MINIREVIEW

Using High-Efficiency Mouse Germline Mutagenesis to Investigate Complex Biological Phenomena: Genetic Diseases, Behavior, and Development (43905)

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Abstract. **A valuable approach to investigating a biological process is to study the effect of mutations in the involved genes. By studying a diverse set of such mutations, one can gain important insights into the roles that the given gene product plays in the biological process. Although this approach has long been recognized, the scarcity of mammalian mutations has largely limited such investigations to simple organisms. It has recently been shown that highly efficient mutagenesis of the mouse germline with a random point mutagen can produce mutations that are valuable in several important ways. First, it can produce numerous different types of mutations. Second, it can be used to mutate genes that have yet to be cloned or characterized. Genes that have been marked by mutation can ultimately yield molecular access after mapping to high resolution and cloning from map position. Such new investigative capabilities will ultimately allow one to gain intimate knowledge of the molecular basis of complex biological processes like behavior and development. Third, mutations can be induced that yield animal models of human heritable diseases. Such disease models allow for intensive research into the etiology of the given disease and also permit the facile evaluation of new therapeutic regimens.** [P.S.E.B.M. 1995, Vol 209]

The laboratory mouse provides an attractive model for human biological phenomena. In addition to the ease with which mice can be bred and maintained in the laboratory, there is a growing body of evidence detailing the similarity between the genomes of mouse and humans (1). These features, while critical for the utility of the laboratory mouse as a human model system, would be insufficient were it not for an experimental feature of the genetics of the laboratory mouse. Two mutagenesis protocols have been developed that allow the induction and recovery of mutations in genes of interest. These two methods are random high-efficiency mutagenesis of mouse germ cells and targeted gene knockout in mouse embryonic stem cells (for recent reviews, see Refs. 2 and 3). This article will discuss a range of applications for high-efficiency chemical mutagenesis with a point mutagen.

A thorough investigation of the role of a gene product in the life of an organism requires a diverse set of mutations. A single mutation often reveals only part of the overall contribution of a gene product in development. Conclusions about the role of a gene product drawn from a single mutation may be very misleading if the mutation produces an atypical phenotype. Upon

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studying a diverse set of mutant alleles, such errors are avoided. Therefore, in order to thoroughly investigate the role of a gene, one would like to carry out mutagenesis with agents that produce many different types of mutations. A key strength of high-efficiency point mutagenesis of the mouse germline is this ability.

A brief description of the discovery of high-efficiency mouse mutagenesis should assist in understanding the capabilities of this method for biological inquiries. Using an assay system based on genetic noncomplementation, called the specific locus screen, the DNA alkylating agent *N*-ethyl-*N*-nitrosourea (ENU) was discovered to be the most powerful mutagen known for the mouse germline (4). This work and other studies that followed have identified exposure conditions that have progressively led to even higher induced mutation frequencies than those originally obtained (Table I, Rows 1 and 2). Other mutagenesis experiments, examining the induced mutation frequency at a single locus (Table I, Rows 3–6), have sometimes noted much higher induced mutation frequencies. However, this seems more likely to reflect locus-specific or mouse line-specific variation in ENU mutagenesis susceptibility rather than a breakthrough in optimizing the treatment protocol.

The specific locus screen requires the availability of a recessive mutant allele at the locus of interest. New mutant alleles can then be isolated after mating mutagenized animals to animals homozygous for the initial mutation. This noncomplementation strategy requires only one generation and is therefore the most efficient. It has long been known that the mutagenic action of ENU in lower organisms and in cultured cells yields almost exclusively single base substitutions (5–12). These observations have been uniformly upheld for ENU-induced mutations in the mouse germline that have been sequenced (13–17; McDonald JD, Charlton C, in preparation). This has very important ramifications for the types of mutations recovered from ENU mutagenesis. Single base changes are capable of inducing all of the different classes of mutations referred to on the basis of their effect on the function of the gene product (Table II). The overall strength of ENU mutagenesis is a combination of this full-featured mutational profile and the extremely high mutation frequency that can be induced through its use. The latter feature bears most directly on the likelihood of successful mutation recovery; the former feature allows for thorough genetic examinations.

The high mutagenic potential of ENU permits one to mutate genes about which very little is known. It is very important that the mutant phenotype be clearly recognizable. Given this minimal requirement, a mutated gene can be recovered by identifying progeny that have inherited it and are displaying the altered phenotype. This provides a way to study the great majority of genes that have yet to be cloned or characterized. Indeed, in this scenario, marking a gene by mutation can represent the first step in a chain of events that ultimately provides molecular access to the gene in question. The genetic map of the mouse is the most highly characterized mammalian map, rivaled only by the human map. Efforts on the part of many different groups have taken what was formerly a difficult and tedious undertaking (reviewed in Ref. 18) and provided mapping strains and strategies that have made the localization of new mutations gradually more facile. These methods range from systems that exploit the polymorphic diversity between different inbred mouse lines (19) to those that use endogenous viral genomes as genetic markers (20) to mapping systems that exploit the polymorphic diversity between laboratory and wild-derived mice (21, 22). The recent development of a PCR-based mapping technique called simple sequence length polymorphism (SSLP) (23) has brought the speed and accuracy of mouse genetic mapping to new levels. As an example, this technique recently proved its enhanced capabilities by allowing the mapping of the ENU-induced mouse mutation *Clock* in several months rather than the much longer periods

