# Transgenic Animals

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**The ability to introduce foreign genes into the germ line and the successful expression of the inserted gene in the organism have allowed the genetic manipulation of animals on an unprecedented scale. The information gained from the use of the transgenic technology is relevant to almost any aspect of modern biology including developmental gene regulation, the action of oncogenes, the immune system, and mammalian development. Because specific mutations can be introduced into transgenic mice, it becomes feasible to generate precise animal models for human genetic diseases and to begin a systematic genetic dissection of the mammalian genome.**

THE INTRODUCTION OF GENES INTO THE GERM LINE OF mammals is one of the major recent technological advances in biology. Transgenic animals have been instrumental in providing new insights into mechanisms of development and developmental gene regulation, into the action of oncogenes, and into the intricate cell interactions within the immune system. Furthermore, the transgenic technology offers exciting possibilities for generating precise animal models for human genetic diseases and for producing large quantities of economically important proteins by means of genetically engineered farm animals.

The first animals carrying experimentally introduced foreign genes were derived by microinjection of simian virus 40 (SV40) DNA into the blastocyst cavity (*1*). The presence of the injected DNA in a number of somatic tissues derived from mice that had been injected as embryos was demonstrated by DNA reassociation kinetics. However, integration of the viral DNA into the germ line was not demonstrated in these early experiments. A later study suggested that some of the SV40 DNA remained episomal in somatic tissues (*2*). Germ line transmission of foreign DNA was detected in subsequent studies when mouse embryos were exposed to infectious Moloney leukemia retrovirus (M-MuLV), which resulted in the generation of the first transgenic mouse strain (*3*).

Infection of mouse embryos with retroviruses constitutes one method of genetically manipulating mouse embryos. Another more commonly used technique for generating transgenic animals is the direct microinjection of recombinant DNA into a pronucleus of the fertilized egg (*4*). Finally, a recently developed technique involves the introduction of DNA by viral transduction or transfection into embryonic stem cells (ES cells), which are able to contribute to the germ line when injected into host blastocysts (*5*). In this article I will not attempt to give a comprehensive review of the field but rather emphasize principles and recent developments. Several detailed review articles have been published over the last 2 years (*6*) that cover the earlier work on transgenic animals.

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## Methods for Introducing Genes into Animals

*Microinjection of DNA into pronucleus.* Microinjection of cloned DNA directly into a pronucleus of a fertilized mouse egg has been the most widely and successfully used method for generating transgenic mice. Typically, multiple DNA molecules arranged in a head-to-tail array integrate stably into the host genome. It is thought that the injected DNA molecules associate by homologous recombination before integration and in most cases insert subsequently at a single chromosomal site. It has been proposed that random chromosome breaks, possibly caused by repair enzymes that are induced by the free ends of the injected DNA molecules, may serve as integration sites of the foreign DNA (*7*). Frequently, rearrangements, deletions, duplications (*8*), or translocations (*9*) of the host sequences occur at the insertion sites. However, the injected DNA does not always integrate into the host genome. Bovine papilloma virus (BPV), for example, either integrates stably into the genome of transgenic mice or is maintained as an episome depending on the structure of the injected DNA (*10*). Episomal replication and transmission to the offspring have also been reported for a rearranged plasmid coding for the polyoma virus large T antigen, although the mechanism responsible for the episomal state has not been resolved (*11*).

The principal advantage of direct microinjection of recombinant DNA into the pronucleus is the efficiency of generating transgenic lines that express most genes in a predictable manner. However, one disadvantage of this method is that it cannot be used to introduce genes into cells at later developmental stages. Moreover, the cloning of the chromosomal insertion site may be difficult because of the multiple copy inserts and the host sequence rearrangements.

*Retrovirus infection.* In contrast to microinjected DNA, retroviruses integrate by a precisely defined mechanism into the genome of the infected cell. Only a single proviral copy is inserted at a given chromsomal site and no rearrangements of the host genome are induced apart from a short duplication of host sequences at the site of integration (*12*). Preimplantation stage mouse embryos can be exposed to concentrated virus stocks (*3*) or cocultivated on monolayers of virus-producing cells (*13*). Methods also have been devised to introduce virus into postimplantation embryos between days 8 and 12 of gestation (*14*). While this allows infection of cells from many somatic tissues, germ cells are infected with a low frequency (*15*). Similarly, when chicken embryos were exposed at the blastodisk stage to avian leukemia virus, infection of germ cells was inefficient (*16*). Because the chick pronucleus cannot be microinjected with DNA, retrovirus infection is probably the only feasible method for generating transgenic chickens.

