Mammalian Genome 11, 500–506 (2000).
DOI: 10.1007/s003350010096

Mammalian Genome
Incorporating Mouse Genome
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Implementation of a large-scale ENU mutagenesis program: towards increasing the mouse mutant resource

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Received: 16 December 1999 / Accepted: 16 December 1999

Abstract. Systematic approaches to mouse mutagenesis will be vital for future studies of gene function. We have begun a major ENU mutagenesis program incorporating a large genome-wide screen for dominant mutations. Progeny of ENU-mutagenized mice are screened for visible defects at birth and weaning, and at 5 weeks of age by using a systematic and semi-quantitative screening protocol—SHIRPA. Following this, mice are screened for abnormal locomotor activity and for deficits in prepulse inhibition of the acoustic startle response. Moreover, in the primary screen, blood is collected from mice and subjected to a comprehensive clinical biochemical analysis. Subsequently, secondary and tertiary screens of increasing complexity can be used on animals demonstrating deficits in the primary screen. Frozen sperm is archived from all the male mice passing through the screen. In addition, tail tips are stored for DNA. Overall, the program will provide an extensive new resource of mutant and phenotype data to the mouse and human genetics communities at large. The challenge now is to employ the expanding mouse mutant resource to improve the mutant map of the mouse. An improved mutant map of the mouse will be an important asset in exploiting the growing gene map of the mouse and assisting with the identification of genes underlying novel mutations—with consequent benefits for the analysis of gene function and the identification of novel pathways.

Introduction

With the birth of the new millennium, the prospect of complete sequences of both human and mouse genomes launches a new challenge for the researcher—how does one systematically assign a function to all gene sequences? Comparative sequence analysis and bioinformatics will undoubtedly further our understanding of gene function (Ashburner and Goodman 1997; Rastan and Beeley 1997), but may not effectively account for the function of novel gene sequences. In parallel with bioinformatics, the use of mutagenesis in the mouse will be a critical tool in functional genomics. Mouse mutants have an important role in discerning mammalian gene function and modeling human disease (Zhang et al. 1994; Gibson et al. 1995; King et al. 1997); however, at present mutants exist for only a small percentage of all mouse genes (Brown and Peters 1996). To address this issue, many researchers have directed their efforts towards increasing the breadth and depth of the mouse mutant resource by using a number of complementary approaches.

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Targeted mutagenesis has had a central role in the discovery of complex molecular pathways in a variety of organisms including the mouse (Capecchi 1989). Although such an approach is invariably successful, it is not amenable to the recovery of large numbers of mutations on a genome-wide basis, particularly in genes where structural and functional relationships have not been at least partially characterized. An additional confounding factor is that investigators tend to carry out only one or two phenotypic assays on mutants to characterize them. Invariably, such assays are selected on the basis of preconceptions of gene function, with the result that important functional data associated with a particular gene may be overlooked. The arrival of gene trap procedures in embryonic stem cells has introduced the possibility of genome-wide generation of targeted mutations (Hicks et al. 1997; Zambrowicz et al. 1998). Although this allows for systematic screening of many important genes, one disadvantage is that a priori assumptions must be made about the likely role and importance of any gene trap sequence that is the subject of further study.

An alternate strategy, already exploited in many organisms including the mouse, involves screening for particular aberrant phenotypes in progeny of chemically mutagenized animals (Nusslein-Volhard et al. 1984; Driever et al. 1996; Haffter et al. 1996; Shedlovsky et al. 1988; Rinchik et al. 1990). For this purpose, the chemical mutagen *N*-ethyl-*N*-nitrosourea (ENU) has been shown to induce mutations in mice at a high frequency (0.0015 per locus per gamete), meaning that screening fewer than 1000 gametes could potentially identify mutations at any given locus (Rinchik 1991; Hitotsumachi et al. 1985). In addition, ENU induces point mutations and is, therefore, ideal for the identification of new single-gene mutations. One further advantage of using ENU is that it is effective in inducing gain-of-function as well as loss-of-function mutations. The prospect of generating allelic series at new and existing gene loci is a particularly appealing prospect in the analysis of gene function. One disadvantage of using chemical mutagenesis as a phenotype-driven approach is that, having identified an aberrant phenotype, it still requires considerable effort to identify the underlying gene. Nevertheless, progress in the advancement and refinement of genome sequencing and bioinformatics tools will help in accelerating the transition from phenotype to gene.

