REVIEW

Dissecting mouse development with toxigenics

Mammalian molecular geneticists frequently are envious of the precision and speed with which the developmental biology of lower eukaryotes can be dissected. Deriving developmental mutants in mice by the use of traditional genetics, in which phenotype is selected followed by the unraveling of genotype, is far more complex and time consuming than in yeast, *Drosophila* and *Caenorhabditis elegans*. However, the advent of technology for directly manipulating the mouse genome promises to contribute an arsenal of techniques for 'reverse' genetics, whereby novel genes may be constructed and introduced into the genome to reveal the resulting phenotype. One of the more exotic approaches for reverse genetics developed in recent years is the insertion of genes that lead to programmed cell death by the developmentally controlled expression of a toxin.

The use of toxins to study normal biology is not a new idea. The eminent physiologist Claude Bernard first suggested the use of toxins as probes for normal physiology, suggesting that, 'Poisons are veritable reagents of life, extremely delicate instruments with which to dissect the vital units' (Bernard 1865). Immunologists have derived and applied 'immunotoxins,' toxic substances coupled to monoclonal antibodies, to induce toxic ablation of specific cells and to attempt immunotoxic therapy of malignant diseases (Vitetta and Uhr 1985). However, the genetically programmed production of a toxin, rather than its pharmacologic use, recently has been applied through the construction of toxigenes. Toxigenes are artificial genes that produce a gene product that is itself a toxin or that has the capacity to metabolize a nontoxic substance to a toxin, leading to an induced 'suicide' of the host cell. The potential of toxigenes for specific cell ablation results from the exquisite temporal and spatial control of gene expression during differentiation and the ability of disembodied regulatory signals to confer this specificity on artificial genes.

Most recent work applying toxigenes as reverse genetic tools in transgenic mice is based on the earlier work of Maxwell et al. (1986), who studied the expression of the gene encoding the toxin produced by *Corynebacterium diphtheriae*, the causative agent in human diphtheria, in transfected tissue culture cells. Diphtheria toxin is the product of the *tox* gene, which is carried by lysogenic corynephages of pathogenic strains of *C. diphtheriae*. As a prototype toxin for genetic ablation, diphtheria toxin consists of a toxic A subunit (DT-A) associated through disulfide bonds with a B subunit that is recognized by a specific receptor. Binding of the B subunit to the cell-surface receptor initiates internalization of the associated A subunit, which then catalyzes the NAD-dependent ADP ribosylation of elongation factor-

2, resulting in the inhibition of protein synthesis and cell death (see Fig. 1).

A second potentially useful toxin for toxigene experiments is ricin, a toxic lectin produced by the castor bean, *Ricinus communis*, which is used widely for the assembly of immunotoxins and immunotoxic therapy. The structure of ricin is analogous to that of diphtheria toxin, in that the B subunit is a specific galactosyl binding protein that adheres to the external cell surface of virtually all cells presenting galactose-containing carbohydrates. The hydrophobic segment of the toxic A subunit (R-A) allows it to penetrate the lipid bilayer and undergo conformational changes that lead to its release into the cytoplasm. Once internalized, R-A acts as a highly specific ribonuclease that catalyzes the inactivation of ribosomes by cleaving the adenosine from the A₄₃₆₅ of the 28S ribosomal RNA (Lamb et al. 1985). The ability of these free R-A subunits to induce cell death in the absence of the cell-surface binding component is often attenuated by two orders of magnitude or more when present outside of the cell membrane. The in vivo toxicity of the ricin A chain in mice is 1500 times less than that of the intact toxin or the A subunit conjugated with an antibody (Jansen et al. 1982). The natural toxin gene may be converted to a toxigene by removing the sequence encoding the signal peptide to allow cytoplasmic expression of the toxin rather than secretion, and expressing the A subunit alone without the associated B subunit. When transcribed, the toxigene kills the host cell; however, because of the low external toxicity of the A subunit alone, surrounding bystander cells are spared, allowing for the elimination of cells based on their developmental stage and capacity for gene expression rather than their anatomical location. The strategy is then to bury in the genome a silent toxigene coupled to an appropriate tissue-specific promoter and enhancer to await appropriately timed gene activation, inducing cell suicide but preventing murder.

