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Mouse Mutagenesis with the Chemical Supermutagen ENU

Frank J. Probst and Monica J. Justice

Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas, USA

Abstract

The generation and analysis of germline mutations in the mouse is one of the cornerstones of modern biological research. The chemical supermutagen *N*-ethyl-*N*-nitrosourea (ENU) is the most potent known mouse mutagen and can be used to generate point mutations throughout the mouse genome. The progeny of ENU-mutagenized males can be screened for autosomal dominant phenotypes, or they can be used to generate multigeneration pedigrees to screen for autosomal recessive traits. The introduction of balancer chromosomes into the breeding scheme can allow for the selective capture of mutations in a specific chromosomal region. More recent work has demonstrated that the use of animals that already have a mutation of interest can lead to the successful isolation of additional mutations that modify the original mutant phenotype. Further, modern molecular techniques ensure that mutations can be readily identified. We describe here the procedures for mutagenizing male mice with ENU and explain the various types of screens that can be performed for different kinds of induced mutations. The currently published research on ENU mutagenesis in the mouse has only scratched the surface of what is possible with this powerful technique, and further work is certain to deepen our knowledge of the role of the individual components of the mouse genome and the myriad relationships between them.

1. Introduction

1.1. A brief history of mouse mutagens

The ability to generate and maintain pathologic mutations in the house mouse (*Mus musculus*) is one of the most powerful tools available to modern scientists. The study of mouse mutations is generally either *genotype-driven*—in which a specific mutation is engineered in a particular gene, and the resulting animals are analyzed for abnormalities—or *phenotype-driven*—in which a population of mice are screened for individuals with abnormal phenotypes, and the subsequent analysis focuses on identifying and analyzing the underlying genetic changes that are responsible for the abnormalities.

Phenotype-driven mouse mutation screens typically begin with a mutagenesis step in order to increase the number of DNA lesions in the population being screened. Early studies on the ability of various mutagens to generate germline mutations in the mouse focused on the use of a *T*(test) stock. The original *T*-stock mice were homozygous for seven different autosomal recessive, viable mutations affecting coat color and ear shape. When a *T*-stock female is bred to a wild-type male, the progeny will usually be heterozygous at all seven loci and will therefore appear wild-type. However, if the male parent transmits a second recessive mutation at one of the seven loci, then the offspring will have an abnormal coat color or ear shape. Analyzing the number of abnormal progeny allows for a determination of

the mutation rate in the male germline at each of the seven loci. This type of experiment is known as a *specific-locus test*.

The specific-locus test was initially used shortly after World War II to demonstrate that X-ray irradiation of male mice can increase the observed mutation rate in their progeny by over 20-fold when compared to nonirradiated mice, which was a substantially greater increase than expected, based on previous work with *Drosophila* (Russell, 1951). Furthermore, it was demonstrated that in the mouse, unlike in *Drosophila*, radiation given in a single acute dose is far more mutagenic than chronic exposure to radiation, even when the total dose of radiation is the same (Russell *et al.*, 1958).

The first experiments using the specific-locus test with chemical mutagens were somewhat disappointing. Most chemicals given to male mice did not result in a large increase in the number of progeny with specific-locus mutations (reviewed in Ehling, 1978). Even procarbazine, a powerful chemotherapeutic agent used to treat cancer, was found to induce mutations at only one-third the rate of X-rays (Ehling and Neuhauser, 1979), and neither ethylmethane sulfonate (EMS) nor diethylnitrosamine (DEN), both powerful mutagens in *Drosophila*, caused a significant increase in the specific-locus mutation rate in mice (Ehling and Neuhauser-Klaus, 1989; Russell and Kelley, 1979).

