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NOVEL STRATEGIES FOR MANIPULATING HEPATIC GENE EXPRESSION *IN VIVO*

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TRANSGENESIS AND GENE TARGETING

For the past two decades, molecular genetic advances have been applied to manipulate the mouse genome with increasing specificity. The first breakthrough involved the development of transgenic mouse technology. Transgenic mice are generated by introducing specifically constructed gene sequences into the germline that target expression of this foreign DNA to one or more cell types (Fig. 63.1). Most commonly, pronuclear microinjection is used to introduce purified double-stranded DNA sequences of up to 50 kilobases (kb) in length into one of the two pronuclei in the fertilized mammalian egg. The exogenous DNA, a *trans*ferred *gene*tic material (*transgene*), may be of prokaryotic or eukaryotic origin, and typically becomes integrated into the mammalian genome by an incompletely characterized mechanism (1). Resulting animals are born with one to many copies of the transgene in each of their cells, although if the transgene integrates into the zygote DNA after cell division has commenced, a mosaic animal can be generated that lacks transgene DNA in some cells. The mouse that develops from the microinjected egg is referred to as the *founder* transgenic mouse. If the germ cells of the founder transmit the transgene to its offspring, then all descendants of this animal are members of a unique lineage or line of mice. All animals within a line will possess the transgene at the same location within the genome, and typically display the same pattern of transgene expression. Interestingly, many copies of the transgene can be joined together and integrated as a single linear array or *concatamer* through what is thought to be a homologous recombination process. However, the number of copies of the transgene present within the genome (*copy number*) may not be correlated with the level of transgene expression in the animal.

A large number of enhancer/promoters have been employed to target transgene expression to hepatocytes with variable specificity, and the resulting transgenic mice have been used to study basic mechanisms of hepatic metabolism, growth regulation and carcinogenesis (reviewed in refs. 2–7). However, for some studies, transgene targeting strategies have limitations. For example, targeting expression of mutant H-*ras* to hepatocytes using the albumin enhancer/promoter (which is activated in fetal hepatoblasts) induces fetal liver hyperplasia and death of most transgenic mice shortly after birth, precluding a thorough analysis of the role of this oncogene in hepatocarcinogenesis (8). To address this limitation, it becomes crucial to have an inducible transgene expression system that can be turned on and off at will.

A second, more recently developed methodology involves gene targeting by modification or "knockout" of endogenous genes. By using this technique, endogenous genes can

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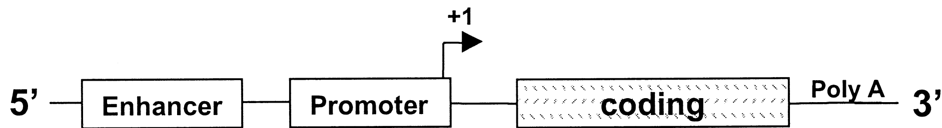

FIGURE 63.1. Molecular components of a typical transgene (double-stranded DNA). "Enhancer" and "Promoter" are gene regulatory elements that direct cell type- and developmental stage-specific gene expression. (*+1*) transcriptional start site; "coding," coding sequence for a gene of interest; polyadenylation (*polyA*) signal, sequence directing addition of polyA tract and often important for mRNA stability. Transgenes also may contain flanking locus control regions to enhance expression (reviewed in refs. 61 and 62). These regions are believed to mediate formation and maintenance of a more "open" conformation of surrounding DNA, thus favoring binding of transcription factors and enhancing transgene expression.

be deleted or inactivated following homologous recombination with gene targeting vectors in embryonic stem (ES) cells (9–12). ES cell lines are derived from the inner cell mass of the blastocyst (embryonic stage corresponding to day 4 post-fertilization), and have the capacity to generate any cell in the mammalian body (totipotency). DNA fragments designed to undergo homologous recombination at selected DNA target sites are introduced into ES cells by electroporation. A gene targeting construct can be designed that is homologous to an endogenous gene but that contains one or more genetic modifications, such as deletions or mutations. Following homologous recombination between the targeting construct and the endogenous gene, these gene differences will be introduced into the ES cell (Fig. 63.2). Cultured ES cells with the appropriate genetic modification then are selected using one of several available selection schemes (reviewed in ref. 11). These ES cells are injected back into a blastocyst, and they can become incorporated into the recipient inner cell mass and subsequently colonize the embryo. ES cell-injected blastocysts are surgically transferred into the uterus of a surrogate mother, in which they resume development. Resulting animals are chimeras, composed of exogenously-derived ES cells and endogenous blastocyst cells. If the introduced ES cells have colonized the recipient mouse germline, some offspring of the chimeric mouse derived from these germ cells will be of the ES cell genotype. ES cell-derived offspring are mated to each other to generate animals that are homozygous for the gene modification (typically only one allele of the target gene will be altered in the ES cell). The strength of this system is that it allows for selection and expansion in culture of the small fraction of ES cells undergoing homologous recombination.

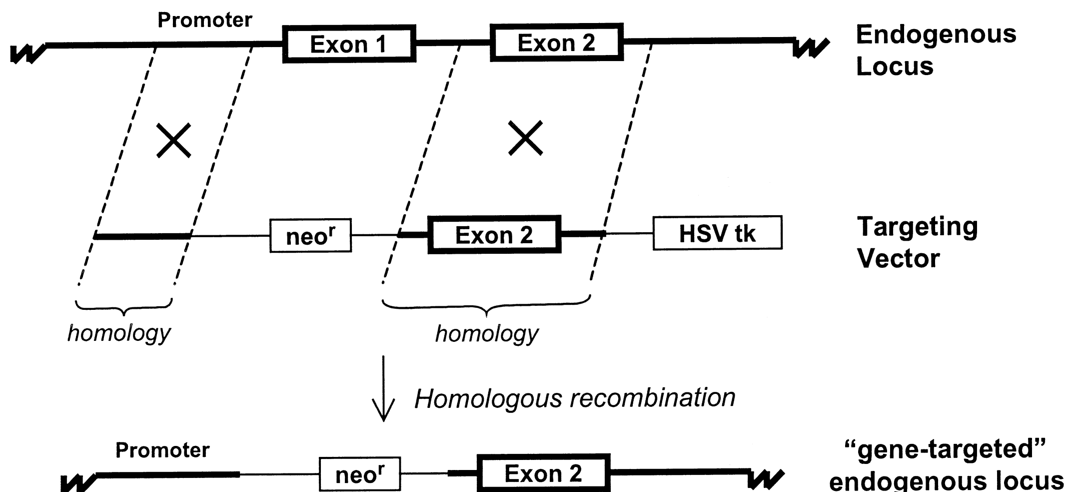

FIGURE 63.2. Targeting modifications to an endogenous locus in embryonic stem (ES) cells. A targeting vector that contains areas of homology to an endogenous gene is introduced into ES cells. The horizontal bold lines indicate endogenous DNA or regions of the vector that are homologous to the endogenous gene. The *X* indicates crossing-over between ES cell genomic DNA and the targeting vector. The most frequent selection scheme employed uses ganciclovir (GCV) and G418. GCV is a substrate rendered toxic by the enzyme thymidine kinase of the Herpes Simplex Virus (*HSV tk*). Following exposure to GCV, cells with nonhomologous integration of the whole targeting vector will be destroyed (negative selection). The cytotoxic antibiotic G418 is detoxified by the neo resistance gene product. Following exposure to G418, cells without the targeting vector will be destroyed, while cells containing the neo^r gene will survive (positive selection).