Table I. Induced Mutation Frequency with ENU

Exposure method	Mutation frequency[a]	Locus[b]	Reference
Single[c]	1/2000	Specific locus group	4
Multiple[c]	1/1000	Specific locus group	42–44
Single[d]	0/105	*Pah*	32
Single[e]	1/167 (3/500)	*Pah*	30, 31
Multiple[f]	0/365	*Pah*	McDonald, unpublished
Pooled single and multiple	1/323 (3/970)	*Pah*	

[a] Mutation frequency is expressed as the number of mutant loci recovered per gametes screened for mutation. This frequency is reduced from total number to lowest terms for ease of comparison. In the case of mutations at *Pah*, actual mutant yield is added parenthetically to give an indication of the total numbers on which the estimate is based.
[b] The specific locus group is a group of seven genes used as reporters in mutagenesis experiments because they yield easily detectable visible traits in homozygous mutants (45).
[c] Mutagenized animals were (101 × C3H)F1.
[d] Mutagenized animals were (C57Bl/6 × CBA/Ca)F1.
[e] Mutagenized animals were BTBR/Pas.
[f] Mutagenized animals were C57Bl/6.

Table II. Classification of Mutations on the Basis of Their Effect on the Function of the Gene Product[a]

Effect of mutation on gene product	Classification
Gene product acts with reduced efficiency	Hypomorphic
Gene product acts with increased efficiency	Hypermorphic
Complete loss of gene product function	Amorphic
Gene product acquires novel functions	Neomorphic
Mutant gene product antagonizes normal gene product function	Antimorphic

[a] Modified from Ref. 46.

typically required for previous methods (24). The SSLP technique is currently capable of locating nearly any genetic locus to an interval of 1 to 2 megabases of DNA and is proceeding toward a map density that will allow the localization of sites to the submegabase level (25). This capability can lead directly to molecular access because it localizes a genetic site to an interval of DNA that can be cloned intact into a large capacity cloning vector like the yeast artificial chromosome system (26, 27). It is precisely this rationale of positional cloning, made possible by the combined abilities to quickly map mutations to high resolution and then clone the indicated interval into large capacity cloning vectors, that makes high-efficiency mutagenesis of the mouse germline a useful method for gaining access to the molecules that underlie complex biological phenomena.

Because most mutations are recessive, one is typically confronted with the need to produce homozygous mutants before significant phenotypic variation is observed. This can be accomplished by a small-scale pedigree analysis of the gametes from an ENU-treated animal. Although such analysis can be rather time and resource intensive, once an initial mutant allele of a gene has been produced, it can be used to isolate additional mutant alleles by noncomplementation in a much less demanding screening protocol (Fig. 1).

The Use of ENU to Produce Animal Models for Human Diseases

ENU mutagenesis has been used to produce a number of mouse models for human diseases (3). The most systematic use so far has been for modeling human phenylketonuria (PKU) (28, 29). Three mutant alleles of the mouse phenylalanine hydroxylase gene, the homolog of the gene responsible for human PKU, have been produced and isolated (30, 31). In addition, this project produced mutations leading to syndromes related to PKU; one mutation causing a deficiency of the enzyme GTP-cyclohydrolase I (32) and two yielding possible models for Hartnup's disorder (unpublished). The success of this project was made possible not only by the high mutation frequency inducible by ENU but also by the existence of a facile and accurate method for detecting the phenotypic alteration most consistently associated with all forms of PKU; elevated blood levels of phenylalanine. The Guthrie assay, a bacterial growth-inhibition assay, provided a phenotypic selection system with a number of important features: ease of use, ability to screen many individuals per assay, and a very low rate of false-positive indications (33). With this selection system in hand, it was possible to easily and accurately screen the num-

