of the earliest and influential examples, they were used to analyze mRNA population changes in yeast cells grown under different environmental conditions (Lashkari et al. 1997; Wodicka et al. 1997). Recently, they have attracted enormous attention because they can distinguish tumor types that are difficult to characterize by histopathology and can sometimes identify the cell types from which tumors originated by the residual, differentiated molecular functions these cells retain (Horvath and Henshall 2001; Kallioniemi 2001; Polyak and Riggins 2001). Because prognosis and response to chemotherapy correlate with a molecular classification of tumors, it is now a major challenge to identify the mouse counterparts of each human tumor type so that they can be used as models for developing therapy.

In sum, expression arrays provide a means of describing cell processes in exquisite molecular detail on a scale never before possible. It is difficult to overestimate their impact.

Genotype-driven Approaches

Experimental biology normally requires a means of perturbing a system and then studying the outcome. The ultimate extension of this strategy is altering the genome itself.

DNA Transgenesis

To introduce a new gene into a mouse, the relevant DNA sequence is injected into the pronucleus of a fertilized egg, where it can incorporate randomly into chromosomal DNA. The embryos are inserted into appropriate female recipients

and develop into live animals, several of which carry new genetic material.

Within a matter of months around the beginning of 1980, six separate laboratories reported constructing transgenic mice (Brinster et al. 1981; Constantini and Lacy 1981; Gordon et al. 1980; Harbers et al. 1981; Jähner and Jaenisch 1980; Wagner et al. 1981a,b). Gordon and colleagues (1980) constructed the first mouse; however, the most conceptually radical one was constructed by Wagner and co-workers (1981b), who documented that a rabbit DNA sequence could interact with the mouse genomic regulatory apparatus to make a mRNA that could be translated into a functional protein, β -globin, all in the appropriate tissue-specific manner.

Before then, no one had understood the extent to which evolution had conserved regulatory processes, and no one had ever been able to transfer genetic material across the species barrier, much less between orders as different as lagomorphs and rodents. Now, 20 yr and innumerable examples later, we accept that evolution has proceeded very conservatively at the molecular level, and that comparative genomics, especially comparing mice and humans, can be a major source of biological insights.

Knockouts

In the late 1980s, mammalian genetic research was transformed again when homologous gene replacement or “knockout” techniques were developed. Because the location and copy number of the transgene in transgenic mice are different in every recipient, the level of gene expression is not uniform in different lines. Therefore, transgenics are primarily informative for dissecting the functional anatomy of a gene. In contrast, homologous recombination replaces an endogenous gene with an altered copy of the same gene, providing a uniform insertion point and copy number and preserving the adjacent DNA regulatory sequences.

The first steps in developing knockout technology involved learning to culture mouse embryonic stem (ES¹) cells in vitro and then using those cells to repopulate blastocysts (Evans and Kaufman 1981; Martin 1981). Among the mice arising from these chimeric blastocysts, the inserted ES cells could contribute to *all* tissues, germline as well as somatic. Taking advantage of the fact that homologous recombination had been shown to occur (albeit at very low frequency) in other cultured somatic cells, several groups applied these latter approaches to ES cells (Doetschman et al. 1985, 1987; Thomas and Capecchi 1987). ES cells could now be cultured and transfected with a specific DNA sequence; and those few undergoing the desired homologous recombination could be selected and introduced into mouse blastocysts, which then developed into offspring carrying the altered gene (Chisaka and Capecchi 1991; DeChiara et al. 1990; Schorle et al. 1991; Schwartzberg et al. 1989; Zijlstra et al. 1989).

Initially, the technique was used to produce “knock-

outs,” null mutants in which the function of a gene was lost in all cells. Very often, the phenotypes of such mice have been surprising and have emphasized our limited understanding of biological processes. In one early example, brilliant experiments with cell cultures had indicated that the *myoD* gene was essential for muscle differentiation (Weintraub et al. 1989); however, when *myoD* gene knockouts were constructed, they were relatively healthy (Rudnicki et al. 1992). The *myoD* gene proved “redundant,” and researchers subsequently discovered alternative means of accomplishing its regulatory processes (Arnold and Braun 1996; Dias et al. 1994; Megeney et al. 1996; Rudnicki et al. 1993). Indeed, functional redundancy has become a recurring theme in mammalian genetics.