Despite the importance of this approach, the inability to manipulate the timing of gene deletion or the specific cell type undergoing gene deletion has limited its usefulness. Certain gene deletions are lethal during fetal development, making it difficult to determine the function of the gene at later developmental stages. In the remainder of this chapter, we describe recently developed methodologies that permit temporal and spatial control of gene expression or deletion, and thereby extend our power to understand the genetic basis of development and function *in vivo*.

INDUCIBLE SYSTEMS

The Evolution of Inducible Systems: Past and Present

Over the last few years, several systems have been developed that permit activation and/or repression of transgene expression by an inducer molecule. Four general categories of regulatory systems have been tested. These include: (a) inducible promoters (responsive to heat shock, inflammatory mediators, or heavy metal ions); (b) ligand-regulated systems (antibiotic-regulated); (c) hormone-regulated systems (steroid hormone regulation); and (d) dimeric ligands (rapamycin-induced dimerization of effectors) (reviewed in refs. 13–15). Most inducible systems are binary, containing as basic elements an *activator protein* and a *target gene*. The activator protein regulates target gene expression by binding to regulatory sequences linked to the target gene (i.e., regulation occurs in *trans*). Therefore, the activator protein is termed a *trans*activator. Expression of the activator protein is targeted to a specific cell type by a tissue-specific promoter. Target transgenes have three elements: a nucleotide sequence to which the transactivator protein binds, a minimal promoter that cannot stimulate transcription initiation on its own, and the coding sequence of a gene whose expression is to be turned on. To function appropriately, an optimal inducible system must possess several characteristics: (a) a nontoxic inducer; (b) production of high levels of transactivator protein; (c) restriction of transactivator protein production to the desired cell type; (d) minimal target gene expression in the absence of transactivator protein or inducer; (e) high-level induction of target gene expression in the presence of transactivator protein and inducer; and (f) no pleiotropy (i.e., nontarget endogenous genes should not be activated or repressed). Not all systems are practical for use in mice due to inducer cost, toxicity, or effects on expression of endogenous genes (commonly upregulation); uneven inducer distribution in tissues; or high background expression of a target gene in the absence of the inducer. Most successful experimental studies in mice have employed the tetracycline-inducible system, which is described in detail below.

Tetracycline-Based Regulatory Systems

The Original Tetracycline Responsive System: Tet-responsive Transactivator Protein

In 1992, Bujard and colleagues demonstrated the ability of the antibiotic tetracycline (Tet) to modulate gene expression in mammalian cells engineered to carry the Tet transactivator system (Tet system) (16). Mechanistically, the Tet system is a binary system based on cell type-specific expression of a Tet-responsive transactivator protein (tTA), and a target gene under the control of a tTA-responsive promoter element. tTA binding to the target gene is regulated by the antibiotic tetracycline or one of its derivatives (doxycyline or anhydrotetracycline, for example), which function as inducer molecules (Fig. 63.3). The tTA protein contains two functional elements: a DNA binding domain from the Tet repressor protein (TetR) of the *Escherichia coli (E. coli) tet* operon, and a

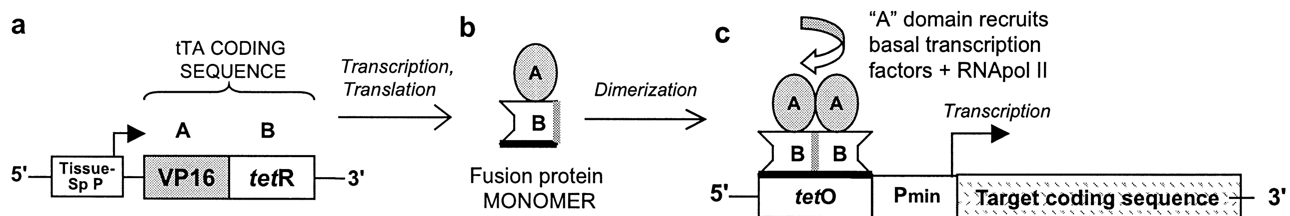

FIGURE 63.3. The original tetracycline transactivator (*tTA*) fusion protein. **(a)** tTA was generated by fusing two distinct functional domains: the transactivation domain of the Viral Protein 16 (*VP16*) from Herpes Simplex Virus (HSV), here depicted as *A* (Activates), and the DNA-binding domain of the TetR repressor protein from the *E. coli Tn10 tet* operon, here shown as *B* (Binds DNA). tTA is expressed in a specific cell type due to the presence of a tissue-specific promoter (*tissue-Sp P*). **(b)** The tTA gene is transcribed and translated to generate a monomer, with "A" and "B" domains. **(c)** the tTA protein functions as a dimer, binding to tet operator (*tet*O) sequences on DNA and activating transcription from the minimal promoter (P_{min}) of the target gene in the *absence* of inducer (Tet or Dox). For convenience, only one of the seven *tet*O repeats is depicted. Shown as a *gray vertical line* is the region that corresponds to the dimerization domain of tTA and as a *black horizontal line* the DNA-binding domain of tTA. (Modified from Kistner A, Gossen M, Zimmermann F, et al. Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. *Proc Natl Acad Sci USA*, 1996;93:10933–10938.)

transactivation domain from the viral protein 16 (VP16, from Herpes Simplex Virus). This fusion protein binds DNA specifically as a dimer at a 19 base-pair (bp) DNA sequence, the *tet* operator (*tet*O). The tTA-responsive target transgene contains *tet*O sequences fused to a minimal promoter upstream of the coding region of the gene of interest. To limit target gene expression in the uninduced state, it is important that the tTA-responsive target gene regulatory element has low or no background expression. For this purpose, a "minimal" promoter is used, which consists of a regulatory element that cannot recruit all necessary transcription proteins on its own. Most often, a human cytomegalovirus minimal promoter (hCMV P_{min}) is employed. It is cloned adjacent to multimerized *tet*O sequences (seven were most efficient). Binding of tTA to the *tet*O element brings the VP16 activation domain next to the minimal promoter, thereby producing a fully active gene regulatory element capable of mediating expression of the linked coding sequence. Binding of Tet to the TetR domain of tTA induces a conformational change that eliminates tTA binding to the *tet*O sequence, thereby turning off target gene expression (Fig. 63.4a). This Tet-responsive system proved to be a breakthrough in inducible gene expression in mammalian cells, since tTA was shown to activate gene expression up to five orders of magnitude in the absence of Tet in cultured cells. Importantly, Tet and its derivatives are relatively inexpensive, and are not toxic to mammalian cells at levels that efficiently turn off gene expression. Furthermore, Tet is widely distributed in tissues (including the central nervous system), and passes from mother to offspring through the placenta and in milk. Use of the inducible transgene expression system in mice requires that both target and transactivator transgenes are present in the same animal. In a typical experiment, separate lines of transgenic mice are generated that carry only one of the transgene pair, and these lines are crossed to generate bitransgenic mice carrying both constructs. A principal limitation of this system involves target gene induction kinetics: it may take several days before *in vivo* clearance of Tet (or Dox) sufficiently reduces inducer concentration so that tTA can bind to *tet*O sequences and initiate target gene expression. This system is available commercially as the Tet-off system (Clonetech, Palo Alto, CA, U.S.A.).