The results with the chemical *N*-ethyl-*N*-nitrosourea (ENU; Fig. 15.1), on the other hand, were stunning. When male mice were given a single dose of ENU, they were initially found to have temporary sterility. After 2–3 months, however, many of the animals recovered their fertility. (This prolonged period of infertility suggests that ENU has its principal effect on spermatogonial stem cells as opposed to postmeiotic cells.) The offspring of these animals revealed a mutation rate five times higher than that seen with X-rays (Russell *et al.*, 1979). Furthermore, the administration of multiple doses of ENU over a period of several weeks yielded a mutation rate 12 times higher than X-rays, 36 times higher than procarbazine, and 200 times higher than the spontaneous mutation rate. At this rate, an average of one mutation can be isolated per locus per every 700 gametes (Hitotsumachi *et al.*, 1985; Russell *et al.*, 1982a,b). ENU was therefore deemed a “supermutagen” and declared the chemical mutagen of choice for generating new mouse mutations (Russell *et al.*, 1979).

1.2. ENU's mechanism of action

ENU causes mutations by alkylating DNA. The ethyl group of ENU (Fig. 15.1) can be transferred to a variety of different target atoms in DNA, including the N-1, N-3, and N-7 groups of adenine; the O² and N-3 groups of cytosine; the N-3, O⁶, and N-7 groups of guanine; the O², N-3, and O⁴ groups of thymine; and the phosphate groups of the DNA backbone (Singer, 1976; Sun and Singer, 1975). In practice, ENU usually causes point mutations (i.e., single base-pair changes) in the mouse germline, though small deletions are occasionally reported. A–T to T–A transversions are the most common base-pair changes, but all possible point mutations can occur (Justice *et al.*, 1999; Kohler *et al.*, 1991).

The fact that ENU is a point mutagen means that it not only generates null mutations (in which no functional gene product is produced) but also missense mutations and regulatory mutations that can have a variety of different effects on the gene product. These changes can

lead to too little or too much protein, a less active or a superactive protein, misexpression of the gene in certain tissues, or even a completely new function for the protein. Thus, in contrast to most targeted mouse gene inactivations (commonly known as “knockout” mice), which have an “all or none” effect on the gene, ENU can produce an allelic series of mutations in a gene, each with a different phenotype, which can provide far more information about the function of a gene than a single null allele.

1.3. Recovering ENU-induced mutations with various breeding schemes

ENU can be used to generate both dominant and recessive mutations, but different breeding schemes must be used to detect the different kinds of mutations. The detection of autosomal dominant phenotypes is relatively straightforward. Male mice are injected with ENU, allowed to recover their fertility, and then bred to healthy female mice (the G_0 generation). As a result of the ENU treatment, the G_0 male mice have germline mosaicism for a number of different ENU-induced mutations. Autosomal dominant mutations caused by the ENU will be apparent in the progeny of these animals (the G_1 generation), so the G_1 animals can be screened for phenotypes of interest, and mutant animals can be identified and bred for further study (Fig. 15.2A). Large-scale mutagenesis programs for mutant phenotypes using this scheme have identified hundreds of new autosomal dominant mouse mutations (Hrabe de Angelis *et al.*, 2000; Justice, 2000; Nolan *et al.*, 2000).

Genome-wide screens for autosomal recessive mutations are more laborious and require more animal breeding. ENU-mutagenized male mice are first bred to healthy female mice (the G_0 generation). The progeny of this cross (the G_1 generation) will be heterozygous carriers for any mutations caused by the ENU, so recessive mutations will not yield an abnormal phenotype in this generation. In order to breed these mutations to homozygosity, G_1 animals (typically only the males, because they have greater breeding capacity) must be bred to healthy mice to produce G_2 animals. If the G_1 animal carries an autosomal recessive mutation, it will be transmitted to 50% of the G_2 progeny. The G_2 animals can then be either backcrossed to the original G_1 animal or intercrossed to one another to produce the G_3 generation. The G_3 animals have the potential to be homozygous for any mutation induced by the ENU, so autosomal recessive mutations can yield a any ENU-induced mutation in the G_1 animal. The G_2 animals can then be crossed back to the original G_1 animal (usually a father/daughter cross) or intercrossed to one another (a brother/sister cross) to produce G_3 animals. ENU-induced autosomal recessive mutations will manifest themselves in this generation (shown here again as a white coat color). However, because only half of the G_2 animals will inherit any given mutation from the G_1 generation, some mutations can be lost if they are not transmitted to those G_2 animals that are selected for further mating. Furthermore, embryonic lethal mutations will only be discovered if numerous G_2 females are sacrificed and examined for abnormal embryos. (C) These problems can be overcome via an autosomal recessive screen using a balancer chromosome, which will trap all ENU-induced autosomal recessive mutations in a region of interest. In this example, the balancer chromosome (shown as a bar with arrowheads, indicating inversion breakpoints) is engineered to confer an autosomal dominant agouti coat color, shown here as a gray animal. In the G_1 generation, only agouti-colored animals (who inherited the balancer from their mother) are selected for further breeding. These animals are bred to animals that are

