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REVIEW

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Functional genomics in the mouse

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Abstract The mouse is the premier genetic model organism for the study of human disease and development. With the recent advances in sequencing of the human and mouse genomes, there is strong interest now in large-scale approaches to decipher the function of mouse genes using various mutagenesis technologies. This review discusses what tools are currently available for manipulating and mutagenizing the mouse genome, such as ethylnitrosourea and gene trap mutagenesis, engineered inversions and deletions using the cre-lox system, and proviral insertional mutagenesis in somatic cells, and how these are being used to uncover gene function.

Keywords Mutagenesis · Developmental biology · Genomics

With the recent availability of near-complete DNA sequence for the human and mouse genomes, attention is now being turned to the question of the function of genes within these genomes, and toward the development of techniques to address this issue on a genome-wide scale. Mutants represent one of the most effective ways to acquire information as to a gene's function. As is usually the case in genetics, technologies for manipulating more complex genomes are being adapted from those established in lower organisms. This is particularly the case for the mouse, for which techniques for mutagenesis, mapping and maintenance of mutations, and the identification of mutant genes, are being developed from similar technologies in use for analysis of yeast, *Drosophila melanogaster*, *Caenorhabditis elegans* and *Danio rerio*. In this review, I present some of the techniques that are being applied to the daunting task of functional genomic analysis in the mouse.

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Why the mouse

The mouse's role in research comprises a long and rich history (Silver 1995; Beck et al. 2000). The development of inbred mouse strains was a significant development that allowed studies in cancer, histocompatability, and inheritance of visible traits (Silver 1995; Beck et al. 2000). Indeed, despite the facility with which other model organisms can be dissected genetically (e.g. *D. melanogaster* and *C. elegans*), the mouse is valued for its relatively unique applicability to the genetic study of immunology, cancer, behavior, and mammalian development. At present, it represents the premier genetic model organism for the study of human disease and development.

Genetic and mutational analysis of mice advanced in the mid-twentieth century due to the interest at that time in the biological effects of ionizing radiation. Studies were undertaken to determine the mutagenic capability of radiation, and, in further studies, the mutagenicity of certain chemicals. A key technical feature in the studies of Russell and colleagues at Oak Ridge National Laboratories in Oak Ridge, Tennessee, was the development of a tester strain of mice that was heterozygous for recessive mutations at seven genes for which the homozygous mutant state was clearly visible. Using this approach, it was possible to quantitate the frequency and ascertain the types of mutations induced by different agents (Silver 1995). From these and other studies it became appreciated that X-ray treatment of male breeders resulted in a rather low mutation frequency, and that the mutations were often complex rearrangements, translocations, or deletions. Two chemicals emerged from these studies as particularly potent mutagens: ethylnitrosourea (ENU) and chlorambucil (CHL). ENU is more efficient at inducing mutations, and tends to cause point mutations (discussed below), while CHL causes large deletions, the difference being due to the stage of spermatogenesis at which the mutagen acts (Silver 1995). Both agents have proven instrumental in inducing mutations in the mouse.

Simple mouse facts

Key features of mouse biology that affect the execution of functional genetic analysis include the following. Mice gestational duration is 19–21 days, with litters being 3–9 pups, depending on the strain. A generation time is generally 10 weeks, allowing for five generations per year. The size of the mouse genome is approximately 3×10^9 basepairs, roughly equivalent to the human genome. Current estimates for the number of genes range from 30,000 to 100,000, this despite newly available sequence information for both species. In the mouse, these are distributed on 19 autosomes and the sex chromosomes, for a total of 20 chromosomes per haploid genome, compared with 23 in the human. Nearly all human genes have a counterpart in the mouse, and the evolutionary time between the two species is estimated at 60 million years, as compared with nearly ten times that when comparing humans and *D. melanogaster* (Silver 1995). The similarity between the human and mouse genomes is also reflected in the occurrence of large segments of synteny between mouse and human. These are segments of 10–20 megabases harboring dozens to hundreds of genes that have the same gene order and similar intergenic distances between the two species (Copeland et al. 1993). In genetic terms, the mouse genome contains 1,600 cM.

One reason for the use of the mouse relative to other model genetic organisms for studies in disease and development is that mammalian development is significantly different than that seen in lower organisms such as *D. melanogaster* and *C. elegans*. Specifically, transcription in mammalian embryos occurs early in development, indicating the relative importance of zygotically transcribed genes as compared with maternally-derived mRNA transcripts. In addition, mice, like humans, utilize a uterus and placenta for development, unlike lower genetic model organisms. Finally, there is evidence of genome duplication in the teleost lineages, bringing into question the translatability of genetic findings in zebrafish to humans. In terms of studies in immunology and human disease, mouse is the closest genetically tractable organism.