The Reverse Tetracycline Responsive System: Reverse Tetracycline Transactivation (rtTA)

The reverse tetracycline transactivation (rtTA) system, which provides a "reverse" transactivator binding phenotype relative to tTA, was first described in 1995 (17). The rtTA protein was derived from tTA by random mutagenesis, and contains four amino acid substitutions in the TetR moiety that allow rtTA to bind *tet*O sequences only in the *presence* of Tet or its analogs (Fig. 63.4b). However, the binding affinity of rtTA for Tet is 100X lower than that of tTA; therefore it becomes important to use the more avidly-binding Tet derivative doxycycline (Dox) (17). This system is useful when animals need to be maintained in the

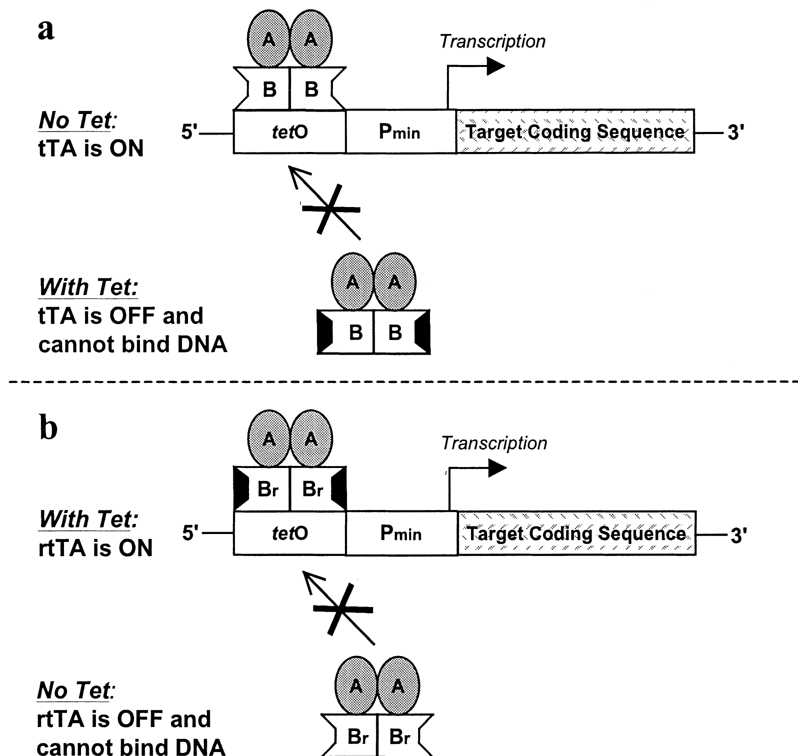

FIGURE 63.4. Mechanism of action of tetracycline transactivator (*tTA*) and reverse tTA (*rtTA*). **(a)** The tTA. The *symbol in black* represents the inducer [tetracycline (*Tet*) or doxycycline (Dox)]. Each dimer of tTA binds to one *tet*O sequence (19 bp) in the *absence* of Tet and recruits the transcriptional machinery to a TATA box-containing minimal promoter via the VP16 transactivation domain. This system is described commercially as the Tet-off system. **(b)** The rtTA. Following binding of Dox or Tet to rtTA, the conformation of the TetR (DNA-binding) domain changes, rtTA now is able to bind DNA at the *tet*O sequence, and transcription can be initiated. This system is described commercially as the Tet-on system. *A*, activator; *B*, binding; *Br*, reverse binding. (Modified from Kistner A, Gossen M, Zimmermann F, et al. Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. *Proc Natl Acad Sci USA*, 1996;93: 10933–10938.)

repressed state for long periods of time, or when rapid target gene induction is desired (which for tTA is limited by the rate of disappearance of Tet from the cell or body). Using the rtTA system, Kistner et al. reported a steady-state 10^5-fold induction of reporter gene activity in the liver of transgenic mice receiving Dox in drinking water, although high background expression was observed in other organs (18). The principal limitation of this system in mice is the frequent presence of significant background target gene expression in the absence of Dox. This system is available commercially as the Tet-on system (Clonetech).

Modifications of the Tetracycline-based Regulatory Systems

Bidirectional vectors for gene expression

Bidirectional vectors have been developed that permit regulation of expression of multiple coding regions simultaneously (Fig. 63.5) (19–20). In this system, the target contains seven *tet*O sequences flanked by two minimal promoters (in opposite orientation to one another). Each minimal promoter directs expression of a separate coding sequence. This unit forms a *bidirectional promoter* (P_{bi-1}), allowing coordinated regulation of two transcriptional units from the centrally located *tet*O sequences. If one coding sequence encodes an easily detected reporter protein, expression of a nonassayable protein encoded by the other target gene can be monitored via the reporter function. Baron et al. described the successful use of this system in cultured HeLa cells, and suggested that the system also worked in transgenic mice (20).

Mutually exclusive two-gene expression system

Baron et al. also have described a mutually exclusive inducible gene expression system (21). This approach combines the tTA and rtTA systems, although with several modifications. Alterations were introduced into both transactivator proteins and into the *tet*O DNA sequence. DNA binding sites were altered by changing nucleotides at different locations in the *tet*O sequence. This produced novel *tet*O sequences, termed *tet*O_{4C} and *tet*O_{6C}, that differed in their TetR moiety binding specificities. Amino acid changes in the regulator proteins (tTA and rtTA) consisted of (a) modifications in the TetR domains that conferred new DNA binding specificities; (b) shortened VP16 transactivation domain, which remained active but was tolerated in higher concentrations by cells (reviewed in ref. 22); and (c) replacement of the class B by a class E dimerization domain in tTA but not rtTA (class B and E domains are naturally occurring dimerization region variants that cannot form heterodimers; Fig. 63.6). The latter modification eliminated the possibility of heterodimerization between tTA and rtTA. These alterations produced $tTA2^{E}_{4C}$ and $rtTA2^{B}_{6C}$, which bound to *tet*O_{4C} and *tet*O_{6C}, respectively. Although their DNA-binding specificities were altered, these tTA and rtTA mutants maintain their original capacity to bind Tet and turn target genes off or on. When used *in vitro* with a graded concentration of an inducer, they provide a system to regulate the expression of two target genes in a mutually exclusive manner (Fig. 63.6). This system has not yet been demonstrated in mice.