heterozygous for the balancer chromosome and also heterozygous for a second autosomal dominant marker on the opposite chromosome, such as the rex (Re) mutation, which produces wavy fur. Animals in the G₂ generation with straight fur (indicating absence of the rex mutation) must have inherited a mutagenized chromosome from their father and a balancer chromosome from their mother. (In this example, the balancer chromosome has been engineered for embryonic lethality in the homozygous state, so this genotype is not seen among the live-born progeny.) Brother/sister matings between G₂ animals with straight agouti fur can then be used to produce the G₃ generation. Animals in this generation are either heterozygous for the balancer and a mutagenized segment of DNA for the region of interest (inherited *en bloc* from the G₁ generation, since the balancer chromosome suppresses the recovery of recombinants that occur between the inversion breakpoints), or they are homozygous for a mutagenized segment of DNA. Any autosomal recessive phenotypes caused by mutations in the region of interest (shown here again as a white coat color) will be apparent in this generation. If *all* of the animals in this generation have an agouti coat color, then all of them must be heterozygous for the balancer chromosome. This indicates the presence of an autosomal recessive embryonic lethal mutation. Pregnant G₂ animals can then be analyzed to determine precisely when and how such a mutation disrupts embryogenesis. (D) In a modifier screen, in contrast to the other types of screens, the G₀ generation already has a known mutation of interest. In this example, both the males and females in the G₀ generation are homozygous for a mutation that causes a white coat color (shown by the diamonds). When male animals are mutagenized and bred to female animals, all of the progeny will be homozygous for the original mutation of interest and should have a white coat color. If the ENU causes a new autosomal dominant mutation that modifies the white coat color phenotype (shown here as a gray animal), this will be detectable in the G₁ generation. (Note that for simplicity, the modifier mutation shown here is on the same chromosome as the original mutation, but modifiers located anywhere in the genome can be isolated.) Screens for suppressor mutations may prove to be extremely valuable for understanding disease modification. Screens for enhancers exploit nonallelic noncomplementation, where a “sensitizing” mutation allows other mutations to be seen as semidominant phenotypes only in the presence of the first mutation. Such mutations may result in an autosomal recessive phenotype without the presence of the “sensitizing” mutation. phenotype in this generation (Fig. 15.2B). Such screens have been valuable in dissecting genes and pathways required for embryonic development (Fernandez *et al.*, 2009; Garcia-Garcia *et al.*, 2005; Herron *et al.*, 2002; Kasarskis *et al.*, 1998; Zohn *et al.*, 2005). However, one of the major challenges encountered in these genome-wide screens for autosomal recessive mutations is the isolation and maintenance of embryonic lethal mutations. Since homozygous mutants will not be seen in the live-born progeny, detecting such mutations usually requires dissecting of a number of pregnant G₂ female mice and observing that a fraction of the embryos are either malformed or deceased in utero. Once this observation has been made, numerous additional animals must be bred to further analyze and maintain the mutation.