There is no doubt that ENU induces mutations at a high efficiency (Hitotsumachi et al. 1985). With this in mind, the next challenge is to design and incorporate suitable phenotypic screens that will match the efficiency of mutation induction. Many research centers worldwide have risen to this challenge (for a review see Justice et al. 1999). To date, two European centers have in-

Table 1. Comparison of single versus fractionated dosage of ENU. Data obtained from BALB/c mice after intraperitoneal injection at 10–12 weeks.

Dosage	1 × 200 mg/kg	2 × 100 mg/kg
Return to fertility (days)	120	90
Average litter size	4.3	5.8
% sterile males	15	<5
% potential mutants	<1	>1

corporated large-scale systematic and hierarchical screens in progeny of mutagenized mice with a common goal of increasing the breadth and depth of the mouse mutant resource. Here, we describe the framework of a large-scale, dominant, genome-wide screen under way at the MRC Mammalian Genetics Unit, Harwell, and incorporating research carried out at multiple research centers within the UK (MRC, Harwell; Queen Mary and Westfield College, London; Imperial College, London and SmithKline Beecham Pharmaceuticals, Harlow). This program incorporates multiple phenotypic screens and will enable us to make a significant contribution to the mouse mutant resource.

Methodology and organization of the program

The animal studies described in this paper were carried out under the guidance issued by the Medical Research Council in "Responsibility In the Use of Animals for Medical research" (July 1993) and Home Office Project License No. 30/1517.

Mutagenesis. Selection of appropriate strains for mutagenesis: It is important to select appropriate mouse strains for mutagenesis and breeding, because major differences have been reported in responses both to the toxic effects and the mutagenic potency of ENU (reviewed recently by Davis et al. 1999). For the present study, male BALB/c mice (Charles River, UK) were used for mutagenesis because this strain is known to retain good fecundity and sustain a high mutation frequency after ENU administration. In addition, follow-up studies, including gene mapping and behavioral analysis, have been established with this strain. Male mice are injected with ENU at approximately 10 weeks of age. Procedures for ENU administration, including detailed safety procedures, are described in detail elsewhere (Nolan et al. 1997). Batches of 300 BALB/c males are injected at six monthly intervals. This will ensure a constant weekly supply of up to 300 progeny for phenotypic screens.

To ensure that repeat mutations are not scored, only 50 progeny are screened per male injected. The most comprehensive calculation on this number was based on data obtained by Rinchik and Carpenter (1999), who carried out a large-scale screen for mutants on a subregion of mouse Chr 7. An exhaustive analysis of non-complementing clusters of mutations in this region demonstrated that duplication of mutations does not arise when an average of 29 G1 females (58 G1 mice) are analyzed per G0 (mutagenized) male. It has also been our experience using multiple phenotypic screens that repeat mutations do not occur with 50 G1 mice screened per G0 male.

ENU dosage and dosage intervals: An assessment can be made on the effectiveness of ENU administration/toxicity based on several simple parameters (Table 1). Using a specific locus test to determine the mutagenic efficiency of ENU, Hitotsumachi et al. (1985) found that ENU appeared to be less toxic and more mutagenic when administered in fractionated doses at weekly intervals. Simple estimates of the toxic effects of ENU can be established by measuring the period of temporary sterility induced by ENU and the average litter sizes upon return to fertility. Since ENU is mutagenic or cytotoxic to all stages of spermatogenic cells, successfully mutagenized animals are rendered temporarily sterile after existing postmeiotic cells have completed spermiogenesis to become mature spermatozoa. We have found an intraperitoneal injection of 200 mg ENU/kg to be an appropriate dose in 10- to 12-week-old BALB/c mice on the basis of these measurements. Moreover, we have found that by fractionating this into two weekly doses of 100 mg/kg, not only is the sterile period per male reduced and the litter size increased, but also the incidence of dominant mutations in progeny of this group appears to be larger. In all future studies, a fractionated dose of 2 × 100 mg ENU/kg is used.