Combining a transcriptional activator (rtTA) and a transcriptional repressor (tTR)

In contrast to other Tet system modifications, the Tet-controlled transcriptional repressor system provides the option of specifically repressing basal target gene transcription. This tet-silencing system requires three transgenes. The first is the target transgene, containing the typical *tet*O multimer, minimal promoter, and gene coding sequence. The two remaining genes produce fusion proteins that bind to *tet*O sequences. The first is rtTA, with the original type B dimerization domain. The second is a new fusion protein that we will refer to as the tet-controlled transrepressor (tTR; 23,24). tTR was derived from the original tTA protein by performing the following two modifications: replacing the type B by the type E dimerization domain in the TetR moiety, and fusing this modified TetR domain to a silencer domain (the N-terminus KRAB repressor domain from the mammalian Kox-1 or Kid-1 protein) (Fig. 63.7; reviewed in ref. 25). The KRAB domain is a Krüpel-associated box (KRAB) present in one-third of all human zinc finger proteins, and is a potent

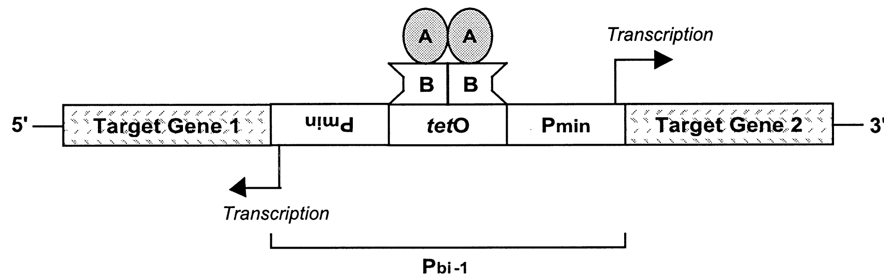

FIGURE 63.5. The bidirectional promoter P_{bi-1}. Tetracycline transactivator (tTA) activates two minimal promoters by binding to a central *tet*O multimer sequence. The *arrows* indicate direction of transcription for each target gene. *A,* activator; *B,* binding. (Modified from Baron U, Freundlieb S, Gossen M, et al. Co-regulation of two gene activities by tetracycline via a bidirectional promoter. *Nucleic Acids Res*, 1995;23:3605–3606.)

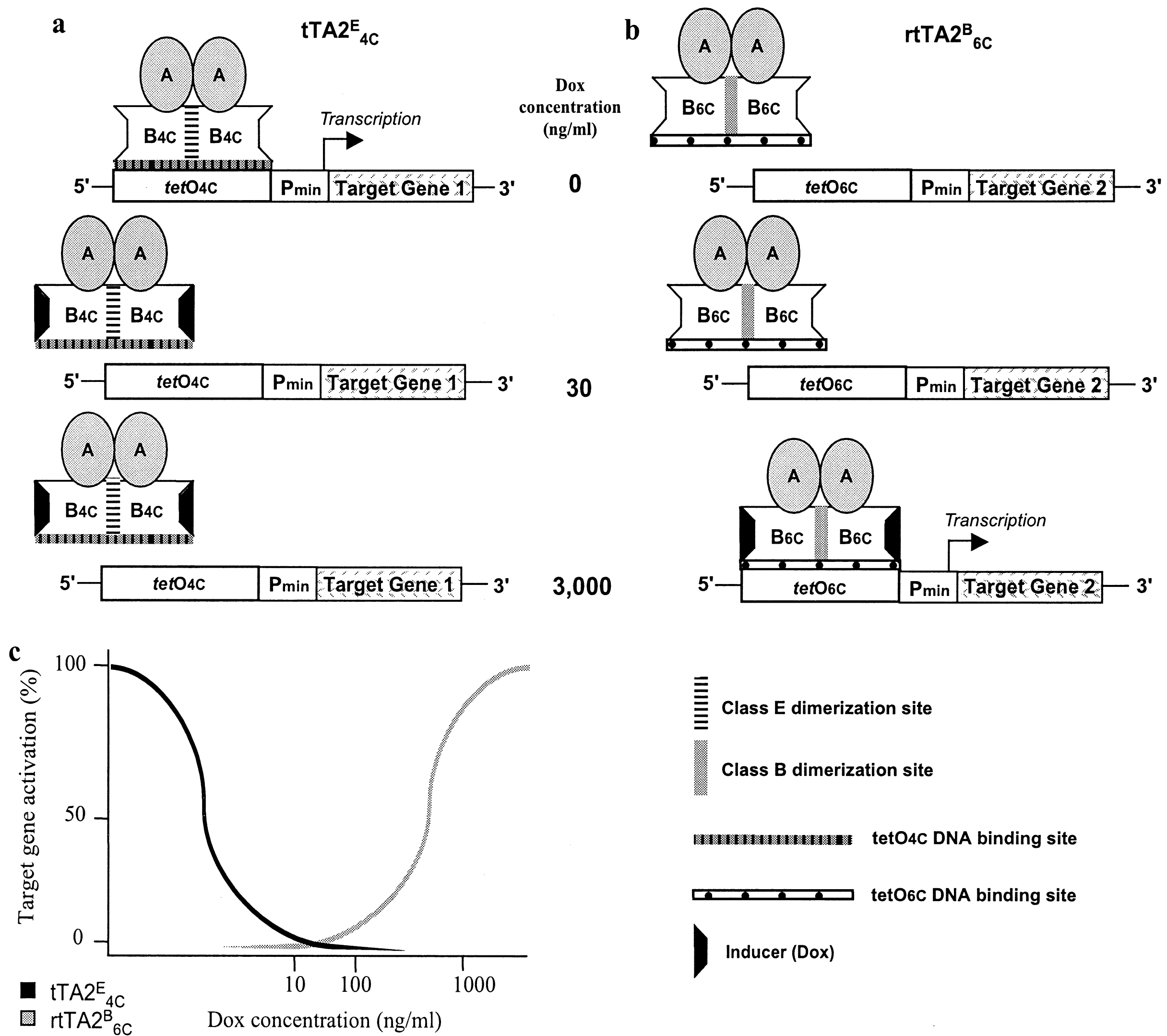

FIGURE 63.6. Mutually exclusive regulation of two target genes by doxycycline (Dox). The transactivator $tTA2^{E}_{4C}$ binds tetO-P_{4C} in absence of Dox **(a)**, whereas $rtTA2^{B}_{6C}$ binds tetO-P_{6C} in the presence of Dox (3,000 ng/ml) **(b)**. At intermediate Dox levels (30ng/ml), neither target gene is activated. Transcriptionally activated genes are indicated by the presence of an *arrow*. $tTA2^{E}_{4C}$ contains the class E dimerization domain, whereas $rtTA2^{B}_{6C}$ contains the class B dimerization domain; therefore, these proteins cannot form heterodimers. The inducer used in this system must be Dox, since rtTA is 100 times more sensitive to Dox than it is to Tet (18). **c:** Graph indicating the independent expression of two different target genes, turned on by either $tTA2^{E}_{4}$ (*black line*), or $rtTA2^{B}_{6C}$ (*gray line*). *A*, activator. (Modified from Baron U, Schnappinger D, Helbl V, et al. Generation of conditional mutants in higher eukaryotes by switching between the expression of two genes. *Proc Natl Acad Sci USA*, 1999;96:1013–1018 and Blau HM, Rossi FM. Tet B or not tet B: advances in tetracycline-inducible gene expression. *Proc Natl Acad Sci USA*, 1999;96:797–799.)

transcriptional repressor (26). Because of these changes, rtTA and tTR cannot form heterodimers, and in the absence of Dox there is reduced basal expression from the target transgene because it now binds a potent repressor molecule. The result can be a 6-fold reduced level of basal transcription, coupled with high-level activation of gene expression by rtTA upon addition of Tet (23). Dox must be used in this system since rtTA has a 100X higher affinity for Dox than it has for Tet. This system overcomes one of the major limitations of regulated gene expression in mammalian systems: low-level background expression. This system is available commercially as the transcriptional silencer system or tTS (Clonetech). *In vivo* use of these systems has not yet been described.