The breeding scheme for isolating embryonic lethal autosomal recessive mouse mutations can be made much more efficient by restricting the analysis to certain chromosomal regions through the use of *balancer chromosomes*. A balancer chromosome is an engineered

construct that ideally has three traits: (1) one or more DNA inversions to suppress the recovery of recombination products when a recombination event occurs within the inversion during meiosis, (2) a dominant phenotype that allows for visual genotyping of animals inheriting the balancer chromosome in subsequent generations, and (3) either an autosomal recessive lethal mutation or an autosomal recessive mutation with an obvious phenotype to remove animals from the population that are homozygous for the balancer chromosome (Hentges and Justice, 2004). The introduction of balancer chromosomes into a mutagenesis breeding scheme allows for the capture of all induced mutations that are linked to the chromosomal region of interest, even if these mutations produce a lethal phenotype (Fig. 15.2C) (Kile *et al.*, 2003). Embryos harboring these mutations can then be analyzed to determine precise effect of the mutation on embryonic development (Boles *et al.*, 2009a,b; Hentges *et al.*, 2006).

If the desire is to capture autosomal recessive alleles at a specific locus, the search can be accomplished in two different ways. First, an ENU-mutagenized male mouse can be bred to a female mouse carrying a known mutation at the locus of interest (as either a heterozygote or a homozygote), and the offspring can be screened for animals that inherited the known mutation from the mother and a new ENU-induced mutation from the father (Cordes and Barsh, 1994; Ebersole *et al.*, 1996; Shumacher *et al.*, 1996). Second, sequence analysis of the locus of interest can be performed on a panel of genomic DNAs from the G₁ males of a large number of ENU-treated fathers. Sperm samples from all of these males have been isolated and stored, so when a heterozygous mutation is detected in the panel, the sperm sample can be requested, and the mutation can be recovered and bred to homozygosity (Quwailid *et al.*, 2004).

A more recent and highly promising use of ENU mutagenesis in the mouse is the generation of mutations that modify a phenotype produced by a well-characterized mutant locus. This type of experiment is known as a modifier screen. All such screens involve mutagenizing and/or breeding to mice that already carry a mutation of interest, with the goal of the screen being to identify mutations that alter the original mutant phenotype (Fig. 15.2D). The first published example of such a screen in the mouse was for suppressors of thrombocytopenia (low numbers of platelets). Male mice homozygous for a null allele in the gene for the thrombopoietin receptor (*Mpl*^{-/-}), which causes thrombocytopenia, were mutagenized with ENU and then bred to *Mpl*^{-/-} females. Platelet counts were performed on progeny of this cross to establish several dominant mutant lines that carried a presumed mutation that suppressed the thrombocytopenia phenotype. Further work revealed that two of these lines had inherited hypomorphic mutations in the *Myb* gene (Carpinelli *et al.*, 2004). A third line had inherited a mutation in the gene for p300, which is known to interact with c-Myb (Kauppi *et al.*, 2008). Subsequent screens have identified modifiers of a GFP transgene with variegated expression (Blewitt *et al.*, 2005), Delta1-dependent Notch signaling (Rubio-Aliaga *et al.*, 2007), neural crest cell development (Buac *et al.*, 2008; Matera *et al.*, 2008), and the growth hormone and TGF-beta signaling pathways (Mohan *et al.*, 2008).

Clearly, ENU mutagenesis in the mouse has been a critical tool in the generation of new, biologically important mouse mutants, but it is still a relatively new research tool and will undoubtedly produce many more interesting animal models in the years to come. With the

recent advances that have been made in both DNA sequence capture technology and nextgeneration sequencing, coupled with the availability of a mouse reference genome, it is now easier than ever to identify the specific DNA lesions that have been induced by ENU and are yielding phenotypes of interest.

2. Materials and Methods

2.1. ENU preparation

Note: ENU is both mutagenic and carcinogenic, and it should be handled with extreme caution. It should only be used inside an efficient chemical hood, and all handlers should wear gloves, lab coats, and masks. Fortunately, ENU has a very short half-life (a few minutes) in alkaline conditions. In the event of a chemical spill, the area should be flooded with 0.1 M KOH to inactivate the ENU. ENU is very sensitive to light, humidity, and pH. It should therefore be prepared fresh prior to each use, and it should be used within 3 h of preparation.