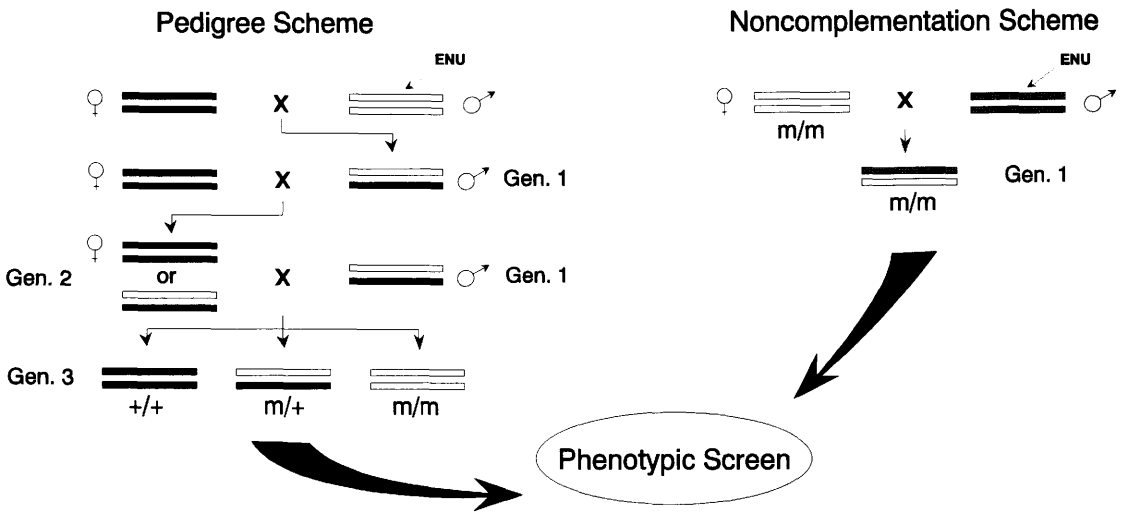

Figure 1. Two different breeding schemes have repeatedly been successful in permitting the identification and isolation of new mutations by the phenotypic variation seen in mutant offspring. In the pedigree scheme, several crosses must be done to produce homozygous mutant offspring. In the noncomplementation scheme, a preexisting mutation allows the more facile production of mutant offspring.

ber of offspring needed to successfully recover numerous mutant mouse strains.

Although the mouse PKU project was highly focused in its goals, its success is encouraging for those undertaking the production of other heritable disease models. Because inborn errors of metabolism typically produce dramatic perturbations in the levels of circulating and excreted metabolites, a potentially mutant offspring could be screened for a wide variety of inborn errors of metabolism by a comprehensive analysis of their blood or urine. Indeed, a short search based on examining perturbations in urinary metabolites quickly led to the isolation of a mouse model for the inborn metabolic error sarcosinemia (34).

The Use of ENU Mutagenesis to Study Mouse Behavioral Genes

Clearly genes affect the behavior of organisms. However, achieving molecular access to these genes has proven very difficult. Nonetheless, the availability of high-efficiency mutagenesis is beginning to permit isolation of mutants when the behavioral phenotype is well defined. For example, important progress has recently been made in the realm of the genetic basis of circadian behavior in higher organisms. The initial indication that this class of genes could be so studied came from investigations into the genetic basis of circadian behavior in lower organisms. It was shown that most such mutations were inherited in a dominant or semidominant manner. This is an extremely important finding because the detection of variant phenotypes for this class of mutations is much more difficult than the simple biochemical assay described for PKU mutants. Because of the likely heritability pattern of this class of behavioral genes, the immediate progeny (Generation 1 animals) could be directly examined, saving considerable labor, time, and space. Thus, the first circadian rhythm mutant ever produced in higher organisms was recently isolated successfully (24). It is important to note that, in this case, while previous genetic work in lower organisms was able to indicate a genetic basis and suggest a likely heritability pattern, no DNA clones or other molecular reagents derived from these organisms were available that allowed access to the genes in higher organisms. This necessitated a mutagenesis approach as a means of identifying and marking involved genes. These genes can now be located and characterized to reveal much more about the molecular basis of circadian behavior in higher organisms (35).

Just as the mouse PKU project suggested likely future successes in the production of mouse disease models, so the mouse circadian behavior project suggests that other behavioral traits may yield to a mutagenic approach.

The Use of ENU Mutagenesis to Study Development

In general any gene can be put into a developmental context. However, it is the genes most directly involved in the ontogenic development of an organism that are typically placed in the category of developmental genes. The most thorough use of high-efficiency mutagenesis to study such genes has been the production of a set of embryonic lethal mutations all located in a region of mouse Chromosome 17 called the *T*-region (36). Some of these mutants were isolated by following a classical inbreeding pedigree emerging from a gamete that carried a recessive visible marker (tufted) in the region of interest. Failure to recover the homozygotes for such a visible marker signaled the presence of a recessive lethal (or detrimental) mutation in the region.

This set is currently allowing a previously impossible investigation into the role of specific genes in embryonic development. Indeed, it is here that the molecular access that can be achieved by mutagenic marking of genes of interest is most evident. Using these mutations along with other cloned markers from the area, a genetic map of the region was constructed and increasingly refined (37–39). This high-resolution mapping information has already aided in the cloning of one important developmental gene from the region (40) and promises to allow soon the recovery of another (41).

Final Thoughts on the Use of High-Efficiency Mutagenesis As a Tool to Study Complex Biological Phenomena

ENU mutagenesis has shown itself to be an effective tool for a number of different research pursuits. Owing to its random mutagenic nature, it is capable of mutating any gene. Indeed, a survey of loci mutated to date does not reveal any class of gene that is exempt from ENU mutagenesis. This makes the agent useful in many different approaches to studying the biology of higher organisms. It is hoped that the description of several specific ways in which it has been used successfully will stimulate life scientists to think about how it could be used to help them gain access to the molecules that mediate the specific biological process in which they are interested.

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