The main advantage of the use of retroviruses or retroviral vectors for gene transfer into animals is the technical ease of introducing virus into the embryos at various developmental stages. Furthermore, it has proved much easier to isolate the flanking host sequences of a proviral insert than those flanking a DNA insert derived from pronuclear injection. This is of considerable advantage when attempting to identify the host gene disrupted by insertion of

the proviral DNA. The main disadvantages of the use of retroviruses for gene transfer are the size limitation for transduced DNA and the unresolved problems of reproducibly expressing the transduced gene in the animal.

*Embryonic stem cells.* Embryonic stem cells are established in vitro from explanted blastocysts and retain their normal karyotype in culture (*17*). When injected into host blastocysts, ES cells can colonize the embryo and contribute to the germ line of the resulting chimeric animal (*5*). Genes can be efficiently introduced into ES cells by DNA transfection or by retrovirus-mediated transduction, and the cell clones selected for the presence of foreign DNA retain their pluripotent character. By means of this approach, mice have been generated from cell clones that were selected in vitro for a specific phenotype (*18*). This opens exciting possibilities for deriving mouse strains carrying specific mutations. Although only a few laboratories have reported successful germ line contribution of ES cells to date, it is likely that this approach will receive increasing attention for the genetic manipulation of mice.

## Expression of Genes in Transgenic Animals

*Pronuclear injection of cloned genes.* The crucial problem has been the predictable and tissue-specific expression of the injected genes. In early experiments only low or extremely variable expression, dependent on the chromosomal position of the inserted gene, was seen. It was soon realized that the presence of prokaryotic vector sequences is highly inhibitory to the appropriate expression of certain genes including β-globin, α-actin, and α-fetoprotein (*19–21*). Therefore, most investigators now remove the prokaryotic vector sequences before injection of the gene into embryos to avoid any possible perturbing effects these sequences might have on gene expression. In contrast, genes such as those encoding immunoglobulin (Ig), elastase, and collagen appear to be less sensitive to the presence of vector sequences and are often expressed independently of the chromosomal position (*22–25*). Table 1 summarizes examples of the successful expression of genes in a wide variety of specific tissues.

Much effort has been directed toward understanding the basis for developmental activation of genes. Transgenic mice have been instrumental in localizing cis-acting sequence elements responsible for tissue-specific gene regulation. Such elements have been found in the 5′ flanking sequences, either proximal or distal to the promoter, within the gene itself, or in the 3′ flanking sequences. In the case of some promoters the tissue-specific enhancer elements have been identified by systematically introducing DNA constructs into embryos with different lengths of flanking sequences. The elastase (*23*), the γ-crystallin (*26*), and the protamine (*27*) genes all have a compact promoter where all information necessary for tissue-specific gene expression is contained within a few hundred base pairs upstream of the start site. For other genes, tissue-specific enhancer elements are spread over considerable distance. For example, an element located 10 kb upstream of the albumin promoter is indispensable for liver-specific expression (*28*), and an element localized 5 kb 3′ of the T cell receptor gene controls T cell–specific expression (*29*). Three distinct enhancer elements responsible for tissue-specific expression of the α-fetoprotein gene are contained within 7 kb of upstream sequences (*21*).