1. Prepare phosphate/citrate buffer: 0.1 M dibasic sodium phosphate, 0.05 M sodium citrate, adjust the pH to 5.0 with phosphoric acid. Filter sterilize using a 0.45 μ m filter.
2. Remove the 1 g ENU ISOPAC (Sigma-Aldrich catalog number N3385) from the freezer and allow it to warm to room temperature in the dark.
3. In an efficient chemical hood, insert an 18-gauge needle into the ENU bottle as a vent. Using a second needle, inject the bottle with 10 ml of 95% ethanol. Remove both needles and gently agitate the bottle until the ENU goes into solution. When completely dissolved, the solution will be yellow but clear. It can take up to 10 min for the ENU to dissolve completely.
4. Reinsert the 18-gauge needle into the ENU bottle as a vent and use a second needle to add 90 ml of phosphate/citrate buffer. Mix thoroughly.

Note: If the amount of ENU to be injected into each animal is small, then 5 ml of concentrated ENU can be removed from the stock bottle prior to adding the phosphate/citrate buffer, and the remaining 5 ml can be diluted with 95 ml of phosphate/citrate buffer. If concentrated ENU is removed from the stock bottle at this step, it should be inactivated by adding it to at least 50 ml of 0.1 M KOH and exposing it to light for at least 24 h.

5. Dilute 400 μ l of the ENU solution with 1.6 ml phosphate/citrate buffer (a 1:5 dilution) in a disposable plastic cuvette. Set up a blank containing 40 μ l of 95% ethanol and 1.96 ml phosphate/citrate buffer in a similar disposable plastic cuvette. Measure the OD_{398 nm} of the ENU solution on a spectrophotometer. An OD_{398nm} of 0.72 corresponds to an ENU concentration of 1 mg/ml. Therefore, you should calculate the concentration (in mg/ml) of your ENU stock solution by multiplying by 5 (the dilution factor) and dividing by 0.72.

Note: Determining the concentration of ENU in the stock vial is very important, because 1 g vials can contain from 0.7 to 1.3 g of ENU.

2.2. Injecting the animals

Note: The optimum dose of ENU varies significantly among mouse strains. Optimum doses have previously been determined for a number of different inbred mouse strains. For C57BL/6J animals, the optimum dose is a total of 300 mg/kg given in three 100 mg/kg fractions administered at 1-week intervals. Animals should be 8–12 weeks of age at the time of the first injection. C57BL/6J animals will have a sterile period lasting 90–105 days from the time of the last injection. The length of the sterile period, like the optimum ENU dose, also varies from strain to strain (Davis *et al.*, 1999; Weber *et al.*, 2000). Researchers working with a previously unstudied strain of mice will need to optimize the dose of ENU for that particular strain.

1. Weigh each animal immediately prior to each injection. Animals tend to lose weight after ENU treatment, so it is particularly important to reweigh animals when giving fractionated doses.
2. The appropriate ENU dose for the animal depends on (a) the ENU concentration in the stock bottle, as calculated above, (b) the weight of the animal, and (c) the strain of animals being used. The appropriate dose should be given to each animal intraperitoneally using a 1 cc tuberculin syringe with a 26-gauge, 3/8 in. needle. Animals should then be placed in a fresh cage with clean bedding. The animals may appear wobbly for about 30 min after the injection, due to the alcohol content of the injection.
3. Keep the mice in an efficient chemical hood for at least 24 h after each injection. Do not change or handle any of the bedding during this time frame.

2.3. Inactivating the ENU

1. When the injections are complete, vent the ENU stock bottle with an 18 gauge needle and fill the bottle with 0.1 *M*KOH with a second needle. Leave the bottle in an efficient chemical hood, exposed to light, for at least 24 h.
2. Treat all equipment and gloves that came in contact with ENU with 0.1 *M*KOH. This includes rinsing all needles and syringes with 0.1 *M*KOH.
3. Discard the contents of the ENU stock bottle into a liquid chemical waste container, rinse the bottle with water, and again discard the contents into a liquid chemical waste container. The bottle should be discarded into a waste container approved for glass. The remainder of the solid waste should be discarded in the appropriate waste containers, including sharps containers for the needles and syringes.

