

Finding the genes that direct mammalian development

ENU mutagenesis in the mouse

The genetic control of mammalian embryogenesis is not well understood. N-ethyl-N-nitrosourea (ENU) mutagenesis screens in the mouse provide a route to identify more of the genes that are required for mammalian development. The characterization of ENU-induced mutations can build on the resources provided by the mouse and human genome projects to help define the tissue interactions and signaling pathways that direct early mammalian development.

The mouse has a rich tradition in developmental genetics. Many of the genes that have provided the special insights into mammalian development were discovered on the basis of the phenotypes of mutant mice¹ (Table 1). For example, *Brachyury*, the founding member of the Tbox gene family, was identified in the 1920s because *Brachyury (T)/+* mice have short tails; shortly thereafter it was found that *T/T* homozygotes die at midgestation and the posterior part of the body axis is truncated².

In the recent past, the strengths of classical mouse genetic studies have been overshadowed, both by targeted mutagenesis in the mouse and by model organism genetics. Targeted mutagenesis currently dominates mouse genetics because of its extraordinary ability to reveal the function of cloned genes. At the same time, the ease of large-scale genetic screens in *Drosophila*, *Caenorhabditis elegans* and Zebrafish has made these organisms popular for phenotype-based gene discovery. However, mammalian development differs in fundamental respects from the development of all these model organisms. For example, transcription in mammalian embryonic nuclei begins days before gastrulation, so the critical events of germ-layer determination and early morphogenesis depend on zygotically transcribed genes rather than on maternal stores. After

implantation, survival of the mammalian embryo depends on the development of a series of physical connections between the developing embryo and maternal circulation, including the placenta, that have no counterpart in invertebrate or fish embryos. In addition, analysis of the Zebrafish genome has led to the surprising finding that there are seven Hox clusters in Zebrafish in contrast to the four clusters in mammals³, suggesting that there might have been an extra genome duplication in the teleost lineage⁴. Thus, there will be no simple relationship between the phenotypes of mouse and fish mutants in homologous genes.

Despite the major contributions of model organisms and gene targeting, mutations have been made in <5% of the genes in the mouse genome. Recent work suggests that ENU mutagenesis screens have the potential to return classical mouse genetics to the forefront of mammalian gene discovery. An arsenal of reagents to characterize mouse genes identified by mutant phenotypes is now available, including bacterial artificial chromosome (BAC) transgenics for mutant complementation and integrated genetic and physical maps for positional cloning. This article focuses on ENU-mutagenesis approaches to identify genes that control mouse development.

TABLE 1. Examples of mouse developmental regulatory genes identified on the basis of mutant phenotypes

Gene	Origin	Phenotype	Protein encoded (cloning approach)	Refs
<i>brachypodism</i> <i>Brachyury (T)</i>	Spontaneous recessive Spontaneous dominant visible: short tail	Skeletal defects in limbs Truncated axis	Gdf5 (candidate gene) T (positional cloning)	20 2, 21
<i>eed</i>	ENU-induced lethal over albino deletion	Lack of anterior development (null); homeotic transformation (hypomorph)	esc homolog; Polycomb-group gene (positional cloning in region defined by deletion breakpoints)	10
<i>Extra-toes</i>	Spontaneous dominant visible: polydactyly	Neural tube and limb defects	Gli3 (candidate gene)	22
<i>Fused/Axin</i>	Spontaneous dominant visible: kinked tail	Axis duplications	Axin (transgene insertion allele)	23
<i>kreisler</i> <i>nodal</i>	Spontaneous recessive Retroviral insertion-induced: recessive lethal	Circling behavior; hindbrain abnormal No mesoderm produced	Transcription factor (positional cloning) Nodal (BMP family member) (retroviral insertion allele)	24 25
<i>short ear</i> <i>situs inversus viscerum (iv)</i> <i>Splotch</i>	Spontaneous recessive Spontaneous recessive Spontaneous dominant visible: white belly spot	Skeletal defects Randomization of left–right asymmetry Neural tube and neural crest defects	Bmp5 (positional cloning within deletion region) Left–right dynein (transgene insertion allele) Pax3 (candidate gene)	26 27 28

Abbreviations: Bmp5, bone morphogenic protein 5; eed, embryonic ectoderm development; esc, extra sexcombs; Gdf5, growth differentiation factor 5; Gli3, Gli-Kruppel family member Gli3; Pax3, paired box gene 3 product.

