Novel Mouse Models in Biomedical Research: The Power of Dissecting Pathways by Quantitative Control of Gene Activities

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Abstract The last decade has seen significant progress in the development and refinement of genetic approaches applicable to the mouse, making this animal the prime organism for the study of mammalian genetics. Particularly, the potential to control individual gene activities in a temporally defined and tissue-specific manner has allowed us to dissect gene functions and pathways in vivo with unprecedented precision, yielding exciting new insights into such complex biological processes as development, behavior, and disease. For biomedical research, the new approaches of mouse molecular genetics open up new perspectives for modeling human diseases. Particularly methods that allow quantitative and reversible control over disease genes will enable the experimenter to not only study the onset of a disease and its progression but also to examine its potential reversibility and to investigate mechanisms of disease regression. In this chapter, we summarize the principle of Tet regulation as a paradigm for a reversible gene control system and we briefly discuss Tet regulation-based mouse models of human diseases in the area of cancer as well as of neurodegenerative and cardiac diseases.

Keywords Conditional mouse mutants · Tetracycline controlled gene expression · Models of human disease

1 Introduction

During the past decade, experimental approaches have emerged which allow the conditional alteration of individual gene activities in higher eukaryotes in a temporally defined and cell type restricted manner. Applying these approaches to the mouse—thanks to transgenesis and embryonic stem (ES) cell technology, the prime model of mammalian molecular genetics—is increasingly providing new insights into fundamental biological processes. For biomedical research, these technologies are of particular interest as they offer new opportunities for modeling human diseases in transgenic rodents. Such models will more faithfully mimic pathologies and, thus, will enhance our understanding of diseases at the molecular and physiological level, thereby facilitating the development of new strategies for interventions and for prevention.

The crucial precondition for the sensible application of gene activation/inactivation approaches is specificity: interference should exclusively affect the gene under study. This demanding prerequisite is met, to different extent, by systems based either on elements heterologous to, e.g., the mammalian cell, or on modified endogenous components. The most commonly applied strategies presently, which apparently fulfill the required criteria best, exploit prokaryotic elements, evolutionarily most distant to higher eukaryotes.

Thus, the CRE/LoxP recombination system of *Escherichia coli* phage P1 has been successfully applied, and the conditional knock-out approach in transgenic mice has yielded a wealth of information. A second widely applied strategy, which aims at controlling transcription of target genes in eukaryotes, is based on regulatory elements of the *E. coli* tetracycline resistance operon.

When the two basic strategies—site-specific recombination and control with gene expression—are compared, genetic analysis via temporally and spatially restricted recombination has proven to be enormously successful. Nevertheless, the potential of reversibly and quantitatively interfering with a gene's activity, e.g., at the level of transcription, adds another quality to the study of gene function in vivo: the well-defined and reversible perturbation of a system allows not only comparisons between the normal and the perturbed state, but also monitoring of the system's reaction when interference is terminated. Therefore, for modeling human diseases in transgenic animals, the fully reversible control of target genes appears to be particularly informative as will be shown below.

For these reasons we restrict our review to what one may call "truly conditional," i.e., reversible approaches. Moreover, being fully aware of the various methods developed, we will focus on the Tet regulatory system as a paradigm for which, due to its widespread application, an extended experience has accumulated. For information on various other approaches, we refer the reader to

some informative recent reviews (Sauer 1998; Lewandoski 2001; Gossen and Bujard 2002; Kuehn and Schwenk 2002).

The Tet regulatory systems meet two essential criteria for genetic conditionality: (1) They allow the experimenter to reversibly alter the expression of a gene and, thus, to study the impact of timely limited perturbations of gene activities; (2) they enable the experimenter to not only switch a gene on and off, but also to adjust its activity to intermediate levels, which often mimics pathological states more closely than total gene inactivation. Thus, the activity of a disease gene can be altered at a given time within an animal's lifespan to different degrees and, if required, for a defined time period only. These features permit us to address a number of important questions regarding such issues as:

- Early events during the onset of a disease
- Disease progression
- Potential reversibility of pathologies upon inactivation of the disease-initiating gene
- Disease regression

Disease models with such properties will be most suitable for the identification and validation of genes and their products as targets for pharmacological interventions. They will also be valuable as in vivo systems for preclinical drug efficacy studies.