Generation of F1 mice for phenotypic analysis: To identify new dominant mutations, screening is carried out on the F_1 offspring and later descendants of mutagenized BALB/c mice crossed to C3H/He females (Charles River, UK). To ensure that enough progeny are produced in the lifespan of a male, two females are housed with each male. Although phenotypic analysis may be complicated in subsequent genetic crosses, screening of F_1 hybrid progeny has many advantages. Firstly, maintenance of dominant mutations on a hybrid background can be more robust than when maintained on an inbred line. Screening of F_1 hybrids is also more advantageous for genetic mapping because the first cross in a genetic backcross will have already been set up. Finally, production of F_1 hybrids in this way will still ensure that a genetically homogeneous population of mice are screened.

Primary screens. The following screens are routinely carried out on F_1 progeny of mutagenized males as well as on mouse lines in inheritance testing, on inbred strains, and on a control cohort of 2000 BALB/c × C3H/He F_1 hybrid mice. A graphical representation of the procedural sequence is shown in Fig. 1.

Dysmorphology screen: The first step in this large-scale program is to systematically screen for anomalies identifiable at birth and weaning. Throughout the program, matings are checked on a daily basis so that information from birth onwards can be accurately recorded. Information accumulated at this stage of the screen will be vital in the eventual phenotypic description of potential mutants. A complete list of classifications used at birth and up to weaning is shown in Table 2. Examples of mutants identified to date include anomalies in size and pigmentation as well as craniofacial, limb, and tail defects. Size defects at birth are recorded by weighing the abnormal neonate plus two siblings of the same sex. For identification purposes and phenotypic analysis at later stages, mice can be identified by toe snipping. All mice are weaned at 3 weeks of age, and at this stage are group housed in cages of up to five animals. Mice are marked with ear punches for identification purposes.

The SHIRPA screen: At 5 weeks of age, all mice are assessed by using the SHIRPA protocol (Rogers et al. 1997). Analysis of data from this test provides a comprehensive profile and can indicate deficits in muscle and lower motoneuron, spinocerebellar, sensory, neuropsychiatric, and autonomic function. The screen, involving a battery of up to 40 simple tests, is semi-quantitative and based on earlier screens by Irwin (1968) developed to classify systematically a range of drug effects in wild-type animals. The procedure is carried out in a simple testing arena and takes approximately 10 min per mouse. It is also possible to add further tests to the current battery to expand the range of phenotypic observations. An advantage of the SHIRPA screen is that, as well as being used to identify novel mutations that are not obvious by simple observation at birth, weaning, or later, it can also be used

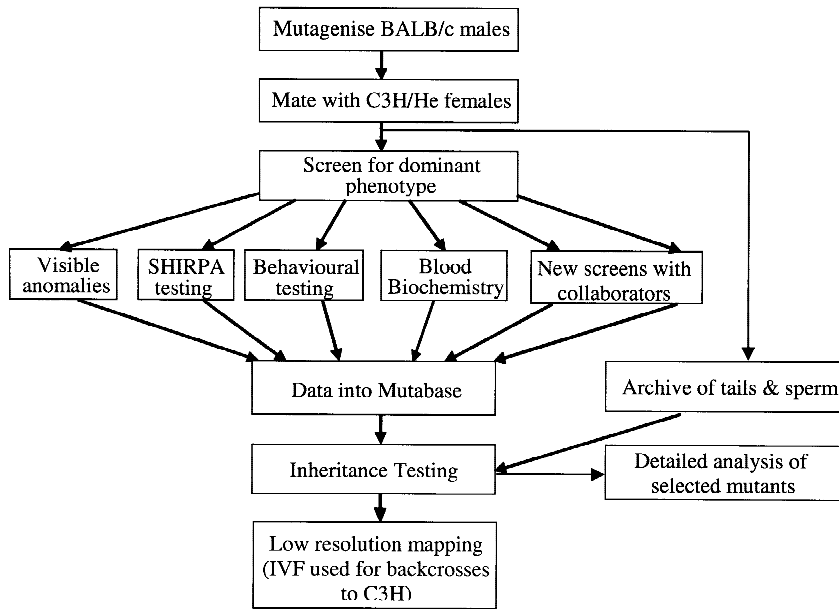

Fig. 1. Flow chart indicating the sequence of current procedures in the ENU mutagenesis program.