Mutagenesis in the mouse: targeted mutations

At present, less than 5% of genes in the mouse genome have been mutated, most of these having been targeted by homologous recombination in embryonic stem cells. The 2001 Lasker awards were awarded for this technology, to Martin Evans, Mario Capecchi, and Oliver Smithies, whose laboratories collectively developed the critical components of gene targeting capability in mice. Technology for the creation of targeted mutations continues to advance (Muller 1999). Hundreds of genes have been mutated by targeted mutagenesis in the mouse, as catalogued at the Jackson Labs. (http://tbase.jax.org/); many of these are available through the Induced Mutation Repository at the Jackson Labs. of knockouts accomplished to date (http://www.jax.org/pub-cgi/imrpub.sh?objtype=stridx). The field of biomedical research has benefited significantly from the study of these mutant mice, and with the availability of sequence for the mouse genome and newly developed techniques in recombinant plasmid generation (see Copeland et al. 2001), the construction of targeting vectors has become significantly easier.

Mutagenesis in the mouse: chemical mutagenesis and phenotypic screens

Despite the power of the targeted mutation technology, for large-scale screening, this approach has limitations. The creation of targeted mutations is still labor-intensive and one needs a compelling rationale for embarking on a knockout project. More importantly, if one is primarily interested in a certain phenotype, a phenotypic screen is appropriate. While one can often predict the phenotype of mice bearing targeted mutations in known genes for which a function is known, this is not possible for genes for which little is known. Another advantage of chemical mutagenesis is its ability to induce single-base changes in the DNA. While targeted mutagenesis has the ability to create the mutation one desires, the basic gene targeting experiment yields a null allele. It is often the case that the more subtle mutations, such as single amino acid changes in the protein, yield more information about gene function than null alleles (for example see Steingrimsson et al. 1994).

With the advent of sequencing the mouse genome, there is renewed interest in mutagenesis screens that focus on phenotypes rather than on a specific gene. In contrast to mutations induced by knockouts or knock-ins, mutagenesis with a chemical that induces point mutations followed by a phenotypic screen has the ability to yield a vast array of alleles that represent a spectrum of degrees of deviation from normal. These mutations can be gain-of-function alleles, hypomorphic alleles, and null alleles that are due to a variety of missense mutations and/or nonsense mutations. ENU-induced allelic series can allow one to distinguish the functional role(s) of individual isoforms generated by a complex locus. Allelic series were created decades ago at genes controlling certain visible features such as coat color, tail length, shape of skeleton and ears, and behavior (Justice 2000).

Thus, the two major strengths of a chemical mutagenesis screen with a mutagen such as ENU are: (1) that the mutations can result in a spectrum of changes to protein structure and function, which allows, in the end, more information about the protein; and (2) that the screen is centered on the phenotype rather than on the gene. All manner of genetic changes that result in a given phenotype are possible, thus allowing for the discovery of new genes that regulate a certain developmental pathway. There are significant technical challenges related to an ENU mutagenesis experiment which derive primarily

from the fact that the mutation most often represents one base change in the mouse genome of 3×10^9 basepairs. A variety of techniques have emerged, and continue to emerge, to deal with this problem.

Types of ENU-induced base changes

The mechanism of ENU mutagenesis involves ethylation of O or N in DNA. ENU predominantly modifies A/T basepairs: 44% A/T→T/A; 38% A/T→G/C; 8% G/C→A/T; 3% G/C→C/G; 5% A/T→C/G; 2% G/C→T/A. Of these 64% are missense mutations, 10% nonsense mutations; 26% splicing errors. The mutation in a single gene of choice is 1 out of 175–655 gametes screened (Justice 2000). In male mice treated with ENU, the effective mutagenesis occurs in the early spermatogonial cells, which means that following an initial sterile period, the males will continue to generate mutated sperm for the rest of their reproductive lives. X-rays tend to create large deletions of unpredictable size. Due to this, and the low efficiency with which mutations are induced, it tends not to be used. Chlorambucil also tends to create large deletions, but in this case, the mutations are induced at a higher frequency. ICR-191 is a acridine-derived frameshift mutagen (McKendry et al. 1991; Taft et al. 1994), and thus is less desirous if one seeks gain-of-function or hypomorphic alleles.

Types of mutagenesis screens

There are two approaches being taken to mutagenesis in the mouse: regional screens and whole genome screens (Anderson 2000). Each has its own advantages and challenges. In a regional screen, one focuses on mutations that fall within a discrete region of the mouse genome by employing mouse strains with characterized chromosomal deletions (to uncover recessive mutations within that interval) or inversions (as balancer chromosomes for mutation maintenance). In a whole genome screen, one uses essentially genotypically normal mice, and screens primarily for dominant mutations, or sets up more elaborate mating schemes to screen for recessive mutations. While the latter allows for a wider pool of mutated genes, it also requires more effort to identify and clone the mutation.