Novel Mutations in rtTA: the $rtTA2^{S}$-M2 Transactivator

Recently, five new rtTAs have been identified in a random mutagenesis screen by Urlinger et al. (27). The most promising new transactivator, $rtTA2^{S}$-M2, functions at a 10-fold lower Dox concentration than the original rtTA, is more stable in eukaryotic cells, is associated with no

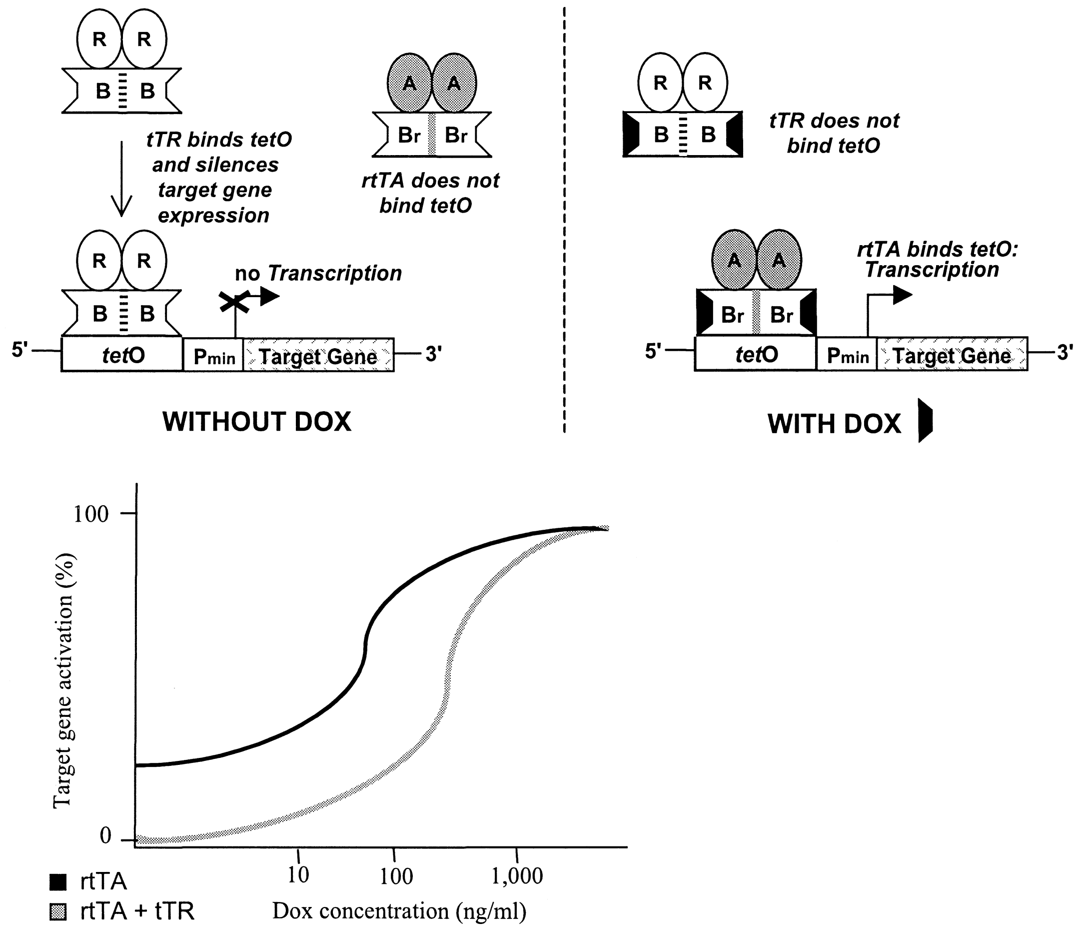

FIGURE 63.7. The "silencing" system: transcriptional transrepressor (*tTR*) and reverse transcriptional activator (*rtTA*) proteins work together to reduce background expression. The "silencing" system is composed of a tTR and an rtTA, both inducer-responsive. The inducer used in this system must be doxycycline (*Dox*), since rtTA is 100 times more sensitive to Dox than to tetracycline (Tet) (17). In the absence of inducer, tTR binds to *tet*O and inhibits target gene expression. In the presence of Dox, rtTA binds to *tet*O and induces target gene expression. tTR and rtTA have different dimerization domains, (*vertical striped* or *gray lines*, respectively), which prevents heterodimer formation. The graph indicates how the basal level of target gene expression can be reduced without affecting the fully induced expression level. (Modified from Blau HM, Rossi FM. Tet B or not tet B: advances in tetracycline-inducible gene expression. *Proc Natl Acad Sci USA*, 1999;96:797–799.)

detectable background expression in the absence of inducer, and allows stringent regulation of target genes over a range of 4 to 5 orders of magnitude in stably transfected HeLa cells. These new versions of rtTA combine tightness of expression control with a broad regulatory range and likely will be used widely in future experimental studies.

THE CRE/LOX SYSTEM

The Cre protein is a 38-kd site-specific recombinase encoded by the bacteriophage P1 genome that mediates intramolecular (excisive or inversional) and intermolecular (integrative) site-specific recombination between specific nucleotide sequences termed *lox*P sites. Its function in phage is to resolve dimeric lysogenic P1 plasmids that arise by general recombination, a process that facilitates the effective partition of the P1 prophage. A *lox*P site (locus of crossing-over) consists of two 13-bp inverted repeats separated by an 8-bp asymmetric spacer region. One molecule of Cre binds per inverted repeat, so that two Cre molecules bind at each *lox*P site. Recombination with a second *lox*P sequence occurs in the asymmetric spacer. Thus, when Cre binds to *lox*P sites engineered to flank part of a gene, there is deletion of the gene sequence that lies between them (Fig. 63.8). The Cre system can be used for two basic purposes: tissue-specific gene deletion or gene activation. This opens up possibilities for elegant control of alterations of gene expression in animals.