Table 2. Screening for visible defects at birth and weaning.

Category	Birth	Pre-weaning	Weaning
Size	Large/small	Large/small	Large/small
Sense organs	Eye size/color		Eye size/color
	Low-set ears		Ear size/position
Skin and hair	Anaemia	Stripes	Skin color/texture
	Skin color/texture	Skin color	Loose/tight skin
	Spots on/under skin	Blotchy coat	Greasy/rough coat
	Curly whiskers		Curly coat/whiskers
			Thin/balding coat
			Dark footpads
Behavior	Activity	Activity	Activity
		Tremors/fits	Tremors/fits
		Circling	Circling
		Head weaving	Head weaving
		Ataxia/gait	Ataxia/gait
Skeleton	Micrognathia		Micrognathia
	Agnathia		Agnathia
	Short head		Short/wide/thin head
	Scoliosis		Scoliosis
			Cleft palate
Tail and extremities	Short/bent tail		Short/bent tail
	Poly- or syndactyly		Poly- or syndactyly
	Fused toes		Fused toes
	Limbs		Extra limbs
	Bent/short limbs		Bent/short limbs
	Puffy limbs/tail		Puffy limbs/tail
Color and white spotting		Belly spot	Belly spot
		Head blaze	Head blaze
		Coat color	Coat color
Various	Blebs/bruising	Hydrocephaly	Hydrocephaly
	Edema		
	Exencephaly		
	Hydrocephaly		
	Spina bifida		
	Chylous ascites		

to categorize mutants detected in other phenotypic screens. For example, a particular mutant identified at birth and weaning with craniofacial defects also had a phenotype at SHIRPA that categorized this mouse as passive to handling. Once abnormal phenotypes at SHIRPA are identified, results are confirmed by an independent operator. To date, close to half of the mutants identified in our screen are classified based on a SHIRPA phenotype alone. However, subsequent analysis of these mutants by using secondary and tertiary screens may be successful in identifying additional anomalies associated with these mutations.

Behavioral screens: Targeted mutagenesis studies have underlined the fact that mutations in single genes can affect components of complex mammalian behaviors (Lijam et al. 1997; Silva et al. 1992; Brown et al. 1996). For the purposes of this program, two screens are carried out on F_1 progeny at 6 weeks of age, based on their simplicity and on the likelihood that they may identify components of human inherited disorders. The first of these, monitoring locomotor activity in mice, can be used to identify models of anxiety disorders as well as deficits in motor function and abnormally high activity levels (Krezel et al. 1998; Saudou et al. 1994). Up to 24 mice at a time can be maintained in cages equipped with beam-splitting devices (Benwick Electronics, UK). Activity is measured by recording the number of beam splits and the number of cage transitions over a certain period of time. Briefly, activity is recorded for 35 min in bins of 5 min duration. Total activity and transitions are measured over the last 30 min of this time period. Values in the first 5-min bin are ignored owing to the extreme variations in results. To date, we have screened close to 3000 F_1 progeny for abnormal locomotor activity (LMA) scores. By expressing our results in a population distribution format and using a cutoff of 3 standard deviations from the population mean, we have identified outliers in 5% of the F_1 population. It is interesting to note that mice expressing particular phenotypes (e.g., circling) are particularly active in LMA. Prior to inheritance testing, outliers are retested one week later and only those mice with consistently abnormal scores are considered for inheritance testing. To date, approximately 20% of our retest scores are consistent with the original score.