Mutagenesis screens – regional screens using chromosome deletions

A regional screen is one in which a discrete area of the genome is subjected to study (Rinchik 2000). These rely on the use of inversions and deletions, which were initially developed as tools in *D. melanogaster* (Judd et al. 1972), and can play an invaluable role in a mutagenesis program in the mouse (Rinchik 2000). To facilitate such an experiment, visible coat-color markers are used to follow the normal counterpart of the deleted or inverted chromosome. An example of this approach is the use of a strain bearing a deletion on chromosome 7 at the albino (*Tyr* or *c*) and pink-eyed dilution (*p*) loci for saturation mutagenesis (Rinchik 2000). This chromosomal deletion is large (about 4–5 cM), and the corresponding interval on the normal chromosome is marked by a coat-color mutation (in this case p^x, an intermediate allele of *p*), which allows one to identify the offspring inheriting the normal chromosome (Fig. 1). In this example, crosses were set up initially between ENU-mutagenized *p*/*p* males and wild-type females to generate G1 mice in which one chromosome (marked by *p*) is derived from the ENU-treated male, which may carry recessive mutations in the interval of interest. To identify such mutations, G1 progeny of this mating are mated to the "selector" mice bearing a deletion of the region of interest and the p^x-marked non-deleted chromosome. Recessive, ENU-induced mutations that lie within the deleted interval are hemizygous in offspring that inherit the deleted interval from the selector strain. These will be pink-eyed, due to the presence of the linked *p* marker (Fig. 1). Because the mutation is hemizygous when it is opposite a deletion, this is called a hemizygosity screen. Regional screens allow one to take advantage of visible markers to

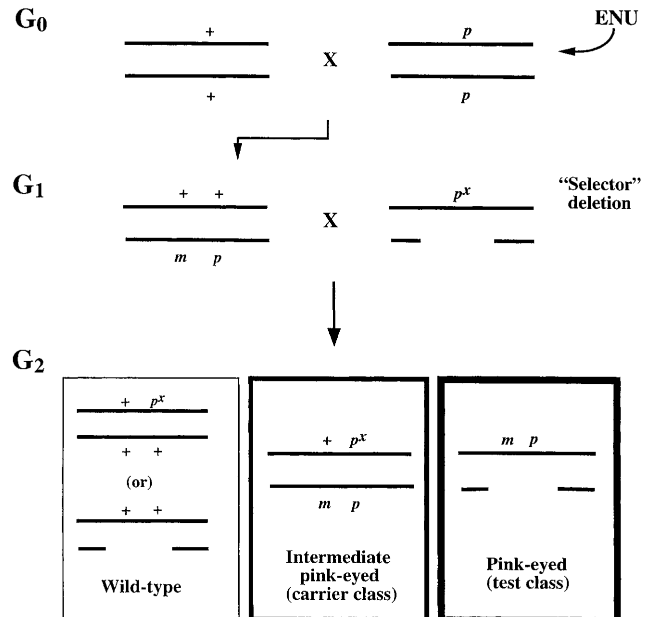

Fig. 1 A regional mutagenesis screen employing visible coat-color markers and a chromosome deletion. This strategy, designed and now being utilized by Rinchik and coworkers (reviewed in Rinchik 2000), uses two alleles of the pink-eye gene (*p* and p^x) to mark the mutagenized and the normal, non-mutated chromosomal regions, respectively. New, ethylnitrosourea- (ENU) induced mutations, denoted by *m*, are uncovered by placing them opposite the deletion in the G_2 generation. As indicated in the boxes at bottom, the three genotypic classes of G_2 offspring can be distinguished by visual inspection of the mice, due to the distinct coat- and eye-color phenotypes of the three possible combinations of *p* alleles. (Reprinted from Rinchik 2000 with permission from Springer, New York)

mark the mutagenized region, which aids in strain maintenance since it obviates the need for molecular analysis of the mice. The mapping of the mutation is facilitated, since one knows at the outset that the mutation falls within the deleted interval; finer mapping can be done by crosses to mice bearing smaller deletions.

Deletions help in confining mutation scans to a given region of the genome; deletion series also help in mapping mutations. Recessive mutations are more easily discovered and recovered using stocks of mice carrying deletions. Such a screen can be accomplished in one to two generations. Deletion stocks are available for about 15% of the mouse genome (http://www.mgu.har.mrc.ac.uk). The capability for creating deletions by targeted insertion of loxP sites in ES cells has been engineered by Bradley and coworkers and is discussed below in greater detail (Ramirez-Solis et al. 1995; Justice et al. 1997; Zheng et al. 1999). An alternative strategy for creating deletions has been designed by You et al., in which a negative selectable marker is inserted randomly into ES cells and, following treatment with a mutagen, one selects for loss of the negative selectable marker. This nearly always occurs through a deletion of variable size (You et al. 1997, 1998). Such cells can be used to generate mice with the same deletion.

Mutagenesis screens – regional screens using chromosome inversions

Inversions, especially those that are homozygous lethal and carry a dominant visible marker, are useful in stock maintenance and in allowing easy identification of mutant carrier classes and homozygous mutant classes of offspring. Because they are often homozygous lethal and fail to recombine with the non-inverted chromosomal counterpart, inversions marked by a dominant visible marker are excellent genetic reagents for stock maintenance. They also facilitate creation of homozygous genotype for induced mutations that reside within the non-inverted chromosomal partner. The key advantage that inversions have over deletions is that often mice carrying large, or even small, deletions are not viable due to haploinsufficiency. The use of inversions has recently been reviewed by Rinchik (2000).