In the following, we will describe the principle of Tet regulation and some recent technological advances before we highlight some conditional mouse models in the area of oncology as well as neurodegenerative and cardiac diseases. Obviously, we cannot give a comprehensive survey and apologize to colleagues whose excellent work could not be included in the context of this chapter.

2
The Tet Regulatory System—Principles, Components, and Approaches

The Tet regulatory systems act at the level of transcription whereby a gene of interest is controlled via an artificial control circuit superimposed on the enormously complex regulatory network of an eukaryotic cell. This circuit can be controlled from outside, preferably by the tetracycline derivative doxycycline (Dox). When properly set up, the circuit by itself will not at all or only marginally affect the metabolism of the host cell, thereby relying on the Tet systems' exquisite specificity. The favorable properties of the Tet systems are based on three crucial parameters (for reviews see Gossen and Bujard 2001; Baron and Bujard 2000):

- The evolutionary distance between the regulatory core elements of the system which are of prokaryotic origin and which appear not to engage in interactions with essential components of the eukaryotic cell

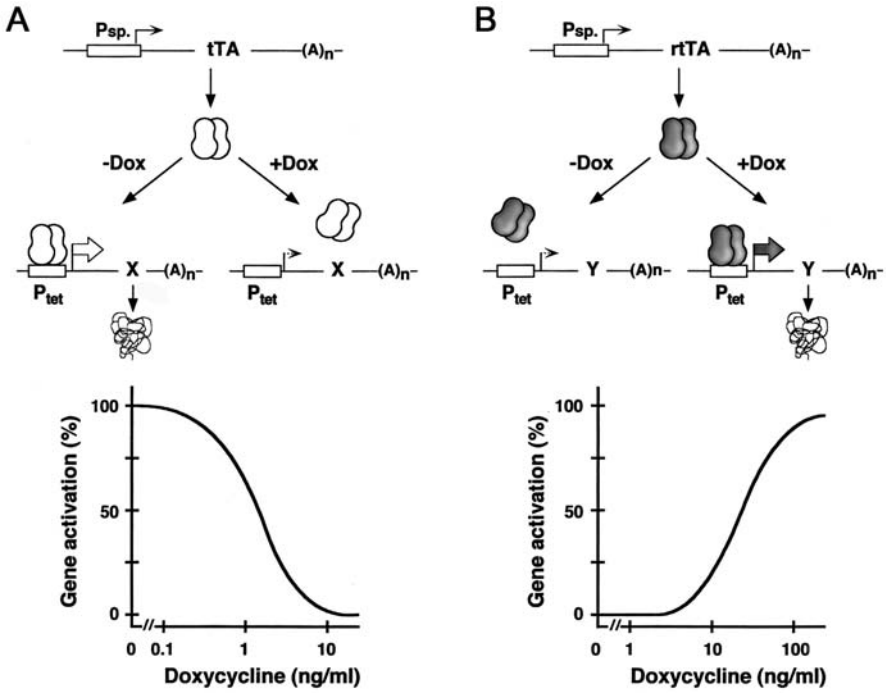
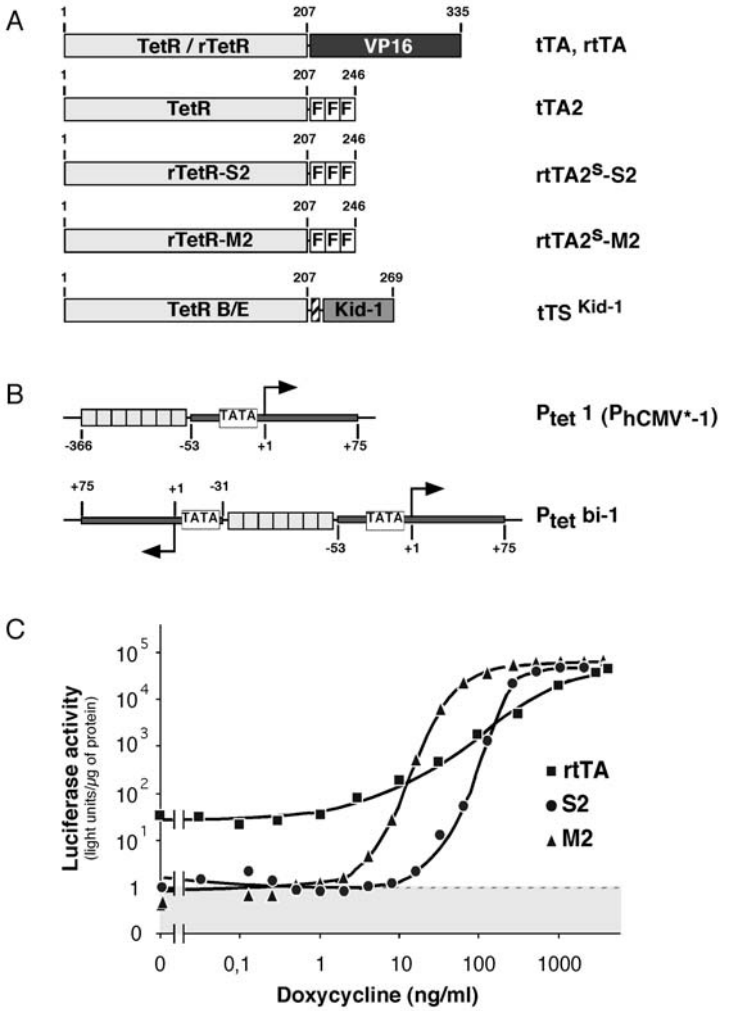