Transgenic animals have been particularly useful in the analysis of developmental activation of the β-globin gene family. During development, distinct "embryonic," "fetal," and "adult" globin genes are sequentially expressed in erythroid cells. Cloned fetal and adult globin genes introduced into the mouse germ line were expressed correctly (*19*), and the stage-specific activation was dependent on enhancer elements at the 3′ as well as the 5′ end of the gene (*30*). Nevertheless, serious problems with β-globin expression remained. Expression of globin genes was always found to be dependent on the site of chromosomal integration and independent of the copy number, and the transgenes were never expressed at a level comparable to the expression of the endogenous gene. When sequences located at distances 20 to 50 kb from either side of the β-globin gene were included, the injected gene was expressed at a level comparable to the endogenous globin gene and directly related to the copy number (*31*). It is therefore possible that these sequences control the accessibility of the β-globin locus to tissue-specific trans-acting factors or that they contain nuclear matrix–binding sites or represent enhancer elements that may exert their effects over very long distances. Genes other than those encoding β-globin also frequently show expression that is position-dependent and not related to copy number. Consequently, it is possible that sequence elements acting at a distance in controlling gene expression are not unique to globin genes and may have to be identified before high expression can be achieved reproducibly.

**Table 1.** Expression of transgenes in specific tissues.

| Tissue | Gene or promoter | Reference |
|---|---|---|
| Brain | MBP, *Thy-1*, NFP, GRH, VP | (*40, 42, 69, 102*) |
| Lens | Crystallin | (*26, 63*) |
| Mammary epithelial cells | β-Lactoglobulin, WAP | (*45, 58*) |
| Spermatids | Protamine | (*27*) |
| Pancreas | Insulin, elastase | (*23, 67, 71, 103*) |
| Kidney | Ren-2 | (*104*) |
| Liver | Alb, AGP-A, CRP, α2u-G, AAT, HBV | (*28, 39, 105*) |
| Yolk sac | α-Fetoprotein | (*21*) |
| Hemopoietic tissues | | |
| Erythroid cells | β-Globin | (*19, 30, 31*) |
| B cells | κ Ig, μ Ig | (*22*) |
| T cells | T cell receptor | (*29*) |
| Macrophages | M-MuLV LTR | (*53*) |
| Connective tissue | MSV LTR, collagen, vimentin | (*9, 24, 106*) |
| Muscle | α-Actin, myosin light chain | (*20, 107*) |
| Many tissues | H-2 (HLA), β2-m; CuZn SOD | (*75, 108*) |

AAT, α1-antitrypsin; AGP-A, αl-acid glycoprotein; Alb, albumin; α2u-G, α2u globulin; β2-m, β2-microglobulin chain; CRP, C-reactive protein; CuZn SOD, Cu/Zn-superoxide dismutase; GRH, gonadotropin-releasing hormone; HBV, hepatitis B virus; HLA, histocompatability antigen class; MBP, myelin basic protein; MSV, murine sarcoma virus; NFP, neurofilament protein; Ren-2, renin-2; VP, vasopressin; WAP, whey acidic protein.

The tissue in which a particular gene is expressed is frequently determined by the combination of a tissue-specific enhancer with a particular promoter. For example, the Ig enhancer directs Ig or *myc* expression to B cells (*22*) but enhances expression of the SV40 T antigen in many tissues (*32*). That sequences localized in introns may also be significant in determining the level of transcription in animals, but not in cultured cells, was demonstrated in a recent study when pairs of genes, either with or without introns (as complementary DNA), were microinjected into embryos (*33*).

For many experimental purposes, it would be highly desirable to be able to modulate expression of a transgene with some external stimulus. Promoters of genes subject to modulation by hormonal or other environmental stimuli have been shown in several instances to properly control expression of transgenes. The metallothionein promoter, for example, has been used to direct expression of many different reporter genes, and in some cases expression could be stimulated by feeding the animals with heavy metals (*34*). Hormone-inducible promoters that function in transgenic mice include the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) (*35, 36*), the transferrin gene (*37*), the H-2 $E_\alpha$ gene (*38*), and

two liver-specific genes (*39*). These results are promising and indicate that transgene expression can be modulated in vivo by external signals. However, at present, the stimuli used to activate inducible genes show toxic side effects that limit their experimental utility.

The ability to introduce and express genes in the animal has opened the door to efforts designed to correct genetic defects. So far "gene therapy," that is, the repair of a mutated gene, has not been accomplished in the animal. Genes introduced into animals invariably integrate at a site distant from the resident defective gene. Therefore, mutant and introduced normal genes will segregate independently in the next generation. Nevertheless, successful corrections of hereditary disorders at the phenotypic level have been accomplished and include hormone deficiencies (*40*), β-thalassemia (*41*), and a myelination defect (*42*). These types of experiments will help to elucidate the molecular deficiency causing the respective hereditary disorder.