Experiments with p53 knockout mice have also given unexpected results. Cell culture studies had suggested that p53, the most frequently mutated gene associated with human tumors, is essential for normal cell cycle function. Knockouts were confidently expected to be cell lethals, causing embryos to die in utero; however, the first homozygous p53 knockouts were born alive, running around their cages a few weeks later and forcing a dramatic re-evaluation of p53 function (Donehower et al. 1992; Jacks et al. 1994). The reassurance that p53 function is indeed important in neoplasia came as the mice developed multiple tumors several months later.

As time progressed, researchers sought more sophisticated knockouts. Mutants that died when a knocked out gene first became essential during embryonic development revealed nothing about gene function later in life, and a gene whose function was missing in all cells revealed nothing about the tissue specificity of its action. In response, timed, tissue-specific deletions were developed by flanking genes with sequences responding to either the Cre or Flp microbial recombinases (Meyers et al. 1998). Mice with such flanking sequences can be crossed with animals expressing these recombinases under the control of tissue-specific promoters. Among these progeny, the desired gene is deleted only in tissues expressing the recombinase.

Genes can also be reversibly controlled by inserting nonmammalian regulatory systems, such as the bacterial tetracycline repressor system, into their promoter regions; the repressor system can be regulated externally by the presence or absence of antibiotic analogs in drinking water. A particularly elegant example of functional analysis involving reversible gene activation is the work on several neural proteins in learning and memory (Malleret et al. 2001; Mansuy et al. 1998a,b; Mayford et al. 1996). Most recently, the bacterial lac repressor system, about which so much is known, has been adapted to regulate *in vivo* gene expression in the mouse (Cronin et al. 2001).

Phenotyping as a Limiting Factor

As powerful as our tools for discovering and analyzing mouse genes have become, they are limited by our ability to

determine phenotypes accurately, especially at the physiological level. Many molecular and cellular processes can be assayed quite well—metabolites and proteins by their activity or immunological characteristics, and thousands of mRNAs simultaneously by expression arrays. However, the technical problems of assaying many physiological and behavioral phenotypes remain a challenge, particularly for QTL analyses, in which robust and reproducible measurements are so important.

Mouse Size

Phenotyping an animal as small as a mouse can present its special challenges. Although methods developed for larger animals often have been adapted to mice, sometimes it has been necessary to develop novel methods specifically for mice. For example, using the mouse to investigate the genes that confer atherosclerosis susceptibility required devising not only an appropriate high fat and cholesterol diet for mice (Paigen et al. 1985) but also a standard and reproducible technique for measuring aortic lesions in the animals (Paigen et al. 1987a,b). Using the mouse to investigate the genetic factors contributing to osteoporosis required imaging and quantifying bone density in volumes of 1 mm³ or less (Nagy and Clair 2000). Using the mouse to identify QTL governing susceptibility to glaucoma and its attendant retinal nerve degeneration required developing a technique to measure intraocular pressure in the 5- μ L volume of the mouse eye (John et al. 1997).

Noninvasive Phenotyping

There is now a steady trend toward noninvasive phenotyping of physiological parameters; indeed, it is the subject of the present volume, and justifiably so. There is also a great deal of current interest in extending the use of noninvasive imaging techniques such as magnetic resonance imaging (Chatham and Blackband 2001; Krishna et al. 2001), ultrasound (Coatney 2001), positron emission tomography (Cherry and Gambhir 2001), and computed tomography for small rodents (Balaban and Hampshire 2001; Berul et al. 1996; Hoit 2001; Ruff et al. 2000). Such techniques can serially measure the same animal, significantly reduce trauma, and be fast and simple.