This strategy has now been applied to the experimental manipulation of several tissues with tantalizing results. The elastase gene encodes a serine protease that is an important digestive enzyme and is transcribed almost exclusively in cells of the exocrine pancreas. The 205 nucleotides present upstream of the initiation site are capable of directing the exocrine pancreas-specific expression of indicator genes in cell culture or in the tissues of transgenic mice (Ornitz et al. 1985). Expression of a toxigene controlled by the elastase regulatory signals might be expected to eliminate the developing exocrine cells at the stage where induction of elastase synthesis first occurs. When a fusion between the elastase promoter and the DT-A gene was introduced by mi-

Figure 1. Techniques for inducing cell suicide using toxigenes. I. Inhibition of protein synthesis through ribosylation of elongation factor-2 using a toxigen expressing the A subunit of diphtheria toxin (DT-A). II. Inhibition of protein synthesis through inactivation of 28S ribosomal RNA using a toxigen expressing the ricin A subunit. III. Inhibition of DNA replication by acyclovir by expressing herpes virus thymidine kinase which, unlike host cell thymidine kinases, phosphorylates nucleoside analogs to toxic intermediates.

croinjection into the genome of single-cell embryos, pancreatic development was arrested in 7 of 24 transgenic mice carrying the construction (Palmiter et al. 1987). The expression of the DT-A gene was associated with a 10- to 100-fold reduction in the number of islet and duct cells, with a complete absence of exocrine cells. Though an impressive demonstration, the inheritance of this phenotype could not be studied due to the lethality of the phenotype in the founder mice.

A similar approach was taken using regulatory signals from lens crystallin genes to control toxigen expression. The numerous crystallins produced by lens fiber cells are responsible for the structural and optical properties of the lens and are the products of a large multi-gene family. Crystallins are produced in a precisely coordinated spatial and temporal pattern during lens development and are produced continually throughout much of embryogenesis. In the case of both the γ 2- and α A-crystallin genes, short regions upstream of the promoters are sufficient for the apparently physiologically regulated expression of attached indicator genes when introduced into the germ line of transgenic animals. Breitman et al. (1987) constructed and utilized a toxigen in which DT-A was expressed from the γ 2-crystallin promoter and Landel et al. (1988) used R-A expressed from an α A-crystallin promoter. In both cases, animals were created that were physiologically normal except for abnormalities in formation of the ocular lens. The γ 2-crystallin-DT-A transgenic mice had a variable microphthalmia present in three of six founder animals

that varied in severity from cataracts and mild lens abnormalities to complete absence of the lens. All animals derived from α A-crystallin-R-A founders demonstrated a similar microphthalmia associated with fluid-filled vesicles filling the lens and with a disorganized lens fiber architecture. As opposed to studies using the elastase promoter, these lens abnormalities were not lethal and thus strains of transgenic mice expressing the toxic phenotype could be established readily for further developmental studies. In addition to the similarities, α A-crystallin-R-A and γ 2-crystallin-DT-A display interesting, and possibly significant, differences in phenotype. α A-crystallin-R-A mice had apparent overgrowth and malformation of the neural retina and optic nerve and ectopic lens fiber cell production, whereas the γ 2-crystallin-DT-A mice had normally appearing retinal structures. These differences could be the result of variations in the expression of the two crystallin promoters, the consequence of ectopic expression of transgenes in some transgenic strains, or variations in the effects of the different toxins. Whatever the mechanism, however, these observations suggest that developmental abnormalities in the formation of lens can lead to associated malformations in other structures of the eye, including the neural retina.

Similar abnormalities delineating interactions of lens, neural retina, and optic nerve have been described in naturally occurring microphthalmic and anophthalmic mutant mice where the primary defect is likely due to perturbations of subtle tissue interactions rather than

cell suicide (Harch et al. 1978; Hogan et al. 1988). Although highly useful to developmental biologists, the generation of sufficient numbers of complementary mutations to enable a complete developmental analysis analogous to that possible with *Drosophila* is unlikely to occur using traditional genetic means. Though the argument can be made that little is to be gained from creating additional eye mutations through the albeit highly artificial approach afforded by toxicogenics, some important questions regarding tissue interactions cannot easily be addressed using the available naturally occurring mutants. The possibility of transdetermination of neural retina to lens in the mouse, a phenomena well known in amphibia, has been suggested on the basis of abnormalities induced in α A-crystallin-R-A transgenic mice and possibly in other toxigene carrying mice as well, providing but one example of additional new information likely to result (Landel et al. 1988).