The effect of loss of certain genes cannot be investigated fully in mice because the null state causes early embryonic

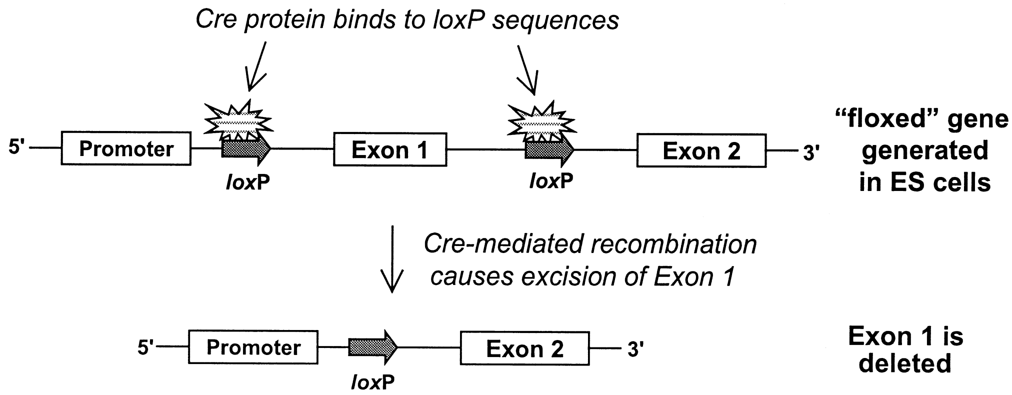

FIGURE 63.8. Knocking out a gene using the Cre/lox system. A selected "floxed" (flanked by *lox*P sites) locus must be introduced into the genome via homologous recombination in embryonic stem cells. The resulting gene-targeted animal then can be mated to a transgenic mouse expressing Cre in a specific cell type. In these cells only, Cre recombinase will bind to the *lox*P sites and excise the intervening DNA. *ES*, embryonic stem.

lethality. To overcome this limitation, these genes can be "knocked out" in adult tissues using Cre/lox. Two mouse lines are required for this "conditional" gene deletion: first, a conventional transgenic line with Cre expression targeted to the cell type of interest, and second, a mouse line carrying the target gene flanked by two *lox*P sites (a "floxed" gene). The latter is generated in ES cells. Cre-mediated recombination occurs only in cells expressing the recombinase, and excision of the target gene ensues (Fig. 63.8). This approach first was demonstrated by deleting a DNA polymerase β gene segment in lymphocytes (28), and has been used since to study gene deletion in hepatocytes (29–31) and other tissues (reviewed in ref. 12). Activation of gene expression also can be accomplished using Cre. In this approach a transgene or an endogenous gene is engineered to contain a floxed sequence that interrupts transcription or translation of the chosen target gene. Cre expression removes the interrupting sequence, thereby "activating" gene expression (Figs. 63.9 and 63.10). This approach has been shown to work in transgenic mice (32–34).

The Tet-inducible and Cre-controlled systems can be combined (Fig. 63.10) (35–37). In this approach, tTA can

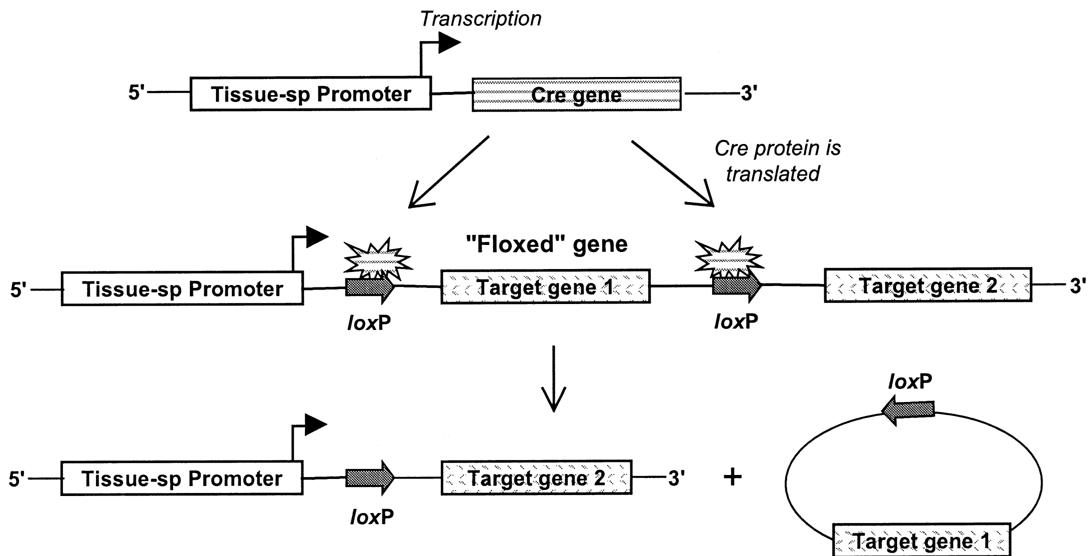

FIGURE 63.9. Activation of gene expression by Cre-mediated gene rearrangement. Cre, represented as a *star*, binds to lox sites in a target transgene. The result is a deletion of Gene 1, bringing Gene 2 under control of the promoter. The circular piece of DNA containing Gene 1 is degraded. Transcription termination sequences could be substituted for Gene 1 as a means to block transcription of Gene 2 in the unrearranged transgene. A complication of this approach is the fact that transgenes typically integrate in multiple copies. Thus, multiple deletion intermediates may be observed.

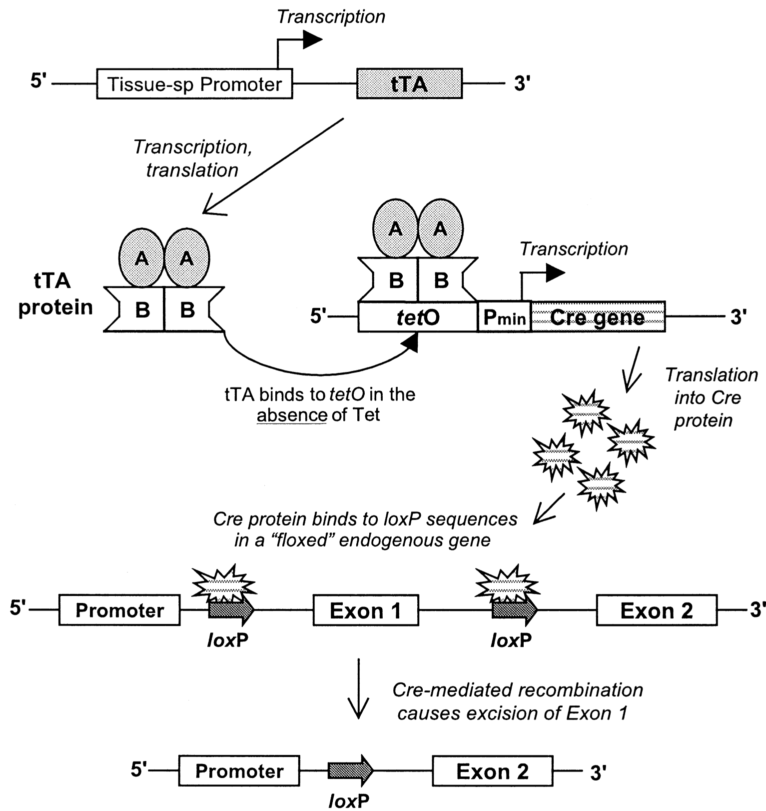

FIGURE 63.10. Inducible Cre/lox: an overview of the combined Cre/lox and Tet systems. Tet-responsive transactivator protein (*tTA*) is expressed in a specific cell type due to the presence of a tissue-specific promoter (*tissue-sp promoter*). In the absence of the inducer Tet, the tTA transactivator binds to the target construct containing *tet*O sequences and a minimal promoter (P_{min}). The Cre gene is transcribed, translated, and mediates deletion of the target gene only in the Cre-expressing cells. *A*, activator; *B*, binding.

regulate expression of the Cre gene from a promoter containing *tet*O sequences. In the absence of Tet, the Cre gene is expressed and induces site-specific recombination between two *lox*P sites in a target gene. In the presence of Tet, the Cre gene is not expressed and recombination does not occur. By establishing inducible expression of the recombinase, which then permanently activates other transgenes, knocks out specific genes, or even switches between the expression of two genes, this combined approach greatly increases the available experimental flexibility.