Fig. 1A, B Outline of the Tet regulatory system. **A** *Upper part*, mode of action of the tetracycline controlled transactivator (tTA). In absence of the effector substance Dox, tTA binds to *tet* operator sequences within P_{tet} and activates transcription of gene X. In the presence of Dox, tTA is prevented from binding and activating P_{tet}. *Lower part* illustrates the response of tTA-dependent gene expression at different concentrations of Dox. Gene activity is maximal in the absence of the antibiotic and is gradually downregulated at the single-cell level by increasing concentrations. **B** *Upper part*, mode of action of the reverse tetracycline controlled transactivator (*rtTA*). The original rtTA differs from tTA in 4 amino acids within the TetR moiety, resulting in the reverse phenotype which requires Dox for binding to *tet* operator sequences and, thus, to P_{tet}. *Lower part*, response of rtTA-mediated gene expression using the improved $rtTA2^S$-M2 to different concentrations of Dox. By increasing effector concentrations beyond 10 ng/ml, transcription is gradually enhanced until it reaches the maximum at 80–100 ng/ml. In both systems, tissue or cell-type specificity is brought about by the promoter P_{sp} driving tTA/rtTA expression

Fig. 2A–C Schematic outline of the tetracycline-controlled transcription factors, their responsive promoters, and induction profiles of rtTAs. **A** Fusions between the repressor protein (TetR) of the *E. coli Tn10* tetracycline resistance operon and domains capable of either activating or silencing transcription. tTA is a fusion between the 207 amino acid TetR and the 128 amino acid long C-terminal portion of VP16 of *Herpes simplex* virus. In all tTA2 and rtTA2 versions, the VP16 moiety is replaced by three 13 amino acid-long minimal activation domains (*F*). $rtTA2^S$-S2 and $rtTA2^S$-M2 are improved versions of



rtTA, based on novel TetR mutants. tTA2 and the new rtTA versions are encoded in synthetic polynucleotides optimized for expression in mammalian cells. tTS^{kid}-1 is a fusion between $TetR^{B/E}$ and a 61 amino acid-long repression domain (KRAB) from the human kidney protein Kid-1. A nuclear localization sequence is placed between TetR and the silencing domain. The $TetR^{B/E}$ version prevents heterodimerization between tTS and rtTA monomers when coexpressed in one cell. **B** tTA/rtTA-responsive promoters. P_{tet} is a fusion between seven *tetO* operators (*gray boxes*) and a minimal promoter derived from the human cytomegalovirus promoter IE (P_{hCMV}). The bidirectional promoter P_{tet}bi-1 consists of a core of heptamerized *tet* operators which is flanked by two divergently oriented P_{hCMV}-derived minimal promoters. Bidirectional promoters allow the coregulation of two genes. Positions spanning promoter regions and operator sequences are indicated with respect to the transcriptional start site (+1). **C** Induction of luciferase activity by rtTA, $rtTA2^S$-S2, and $rtTA2^S$-M2 at different Dox concentrations. HeLa cell lines were used, which stably synthesize the respective transactivators under P_{hCMV} control and which also contain the stably integrated luciferase gene under P_{tet} control. Both new rtTAs (S2, M2) do not show intrinsic background activities under these conditions. $rtTA2^S$-M2 exhibits, in addition, an around tenfold higher sensitivity towards Dox