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TABLE 2. Examples of mouse developmental mutants identified in ENU^a mutagenesis screens

Screen	Examples of mutations identified	Refs
Lethals <i>in trans</i> to <i>albino</i> deletions	<i>eed</i> (3 alleles); five additional prenatal lethal loci	7
Lethals in <i>T/t</i> region	<i>quaking</i> (4 lethal alleles); new alleles of <i>T</i> ; nine additional prenatal lethal loci	29–31
Failure to complement the behavioral defect of a spontaneous <i>kreisler</i> allele	<i>kreisler</i> allele	24
Dominant behavioral mutants	<i>Wheels</i> (inner ear defects)	14
Morphological abnormalities at midgestation (9.5 dpc)	5 embryonic lethal mutants	11
Forebrain defects at midgestation (10.5 dpc ^b)	<i>flat-top</i> ; 4 other mutants	12

^aN-ethyl-N-nitrosourea.
^bdpc: days *post-coitum*.

the most efficient ENU dosage regime gave an average of one new mutation per gene in only 700 first-generation (F1) progeny in seven loci that produce visible phenotypes (the specific locus test)⁵. Assuming that there are ~70 000 genes in the mouse genome, each F1 animal is heterozygous for ~100 new inactivating mutations. This high efficiency of mutagenesis makes it possible to identify the small fraction of genes that affects any particular biological process. For example, in a screen of only 100 ENU-treated lines, ~10 000 different new mutations can be tested.