2.4. Breeding the mutagenized males

ENU-treated male mice typically go through a period of infertility that lasts for several weeks after the last ENU injection, and some animals will never recover their fertility. For this reason, it is strongly advised that all mutagenized animals be tested for fertility by placing them in cages with multiple female mice approximately 8–10 weeks after the last

ENU injection. Only males that successfully impregnate a female should be used for further experiments.

Fertile males can then be introduced into a breeding scheme that is appropriate for the kind of mutation that is being screened for (Fig. 15.2). Mutagenized males are expected to have germline mosaicism for many different ENU-induced mutations but may carry repeat mutations or clusters, because ENU is a premeiotic mutagen. In order to avoid oversampling the gametes of a single mouse (and isolating the same induced mutation multiple times), males should be discarded after a certain number of gametes have been sampled (~50 offspring per mutagenized male for dominant mutation screens, and ~30 offspring per mutagenized male for recessive mutation screens). To generate these offspring as efficiently as possible, rotation matings are strongly recommended. In rotation matings, each male is placed with two new females at the beginning of each week. Mouse gestation takes approximately 3 weeks, and mice can be weaned at 3 weeks of age, for a total of 6 weeks from the time of conception to the time of weaning. Thus, we recommend rotating each mutagenized male through seven sets of females at 1-week intervals, and then starting this whole cycle again at the end of 7 weeks. Female breeders should be retired at 6–9 months of age and replaced with younger animals. Mutagenized males will have a markedly reduced lifespan as a result of the ENU treatment, and if rotation matings are not used, it is very likely that the male will die prior to producing the desired number of offspring.

2.5. Screening for abnormal phenotypes

Like the breeding scheme, the phenotype screening that is performed will vary from experiment to experiment, depending on the overall goals of the research. Phenotypes that produce a visible abnormality are obviously the easiest to detect and are seen in virtually all ENU mutagenesis screens. More subtle phenotypes must be ascertained with more sophisticated analyses, such as biochemical testing of blood and urine, imaging, histologic analysis, or behavioral testing. The ideal phenotype screen is inexpensive, rapid, easy to perform, and reproducible. Mice that are scored as abnormal in the first round of screening can be assessed with further testing or immediately bred to assess for transmission of the phenotype to later generations. For a review of the challenges of accurate phenotyping, see Justice (2008) and Brown *et al.* (2009).

Even the most rugged phenotyping screens will sometimes identify animals that fail to transmit the phenotype to successive generations. There are several possible explanations for this phenomenon. First and foremost, the mutant phenotype may represent a simple outlier as opposed to a true mutant phenotype. Second, the phenotype may have been the result of an epigenetic change rather than a base-pair change, as ENU can yield epigenetic changes in the genome (including DNA methylation and histone modifications) as well as point mutations. Third, the phenotype may be penetrant on some genetic backgrounds but not on others. This should always be a consideration if potential mutants are immediately bred to a different mouse strain to initiate genetic mapping of the mutation. Absence of the phenotype in such a cross does not confirm that a particular mutant phenotype is not heritable—if this occurs, the original abnormal animal should be crossed to an animal with the same genetic background to determine if the phenotype can be transmitted on the original background. If

this is the case, then special consideration must be given to the particular strains that are subsequently used to maintain and map the mutation.

2.6. Mapping mutant phenotypes and identifying mutations

Once a particular phenotype has been identified and confirmed to be heritable, the underlying mutation responsible for the phenotype must be determined. This is typically done by the traditional positional cloning approach of breeding the mutation to a different strain (or strains) of mice and analyzing the offspring to determine which genetic markers consistently segregate with the abnormal phenotype. Candidate genes in the region of interest can then be sequenced to identify the causative mutation. This was previously a laborious process that could take years, but the completion of the mouse genome, the availability of single nucleotide polymorphism (SNP) panels, and the falling cost of DNA sequencing have allowed most mutations to now be identified over a period of weeks to months of focused research (Fig. 15.3). In the past, candidate gene analysis typically progressed by sequencing gene after gene until a causative mutation was identified. More recently, as DNA sequencing has become less and less expensive, it has become cost-effective to simply sequence all of the exons in the critical interval harboring the mutation (Boles *et al.*, 2009b). In the near future, it is likely that DNA capture technology and next-generation sequencing will allow researchers to simply sequence the entire genomic interval of interest to identify the mutations responsible for their mutant phenotypes.