Similarly, screens for abnormal acoustic startle response and for deficits in prepulse inhibition of the acoustic startle response (PPI) can be screened for systematically and efficiently in F_1 progeny of mutagenized males. The acoustic startle response is characterized by an exaggerated flinching response to unexpected auditory stimuli. This response can normally be attenuated when it is preceded by a weaker prestimulus—PPI. Deficits in PPI have been associated with a number of human psychiatric disorders such as schizophrenia (Geyer and Braff 1987), and recently mouse models with single-gene defects exhibiting deficits in PPI have been identified and characterized (Lijam et al. 1997; Gogos et al. 1999). The screen for potential mutants is similar to one described earlier (Nolan et al. 1997). Briefly, up to 12 mice at a time are tested in soundproofed startle chambers. Startle chambers contain an inner chamber equipped with a loudspeaker and startle platform linked

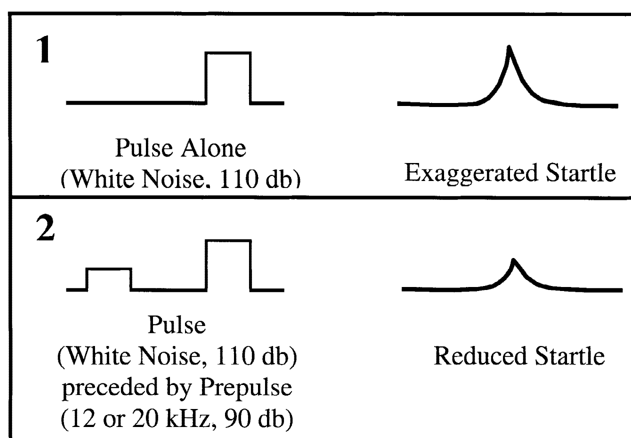

Fig. 2. Prepulse inhibition of the acoustic startle response. 1) Mice, when subjected to a white noise sound pulse at 110 db, typically respond with an exaggerated startle response. Mice are screened for abnormally high or abnormally low startle. 2) When this sound pulse is preceded by a weaker prepulse (at a frequency of 12 or 20 kHz and a reduced amplitude of 90 db), the startle response is reduced dramatically. Mice are screened and individuals identified where this reduction does not occur.

to a computer via an accelerometer. Tests are divided into startle and PPI sessions, and the entire procedure lasts approximately 40 min. For the startle response, a session consists of a 5-min acclimation period followed by three types of trials presented a total of 15 times each in pseudorandom order: 110 db pulse at a frequency of 12 or 20 kHz or white noise. Responses are expressed in arbitrary units and averaged for each type of trial. Duration of pulses are 10 ms and the interval between pulses is 25 s. For PPI (Fig. 2), animals receive either a pulse alone (white noise, 110 db) or pulse preceded by a 90-db prepulse at a frequency of either 12 or 20 kHz. The interval between prepulse and pulse is 100 ms. Again, each trial is presented a total of 15 times in pseudorandom order. Startle responses of mice when given a pulse after a prepulse are expressed as a percentage of the pulse response alone, and values are averaged over the trial period. As with the LMA studies, results for F_1 progeny are expressed in population distribution format and outliers identified as being greater than 3 standard deviations from the population mean. Outliers are retested after 1 week, and only those with consistently abnormal scores are considered for inheritance testing. Interestingly, these numbers are similar to those in the LMA screen; 5% of mice are outliers, and approximately 15–20% of these show consistently abnormal parameters.

Hematology and clinical biochemistry: In addition to abnormal visible, neurological, and behavioral parameters, cohorts of mice are tested for abnormal hematological and blood biochemical profiles. Biochemical markers have been selected to assess hepatic, renal, bone, muscle, and lipid function, and a summary of tests used is indicated in Table 3. In total, 22 parameters are measured for each mouse. Blood tests are carried out at between 6 and 10 weeks of age, and data are analyzed in groups of male and female animals. This allows us to account for sex differences in any of the parameters tested. Mice are prewarmed in Thermacages (Datesand Ltd, UK) prior to tail tipping, and blood is collected by capillary action into lithium-heparin-coated tubes (Sarstedt Ltd, UK). Throughout the procedure, mice are kept in suitable restraining devices, and tails are anaesthetized with an ethyl chloride spray. Not more than 300 μl of whole blood is collected in this way. For clinical biochemistry, whole blood is centrifuged at 3000 rpm for 10 min in a refrigerated centrifuge. Ideally this should be done immediately, but not later than 4 h after bleeding to prevent *in vitro* changes in the parameters of interest. 130 μl of plasma is required to perform the current standard profile of assays. Hematology, full blood count and differential, requires 100 μl of whole blood. Hematological analysis will be incorporated at a later date. For automated analysis of blood biochemical profiles, an Olympus AU400 analyzer is used. Two criteria are used to identify potential outliers—mice with values greater than 3 standard deviations from the mean for any one parameter or greater than 2 standard deviations from the mean for groups of related parameters. Mice meeting either of these criteria are retested after 1 month, and those with consistently abnormal parameters are selected for inheritance testing. Currently abnormal parameters have been identified in approximately 10% of mice tested, and 14% of mice retested show consistently abnormal parameters.