Creation of inversions and deletions with the cre-loxP system

Deletions and inversions can be created in the chromosome in ES cells by chromosome engineering using the vectors and techniques developed by Bradley and coworkers. This is accomplished by targeted insertion of *loxP* sites at the ends of the region to be deleted or inverted, where the insertion construct is such that one can select for cre-mediated recombination of the *loxP* sites (Ramirez-Solis et al. 1995). One can tag these mutations with a dominant coat-color marker (e.g. K14 agouti) allowing for ease in mapping, stock maintenance, and genetic screens (Fig. 2). The same group have created two lambda libraries of 129SvEv mouse DNA within a vector that has several features that allow for rapid execution of homologous recombination-mediated insertion into the mouse genome, and subsequent use for deletion of large sections of mouse chromosomes (Zheng et al. 1999). Importantly, the lambda construct has dominant coat-color markers (agouti and tyrosinase) that allow one to follow the insertionally mutated allele. Second, there are libraries in two different vectors, each of which has a portion of the *Hprt* minigene: a 5′ *Hprt* vector, and a 3′ *Hprt* vector. Third, they harbor *loxP* sites for cre-mediat-

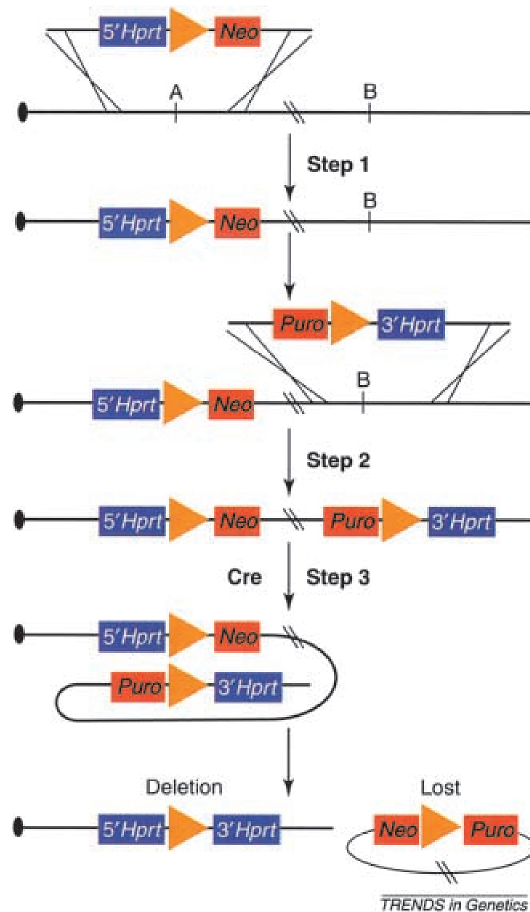

Fig. 2 A three-step scheme to generate defined mutations in embryonic stem cells. This system, designed by A. Bradley and coworkers (reviewed in Mills and Bradley 2001), requires insertion, via homologous recombination, of two parts of an *Hprt* minigene at the 5′ and 3′ ends of the region to be deleted (*Steps 1 and 2*). These steps are facilitated by the presence of positive selection markers Neo and Puro. The minigenes contain either the 5′ or 3′ portions of the selectable *Hprt* gene, as well as a *loxP* site (*arrowheads*). In *Step 3*, *Cre* recombinase is introduced, which mediates recombination of the *loxP* sites. This results in deletion of the DNA between the *loxP* sites and the creation of a functional *Hprt* gene. Additional features, described by Zheng et al. (1999), include the tagging of the 5′ and 3′ cassettes with coat-color markers *Ty* and *Ag*, for identifying mice carrying the altered chromosomal region. (Reprinted from Mills and Bradley 2001 with permission from Elsevier Science

Table 1 Regional mutagenesis screens

Chromosome	Authors[a]	Location	Website
11	M. Justice, R. Behringer and coworkers	Baylor College of Medicine	http://www.mouse-genome.bcm.tmc.edu/ENU/ENUHome.asp
7, 10, 15, and X	E. Rinchik, D. Johnson and coworkers	Oak Ridge National Laboratories	http://bio.lsd.ornl.gov/mouse/
13	S. Brown, P. Nolan and coworkers	Mammalian Genetics Group, Harwell, UK	http://www.mgu.har.mrc.ac.uk/mutabase/
5	J. Schimenti and M. Bucan	Jackson Labs. and U. Penn	n/a

[a] For references, please see text

ed recombination through which one can eliminate the sequence between the two *loxp* sites. The vectors are designed to be integrated at the ends of a chromosomal region that one wishes to delete. One isolates lambda clones corresponding to sequences at the ends of this region, recombines these into the genome of *Hprt*⁻ ES cells, and uses cre-mediated recombination to eliminate the intervening sequences. This will leave the dominant coat color markers and a functional hprt gene for which one can select in hypoxanthine aminopterin thymidine (HAT) medium. The agouti marker appears to be the preferable marker, since it can more easily be distinguished on nonagouti and black agouti backgrounds. These libraries should facilitate the creation of chromosome deletions and inversions.

Large scale regional mutagenesis screens

At least four regional mutagenesis screens are currently being conducted (Table 1). Using a Cre-*loxP*-generated inversion on chromosome 11, Justice, Behringer and colleagues at Baylor College of Medicine are conducting a screen for recessive mutations that affect a variety of measurable phenotypes, including clinical chemistry, hematologic, and developmental (http://www.mouse-genome.bcm.tmc.edu/ENU/ENUHome.asp). At Oak Ridge National Laboratories, Rinchik, Johnson, and colleagues are conducting regional homozygosity screens for regions of chromosomes 7, 10, 15, and X (Rinchik 2000); http://bio.lsd.ornl.gov/mouse/. In these experiments, they are screening for mutations affecting a variety of behavioral and central nervous system phenotypes. For this study, phenotypic analysis is being performed by the Tennessee Mouse Genome consortium. The Mammalian Genetics Group at Harwell, United Kingdom, is performing a regional saturation mutagenesis screen for recessive disorders within the 36H deletion region on chromosome 13, a region homologous to 6p22–23 in the human genome (Nolan 2000). Schimenti and Bucan are conducting a regional mutagenesis screen on chromosome 5 (Schimenti and Bucan 1998).