APPLICATIONS OF TET AND CRE/LOX SYSTEMS TO THE STUDY OF LIVER GROWTH AND DISEASE

Experiments employing traditional approaches to modifying the mouse genome have provided important information about regulation of liver growth and metabolism (2–7). Transgenic technology has been used to identify the effects of overexpression of growth factors and/or oncogenes on liver development, regeneration, and cancer, and to assess the metabolic consequences of changing patterns of gene expression. Gene-targeted mice have been used to identify developmental and pathobiological effects of the deletion of single or multiple genes in liver and many other tissues. Nevertheless, questions that can be answered by these models are limited, principally due to the constitutive nature of transgene expression, which must follow the expression pattern dictated by its gene regulatory element, and the global nature of gene knockouts, which are present in all cells throughout all stages of development. These obstacles can be overcome by employing an inducible transgene system or a conditional gene targeting system. The remainder of this chapter discusses potential applications of these methodologies to studies of liver biology and disease.

Liver Development

The ability to knock out activity of specific genes has provided an extremely powerful tool to identify genes whose expression is essential for tissue development and to assess how these genes influence development (see chapter 2). In mouse, liver development initiates at day 9.5 of the 19.5 day gestation period, thus inactivation of genes that are essential for mouse development at an earlier gestational stage will be lethal before liver development begins. To bypass this limitation, an essential part of the target gene can be flanked by *lox*P sites (Fig. 63.8), and expression of Cre can be targeted to the early hepatoblasts. Both albumin (AL) and α-fetoprotein (AFP) genes are expressed in hepatoblasts from the earliest stages of liver development (38,39); either can be used to direct Cre expression, although AFP also is expressed in yolk sac and developing

gut and so provides less cell-type specificity. (Unfortunately, cell-specific promoters to target other liver cell types have not been identified). Cre-expressing cells then undergo deletion of floxed DNA, leading to loss of target gene activity. Typically, experiments are designed so that mice carry the Cre-expressing transgene, one null allele of the target gene, and one floxed allele of the target gene. In this way, fetal survival is ensured (since usually, though not always, the floxed allele will be expressed normally), and the likelihood of target gene deletion is maximized (because only one allele needs to undergo Cre-mediated DNA deletion). Target gene inactivation is restricted to Cre-expressing cells, and the consequences can be assessed without the complication of gene loss in other cell types.

This approach still has limitations. The most important relates to the efficiency of Cre-mediated target gene deletion. Any variation in Cre transgene expression in the target cell population also will affect the kinetics of deletion, since binding of the Cre protein to the *lox*P sites is influenced by intracellular Cre concentration. Thus, target gene rearrangement may not display identical kinetics in all target cells. In fact, if gene deletion kills or injures target cells, a selective process may favor survival and outgrowth of cell populations with inefficient Cre-mediated deletion, and experimental interpretation must recognize this possibility.

Inducible transgene approaches can be employed to identify the effects of upregulated gene expression on liver development. To study developmental gene effects, gene induction must be rapid, as provided by the Tet-on system. Gene induction kinetics in the converse Tet-off system are far too slow following removal of inducer (and likely variable among individual mice) to permit reliable transgene modulation during fetal liver development. Candidates for inducible transgene expression to study fetal liver development include genes active in growth or metabolic pathways, genes encoding dominant-negative proteins that may interfere with these pathways, or even toxins to selectively damage or ablate liver cell populations at specific stages of development. A second important use of inducible systems in this context will be to suppress expression of transgenes in fetal liver. As noted earlier, fetal hepatoblast-targeted expression of mutant H-*ras* induces diffuse hepatic hyperplasia and perinatal death of transgenic mice (8). Keeping transgene expression turned off in fetal and young mouse hepatocytes should permit identification of mutant H-*ras* effects in adult mouse liver.

Liver Regeneration

Liver regeneration, particularly after two-thirds partial hepatectomy, is a highly studied process for which many changes in the pattern of gene expression have been identified (reviewed in refs. 7,40,41) (see Chapter 42 and website chapter 💻 W-31). Following surgical removal of two-thirds of the liver mass, most remaining hepatocytes synchronously enter the cell cycle, replicate DNA, and divide, restoring liver mass within a week to 10 days. Nonparenchymal cells also replicate within days of hepatectomy. During this process, the liver continues to function. Despite our knowledge of gene expression changes occurring throughout this process, the critical regulatory pathways remain incompletely defined. Recent progress in identifying these pathways has relied upon use of gene-targeted mice. For example, mice lacking functional interleukin-6 (IL-6) or tumor necrosis factor receptor type I (TNFR-I) display markedly attenuated liver growth responses to partial hepatectomy, implicating IL-6 and TNFα pathways as important determinants of liver regeneration (42,43). Fausto has reviewed in detail recent studies of liver regeneration in gene-targeted mice (44). He concluded that further progress will require selective deletion of candidate regulatory genes in subsets of cells or after completion of fetal development to avoid the lethality associated with global deletion of certain genes. For example, hepatocyte growth factor (HGF) is believed to be a key regulatory molecule in liver regeneration, but the effect of its loss on regeneration cannot be assessed because HGF-null mice die before or shortly after birth (45,46). Conditional HGF gene deletion in appropriate target cells of adult mice should permit evaluation of its role in this process.

This example also illustrates several of the current limitations of this approach. HGF is expressed by nonparenchymal cells in liver. Efficient delivery of Cre to these target cells, using transgenic or viral vector approaches, is not currently possible due to lack of characterized targeting elements. To address this problem, target gene deletion can be engineered to be less tissue-specific but more developmental stage-specific. A ubiquitously expressed transactivator can be designed to regulate an inducible Cre transgene (such as *tetO*-P_{min}-Cre). The ROSA 26 gene regulatory element (R26) is expressed in all cells of the body in fetal and adult mice (47,48). Mice expressing R26-rtTA can be administered doxycycline as adults, activating rtTA binding to *tetO*-P_{min}-Cre. In theory, all cells in these adult mice should express Cre protein and delete the floxed target sequence. If ubiquitous deletion of the target gene in adults is not lethal, the resulting mice can be used to study the role of that gene in hepatic regeneration.