Behavioral Phenotyping

Mouse behaviors can be particularly difficult to phenotype because they are highly sensitive to environmental factors, which may be difficult to control. In a much-discussed study of the results from three laboratories, Crabbe and colleagues (1999) showed that the robustness with which behavioral phenotypes can be determined varies widely. The three laboratories, at different geographical locations, used pro-

protocols that were as identical as possible to measure the same traits among mice of the same age, from the same inbred strains and obtained from the same source. Although the results for some traits (e.g., alcohol preference) were quite reproducible, others were not, suggesting that local factors (perhaps technician behavior) can strongly influence some behavioral measurements. Solutions to the problems of reproducibly determining behavioral phenotypes have been reviewed (Crawley 1999; Crawley and Paylor 1997; Crawley et al. 1997; Logue et al. 1997; Rafael et al. 2000; Rogers et al. 1997, 1999).

Partitioning the Phenotype

Many of the traits most relevant to human health problems are quite complex physiologically and genetically. The progression of cancer, atherosclerosis, or diabetes depends on many components, and there is much utility in approaching the genetics of each component individually. Often, a subphenotype is more easily and robustly measured, and the genetics are much simplified because they involve only a segment of the whole. Cancer, for example, can be explored in terms of somatic mutation rates, the development of aneuploidy, angiogenesis, host immune responses, and metastatic potential, each as an experimental entity. Determining the genetics of subphenotypes has been successfully applied to the study of autism-related behaviors in mice (Tarantino and Bucan 2000; Tarantino et al. 2000), type I insulin-dependent diabetes (Colucci et al. 1997; Dallas-Pedretti et al. 1995), type II diabetes (Leiter et al. 1998; Reifsnnyder et al. 2000), and osteoporosis (Beamer et al. 1999, 2001; Rosen et al. 2000).

Humanized Mice

Gene Transplants

Scientists use mice and other experimental animals because of the many difficulties and ethical constraints in human experimentation. In part, these difficulties and constraints can be overcome by introducing human genes, proteins, and tissues into appropriate animal hosts.

It is possible to introduce genes and proteins into animal hosts due to the evolutionary conservation of genetic regulatory mechanisms discussed above. Thus, a transgenic mouse with the sickle form of the human β globin gene was produced from a knockout in which the mouse β globin gene was deleted (Chang et al. 1998). The mouse provided an opportunity to explore the pathobiology and potential therapy of sickle cell anemia in a more facile experimental system. In an additional, more simple experiment, it was necessary only to construct a transgenic mouse to study Alzheimer's disease because the human Apo E4 lipoprotein variant that influences Alzheimer's is a dominant allele (Xu et al. 1996).

Transplanting genes in mice, which is likely to become increasingly important, may help identify modifier genes. Using either genetic engineering and/or breeding strategies, the same genetic alteration may be placed in several inbred strains, and strains showing quite different outcomes can be crossed to identify the modifiers (for a review, see Nadeau 2001). Given the wide range of expression of human congenital defects, the identification of modifiers can substantially expand the range of therapeutic possibilities.

Tissue Transplants

Immune-deficient mice, being unable to reject xenografts, can sometimes accept human tissue transplants and provide new and useful experimental models. For example, angiotensin II was first shown to inhibit growth of human tumors transplanted into immunodeficient mice (O'Reilly et al. 1996). Sometimes, success may require that the recipient also possess human cytokines or other growth factors. This has been the case in the drive to graft human pluripotent hematopoietic stem cells into immunodeficient severe combined immunodeficiency disorder (SCID) mice, wherein the cells can differentiate into all the formed elements of blood, including cells of the immune system (Greiner et al. 1998). Such mice would be useful in studying the differentiation of human lymphoid and myeloid lineages. Importantly, they could also be used as a small animal model for AIDS, which is desperately required.

Stem Cell Biology

First identified in bone marrow, stem cells are now known to be quite ubiquitous in adult tissues, including brain, muscle, and adipose tissue (Svendsen and Smith 1999; Zuk et al. 2001). Remarkably, these stem cells can differentiate across the early embryonic distinctions between ectoderm, mesoderm, and endoderm. Brain cells can give rise to muscle and vice versa (Magli et al. 2000). Stem cell research, particularly studies on how to drive the differentiation of ES cells in culture, has increased considerably in recent years (Jones and Thomson 2000; Repin 2001; Vescevi and Snyder 1999). Because of governmental and political constraints, studies of human ES cells are occurring primarily in the private sector (Denker 1999; Dresser 2001; Robertson 2001).