- The unusual specificity of interaction between the Tet repressor (TetR) and the *tet* operator (*tet*O) as well as between TetR and its inducer, Dox
- The well-studied chemical and physiological properties of the inducing agents, i.e., various tetracyclines of which some, particularly Dox, have been widely used in human and animal medicine

As outlined in Figs. 1 and 2, there are two complementary Tet control systems which consist of three elements each: (1) the tetracycline-controlled transactivators (tTAs) or reverse tetracycline-controlled transactivators (rtTAs) which are fusions between TetR and transcriptional activation domains; (2) a minimal, i.e., enhancerless RNA polymerase II promoter, fused downstream to an array of seven *tet*O sequences designated P_{tet}; and (3) the inducing compound Dox or other tetracycline derivatives. In the tTA system, Dox prevents binding of tTA to P_{tet} and, thus, abolishes transcription (Gossen and Bujard 1992). By contrast, rtTA requires Dox for binding to and activation of P_{tet} (Gossen et al. 1995).

In transgenic animals, the rtTA approach is to be preferred whenever a gene should be activated rapidly, since saturating a system with a small effector substance is an intrinsically more rapid process than its depletion (as required for the tTA system). For the same reason, the tTA principle is advantageous whenever a fast shut off of a gene's expression is required.

2.1 Operating the Tet Control Circuit with Dox

Dox penetrates cell membranes by diffusion. Therefore, P_{tet}-controlled genes can be activated at the single-cell level in a ridgeless manner by properly adjusting Dox concentrations. In cultured cells, Dox concentrations far below toxicity levels (5–10 μg/ml for HeLa cells) are required. Thus, tTA is gradually inactivated between 0.05 and 5 ng/ml, whereas 20–100 ng/ml are sufficient for activating the novel rtTA2^S-M2 (Fig. 2 and below). To establish proper Dox concentration in the mouse, the antibiotic is most conveniently supplied via drinking water where 50–200 μg/ml are sufficient for switching off the tTA system in any organ (Kistner et al. 1996). Higher concentrations are required even for the rtTA2^S-M2 system (Fig. 2), but 2 mg/ml (in the water supply), an amount unproblematic also over long periods of time, are usually sufficient. Rapid induction may be achieved by i.p. injection (Fig. 3) whereby up to 2 mg of Dox in 0.2 ml of isotonic saline every 24 h is a well-tolerated dosage in short term experiments (1 to 2 weeks; Hasan et al. 2001). Due to the excellent cell and tissue penetration properties of Dox, appropriate concentrations for tTA inactivation and activation of rtTA2^S-M2 are readily achieved in different compartments of the animal including the placenta and the milk of lactating mothers. Thus, Tet regulation can be imposed onto the developing embryo as well as on offspring before and during weaning. Partial induction of Tet-controlled genes may be achieved by adjusting the amount of Dox delivered, though it has to be reconciled that tissue distribution and biological half-life of Dox differ from organ to organ (Kistner