Furthermore, whichever Cre delivery system is employed, excision of HGF may not occur in all relevant target cells. Thus, interpreting experiments using cell-specific deletion methodology will require evaluation of deletion efficiency in the target cell population. To address this problem, floxed target gene loci can be engineered so that deletion of the floxed DNA segment activates expression of a previously silent marker gene, such as the bacterial *lacZ* gene, thereby permanently identifying cells that no longer can express the target gene. Using the example of HGF, an experiment may suggest that deletion of the HGF gene in adult mice does not affect liver regeneration. However, if

deletion occurred only in 50% of liver nonparenchymal cells, this finding would not support a conclusion that HGF produced in liver had no role in regeneration.

Tet-inducible transgene approaches may be useful in additional ways to define the effects of genes that influence hepatic regeneration. Expression of putative growth stimulatory or inhibitory genes can be induced prior to or at defined times during posthepatectomy regeneration. This approach may be particularly effective in mice receiving a one-third partial hepatectomy, which is reported to "prime" or induce replication competence in hepatocytes without inducing widespread passage through the G_1-S boundary (49–51). Tet-induced genes or gene combinations can be assessed for their "sufficiency" to promote cell cycle progression in one-third hepatectomized mice. Past approaches to defining sufficiency have involved intravenous infusion of growth modulatory substances, often into the portal vein; the inducible transgene approach would complement these studies by permitting localized production within the tissue microenvironment. The potential usefulness of this approach would be influenced by the kinetics of transgene induction.

In certain forms of liver disease, parenchymal regeneration may depend upon activation of a nonhepatocytic progenitor or "stem" cell lineage (52). This type of regeneration is proposed to follow hepatic injury in which replication of surviving hepatocytes is inhibited. The Tet-inducible transgene approach could be used to ablate putative precursor cell populations, using, for example, targeting of the cytotoxic diphtheria toxin gene, thereby identifying the effect on regeneration of loss of that cell type. A gene regulatory element expressed in target cells but not in other cell types would be needed to direct transgene expression. The AFP gene is one candidate: in adult liver, AFP expression normally is low, but in several rat models of severe hepatic injury AFP expression is reactivated in a subset of cells that are proposed to represent hepatocyte progenitors. A noninducible AFP-diphtheria toxin transgene could not be used for this purpose because it would cause fetal death secondary to transgene-mediated destruction of fetal yolk sac, gut, and liver.

Liver Growth Disorders and Cancer

Cancer is largely a genetic disease, although its course has many environmental influences. Typically, multiple genetic changes collaborate during carcinogenesis to produce a fully malignant cell, and malignant cells from different neoplasms may be genetically distinct. Identifying and characterizing causative genetic changes represents at least as complex a task as characterizing liver regeneration after partial hepatectomy, and the approaches to identifying relevant growth signaling pathways in each context will be similar.

Deletions of one or more growth inhibitory or DNA protective genes, termed "tumor suppressor genes," is a universal theme in cancer genetics. In some cases, inheritance of one defective copy of a tumor suppressor gene predisposes an individual to cancer in one or more tissues. Actual development of the disease is associated with loss or inactivation of the remaining allele. Examples include childhood retinoblastoma (the pRb gene) and Li-Fraumani syndrome (the p53 gene). These conditions can be modeled in gene-targeted mice (reviewed in refs. 53,54), but often deletion of both copies is lethal during fetal development (as for pRb) (55), or produces a spectrum of cancers that may not include the tissue of interest (p53 null mice do not typically develop liver cancer) (56,57). These concerns can be addressed by restricting conditional gene knockouts to hepatocytes (or any other cell type to which Cre protein can be delivered). Although it remains important to assess penetrance of Cre-mediated deletions in the target cell population, incomplete penetrance is not as great a concern in this experimental context, since loss of both copies of a cancer-relevant tumor suppressor gene may produce a selective growth advantage in, and subsequent amplification of, affected cells.

Inducible transgene strategies may become critical for *in vivo* study of growth stimulatory oncogenes. Fetal hepatoblast-targeted expression of mutant H-*ras* induces diffuse hyperplasia, not neoplasia (8). Restricting expression to adult hepatocytes will be necessary to identify the effects of this oncogene in the adult tissue context. Perhaps even more exciting, availability of inducible gene expression systems (including inducible cell type-specific Cre transgenes) provides the means to conduct a detailed analysis of interactions between oncogenes, tumor suppressor genes, and growth factors during hepatocarcinogenesis. Analysis can include assessing (a) the combined effects of two or more genetic changes (already established for some combinations without inducibility); (b) the phenotypic consequences of the relative timing or order of expression or deletion of cancer genes; and (c) the effect of turning on and off expression of specific oncogenes, to determine whether persistent expression is necessary for maintenance of the neoplastic state or whether neoplastic cells can progress to a state of independence from the initiating neoplastic stimulus. The latter has been accomplished for skin in a study demonstrating that down-regulation of mutant H-*ras* expression in papillomas caused these lesions to regress (58). The tremendous flexibility of experimental design provided by this technology will dramatically improve our understanding of the complex genetic underpinnings of abnormal liver growth.

Other Conditions

The liver has primary functional roles in detoxifying endogenous and exogenous toxins and in regulating metabolism (see website chapters 💻 W-14 and W-15). Proper functioning requires the concerted actions of multiple genes. Gene deletion experiments can address the role of specific genes in each process. Restricting gene deletion to hepatocytes improves the accuracy of this assessment when the target gene also

influences the development or function of cell types in multiple tissues. Global deletion of the aryl hydrocarbon receptor (AhR) gene, which is involved in metabolic breakdown of chlorinated hydrocarbons such as dioxin, produces a spectrum of abnormalities in the mouse liver but also in the immune system (59,60). Hepatocyte-specific deletion will help to clarify the specific functions of this gene in liver. The importance of cytoskeletal and other structural molecules to hepatocyte function and viability similarly can be determined using conditional, cell type-specific gene deletion, without complications introduced by alterations in other tissues. The genetic basis of any process specific to mouse liver can be addressed with ever-increasing sophistication using the tools of conditional gene targeting and inducible transgene expression.

Future Directions

Relatively few reports describing the use of inducible and conditional deletion methodology have been published, but this condition will change dramatically during the next few years. Several trends are likely to be observed. First, as experimental design catches up with currently available technology, studies more frequently will employ methodological refinements such as the tet-transrepressor system, new-generation transactivator molecules, and combined inducible/conditional deletion systems. Second, the technology itself will change rapidly, permitting increasingly precise control of gene expression and deletion. This change will include refinement of current and development of new systems that will become efficient, practical, and cost-effective for use in animal models. Third, with the availability of multiple, independently regulatable systems, researchers will begin to manipulate simultaneously or sequentially the expression and/or deletion of large collections of genes, permitting mechanistic study of multigenic traits. These experiments will provide a more thorough understanding of the genetic basis of health and disease in mice, improving our knowledge of the corresponding conditions in humans.

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