Genes have also been microinjected into rabbit, sheep, and pig embryos (*43*). The success rate of generating transgenic domestic farm animals is, however, much lower than that obtained with mice, in large part because of technical difficulties in visualizing the pronucleus in the embryos of these species. A human growth hormone gene successfully introduced into the pig in spite of these difficulties was nevertheless unable to increase growth, perhaps because the human hormone was not biologically active in pigs (*43*). Although the importance of genetic engineering for improving livestock has been questioned (*44*), it is likely that transgenic farm animals may become a source of economically valuable proteins. For example, medically relevant proteins, whose expression has been targeted to the mammary epithelial cells, may be harvested from the milk of transgenic cows as has been shown to be possible for transgenic mice (*45*).

*Retroviruses.* Early experiments have shown that preimplantation mouse embryos (*46*) or embryonal carcinoma (EC) cells (*47*) are not able to support the expression and replication of retroviruses. In contrast, virus is expressed efficiently in later stage embryos (*48*) or in differentiated EC cells. Virus replication in early embryonic cells is restricted because the viral LTR, which contains the viral promoter and transcriptional control elements, does not function at that stage. Transcriptional inactivity has been correlated with de novo methylation of the provirus (*47, 48*), with nonfunctioning of the viral enhancer or downstream sequences (*49*), with the presence of repressor activity, and with lack of activating factors (*50*) in the nonpermissive embryonal cells. In most cases, the block, once established in the embryo, is maintained by a cis-acting mechanism at later stages of development (*48*). This likely involves DNA methylation, because injection of postnatal animals with the drug azacytidine (aza C) leads to expression of the previously inactive provirus (*51*). However, some proviruses carried in transgenic mice are expressed in specific tissues (*52*) or at specific stages of development, and it has been shown that virus activation in those cases is influenced by the chromosomal position of the provirus (*13*). A puzzling observation concerns the expression of genes under LTR control in certain tissues when introduced by pronuclear injection (*9, 53, 54*). This suggests that the viral control elements are able to respond to trans-acting transcription signals in the developing organism when they are introduced by a mechanism other than the normal, viral enzyme–mediated integration process.

The inactivity of the viral LTR in embryonic cells has reduced the utility of retroviral vectors for gene transfer into the germ line (*55*). An alternative to expressing virus-transduced genes from the viral LTR is to place the gene of interest under the control of an internal promoter. Transgenic mice carrying viral vectors with the human β-globin gene under control of its own promoter (*52*) or the *neo* gene under that of the thymidine kinase promoter (*56*) expressed the transduced gene in hemopoietic cells or in many tissues, respectively, as would be expected from the two types of promoters. These results are promising and suggest that genes transduced into embryos by viral infection can be expressed when controlled by an appropriate internal promoter rather than the viral LTR.

## Applications of Transgenic Technology

*Models for oncogenesis and diseases.* The potential for using specific promoters or enhancers to direct expression of heterologous genes to a specific cell type has stimulated numerous attempts to change the physiology of an animal experimentally. The transgenic technology has been particularly valuable for studying the consequences of oncogene expression in the animal (*57*). With the use of transgenic mice, problems can be addressed that cannot be approached satisfactorily in cell culture: for example, the spectrum of tissues that are susceptible to the transforming activity of an oncogene, the relation between multistep oncogenesis and cooperativity of oncogenes, and the effect of oncogenes on growth and differentiation.