A major goal in stem cell research is to produce and use differentiated cells of the donor's genotype to treat a variety of diseases, from Parkinson's to leukemia. Success requires understanding the essential environmental conditions, especially the extracellular signals, for stem cells to replicate and then differentiate appropriately. For both practical and legal reasons, much of stem cell research necessarily will be conducted in mammalian models, primarily mice. In some cases, murine stem cells will serve as human surrogates; in

others, immunodeficient mice will serve as hosts for human cells themselves.

The Future

It is obviously impossible to predict future research discoveries in any detail, especially in a field as fast moving as mammalian genetics. To quote Albert Einstein, "If we knew what we were doing, it wouldn't be called research, would it." However, there are some trends that are apparent and worth noting.

Experimental Strategies

Completion of the human and mouse genome sequences is dramatically altering our ability to carry out molecular genetics. We can confidently expect rapid increases in the speed and efficiency of every research strategy that is sequence dependent, especially positional cloning and genetic engineering. We can also expect insights into the functional significance and evolution of the large-scale (megabase) structure of mammalian chromosomes, although we do not know what these insights might be. We will likely see a very substantial increase in the association of genes with biological processes. In some fields, such as cancer and diabetes, these associations will accelerate long-standing approaches. In neurobiology, in which molecular approaches are more recent, they should result in dramatic advances in our understanding of molecular function in areas as diverse as learning, memory, seizure disorders, perception, behavior, and neurodegenerative disorders. It is unclear how long it will take for these associations to extend into an understanding of behavioral pathology, but the effort to discover the associations is certainly justified.

The steadily increasing output of information per scientist per year will likely continue for a long time because a substantial fraction of the world's investment in basic research is continuously devoted to improving and expanding research technologies. This situation generates two trends. One trend is a move toward very large-scale experimental programs, such as occurred first in the genomics arena, later in mutagenesis, and now with multi-institutional consortia centered on particular physiological processes. Another trend is a huge increase in the demand for bioinformatics and computational biology to cope with exponential increases in our needs for data storage, retrieval, and analysis.

The massive increases in our knowledge of the multiple components of biological processes will also pressure researchers to move past the reductionism that has been so productive for decades to a systems analysis approach, which describes and analyzes the complex interactions among the many molecular components contributing to a physiological process. How these often-antagonistic cultures, the atomistic and the holistic, can be productively

conjoined presents an interesting challenge (Greenspan 2001).

Medical Outcomes

Entirely new therapeutic modalities appear in the offing. Some will come from pharmaceuticals directed at "rational" targets. Existing examples include inhibitors of HMG CoA reductase, Cox-2, the gastric proton pump (the world's most prescribed drug), and, in the cancer field, neu-2 and Abl. Some therapeutic modalities will represent entirely new classes of pharmaceuticals directed at modulating gene and mRNA activities; and some will come from the gene replacement technologies that are much discussed but difficult to achieve.

We can expect that therapeutic and preventive medicine will increasingly reflect our unique genetic constitutions. Deciphering these genetic differences is likely to be greatly accelerated by the observed concordance in the chromosomal location of natural human and rodent genetic polymorphisms affecting disease. A recent impressive and profound extension of the concept of genetic identity has been the molecular characterization of human tumors, revealing that histologically similar tumors can be classified by their expression profiles and that these expression profiles correlate with therapeutic responses and eventual prognosis. A challenge at the time of this writing is to find the mouse tumors that correspond to each of the tumor types defined molecularly and then to provide them for therapeutic research.

As a result of the advances in molecular and mammalian genetics now under way, the next generation's medicine will likely be as different from ours as ours has been from a century ago. The 20th century largely conquered infectious diseases, at least in first world countries. The first quarter of the 21st century will likely conquer the complex intrinsic diseases, including cancer, cardiovascular, diabetes, neurodegeneration, and osteoporosis, which now plague us.

What these advances will not provide are solutions to their ethical, social, and economic consequences. These solutions will have to come from elsewhere.

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