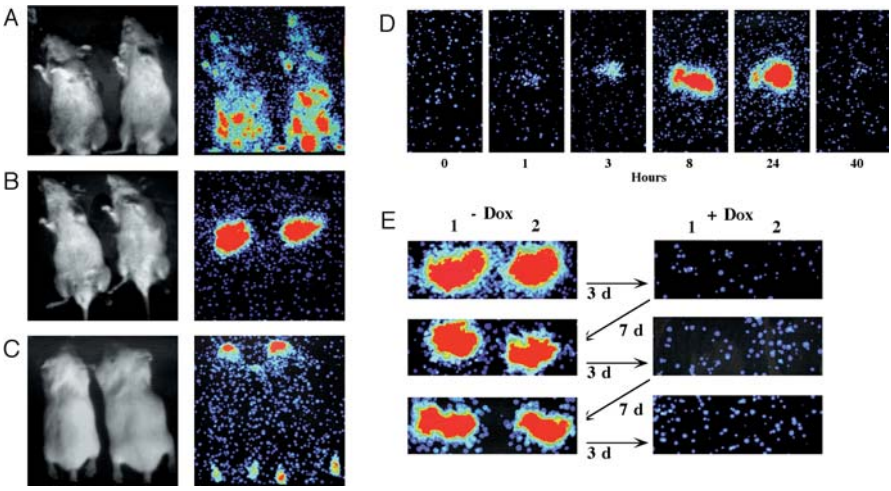


Fig. 3A–E In vivo imaging of Tet-regulated luciferase expression in live animals. **A–C** Animals of 3 mouse lines expressing the tTA gene under the control of P_{hCMV}, P_{LAP} and P_{CamKII}, respectively, were crossed to individuals of the LC-1 strain (Schoenig et al. 2002) where the luciferase and the *cre* gene are coregulated by P_{tet}bi-1 (Fig. 2). To demonstrate organ-/tissue-specific expression directed by the different promoters driving the transactivators, luciferase expression of double transgenic animals was analyzed non-invasively via luciferase bioluminescence. **A** P_{hCMV}, several organs/tissues. **B** P_{LAP}, hepatocytes. **C** P_{CamKII}, brain. **D** Kinetics of induction for hepatocyte-specific luciferase synthesis. Luciferase of rTA^{LAP}/LC-1 double transgenic animals was non-invasively monitored after i.p. injection of 2 mg of Dox at the times indicated. First signs of luciferase activity can be detected after 1 h. **E** Switching luciferase expression in the liver. As shown in multiple cycles of gene activation, exposure of TA^{LAP}-2/LC-1 mice to Dox abolishes luciferase activity within 3 days. Reactivation can be monitored after 2 days (in the experiment shown 7 days) after Dox withdrawal. The identical cycle of activation/inactivation is observed when the same animals are re-examined after 3 months

et al. 1996). Accordingly, proper calibration of experimental conditions is required. The various organs differ also in the kinetics of induction (Kistner et al. 1996), kidney and brain being the slowest, but as is shown in Fig. 3, luciferase activity in the liver is detectable already 1 h after Dox injection (Hasan et al. 2001).

The brain poses a particular problem, as Dox penetrates the blood–brain barrier less efficiently. For operating the tTA system, the amount of Dox that reaches the brain under the above feeding conditions is, however, sufficient (see work described below) while, for the rtTA system, the novel M2 transactivator version which is tenfold more sensitive towards Dox (Fig. 2) is expected to ameliorate problems encountered with the originally described rtTA.

2.2 Modifications of the Tet System

Several significant modifications and improvements of the original tTA system have been developed over the years, particularly the generation of the rtTA,

which is based on a TetR mutant that exhibits fundamentally different DNA binding characteristics: it requires Dox or anhydrotetracycline for its association with *tet*O. The originally described rtTA has been replaced by greatly improved versions (Urlinger et al. 2000) which are more sensitive towards Dox and which exhibit negligible residual affinities towards *tet*O. Particularly, the transactivator version rtTA2^S-M2 senses Dox at around tenfold lower concentrations when compared to the original rtTA (Fig. 2).

Several further modifications are worth mentioning. For a number of reasons, the VP16 domain of the original tTA/rtTA was replaced by minimal activation domains yielding a set of transactivators with graded activation potential (Baron et al. 1997). Moreover, all tTA/rtTAs of the new generation are encoded in synthetic sequences optimized in various ways for expression in human cells (Urlinger et al. 2000).

A development that had considerable impact was the generation of bidirectional promoters, P_{tet}bi (Baron et al. 1995). Here, the heptamerized *tet*O sequences are flanked on both sides by minimal promoters (Fig. 2) which allow for the simultaneous regulation of two genes, whereby the respective transcription units face in opposite directions. These constructs are particularly useful when one gene is used for a reporter function. Thus, the initial characterization of functional transgenic animals is facilitated, particularly when expression of the actual gene of interest is difficult to detect. By using the luciferase gene as reporter, the range of regulation and the tightness of control can be readily assessed, whereas, e.g., the *lacZ* or GFP gene allows monitoring of expression in situ. As will be shown below, the luciferase gene appears particularly useful since it permits the monitoring of gene activities in live animals in a non-invasive way (Hasan et al. 2001).

A problem sometimes encountered is the so-called "leakiness," which refers to a basal level of expression of the P_{tet}-controlled transcription unit in the uninduced state. Although this issue caught considerable attention, it has only rarely been addressed in a correct way. A thorough discussion of this topic can be found elsewhere (Freundlieb et al. 1999) but in essence, one has to discriminate between (1) P_{tet}-dependent and (2) integration site-dependent background activity.