Table 3. Screening for abnormal hematological and blood biochemical profiles.

Hematology	Hepatic	Renal
White cell count	ALT	Sodium
Red cell count	AST	Potassium
Hemoglobin	Total protein	Chloride
Platelets	Albumin	Creatinine
3 Part differential		Urea
Lipid	**Bone**	**Other**
Total cholesterol	ALP	Glucose
HDL cholesterol	Total calcium	Bicarbonate
Triglyceride	Inorganic phosphate	

Tail tipping and genotype based screens: The high rate of per locus mutation induction by ENU can also be exploited to incorporate a genotype-based screening approach. With this approach, it will be possible to screen a DNA archive for mutations at a particular locus and then recover live animals carrying these mutations. The animals can then be assayed for the biological effects of the mutations on heterozygous or homozygous phenotype. In preparation for the incorporation of such screens, an archive of F_1 tail tips is being generated. When mice are tail bled, 1 cm of tail tip is removed from each F_1 male, cut to give duplicate samples and frozen at −70°C. Tail tips can be recovered at a later date and genomic DNA prepared for sequence analysis or for PCR-based assay methods.

Aging phenotypes: As part of the ongoing mutagenesis program, 10% of all mice screened are being maintained in-house for up to a year and retested for late-onset phenotypes. Screens will include repeats of all those carried out to date with a particular emphasis on important age-related parameters such as late onset weight gain, atherosclerosis, osteoporosis, cardiovascular anomalies, and deficits in cognitive function and behavioral performance in general. Mice to be included in this screen will be selected on a random basis from groups of mice exhibiting no earlier phenotypes.

Archiving and data mining: One of the major functions of this phenotypic screen will be to alert researchers to this new mutant resource. For this reason, all information on the results of phenotypic screens is available via our website (http://www.mgu.har.mrc.ac.uk/mutabase/). Phenotypic data are posted as soon as they become available, and mice are maintained on the shelves for 5 weeks after the posting of these data. If, at the end of this period, no requests for mutants have been received, mice will be either terminated or archived.

Sperm and ovary freezing: Currently, sperm from all males (quintuplicate samples) are being archived under liquid nitrogen vapor (including sperm from mice with no identifiable phenotype). This will enable us to recover mutant lines if abnormalities are

identified in, for example, a genotype-based screen (Thornton et al. 1999). Currently, ovaries from females with abnormal phenotypes are also being frozen for future recovery. To date, no plans for archiving tissues from females without a detectable phenotype are proposed.

Mutabase: A critical aspect to the mutagenesis program is that all data be readily accessible and available for cross-referencing. Information on all data, from treatment with mutagen to reports on all phenotypic screens, is archived with Mutabase, a distributed data recording system specifically developed for this program (Strivens et al., this issue). Mutabase is used, for example, to determine duration of sterility and litter sizes associated with a particular batch or dosage of mutagen, to record all birth and weaning data, and to cross reference abnormal phenotypes for a particular mouse in the various screens currently being utilized.