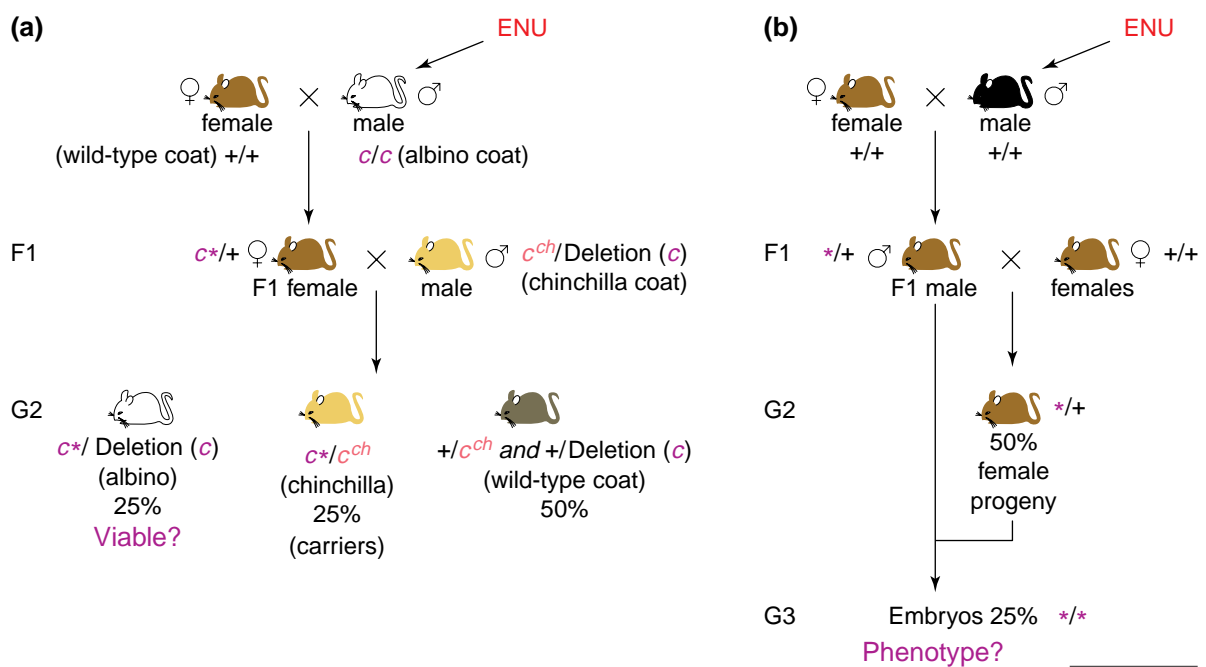
Region-based screens

The potency of ENU as a mutagen opens up a variety of approaches to identify genes that play important roles in mammalian development (Table 2). One strategy is to look for mutations in a defined region of the genome. The simplest version of this type of screen is to identify lethal mutations that fail to complement a particular chromosomal deletion (Fig. 1a). For example, 24 ENU-induced mutations that are lethal when *in trans* to particular *albino* deletions have been identified in large-scale experiments^{6,7}. It is interesting to note that only one of the genes defined in these screens (*eed*) is known to be homologous to a gene

ENU: an extraordinarily efficient mutagen in the mouse

Efficient chemical mutagenesis in the mouse using the point mutagen ENU was developed more than 20 years ago. The unique strength of ENU mutagenesis is its great efficiency:

FIGURE 1. Two kinds of screens to identify recessive mutations that affect embryonic development of the mouse



trends in Genetics

An asterisk represents a newly induced mutation. (a) A region-based screen⁶. The males that are treated with ENU are homozygous for a recessive point mutant allele of *albino* and hence have albino-colored coats. Single F1 animals, which are each heterozygous for a *c* chromosome that might carry new mutations, are mated with animals that are heterozygous for a weak allele of *c* (*c^{ch}*) and an *albino* (*c*) deletion, which removes the *albino* locus and a set of flanking genes. Newly induced lethal mutations in genes near *albino* are identified in the G2 generation because there are no viable *albino* (pure white) mice, which would have been heterozygous for the *c* deletion and the mutagenized *c* chromosome. The surviving mice with *chinchilla* colored coats carry the mutagenized *c* chromosome *in trans* to the *c^{ch}* chromosome; these animals are carriers that can be bred to perpetuate the new recessive lethal mutation. Once a line of animals that are heterozygous for the lethal mutation is established, whether the lethal mutation produces an interesting developmental phenotype can be tested. (b) A genome-wide screen for recessive embryonic lethal mutations that produce a clear morphological phenotype⁹. Single F1 males, which are each heterozygous for many different newly induced mutations distributed throughout the genome, are mated to produce a number of G2 females. Each G2 female has a 50% chance of inheriting any single mutation carried by the F1 male. If she does carry a particular mutation, then 25% of the embryos from the cross between that G2 female with her father will be homozygous for the mutation. In this scheme, mutations are identified on the basis of abnormal morphology at a particular developmental stage, rather than being first identified on the basis of homozygous lethality. Mapping of mutations using polymorphic simple sequence repeat markers is facilitated if the mutagenized males are of one inbred strain and the females used in outcrosses are from a different inbred strain.

that was previously shown to be important in the development of one of the model organisms. Methods for making targeted chromosomal deletions throughout the genome will make region-based screens possible in many genomic regions^{8,9}. In the near future, one important application of region-based screens will be to identify essential genes in sequenced regions of mammalian genomes.

eed is an especially good example of an embryonic patterning gene identified in a region-based screen⁷. Three alleles of the *eed* gene were identified as failing to complement large deletions in the *albino* region. Although the *eed* gene would have been eventually identified based upon its 40% sequence identity to the *Drosophila* Polycomb-group gene *esc*, two of the ENU-induced *eed* alleles are null and cause arrest at gastrulation, and one hypomorphic allele causes homeotic transformations¹⁰. This allelic series of ENU-induced point mutations reveals that *eed* is required both for cell-type specification at gastrulation and, later, in anterior–posterior patterning, which would not have been apparent from the phenotype of a standard targeted allele.