3. Conclusion

The extreme mutagenicity of ENU on mouse gametes was not discovered until the late 1970s (Russell *et al.*, 1979), and the first large-scale genome-wide mouse ENU mutagenesis screens for autosomal dominant mutations were not published until 2000 (Hrabe de Angelis *et al.*, 2000; Justice, 2000; Nolan *et al.*, 2000). Since then, there has been an explosion in research using ENU mutagenesis in the mouse, with the publication of genome-wide screens for autosomal recessive mutations (Fernandez *et al.*, 2009; Garcia-Garcia *et al.*, 2005; Herron *et al.*, 2002; Kasarskis *et al.*, 1998; Zohn *et al.*, 2005), screens for autosomal recessive mutations linked to balancer chromosomes (Boles *et al.*, 2009a; Hentges *et al.*, 2006; Kile *et al.*, 2003), and screens for dominant modifiers of other genetic mutations (Blewitt *et al.*, 2005; Buac *et al.*, 2008; Carpinelli *et al.*, 2004; Kauppi *et al.*, 2008; Matera *et al.*, 2008; Mohan *et al.*, 2008; Rubio-Aliaga *et al.*, 2007). A large number of mouse ENU mutagenesis screens are currently ongoing, many of which have generated very promising preliminary results, so we anticipate a bright future for the role of ENU-induced mouse mutations in biological research in the years to come.

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Figure 15.1.
Chemical structure of *N*-ethyl-*N*-nitrosourea (ENU).

Figure 15.2.

Breeding schemes for the isolation of various kinds of ENU-induced mutations. (A) In an autosomal dominant screen, a male mouse is mutagenized with ENU and bred to a healthy female mouse (the G₀ generation). For each mouse, the two bars under the animal represent the two copies of any given autosome. A germline mutation in the ENU-mutagenized male is shown on one of the two chromosomes. If this mutation leads to an autosomal dominant phenotype (in this example, a white coat color), it can be detected in the G₀ male's progeny (the G₁ generation). (B) Autosomal recessive screens require more breeding. G₁ animals will be heterozygous for any ENU-induced mutations and will therefore not show an autosomal recessive phenotype. Mutations must be bred to homozygosity by breeding G₁ animals (typically only the males, who can produce more progeny) to produce a second generation of animals (the G₂ generation). Half of the G₂ animals will have inherited

Figure 15.3.

Mapping and identifying mutations of interest. In order to identify the mutation responsible for a particular phenotype, the mutation must first be localized to a specific segment of the genome. This is most often done with a mapping cross (A). This example shows how this is accomplished for an autosomal recessive mutation causing a white coat color. Mice homozygous for the mutation are bred to a genetically different strain of mice to produce F₁ animals. Each F₁ animal will have inherited one of each pair of chromosomes from the mutant animal and the other from the other strain of mice. They will therefore be heterozygous for both the mutation and for numerous genetic markers scattered throughout the genome. When F₁ animals are intercrossed to one another, they produce the F₂ generation. Animals in the F₂ generation can then be analyzed to determine which genetic markers are being coinherited with the mutation. This is known as a *genome scan*. Once a particular segment of the genome has been identified, the *recombinant progeny*—animals that inherited a meiotic crossover event very close to the mutation—can be used to determine the narrowest possible region that the mutation must lie in, which is known as the *nonrecombinant interval* (B). Once a nonrecombinant interval has been established, the genes in the interval can be identified by examining the published sequence of the mouse genome, typically by using one of the publicly available genome browsers, such as the UCSC Genome Browser or the Ensembl Genome Browser. The classic technique for identifying a mutation was to design polymerase chain reaction (PCR) primers to the exons of each gene in the region and then sequence the PCR products. This was typically done one gene at a time, until the mutation was identified. In the near future, researchers will likely use sequence capture technology to isolate the DNA from a mutant animal that covers the

nonrecombinant interval, followed by next-generation sequencing to establish the sequence of the entire nonrecombinant interval.

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