Types of mutagenesis screens: whole genome

Under certain circumstances, one may wish to screen the entire genome for mutations that yield a particular phenotype or one of many phenotypes that one can assess. For whole-genome screens, one need not employ genetic reagents such as deletions or inversions. Justice has recently reviewed the two types of whole-genome genetic screens: one generation screens for viable/fertile mutants that represent allele series, modifiers, or dominant mutations; and three-generation "pedigree" screens for recessive mutations. Logistically, screens for dominant mutations are far easier, since one need only treat males with ENU, mate with wild-type females, and analyze the offspring for mutations. This approach is exemplified in the identification of *Clock*, the first murine circadian rhythm mutation (Vitaterna et al. 1994). Recessive mutations require a three generation cross: treatment of males with ENU which are then mated to wild-type females to obtain G1 males. Each of these will carry, on average, ten new ENU-induced mutations. To make these homozygous, female offspring of G1 males mated to wild-type females are mated back to their fathers. Fifty percent of these matings will involve carrier females; to attain 95% assurance that a mutation is present in the female, 4–5 daughters need to be mated back to the father. One fourth of the offspring from half of these matings should be homozygous for a given mutation. With 3,000 gametes, one could have 98% confidence of having a mutation in every gene (Schimenti and Bucan 1998). To assess the phenotype in homozygotes for this number of genes would require over 160,000 mice (Schimenti and Bucan 1998).

A small-scale whole-genome screen for ENU-induced recessive developmental mutations has been performed by Anderson and coworkers (Kasarskis et al. 1998). They employed a three generation cross to detect recessive mutations that disrupt mid-gestational development in a genome-wide mutagenesis screen. From 130 male first generation (G1) mice derived from mutagenized males they obtained 86 lines. For each line, 5–10 second generation (G2) females were mated back to the male parent (G1) mouse, and the pregnant females were sacrificed at 9.5 days post-coitum to identify abnormal em-

Table 2 Genome-wide mutagenesis screens (*n/a* not applicable)

Investigator	Location	Phenotypes	Reference	Website
G. Carlson and coworkers	McLaughlin Institute, Great Falls, Mont., USA	Sensitized screen on mutant bAPP background	n/a	http://www.montana.edu/wwwmri/enump.html
S. Brown, P. Nolan and coworkers	Mammalian Genetics Unit at Harwell, UK	Varied, including neurological and behavioral	(Nolan et al. 2000)	http://www.mgu.har.mrc.ac.uk/mutabase/
R. Balling, M. Hrabe-deAngelis and coworkers	GSF-National Research Center, Munich, Germany	Dominant and recessive; various parameters	(Hrabe de Angelis et al. 2000)	http://www.gsf.de/ieg/groups/enu-mouse.html
Toshihiko Shiroishi and coworkers	Genome Sciences Center, RIKEN, Konagawa, Japan	Dominant and recessive; various parameters	n/a	http://www.gsc.riken.go.jp/Mouse/
J. Rossant and coworkers	Mt. Sinai Hospital, Toronto, Ont., Canada	Varied	n/a	http://www.cmhd.ca/default.asp
C. Goodnow and coworkers	Australia National University, Canberra, Australia	Various parameters, including immunologic	n/a	http://jcsmr.anu.edu.au/group_pages/mgc/MedGenCen.html

bryos. Out of 86 lines analyzed, 5 had a 12.5% rate of embryo abnormalities when all embryos were considered, with half of the litters showing no abnormalities, and half showing 25% incidence. For each of these lines, the mutant phenotype was transmissible for multiple generations and behaved as a normal Mendelian trait. Mapping of the genes was performed by simple sequence length polymorphisms present on each chromosome with linkage to phenotype. The success of this experiment indicates that ENU mutagenesis is a viable approach for obtaining recessive mutations causing an assayable phenotype.

Other small-scale screens that have been done include "sensitized pathway" screens, such as that being performed for mutations affecting the phenylalanine metabolic pathway uncovered by sensitizing with phenylalanine injections (Symula et al. 1997). It has also been possible to obtain allelic series at a known gene in a small-scale screen such as those identified at the *quaking* (*qk*) locus (Cox et al. 1998).