Genome-wide, phenotype-based screens

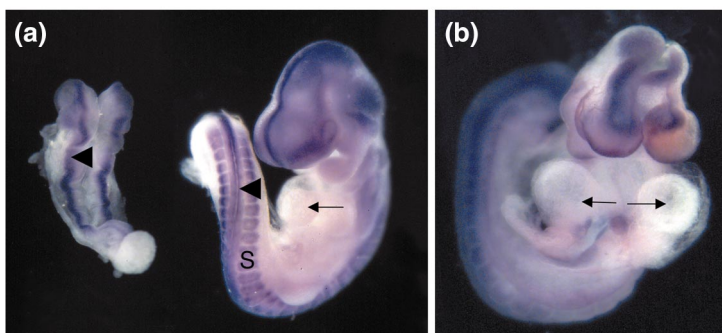
An alternative to the region-based strategy is to use phenotype-based screens to identify genes that are required for a particular process of interest. Large-scale genome-wide screens for dominant ENU-induced mutations that affect behavior and blood chemistry of the mouse are currently underway, and hundreds of candidate mutants have already been identified (<http://www.mgu.har.mrc.ac.uk/mutabase/>; <http://www.gsf.de/isg/groups/enu/>). Although some dominant mutations, like *Brachyury*, have strong developmental phenotypes when made homozygous, the vast majority of genes required for development will not be identified by dominant alleles.

A larger fraction of the genes that are important for mouse development can be discovered by looking for recessive mutations that disrupt normal embryonic development. A genome-wide screen for recessive phenotypes requires one more generation of crosses than the region-based screen (Fig. 1b), but a phenotype-driven screen can identify genes scattered throughout the genome that control a single process.

Several modest-scale screens indicate that recessive mutations can be recovered relatively easily. In screens for recessive mutations that cause morphological abnormalities at midgestation [9.5 days *post-coitum* (dpc)], one mutant was identified in every 5–10 lines screened¹¹ (and K. Anderson, unpublished; Fig. 2). In a screen to look for a more specific phenotype – abnormalities of the forebrain of the 10.5 dpc embryo – four mutants were identified in 150 lines screened¹². Of the five mapped mutants from these screens, none corresponds to a gene that was previously characterized by a targeted mutation. Thus, both phenotype-based and region-specific ENU screens have identified genes that were not previously known to play a role in development.

The goal of large-scale mutagenesis is to identify a large fraction of the genes that contribute to any process of interest. With a set of genes that share related phenotypes in hand, it becomes possible to define the tissue interactions and genetic pathways that control a specific developmental process. Assuming the hit rate defined in the specific locus test, a screen of 1000 lines would identify an average of 1.3 hits per gene. Assuming a Poisson distribution, this would represent ~70% of loci that could be identified in a

FIGURE 2. Some phenotypes of ENU-induced embryonic lethal mutations



trends in Genetics

(a) The 9.5 days *post-coitum* (dpc) embryo on the left is from a mutant line and the embryo on the right is a wild-type sibling; both have been hybridized to reveal the expression of the *Pax3* gene. In the wild-type embryo, *Pax3* is expressed in the dorsolateral cells of the closed neural tube in the wild-type embryo (broad arrow head) and in the somites (S). Arrow marks the heart of the wild-type embryo. In the mutant, *Pax3* is expressed in the dorsolateral cells of the neural plate (broad arrow head), which has not folded into a neural tube. The lack of expression of *Pax3* in the mutant somites reveals a failure of somite development. (b) A 10.5 dpc mutant embryo from another mutant line, stained for endogenous alkaline phosphate activity. The most prominent defect in this embryo is cardia bifida: the two lateral anlage (arrows) of the heart failed to fuse on the midline, and each lateral region has formed a heart tube independently.

saturation screen. These numbers indicate that productive mutant screens could be carried out in any mouse laboratory interested in the genetic analysis of a particular aspect of development.