A recent study by Behringer et al. (1988) suggests that toxicogenics may be useful in clarifying some aspects of the developmental biology of terminal differentiation. The precise lineal relationships among the various hormone-producing cells of the anterior pituitary are unknown. On the basis of the observation that some cell lines established from pituitary tumors produce both growth hormone and prolactin, and that some cells producing both hormones can be detected in adult pituitary tissue, it was proposed that lactotrophs and somatotrophs may be derived from a common precursor. Toxigenic ablation provides one approach for addressing this hypothesis. Fusion of the DT-A gene with a 310-bp promoter fragment of the rat growth hormone gene resulted in 3 of 21 transgenic founder mice that lacked detectable circulating levels of growth hormone and demonstrated impaired growth and a nearly complete absence of somatotrophs in the pituitary. A severe decrease in the number of lactotrophs accompanied the absence of somatotrophs, suggesting a developmental relationship between the two. Although this result by no means proves the lineal relationship between the two, it certainly is consistent with suspicions derived from more classical studies. The authors noted that rare islands of growth hormone-producing cells (0.01% of normal numbers of somatotrophs) that apparently escaped the expression or effects of the toxigene remain in the ablated pituitary. The failure to kill 100% of the targeted cells may represent the natural frequency with which cells fail to express a particular gene or may be due to modification events, methylation, or deletion, due to the site of integration.

These studies serve to demonstrate the success as well as the difficulties of using these techniques to approach biological questions. The toxicity of the gene products of DT-A and R-A fusions may be extreme to the point that single molecules of DT-A or R-A may be sufficient to induce cell suicide (Yamaizumi et al. 1978). Thus, the toxicity resulting from transient expression of the transgene in embryos after microinjection might be expected to lead to embryonic 'suicide', thus lowering the numbers of resulting transgenic animals. Although

normal numbers of transgenic animals were obtained in some studies (Behringer et al. 1988; Palmiter et al. 1987), the low numbers of animals resulting from other studies suggest that early lethality may occur in some instances (Landel et al. 1988). If this is found to be a general phenomena, additional attenuating mutations of the DT-A gene may prove useful for assuring the success of the experiment. In most studies, only a low percentage of animals that carry the integrated toxigene actually express the toxic phenotype.

An approach that presents an attractive alternative to the use of toxin genes involves the expression in transgenic mice of genes that are not of themselves toxic but that sensitize cells to the effects of drugs. Thymidine kinase of herpes simplex virus type 1 (HSV-tk) is not harmful to most terminally differentiated cells and has been used widely as a selectable gene in cell culture studies. Nucleoside analogs have been developed for viral chemotherapy that can be metabolized by the viral enzyme to potentially toxic compounds, but are not recognized by the host cell's metabolic machinery. Acyclovir, FIAU [1-(2-deoxy-2-fluoro- β -D-arabinofuranosyl)-5-iodouracil], gancyclovir, and related drugs display little toxicity for normal cells at low dosage but block the replication of herpes virus at the appropriate drug level. Unlike mammalian thymidine kinases, HSV-tk is capable of phosphorylating these nucleoside analogs to nucleoside monophosphates, which can be further phosphorylated to nucleoside triphosphates by host enzymes, leading to an inhibition of DNA synthesis (Furman et al. 1980). The approach taken by Borrelli et al. (1988) was to create transgenic mice that expressed the HSV-tk gene from immunoglobulin light-chain promoter and heavy-chain enhancer elements, regulatory elements normally active in mature lymphocytes and their immediate progenitors. Transgenic mice were derived that expressed high levels of HSV-tk enzymatic activity in spleen and thymus and very low levels in other tissues. Animals derived from the transgenic founders were physiologically and anatomically normal, but demonstrated a dramatic reduction in weight and atrophy of the spleen and thymus when infused intravenously with FIAU. The drug treatment resulted in an estimated loss of 83% of cells of the thymus and 57% of cells of the spleen within 1 week. Additional transgenic animals produced by this group using the Ig-HSV-tk fusion gene (Heyman et al. 1989) suggests that the ability to eliminate certain hematopoietic cell populations with nucleoside analogs may vary from one founder strain to another, probably relating to variations in the tissue specificity of transgene expression. Drug infusion or injection was sufficient to eliminate more than 99% of thymocytes yet allow repopulation of all T-cell lineages after withdrawal, suggesting that the hematopoietic progenitor cells are relatively spared. Unfortunately, the HSV-tk-mediated cell ablation technique depends entirely on cell division in the target tissue and thus is most applicable to tissues where cells are dividing rapidly and continually throughout the adult life of the mouse, as in the immune system. It is unlikely that targets such as neu-

rons would be susceptible to tk-mediated killing, unless the drug is applied during neurogenesis where more complex developmental interactions might be affected.