Characterization of identified mutants. Inheritance testing: Identification of abnormal phenotypes in F_1 progeny of mutagenized males is the first step in expanding the mouse mutant resource. Inheritance testing on selected potential mutants is carried out by backcrossing to the C3H/He strain and assessing phenotype in 20 backcross progeny. If the phenotype is inherited in a dominant fashion, then 50% of backcross progeny should also express the abnormal phenotype. Often, however, we have found this number to be lower than 50%, indicating that the abnormal phenotype may not be fully penetrant in the backcross being tested. Also, in particular cases, classification of an abnormal phenotype may be difficult in mice backcrossed to C3H/He. In these situations, mice can be alternatively backcrossed to the BALB/c strain if classification is easier. The proportion of lines where phenotype was not inherited includes mice that were sterile, that did not breed well, that died owing to illness, or that were simply too difficult to classify in either backcross.

Genetic mapping: One of the first steps in characterizing a novel mutation is assigning a map position. An additional challenge for the mutagenesis program was to generate mice for backcrosses at a rate that would complement the rate of mutation induction. This has been partially handled by the way the screen has been set up. Crosses for progeny testing in this program can also be used for backcross analysis. In addition, as soon as a mutation is confirmed as being inherited, it is possible to generate immediate large backcross stocks by using in vitro fertilization (IVF) from fresh or frozen sperm of affected males (Thornton et al. 1999). In a typical IVF, we can generate approximately 100 mice for genotypic analysis. If the F_1 is a male, sperm can be recovered from this mouse once inheritance is confirmed. In cases where the F_1 is a female, we can use one of the affected males from the N2 generation to produce a large stock for genome scans. To map mutations, following confirmation of an autosomal dominant mode of inheritance, backcross mice are scored for abnormal phenotype, and DNA is prepared from tail tips. As a precautionary measure, only DNA from affected mice is used, thus ensuring that mistyping owing to incomplete penetrance of abnormal phenotypes need not be considered. Equimolar aliquots of DNA from 30–50 mutant backcross mice are combined and DNA pools scanned with 200 fluorescently labeled microsatellite markers spanning the mouse genome with an average spacing of 10 cM. With this system, both DNA samples and fluorescently labeled amplicons can be multiplexed on genotyping gels. Linkage is determined by measuring the peak heights of fluorescently labeled alleles and comparing these with peak heights of the F_1 (or founder) mouse. Differential amplification of BALB/c and C3H/He alleles is accounted for by comparison with individual F_1 genotypes and appropriate adjustment of signal strength. The ratio of allelic peak heights from linked markers should approach those of the founder mouse. Subsequent to linkage determination, map positions can be confirmed by using individual DNAs and by genotyping with additional markers in the critical region and/or additional tail DNAs from affected mice. Genetic analysis can be augmented by determining the phenotype of homozygous mice. By genotyping progeny of intercrosses between affected mice with linked markers, we can determine whether the mutation is dominant or semi-dominant, or indeed whether the mutation is homozygous lethal.

Secondary and tertiary screens on selected mutants: Generation of large backcross stocks using IVF has the additional advantage that large-scale phenotypic analysis can be carried out rapidly on a mutant stock. The major advantage of this is that in one session we can screen large numbers of mutants and wild-type siblings where the parents are virtually identical. A major goal of the current program is to characterize mouse lines in all screens detailed above to get an accurate and complete phenotypic description for each line. In general, we aim to screen five mutants and five littermate controls in each test (e.g., for overt anomalies, SHIRPA, locomotor activity, PPI, hematology and blood analysis). In addition to this, histology will be carried out on all major organs. Where appropriate, mice have also been tested on a rotarod apparatus and some preliminary brain neurochemistry has been carried out. Eventually, tertiary screens will also be carried out and will include more sophisticated investigations employing, for example, electroencephalography (EEG), nerve conduction, electromyography, and magnetic resonance imaging (MRI).