Ad (1): P_{tet}-1 is not necessarily silent in all cellular environments. It may have an intrinsically elevated activity in cells which contain particular transcription factors capable of binding to the promoter itself or to neighboring sequences of the vector. This leakiness is, however, typical for episomal states of the DNA carrying the transcription unit and for transient expression situations in cell culture. As pointed out previously (Gossen and Bujard 1992), an adaptation of P_{tet} to particular cellular environments may sometimes be advantageous.

Ad (2): The basal activity of a chromosomally integrated minimal promoter depends strongly on its integration site. When inserted into an appropriate locus, P_{tet}-1 will be transcriptionally silent even though it may

be activated by tTA or rtTA to high levels resulting in regulation factors of up to 10^6 (Kistner et al. 1996; Schoenig et al. 2002). By contrast, when the P_{tet}-controlled transcription unit is located close to a nearby enhancer, it may be activated even in the absence of its cognate activator tTA or rtTA. This problem can be circumvented by increasing the number of clones (i.e., the number of independent integration events) to be screened, or, more actively, by reducing the activity of P_{tet}-1 in the uninduced state by shielding it via Tc-controlled transcriptional silencer (tTS) proteins (Freundlieb et al. 1999). Ideally, tTS (Fig. 2) and rtTA bind in a mutually exclusive manner to the *tet*O sequences within P_{tet}, depending on the presence and absence of Dox. This approach was shown to substantially reduce the basal activity of P_{tet} in a number of cell lines (Freundlieb et al. 1999) and in transgenic mice (Zhu et al. 2001a).

As the latter approach allows active suppression of potential unregulated background activity of P_{tet} even when caused by elements which are proximal to the integration site of the minimal promoter, it seems to be more versatile than the adaptation of P_{tet} as proposed above. However, it requires the inclusion of one more component.

An issue that has raised concern is the potential toxicity of tTA and rtTA. Like any other transcription factor, tTA and rtTA, when overexpressed induce undesired pleiotropic effects by "squelching" (Gill and Ptashne 1988) which may even lead to cell death. Thus, tTA or rtTA must not exceed a certain intracellular concentrations. When generating tTA/rtTA in cell or mouse lines, there will automatically be a selection for well-tolerated, integration site-dependent expression levels. Thus, simple screening for proper clones or founder animals will be sufficient as demonstrated by dozens of well functioning tTA/rtTA cell lines and mouse strains. The situation changes, however, when a tTA or rtTA encoding gene is to be integrated into a specific chromosomal locus, e.g., by homologous recombination following the so-called knock-in strategy. Here, the result of integration is not subject to subsequent physiological pre-selection of a proper integration site. Instead, the expression level of the *tTA*/*rtTA* gene is locus-specific and might be too high to be tolerated or too low to be effective. In such cases, tTAs and rtTAs with a graded activation potential may be chosen which compensate for different expression levels (Baron et al. 1997).

Finally, a remark regarding strategies for setting up Tet regulation is helpful at this point. It is frequently attempted to establish the Tet regulation in cultured cells or in mice in a one-step procedure, i.e., by coinjection (or cotransfection in case of a cellular system) of the two essential DNA constructs. Even though such an experimental shortcut may appear attractive, one should be aware that the two DNAs preferably cointegrate at the same chromosomal locus—frequently in multiple copies—which generally leads to considerable crosstalk between enhancers driving the expression of the transactivator gene and the minimal promoter within P_{tet}, resulting in elevated background activities. This approach

is, therefore, only sensible if efficient screening or selection systems are available. In addition, it has to be kept in mind that it is of great value to separately generate and characterize mouse strains which tissue-specifically produce tTA or rtTA as well as mouse lines containing various genes of interest under P_{tet} control as the combination of these various lines offer a higher degree of freedom in the design of experiments.