At present there are seven major large-scale whole-genome mutagenesis screens (Table 2; reviewed by Rathkolb et al. 2000; Justice 2000; Nolan 2000). One of the most ambitious is that being executed by Balling and Hrabe de Angelis in Munich (Hrabe de Angelis et al. 2000; http://www.gsf.de/ieg/groups/enu-mouse.html). This is being conducted at a research center within the German human genome project, and its goal is to find mouse mutants relevant to human disease. The scheme is a three-generation genome-wide screen in which homozygosity to reveal recessive alleles is achieved by backcrossing G2 offspring to their G1 parent. This is followed by a comprehensive screen for phenotypes in the G3 mice. To assure that all observable phenotypes' recessive mutations are identified, they are examining 40 G3 offspring from a G2 × G1 cross. G1 and G2 mice are also screened for dominant and semi-dominant phenotypes. The screen for phenotypes involves nine different areas and a wide variety of individual tests. The areas are: (1) clinical parameters; (2) clinical chemistry; (3) metabolite profile; (4) hematology; (5) immunology; (6) dysmorphology; (7) allergy; (8) neurology; and (9) behavior. Blood is analyzed by electron spray-mass spectrometry (ES/MS) for abnormalities in serum proteins. There are 39 parameters that are used for identifying mutations affecting growth and development (dysmorphology screening).

Types of phenotypic analyses utilized in mutagenic screens

Current phenotypic testing protocols allow assessment of a wide variety of parameters that cover nearly all major organ systems. Screens should be broad and inexpensive, since many mice need to be screened. Externally apparent features, such as coat color, hair and tail morphology, skeleton, limb appearance and function, mobility, behavior, and fertility are part of nearly all large-scale screens. More specialized screens, such as hematologic parameters, clinical chemistry, intrauterine developmental, urinalysis, and more specialized neurological functions are being tested by several centers, with what is often very sophisticated instrumentation(e.g. Rogers et al. 1997). Over 300 confirmed mutations have been identified between the Harwell group and the Munich group. As of November 2001, the RIKEN effort is beginning to accumulate mutations. Due to the fact that these screens not-

ed repeated mutations in the same genes, it may be that they are reaching saturation of the dominant class of mutations. Many of the mutations had phenotypes that mimicked human diseases, thus supporting the contention that the ENU mutagenesis approach will prove highly relevant to the study of human disease.

Emerging techniques for finding the mutations: tricks of the trade

A significant bottleneck at this stage is mutation confirmation and chromosomal localization. A recent review by Wells and Brown describes recent advances in the technology needed to map mutations (Wells and Brown 2000). Mapping is achieved by performing backcrosses or intercrosses, with genotyping of the offspring and correlation of the phenotype with genotype. Wells and Brown identify three constraints to current mapping techniques: (1) generating enough backcross mice; (2) identification and validation of polymorphic markers; and (3) speed of marker data acquisition and analysis acquisition. Generation of sufficient numbers of animals is important: 50 animals allow mapping within 10–20 cM while 500 animals are needed for 1 cM resolution (1 cM = approximately 1.5 Mb). Thus, there is a need for large numbers of animals (unless deletions are used in the mapping). To speed up the generation of backcross animals, in vitro fertilization is now possible, allowing the production of large numbers of offspring from one mouse within a few weeks. To achieve the density of markers needed for genotype analysis, Wells and Brown propose relying on single nucleotide polymorphisms (SNPs) rather than sequence tagged sites (STSs) or simple sequence length polymorphisms (SSLP)s. This is because of the high density of SNPs in the human: 1 per kb. Eight different assays for SNP detection are discussed, none of which is perfect. However, it is apparent that some have real potential. One is microarrayed oligos bearing different SNPs. Regardless of the analysis method, the rate-limiting step is PCR amplification of the mouse genomic DNA for each marker for each mouse to be genotyped.

Once a gene is localized to a few centimorgans, strategies for identifying the mutation are several. The most powerful is complementation with cloned wild-type DNA on BAC vectors (Antoch et al. 1997). The use of YAC, BAC, and PACs to create transgenic mice has constituted a major advance in our ability to manipulate the mouse genetically. The fragments of mouse genomic DNA within these vectors is substantial (200–500 kb), enough to contain most genes, including both coding and regulatory regions. The use of such pieces to create transgenic mice has obviated the problem of position effect that had beset the technique, making interpretation of experiments difficult. The expression of a gene contained within the context of a BAC or YAC in a transgenic mouse closely mimics that of the endogenous gene and is largely independent of transgene insertion site and the presence of vector sequences (Kaufman et al. 1999). It also allows one to then rescue mutant phenotypes by complementation (Antoch et al. 1997; Majumder et al. 1998), thereby allowing identification of essential genetic components, be they coding or regulatory. The preparation of YAC DNA requires specific skills and expertise, and since they have problematic features such as a high degree of chimerism, potential for internal rearrangement and instability, and difficulty in purification away from the yeast chromosomes. BACs (derived from the F factor of *E. coli*; Shizuya et al. 1992) and PACs (derivatives of P1 phage; Ioannou et al. 1994) are preferable vectors for the creation of mouse transgenics due to their stability and lower rate of chimerism (reviewed by Giraldo and Montoliu 2001). These can be injected into mouse zygotes as supercoiled molecules (Antoch et al. 1997) or as linear molecules (Mullins et al. 1997).

BAC libraries for the mouse are readily available, and the ordering of BACs across the genome is largely complete. Through this approach, candidate genes can be identified, which can be confirmed by single strand conformation polymorphism (SSCP) and sequencing. In the absence of a candidate gene, one can rely on synteny between mouse and human to guide the identification of candidate genes.