Discussion

Although there is undoubted evidence as to the power of the phenotype-based approach from large-scale screens in model organisms such as *Drosophila,* time and space constraints make this a more difficult prospect in the mouse. We may never be able to produce such large populations of subjects for phenotypic analysis necessary for the generation of large allelic series/complementation groups. Nevertheless, an alternative strategy in the mouse appears to be the generation of a smaller number of subjects that are mutagenized by using a highly efficient mutagen such as ENU and the design and incorporation of as many phenotypic screens as possible in this same set of mice. On an ever-increasing basis, new reports are being published on the utility of ENU mutagenesis in uncovering novel mutations in a range of screens for phenotypes as diverse as circadian rhythms (Vitaterna et al. 1994) and mouse development (Kasarskis et al. 1998). This mutagenesis program benefits from the incorporation of diverse phenotypic screens; however, it is still likely that many subtle phenotypes could be uncovered by the incorporation of additional screens on the same set of mice. These mice are currently available for use by outside investigators to develop new tests. Additional screens have already been proposed and instigated on these mice. One project involves screening for deafness mutants by measuring a subject's responsiveness to a 20-kHz tone, a sensitive frequency for the mouse. Data from this screen can subsequently be compared with startle and PPI data where different frequency tones are used. An additional project carries out behavioral and clinical screens related to the mouse visual system. Additional proposals include systematic x-ray analysis. With this screen, x-rays can be scanned onto computer and analyzed by any number of collaborators/researchers. The major benefits of such multilayered screening includes not only the fact that an important mouse resource will be utilized to its full effect, but also that complete phenotypic descriptions of mouse mutations can be generated and disseminated to the scientific community.

ENU mutagenesis can be used to exploit the existing mouse mutant resource and further the cause of functional genomics by

using several adaptations of the dominant screen. The use of modifier screens and/or sensitized screens can be established by crossing mice with existing mutations (deletions, null mutations) to ENU-mutagenized mice. Progeny of these crosses with an abnormal phenotype will fall into one of three classes: 1) new dominant mutations, 2) new alleles of the existing mutation or 3) modifiers of the existing mutation. Simple additional crosses can be set up to establish the class of new mutation. The generation of an allelic series in addition to null alleles will allow a better insight into the function of a gene (Mburu et al. 1997; Cordes and Barsch 1994). The identification of modifiers of a particular phenotype will contribute towards the elucidation of complex molecular pathways associated with many physiological processes.

The relative merits and drawbacks of dominant versus recessive screens have been well documented (Brown and Nolan 1998; Schimenti and Bucan 1998; Justice et al; 1999). As a compromise between these two approaches, many researchers have documented proposals for region-specific mutagenesis on a genome-wide basis (Justice et al. 1997; Schimenti and Bucan 1998). As these screens are implemented, they should provide a key to systematic functional genomic analysis. The region-specific mutagenesis screens make use of chromosomal deletion mutations generated throughout the genome and require two generations of crosses rather than three, thus alleviating many time and space constraints. This approach should identify recessive mutations within a particular genomic region and, depending on the number of gametes screened, should approach saturation. The success of these screens will nevertheless depend on a number of factors. The presence of nested deletions within a particular region will simplify positional cloning efforts and complementation analysis. One problem to be tackled is that the genetic background of the screening population (G2) will be a mixture of the genomes of the mutagenized male and that of the deletion stock, which may modify the particular phenotype being studied. Again, the use of substantially diverse phenotypic screens is recommended to identify all potential mutations. In a region-specific screen carried out on a 6- to 11-cM region of mouse Chr 7 (Rinchik and Carpenter 1999), over 4500 gametes were screened. Although mutations were identified at new loci as well as new alleles of existing mutations, the authors were unable to recover phenotypes corresponding to those seen in homozygous or partially complementing deletions in the region. Although the deletion phenotypes could be the result of a contiguous gene syndrome, it was recommended that all possible phenotypes be screened to approach saturation in any genomic region. As part of the mutagenesis effort, a similar region-specific screen is proposed for a Chr 13 region defined by the 36H deletion (Lyon et al. 1996). As this region is also a target for sequencing in the corresponding human region, phenotypic and bioinformatics resources will be combined in the functional analysis of this region of Chr 13.

The indications from this mutagenesis screen are that large-scale phenotypic screens will be informative in the new area of functional genomics. Dominant screens as well as modifier screens and region-specific screens can be used effectively to assign function to gene sequences and to identify important molecular pathways. This, in combination with bioinformatics and sequence analysis, will be a powerful tool in the analysis of gene function.

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