When different promoters or enhancers were used to direct *myc* or *ras* oncogene expression to different tissues, tumor formation resulted in most cases. Oncogenes were expressed frequently in many tissues and this usually preceded tumor formation by many months. However, many tissues in which a given oncogene was expressed never developed tumors. For example, long latencies and variable penetrance were observed when *myc* or *ras* expression was controlled by the MMTV LTR (*36*), the Ig enhancer (*22*), a fusion between the Ig enhancer and the SV40 promoter (*32*), or the whey acididic protein (WAP) promoter (*58*). This is consistent with the concept of oncogenesis as a multistage process (*59*) with activation of an oncogene likely to be the first of several steps in tumorigenesis. However, even though coexpression of *myc* and *ras* resulted in

**Table 2.** Mutations in transgenic mice.

| Type of mutation and procedure | Phenotype (number of mutants) | | Identified mutant genes | Reference |
|---|---|---|---|---|
| Recessive (insertional mutagenesis) | | | | |
| Retrovirus infection of | | | | |
| Embryos | Embryonic lethals | (3) | α1(I) collagen | (*84, 85*) |
| | Kidney failure | (1) | | (*88*) |
| EC cells | Enzyme defect | (1) | HGPRT | (*17*) |
| Microinjection of DNA | Embryonic lethals | (5) | | (*8, 21*) |
| | Limb disturbance | (3) | | (*9, 86*) |
| | Transmission distortion | (1) | | (*87*) |
| Dominant (expression of variant subunit in multimeric protein) | | | | |
| Microinjection of mutant gene | Perinatal lethal | (1) | α1(I) collagen | (*24*) |

accelerated tumor formation (*60*), additional somatic events appeared necessary for the realization of the malignant phenotype. Whereas pancreatic acinar cells seemed highly susceptible to the transformation by *ras*, no tumors developed when *myc* was expressed from the same promoter (*61*). Conversely, neoplastic transformation of mammary gland cells was efficiently induced by *myc* expression but rarely by expression of *ras* (*58*). This strengthens the view that the consequences of oncogene expression also depend on the particular cell type and may differ dramatically between one tissue and another.

When a cellular gene is introduced into the germ line under the control of a heterologous promoter, it is assumed that the phenotype arising in the transgenic animals will reveal not only the pathological consequences of unregulated or ectopic expression of the transgene; it will also help the analysis of its normal function in development and differentiation. Experiments of this kind have been done with proto-oncogenes, growth factor genes, and genes encoding cell surface antigens. For example, deregulated expression of the *fos* proto-oncogene in many tissues failed to induce tumors but rather interfered with normal bone development (*62*), whereas expression of the *mos* gene in the lens resulted in disturbance of lens fiber formation (*63*). Similarly, unregulated expression of a hemopoietic growth factor in macrophages (*53*) or of the *Thy-1* cell surface antigen in many tissues (*64*) caused fatal proliferative abnormalities. Also, expression of a mutant dihydrofolate reductase gene resulted in general growth abnormalities (*65*). Almost any cell type appears to be susceptible to transformation by SV40 T antigen, including cells of the exocrine and endocrine pancreas (*66*), the choroid plexus (*67*), the lens (*68*), the thymus, and the pituitary gland (*32, 69*). T antigen–induced proliferation has also been used as a convenient marker for the study of pancreatic differentiation and the development of immune tolerance (*70*).

Phenotypes induced in transgenic mice by expression of various viral gene products are providing model systems for pathological conditions, some of which resemble human diseases. The small DNA tumor viruses, BPV and polyoma virus, appear to be less promiscuous in their transforming potential than SV40. Introduction of BPV into the germ line resulted in skin tumors (*9*), whereas polyoma virus caused hemangiomas (*71*). A demyelinating disease that resembles progressive multifocal leukoencephalopathy appeared as a consequence of JC virus expression, and a neurofibromatosis resembling von Recklinghausen's disease was observed in mice carrying the human T cell leukemia/lymphoma virus (HTLV-I) *tat* gene (*54*). The latter two viruses are thought to be neurotropic in humans, and the relevant transgenic mice are likely to provide valuable model systems for studying the pathology of virus-induced diseases of the central nervous system.