BAC mutagenesis

One powerful method for confirming the effect of a mutation is to recapitulate it in the context of the normal gene. Recombination-based BAC mutagenesis in *E. coli* enables one to rapidly alter the structure of genes within their native context on large (200 kb) cloned pieces of mouse DNA. While techniques to mutagenize YACs in yeast have existed for some time, analogous techniques for BACs have only more recently been developed. These include systems based on the *E. coli* recA system (Yang et al. 1997), and on bacteriophage (Lee et al. 2001; Muyrers et al. 2001), which are the topic of a recent review (Copeland et al. 2001). The systems based on bacteriophage are more efficient. Technologies now exist for mutagenizing BAC DNAs without the need for selection (Swaminathan et al. 2001) and without leaving exogenous DNA at the mutated site (Muyrers et al. 2000; Nefedov et al. 2000).

The modified BACs created with either the recET system of Stewart and colleagues (Zhang et al. 1998) or the exo/bet system described by Lee et al (Lee et al. 2001) can then be used to create transgenic mice, allowing for structure-function studies through complementation. They can also be used to create knockout constructs (Angrand et al. 1999), and to rapidly subclone a specific fragment from BAC-derived genomic DNA and perhaps from genomic DNA (Zhang et al. 2000). These systems vastly improve the facility with which one can create DNA constructs for complementation and mutation in the mouse.

Other approaches: ENU mutagenesis of ES cells

As an alternative to mutagenesis of mice, Magnuson and coworkers are exploring mutagenesis of ES cells. This offers the practical, logistical advantage of being able to preserve the mutant "organism" easily as a frozen vial of totipotent cells. Also, the cells can be mutagenized in the presence of an agent that blocks the DNA repair pathways, thus allowing a better chance of achieving saturation mutagenesis. Chen et al. looked at mutations in the *Hprt* locus in ES cells using ENU, and found 1 mutation per 200 mutagenized clones (Chen et al. 2000b). By treating with O^6-benzyl-guanine, they inhibited the DNA repair enzyme O^6-alkylguanine-alkyltransferase, which increased the mutation frequency two- to fourfold. Using automated analysis of DNA derived from mutagenized ES cells, it is possible to identify clones bearing mutations in a gene of interest. Based on preliminary data with *Hprt* and HSV*tk*, analysis of 2,000–4,000 ES cell clones should yield 10–20 mutant ES cells. For mutation detection, some high throughput techniques are now emerging, such as denaturing, high-performance liquid chromatography (DHPLC; Oefner and Underhill 1998). These and other high throughput techniques are now in development (reviewed by Chen et al. 2000a). Although technically challenging, this is achievable. This will allow for the generation of allelic series. Towards implementation of this approach, Magnuson and colleagues have generated 2,500 single-cell-derived ENU-mutagenized ES cell clones that can go germline, and have frozen these and extracted DNA from each of them. There is 1 null for every 1,000 clones and 1 hypomorphic (assumed not proven) for every 150.

With mutagenized ES cells one can do in vitro selections, and thus obtain mutants in a particular biochemical or developmental pathway prior to the creation of mice. This approach is being employed by Stanford and coworkers using gene-trap mutagenized ES cells as described in the next section.

Gene-trap mutagenesis

Following the pioneering work of Jaenisch (Kratochwil et al. 1989) on mutagenesis of the mouse germline with retroviruses, Friedrich and Soriano (1991) reported mutagenesis of embryonic stem cells with Moloney leukemia virus-based vectors. A variety of other vectors and approaches to perform mutagenesis with DNA were developed (see http://cmhd.mshri.on.ca/sub/genetrap/references.htm), in which the mutagenizing DNA disrupts genes randomly, and in doing so generates a fusion mRNA transcript. The fusion transcript can be cloned by various PCR-based techniques and sequenced. This approach has been taken by a number of labs., both academic (e.g. German Gene Trap Consortium: http://tikus.gsf.de/ and http://cmhd.mshri.on.ca/sub/genetrap.htm) and commercial (e.g. Zambrowicz et al. 1998; http://www.lexgen.com/). The German gene trap consortium has mutagenized over 4,500 genes in ES cells. Forty three percent of these genes are not in the public databases. These ES cells are freely available to academic researchers. Lexicon has mutated over 20,000 genes in ES cells which are available for a fee (http://www.lexgen.com/). Mutagenesis can also be done with DNA constructs that are designed to specifically select for mutation in certain classes of proteins (Mitchell et al. 2001; http://socrates.berkeley.edu/~skarnes/resource.html). Stanford and colleagues have combined gene-trap mutagenesis with an in vitro phenotypic screen based on the differentiation capacity of ES cells. Their gene-trap vector insertion results in *lacZ* expression when the targeted gene is expressed. This screen allows for genes whose expression is induced upon differentiation of the ES cells into different types of tissues (Stanford et al. 1998).