2.3
Non-invasive Monitoring of Tet-Controlled Transcription Units

The possibility of detecting luciferase activity via bioluminescence in life animals (Contag 1997) can be exploited to monitor the activity of a target gene noninvasively. By coregulating a gene of interest with the luciferase gene via a bidirectional promoter like P_{tet}bi (Fig. 2), luciferase bioluminescence can be exploited as an indicator for the activity of the target gene (Hasan et al. 2001; Fig. 3). This approach is particularly useful in long-term experiments in which the expression of a target gene may be subjected to regulatory regimens with repeated on-and-off cycles or in which partial induction of a gene is intended. Furthermore, since the intensities of target gene expression can vary considerably even among litter mates (Kistner et al. 1996), quantifying luciferase bioluminescence and correlating this activity with the expression of the target gene in individuals enrolled in an experiment will permit a more precise interpretation of the action of the gene under study.

For further methodological and technical information, we like to refer the reader to some recent reviews dealing with various aspects of Tet regulation (Baron and Bujard 2000; Gossen and Bujard 2001; Gossen and Bujard 2002; Schoenig and Bujard 2002).

3
Mouse Models for Human Diseases

Pathologies generally arise through mutations that result in either misexpression of genes or the synthesis of mutated gene products, whereby misexpression refers to deviations from physiological expression levels as well as from temporal programs and spatial (cell-type) restrictions, while mutated gene products may be effective by loss of function or by gaining dominant qualities.

Experimental approaches, which permit the quantitative control of individual gene activities in a temporal and cell type-specific manner, are, therefore, suited to model pathological condition that may rather faithfully mimic respective diseases in transgenic animals. Like most conditional systems acting at the level of gene expression, the Tet regulatory principle is binary in nature. Accordingly, most researchers have followed the strategy of separately generating transgenic tTA or rtTA mouse lines that produce the respective transactivator under the control of a promoter which should relay tissue or cell-type specificity to the system. As the function of promoters is frequently affected by the site of inte-

gration, they may loose their specificity, to a different extent, the most common result being position-effect variegation. Sometimes, loss of specificity is accompanied with a highly defined artificial activity pattern, which may be exploited for studying respective subpopulations of cells.

The second mouse line required should contain the target gene under P_{tet} control. The function of P_{tet} is again dependent on the integration site as discussed above. Thus, for tight control and wide range of regulation of a target gene, one has to screen for an appropriate founder line. The identification of such lines is greatly facilitated by the bidirectional promoter P_{tet}bi where, e.g., the luciferase gene is coregulated with the gene of interest and used for monitoring the regulation potential of a newly generated mouse line (Hasan et al. 2001; Schoenig et al. 2002). In any case, numerous mouse lines were described where tight regulation was achieved, of which, in this regard, the most spectacular one was generated by Glenn Fishman and colleagues (Lee et al. 1998) who placed the gene encoding the diphtheria toxin A chain under Tet control in order to induce ablation of specific cell populations.

More than 50 tTA/rtTA and about 80 "receiving" mouse lines containing target genes under P_{tet} control have been published so far (Tables 1, 2, and 3), and numerous further lines are in the state of being characterized in various laboratories. The synergism that could be created when these rapidly increasing pools of mouse lines would be freely exchangeable within the academic community is certainly worth a call to our colleagues and to organizations maintaining repositories for cooperation.

The generation of useful transgenic animals will greatly profit from experimental approaches that would allow us to generate mouse lines with defined properties in a more efficient and predictable manner. In case of transactivator lines, the integration of tTA/rtTA genes behind specific promoters via homologous recombination may sometimes be of advantage as it has led to animals that express the transactivator faithfully (Bond et al. 2000; Perea 2001). But even with this apparently safe approach, it cannot be excluded that the insertion of the transactivator genes in close vicinity of the promoter may disturb the functional program of some regulatory regions. For "receiving" mouse lines, it appears feasible to identify "silent but activatable loci" where a P_{tet}-controlled transcription unit may, upon integration, function in a predictable manner (K. Schoenig, PhD dissertation 2003). Such loci could be targeted via homologous recombination or by using the BAC technology converted into a vector for transgenesis. Eventually, we might learn more about transcriptional insulators in mammalian systems, which could then be used with similar success, as in drosophila (Burgess-Beusse et al. 2002). Thus, it appears likely that the integrated use of various novel approaches will in the near future significantly increase the efficiency of generating defined mouse lines with largely predictable regulatory properties.