*Immune system.* Transgenic mice have also been important to the study of Ig gene expression. Several groups showed functionally rearranged Ig genes introduced into the germ line to be correctly activated and to alter the expression of the endogenous immunoglobulin repertoire (*22*). These and similar types of studies indicate that light and heavy chains, when expressed at a sufficient level, may interfere by some feedback mechanism with further Ig gene rearrangement. Recent evidence also obtained with transgenic mice suggests that expression of functional Ig genes can cause complex abnormalities in the immune system, that allelic exclusion may be mediated by expression of the membrane-bound form of the human μ chain, and that somatic mutations and gene rearrangement are not concomitant processes (*72*). Chicken or rabbit Ig genes in germ line configuration became rearranged in transgenic mice and form functional hybrid Ig molecules (*73*), suggesting that the production of interspecies monoclonal antibodies may be possible in genetically engineered mice. A detailed review discussing the implications of these results for our understanding of the development of the immune system has recently been published (*74*).

Transgenic mice have also been used to study the function of both class I and class II genes. Introduction of porcine or murine class I major histocompatibility antigens into mice showed that the foreign protein was able to associate in both cases with the endogenous murine β2-microglobulin chain and could form a functional transplantation antigen, whereas the human counterpart was unable to do this (*75*). Similarly, murine class II genes were correctly expressed and functional in transgenic mice (*38*). Furthermore, transfer of a functionally rearranged T cell receptor β-chain gene into transgenic mice showed that expression of the transgene inhibited rearrangement of the endogeneous β genes in analogy to results obtained with Ig genes (*29*).

*Lineage marker.* A central issue in contemporary biology is the construction of fate maps to assess cell ancestry, cell location, and cell commitment in the developing embryo. Visual observation and injected lineage tracers have been used to study the early stages of mammalian development because the preimplantation embryo is easily amenable to experimental manipulation (*76*). However, the inaccessibility of the embryo once it has implanted in the uterus impedes the study of cell lineage at later stages and has prevented the use of direct lineage tracers. Therefore, the study of cell lineage in the postimplantation embryo necessitated the development of stable markers of individual progenitor cells that leave the embryo undisturbed.

The introduction of exogenous DNA into embryos after the one-cell stage generates genetic mosaicism that may be used to analyze such lineage relations. In the first study of this type, preimplantation mouse embryos were infected with retroviruses that served as genetic markers for the progeny of an infected blastomere (*15*). Quantitation of proviral copies in somatic tissues and the germ line of the resulting mosaic animals indicated approximately eight cells to be allocated to the formation of the embryo with each of them contributing equally to all somatic tissues. Germ cells appear to be set aside before allocation of the somatic lineages. Similar lineage studies were performed with genetically mosaic mice generated by microinjection of plasmid DNA into a pronucleus (*77*).

Another approach often used in lower vertebrates or in invertebrates in cell lineage studies has been the removal of cells by microdissection or laser ablation. Recently, specific cell lineages were genetically ablated in transgenic mice by expressing the A chain of diphtheria toxin (DTA) under the control of lineage-specific enhancers. Mice carrying an elastase promoter/toxin construct lacked a normal pancreas as a result of expression of the toxin in pancreatic acinar cells, whereas expression of the DTA gene under the control of the γ-2 crystallin promoter resulted in mice with lens defects (*78*). This strategy should allow the elimination of any cell type for which a specific promoter or enhancer can be used to express the toxin, and live animals should be obtained as long as nonessential lineages such as lens or pancreatic cells are ablated. Drug-inducible ablation of specific lymphoid cells was recently reported in mice expressing the herpes thymidine kinase gene under Ig gene promoter control (*79*). Because dose and time of drug delivery can be experimentally controlled, this approach should permit the ablation of any lineage, including those that are essential for survival of the animal.

*Markers for chromosomal regions.* Inserted foreign DNA sequences may serve as convenient molecular markers for the flanking host loci for which no probes would be available otherwise. For example, a proviral genome integrated into the pseudoautosomal region of the mouse sex chromosomes proved to be a unique molecular marker for the analysis of this region, which is composed of highly repetitive sequences (*80*). The genetic analysis revealed a high frequency of

unequal crossing-over as well as double cross-over events in the pairing region of the sex chromosomes. Transgenes carried on the X chromosome were found either to escape the normal X-inactivation process or to behave like an X-linked gene (*81*). The cloning and characterization of the host sequences flanking these inserts may contribute to an understanding of the molecular control mechanisms of chromosome pairing and mammalian X inactivation.