In addition to identifying important new genes, the random sampling of the genome provided by ENU mutagenesis experiments should help answer fundamental questions about the genetic regulation of mouse development. How many phenotypic classes exist and what are those phenotypes? What is a common phenotype and what is a rare phenotype? How many genes have essential functions in embryogenesis?

Taking advantage of mouse genetic resources

The recent ENU screens reveal that it is relatively easy to isolate interesting mouse developmental mutants. However, the real work in understanding gene function begins when a mutation has been identified. Genetic characterization, molecular characterization and phenotypic analysis are the steps that require time, effort and ingenuity. Fortunately for the mouse geneticist, a rich set of tools is available to make mutant characterization easier.

The first step in characterization of an interesting mutant identified in a genome-wide screen is to map the gene responsible for the phenotype. The well-characterized inbred mouse strains and the physical map of the mouse genome make mapping of ENU-induced mutations relatively straightforward. Only ~24 heterozygous carrier animals are required to map a mutation to a 20 centimorgan (cM) interval (1% of the genome), using markers selected from the 8000 mapped polymerase chain reaction (PCR)-based polymorphisms available^{11–14}. Approximately 2000 PCR reactions will localize the gene to a single genomic region; automated genotyping can reduce the tedium of genome scanning.

Given that the ENU mutagenesis protocol introduces ~100 mutations per line, it could be a challenge to map the

single mutation responsible for a given phenotype. Despite this potential problem, the phenotypes of five of the six developmental mutants from genome-wide screens mapped unambiguously to a single locus^{11,12}. Thus, in most lines, there is a single mutation responsible for the observed developmental phenotype.

The mutations produced by ENU are generally point mutations, so cloning the gene responsible for the phenotype of an ENU-induced mutation relies on map-based methods. Fortunately, positional cloning in the mouse is no longer a Herculean effort. With >7000 genes on the mouse genetic map (<http://www.informatics.jax.org>), candidate genes can be identified for complementation tests and/or sequencing as soon as a mutation is mapped to a small interval. If no mouse candidate gene is identified, the defined synteny between the mouse and human maps can provide a wealth of information on potential candidate genes. With the expected completion of a draft of the human genome sequence within the next year, identification of candidate genes should become nearly instantaneous. If no likely candidate is identified, BAC transgenics can be used to identify DNA sequences that complement the mutant phenotype¹⁵.

Phenotypic analysis of any ENU-induced mutation employs the same wealth of cell-type specific molecular markers used to characterize targeted mouse mutations. Similarly, analysis of chimeras and double-mutant analysis are well-developed tools to define the cell types and pathways affected by a mutation. Thus, many inferences about the developmental role of the gene can be made, even before the gene is characterized molecularly.

Given the efficiency of ENU mutagenesis, there will be more mutations identified than can be easily characterized at one time. Using widely available techniques, mutant stocks can be preserved as frozen embryos. In addition,

sperm-freezing techniques have recently improved, and recovery of animals by *in vitro* fertilization with cryopreserved sperm works well for several strains^{16,17}. New techniques to make balancer mouse chromosomes will also simplify strain maintenance and breeding programs¹⁸.

A mouse is not a man, but it is as close as an experimental geneticist can get

The use of ENU mutagenesis to search for genes that are important in mammalian physiology and human disease has been reviewed recently¹⁹. Developmental genetic studies will also identify new molecules and pathways that are important in aspects of human health. For example, our understanding of the importance of cell-signaling pathways in cancer has been profoundly influenced by *Drosophila* genetic studies; new signaling pathway components identified because of their importance in mammalian development are likely to be important in controlling mammalian cancer cell growth. Most directly, understanding more about mouse developmental genetics will help define the causes of human birth defects. In 1998, approximately one in 30 children born in the USA had one or more birth defects, many of which have a genetic component (<http://www.modimes.org/>). With the vast majority of the genes that direct mammalian development waiting to be identified, ENU mutagenesis in the mouse provides one clear route towards a greater understanding of the genetic control of human development.

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