Retroviral mutagenesis of somatic cells

Proviral insertional mutagenesis using leukemogenic or mammary cancer-inducing retroviruses is a powerful and efficient way of identifying genes whose deregulation can contribute to hematopoietic or mammary cancers in mice (Callahan and Smith 2000; Largaespada 2000). MMTV is passed to nursing pups via milk in several strains including C3H and induces mammary carcinomas with a latency of 6–10 months, depending on the strain of mouse (Zelazny et al. 2001; Chatterjee et al., submitted for publication). MMTV proviral insertions typically occur within 5′ and 3′ non-coding regions, from which location they typically activate gene expression. This unregulated expression of native growth regulatory proteins can contribute to oncogenic transformation. Thus, knowledge of a gene as a site of insertion suggests that the encoded product plays a regulatory role in growth and/or differentiation. Proviruses are also known to insert within intron sequences, and can cause the production of truncated transcripts and, presumably, protein products with altered structure (Dievart et al. 1999; Chatterjee et al., submitted for publication). Such a finding can give information about the portions of the protein that harbor growth-inhibitory and growth-promoting signaling potential. With either insertion within the gene or within non-coding 5′ or 3′ regions, the insertional event provides a convenient molecular tag for the cloning of genes located adjacent to the insertion site. Experimental approaches in proviral insertional mutagenesis have evolved in the last decade. Initial studies examined the insertion sites occurring in inbred strains and used DNA cloning in lambda phage vectors to identify sites of insertion (reviewed in Nusse 1991; Callahan and Smith 2000). These studies resulted in the identification of a handful of genes that fell into three groups: those in the Wnt family of signaling molecules; those in the Fgf family of extracellular signaling proteins, and two in the Notch family of developmental/regulatory proteins (Callahan and Smith 2000). Early studies noted that the frequency of proviral insertion at specific genes depend-

ed on the strain of mouse, indicating that host genetic factors influence the proviral targets selected in tumorigenesis (Callahan and Smith 2000). More recent studies have used a variety of background mouse strains in which to conduct MMTV proviral tagging studies, and these include hybrid mice (Zelazny et al. 2001; Chatterjee et al., submitted for publication), and transgenic mice (Bouchard et al. 1989; Shackleford et al. 1993; Zelazny et al. 2001; Chatterjee et al., submitted for publication). The use of such strains in essence constitutes a "sensitized" genetic screen, and has allowed the identification of a broader spectrum of potential cancer genes, including ones that had not been found as sites of insertion in inbred strains (e.g. Lee et al. 1995; MacArthur et al. 1995). Thus, it is likely that by modifying host genetic background, one can elicit new sets of proviral insertion targets in MMTV-induced tumors.

A second innovation that has allowed greater exploitation of the proviral insertional tagging system is inverse PCR. This technique, which involves PCR amplification of circularized genomic DNA templates using virus-specific primers, allows for the rapid cloning of sites of proviral insertion (van Lohuizen et al. 1991; Li et al. 1999). By sequence analysis, one can easily determine the genomic DNA sequence at the insertion site. Now that substantial and growing sequence information is available for mouse and human in both public and commercial databases, one can rapidly identify the genes most likely affected by proviral insertion by searches using DNA sequence for the site of insertion. The chromosomal location of human homologs can easily be determined through LocusLink (http://www.ncbi.nlm.nih.gov/LocusLink/), Ensembl (http://www.ensembl.org/), or the NCBI Genome Browser (http://www.ncbi.nlm.nih.gov/genome/guide/human/). One can also ascertain, by use of these web sites, whether any human cancers have known genomic alterations in the area. Knowledge that a gene is a site of proviral insertion in a retrovirally induced murine cancer can provide information to warrant further investigation of the gene product and its potential for regulatory function in the cell.

Bioinformatics

Web-accessible databases are facilitating the dissemination of information about the mutagenesis projects (e.g. see Tables 1 and 2), which is an essential aspect of mouse mutagenesis projects. The facility with which the ENU mutagenesis is performed means that the real bottlenecks in the process are the characterization of mutant phenotypes and mapping and ultimately the cloning of the genetic defect. The expectation is that groups outside of the mutagenesis project will perform much of this work. This, as well as the sheer magnitude of the information management work, necessitates web-accessible databases of mutant mouse strains.

Conclusions and future challenges

Technical challenges remain in several areas. While the strength of the mouse as a genetic organism and as a model for human disease is significant, it has a generation time and maintenance cost that complicate certain experimental tasks. One is stock maintenance. It is expensive to maintain lines of mice, especially if the identification of mutant carriers requires a DNA-based test rather than a coat-color marker. This presents the question: are all mutant lines going to be maintained, or will certain lines be cryopreserved? Cryopreservation of sperm is now technically feasible, though the technology is not universally available. Freezing of embryos is much more costly. In addition, how will decisions be made as to which lines to maintain? Will mutations be mapped routinely, and if so, will mapping of mutations be performed prior to freezing? This will likely depend on the demand for specific mutant phenotypes, which at present is not clear. Other questions concern characterization of mutant lines: how much will be done prior to mapping, prior to freezing?

Much of the success of this endeavor rests on cloning of the mutant genes. While the cases where success has been achieved are well known, these cases are limited in number, but are growing. There is a significant challenge in performing mutation detection in a high-throughput manner. This will be especially challenging when analyzing a large genomic area or a large gene or transcript. Certain techniques are now coming on line, such as a temperature gradient method for detecting mutations (Bjorheim et al. 1998), but these remain expensive.

In conclusion, this is an exciting time in mouse genetics, as new and potentially powerful tools are coming on line to aid in the genetic dissection of this old favorite of geneticists.

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