Chromosomal markers provided by transgenic mice have also given clues as to the molecular nature of genomic imprinting, that is, the hypothesis of differential expression of maternal and paternal genomes and the requirement for both in mammalian embryogenesis (*82*). When methylation and transcription of foreign DNA sequences in transgenic lines were analyzed, differences in the extent of DNA modification and of gene expression that depended on the parental origin of the respective sequences were found (*83*). These results provide the first evidence for functional and heritable molecular differences between maternally and paternally derived alleles on mouse chromosomes. Cloning of the host sequences flanking the exogenous DNA should reveal whether these differences are due to differential imprinting of the host locus or are a consequence of the insertion of the foreign DNA.

*Mutations in transgenic mice.* The insertion of foreign DNA sequences into the cellular genome can cause mutational changes by disrupting the function of an endogenous gene. Most insertional mutations in transgenic mice are recessive and have been induced by infection of embryos or ES cells with retroviruses or by microinjection of recombinant DNA into the pronucleus (Table 2). The majority of the mutant strains have an embryonic lethal phenotype (*8, 22, 84, 85*). Other phenotypes include defects in limb formation (*9, 86*), transmission distortion (*87*), or disturbance of kidney function (*88*). Insertional mutations can also occur spontaneously by germ line insertions of endogeneous viruses that are activated in certain strain combinations (*89*). We have identified four retrovirus-induced mutations after inbreeding of 70 transgenic mouse strains (*84, 85, 88*), and similar frequencies have been observed in other laboratories (*90*). Thus, the available data suggest that retroviruses induce mutations at an overall frequency of 5 to 6%; pronuclear injection of plasmid DNA may be slightly more mutagenic (*91*). This figure is likely to be an underestimate, because some mutations may have only subtle phenotypes that are not easily detected.

The generation of mutants by insertional mutagenesis is attractive because the introduced DNA can serve as a probe for isolating the integration site and the flanking host sequences. Flanking sequences have in fact been cloned for a number of mutants but the mutated gene has been identified in only one case, the Mov13 strain, which carries an M-MuLV proviral genome in the first intron of the α1(I) collagen gene (*84*). The proviral insertion induces changes in the methylation pattern and chromatin conformation of the collagen gene and was shown to interfere with transcriptional initiation, causing a complete block in type I collagen synthesis in homozygous embryos (*92*). This results in death at midgestation after the rupture of major blood vessels (*93*). The Mov13 strain has been useful for studying the role of collagen in development and for the molecular analysis of structural mutations in the α1(I) collagen gene (*24, 94*). In another mutant strain, Mov34, the host sequences flanking the proviral insertion have been isolated and were shown to correspond to an abundantly expressed gene that has not yet been further characterized (*85*). Like Mov13, the provirus insertion interfered with transcription of the gene presumably causing the lethal phenotype.

Recent evidence indicates that retrovirus integration is not entirely random but occurs preferentially into regions close to deoxyribonuclease I–hypersensitive sites (*95*) that are characteristic for active genes (*96*). It is possible therefore that the chromatin conformation of a given gene can influence its chance of being mutated. Expressed genes with an opened chromatin conformation may represent a more likely target for integration and insertional mutagenesis than inactive heterochromatic genes.

Many integration sites in mutants induced by DNA microinjection have been cloned. However, in contrast to retrovirus-induced mutations, no transcripts of host sequences corresponding to the mutated gene have been reported to date. The analysis of junction fragments has revealed that deletions, duplications, rearrangements, and translocations have frequently occurred at the site of integration (*8, 9*). This contrasts with retrovirus integration, which leads to a short direct duplication of host sequences at the site of the single proviral insert but does not result in other rearrangements in the host genome (*12*). The sequence rearrangements seen in insertional mutants induced by microinjection of DNA are likely to complicate the analysis of the primary molecular defect that caused the mutant phenotype. This is particularly serious if the rearrangements involve genes distant from the integration site of the exogenous DNA.