Are techniques for genetic ablation useful modalities for developmental biologists or are they tricks with impressive results but generating little useful biological information? Clearly, the usefulness of animals with a selective loss of certain cell types cannot be questioned for certain practical applications. The value of naturally occurring mouse mutants as hosts for human immune cells and targets for immunodeficiency viruses has already been demonstrated (Namikawa, et al. 1988), and the use of transgenics with engineered immune defects undoubtedly will follow. The use of transgenic mice as models for human malignancies, immunological disease, and hereditary disorders has also been well established (Stewart et al. 1984; Allison et al. 1988). Stacey et al. (1988) used a mutant pro- $\alpha 1(I)$ collagen gene bearing an amino acid substitution to induce developmental abnormalities mimicking human osteogenesis imperfecta, and Dyaio et al. (1988) developed a mouse model of the hepatic cirrhosis of $\alpha 1$ -antitrypsin deficiency by inserting a defective human gene into the mouse germ line. However, toxigenics might present an additional technique for modeling more complex degenerative disorders. Huntington's disease is associated with specific neural cell degeneration involving large spiny neurons of the striatum as a consequence of a dominant mutation on the tip of chromosome 4, and toxigenic ablation of these cells in transgenic mice might allow a model for experimentation and dissection of the pathogenesis of the disease, irrespective of the mechanism of cell death.

The existing examples of toxigenic manipulation of mouse development emphasize the exquisite specificity for gene expression possible using eukaryotic enhancers in transgenic mice. The use of promoter fragments as small as 205 (elastase), 310 (growth hormone), or 367 (αA -crystallin) nucleotides fused to toxigene sequences can generate mice that are physiologically normal except for the programmed death of the targeted cells and, in some cases, their immediate progenitors (Breitman et al. 1987; Palmiter et al. 1987; Behringer et al. 1988; Landel et al. 1988). These observations confirm the initial assumption on which this strategy is based—that transgenes in general, and toxigenes in particular, are not expressed in a 'leaky' manner in any other cell type during development. A single molecule of toxin, and thus a single RNA transcript, appears to be sufficient to induce cell suicide and attests to the fine regulation afforded by the cellular enhancers. If one assumes that an average cell produces about 10^6 transcripts per day, and one toxin transcript can induce cell death, then the level of aberrant transcription must necessarily be less than $1 : 10^6$ and, since some cells live for many months, may be less than $1 : 10^8$. Thus, these experiments provide the best evidence to date for the tight regulation afforded by eukaryotic enhancers.

A significant question is whether toxigenics will contribute to our understanding normal physiology or

mammalian development in a convincing way (Bedington 1988). It has been argued that little useful information has come from the studies done to date because the targeted cells are those that express large amounts of a protein product of terminal differentiation—elastase, crystallins, growth hormone, and immunoglobulins. These tissues are the easiest to target because the gene products and their *cis*-regulatory elements are understood in the greatest detail. With the current technology, the use of promoters with absolute tissue specificity and low levels of nonspecific expression is critical for the success of these types of experiments. Regulatory elements for more interesting developmental genes currently are being characterized. However, the complexity of mammals is such that many experiments using constitutively active toxigenes will lead to early embryonic death or will generate founder animals that cannot be bred to establish a strain. Thus, toxigenes that are innately silent but can be activated by exogenous agents or by breeding them to mice carrying an appropriate activator function are needed and may provide the solution to these technological difficulties. Additional approaches to cell 'suicide' using perhaps DNases, RNases, or other inhibitors of cell physiology may provide additions to the arsenal.

The most precise approaches to molecular developmental analysis today may involve the use of markers for normal gene expression detected by *in situ* hybridization, or the detection of neutral reporter genes after gene transfer into progenitor cells. These approaches clearly provide immensely powerful tools for determining cell lineage relationships, particularly in tissues with a fixed and complex architecture such as the brain. Inactivity or inappropriate expression of particular genes, both of which can now be engineered in transgenic animals, also promise to provide valuable information about the significance of the various gene products for development. What toxigenics adds to this impressive list of techniques is the potential ability to eliminate a cell type and ask questions about the function of that cell, or about the functions and developmental interactions of surrounding cells in its absence. This approach will generate complementary functional information that cannot easily be derived by other methods familiar to most developmental biologists. Clearly, we have only begun the dissection of development by eliminating cell types that can be easily targeted through the use of well-characterized promoters and enhancers. However, the use of powerful subtractive hybridization techniques promises to provide a wide variety of specific genes, and associated promoters, with which to target more restricted and potentially interesting cell types. If this can be accomplished using a precisely controllable toxic phenotype, toxigenics may be refined from an elegant sledgehammer to a fine, useful needle.

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