Many mutant mouse strains express phenotypes that resemble genetic diseases in humans, but the molecular basis of the mutation is understood in only a few cases. Insertional mutagenesis by introduction of exogenous DNA into the germ line provides a means for inducing new mutations whose molecular defect is more easily analyzed. Such an approach, however, has serious limitations for those interested in developing a systematic mutational dissection of the mammalian genome, because the gene to be mutated cannot be specified. Recently, strategies have been developed that may substantially improve our ability to generate mutants with a predetermined defect. In initial experiments designed to mimic a human disease, ES cells were mutagenized and clones selected that had lost the ability to produce hypoxanthine-guanine phosphoribosyltransferase (HPRT). However, the HPRT-deficient mice generated from these cells were phenotypically normal, in contrast to patients with Lesch-Nyhan syndrome, a severe neurological condition caused by HPRT deficiency in humans (*18*). This disappointing result most likely reflects differences in the purine metabolism between humans and mice. The result is nevertheless highly encouraging since it demonstrates that mutant mouse strains can indeed be developed from cell clones selected in vitro. Gene targeting by homologous recombination should allow mutation of any chosen gene and has been used in ES cells either to correct a mutated HPRT gene or to disrupt the wild-type HPRT gene (*97*). In each case, the desired cell clones were obtained by using selection for or against HPRT activity. Attempts at mutating genes in the absence of selection schemes require sensitive screening procedures to identify cell clones carrying the exogenous DNA in the target gene. One possible strategy may be the analysis of DNA from pooled cells by the polymerase chain reaction (*98*).

A new strategy for generating mutants with a precisely predetermined phenotype is to alter a cloned gene by site-directed mutagenesis so that it encodes a mutant product capable of inhibiting the function of the wild-type gene. Such mutations have been termed "antimorphs" or, more recently, "dominant negative mutations" (*99*). In the case of multimeric proteins, such mutations may cause the formation of nonfunctional multimers (*100*). The main advantage of this strategy is that it requires only expression of the mutant gene product and not the inactivation of the endogenous wild-type gene in order to realize the mutant phenotype in a cell. To test the feasibility of this approach in the animal, a point mutation analogous to mutations seen in patients with osteogenesis imperfecta II was introduced into the murine proα1(I) collagen gene in vitro. Substitution of a single glycine residue in the proα1(I) collagen gene was shown recently to be associated with this dominant perinatal lethal disease in humans (*101*). When introduced into

transgenic mice, expression of as little as 10% mutant RNA of the total proα1(I) collagen RNA caused a dominant perinatal lethal phenotype that resembled the human condition (*24*). This kind of approach is likely to be useful for the genetic analysis of many other proteins that form multimeric structures, such as proteins of the cytoskeleton, and could provide defined animal models for human diseases in the absence of mutations in the endogenous gene of interest.

## Conclusions and Prospects

The last few years have witnessed an extraordinary increase in the use of transgenic animals. Methods of manipulating embryos and transferring genes have been refined and now constitute standard procedures used for a variety of purposes. Each of the three methods for generating transgenic animals has distinct advantages for some and disadvantages for other applications. Pronuclear injection of recombinant DNA is the method of choice for obtaining expression of a foreign gene in almost any specific tissue (Table 1). Retroviruses or retroviral vectors are superior when genetic tagging of chromosomal loci, for example, for insertional mutagenesis, or of cells for lineage studies are desired. Finally, the most recently developed method of generating transgenic animals from ES cells allows in principle the derivation of mice with any genetic or phenotypic characteristics for which in vitro screening or selection methods are available.

It is likely that rapid advances will occur in the following areas. (i) It will be important to isolate and characterize chromosomal regulatory elements controlling developmental gene activation over large distances (*31*). Inclusion of such elements in gene constructs should guarantee predictable and efficient expression independent of the chromosomal integration site. This will be particularly important for genetic engineering of large farm animals where cost constraints limit the number of transgenic lines that can be generated and evaluted. (ii) The various possibilities of marking early embryonic cells or ablating specific lineages give experimental access to stages of mammalian development as yet not amenable to easy experimental manipulation. This undoubtably will accelerate our understanding of the complex cell interactions in mammalian development. (iii) The prospect for generating recessive or dominant mutations in preselected genes not only will permit the derivation of precise animal models for human hereditary diseases but also will mark the beginning of a systematic genetic dissection of developmental processes that will radically change the future of experimental mammalian genetics.

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