Table 1 Mouse lines expressing tTA or rtTA genes

Promoter	Tissue specificity	tTA	rtTA	Reference(s)
Albumin	Liver	+		Manickan et al. 2001; Raben et al. 2001
α CaMKII[a]	Brain	+		Mayford et al. 1996
α CaMKII	Brain		+	Mansuy et al. 1998
CD2	T cell lineage		+	Legname et al. 2000
CD34	Early bone marrow Progenitor and stem cells	+		Radomska et al. 2002
Clara cell 10-kDa protein (CC10)	Lung airway (parenchyma)		+	Mehrad et al. 2002
Clara cell secretory protein (CCSP)	Respiratory epithelial cells		+	Tichelaar et al. 2000
hCMV	Various tissues	+	+	Kistner et al. 1996; Wiekowski et al. 2001
Endothelin receptor B (EDNRB)	Melanocytes, neural crest	+	+	Shin et al. 1999
Fatty acid binding protein (Fbp)	Small intestine, cecum, colon, bladder		+	Saam et al. 1999
Growth hormone (GH)	Pituitary gland		+	Roh et al. 2001
Intronic IgH enhancer, minimal promoter	Thymus, bone marrow	+		Hess et al. 2001
Immunoglobulin heavy chain enhancer and SRα promoter	Hematopoietic system	+		Felsher et al. 1999
Insulin	Pancreatic β-cells	+		Efrat et al. 1995
Insulin	Pancreatic β-cells		+	Thomas et al. 2001; Milo-Landesmann et al. 2001
Interphotoreceptor retinoid-binding protein (IRBP)	Photoreceptor cells		+	Chang et al. 2000
Keratin 5 (K5)	Epidermis, hair follicle	+	+	Diamond et al. 2000
Keratin 6 (K6)	keratinocytes	+		Guo et al. 1999
Keratin 14 (K14)	Epidermis, squamous epithelia		+	Xie et al. 1999
Keratin 14 (K14)	Mammary gland	+		Dunbar et al. 2001
Keratin 18 (K18)	Trachea, upper bronchi, submucosal glands		+	Ye et al. 2001
Lck	T cell lineage	+		Leenders et al. 2000; Labrecque et al. 2001
Liver-enriched activator protein (LAP)[a]	Liver	+		Kistner et al. 1996
Liver-enriched activator protein (LAP)	Liver		+	Schoenig et al. 2002
Major histocompatibility complex (MHC) class II Eα	Thymic, epithelial, Dendritic, B cells, macrophages,	+		Witherden et al. 2000
α-Myosin heavy chain (MHCα)[a]	Heart muscle	+		Passman et al. 1994
α-Myosin heavy chain (MHCα)	Heart muscle		+	Valencik et al. 2001

Table 1 (continued)

Promoter	Tissue specificity	tTA	rtTA	Reference(s)
Major urinary protein (MUP)	Liver	+		Manickan et al. 2001
MMTV long terminal repeat[a]	Mammary gland, salivary gland, seminal vesicle	+		Henninghausen et al. 1995
MMTV long terminal repeat	Mammary gland, salivary gland, seminal vesicle		+	D'Cruz et al. 2001; Hsu et al. 2001; Gunther et al. 2002
Muscle creatine kinase (MCK)	Muscle	+		Ghersa et al. 1998; Ahmad et al. 2000
Nestin	Various tissues		+	Mitsuhashi et al. 2001
Neuron-specific enolase (NSE)[a]	Brain	+		Chen et al. 1998
P0	Schwann cells		+	Pot et al. 2002
P2	Olfactory sensory neurons		+	Gogos et al. 2000
pdx-1	Pancreas	+		Holland et al. 2002
Peripheral myelin protein 22 (PMP22)	Schwann cells	+		Perea et al. 2001
PrP	Brain	+		Tremblay et al. 1998
Prolactin	Pituitary gland	+		Roh et al. 2001
Retinoblastoma gene (Rb)	Brain, lung, spleen	+		Nikitin et al. 2001
Rhodopsin	Photoreceptor cells		+	Chang et al. 2000
SM22α	Smooth muscle cells	+		Ju et al. 2001
Surfactant protein-C (SP/C)	Respiratory epithelial cells		+	Tichelaar et al. 2000
Tek	Endothelial cells	+		Sarao et al. 1998
Tie	Endothelial cells	+		Sarao et al. 1998
Tie2	Vascular endothelium		+	Teng et al. 2002
Tyrosinase	Melanocytes		+	Chin et al. 1999

[a] Mouse line available at The Jackson Laboratory, Bar Harbor, ME 04609, USA.

Table 2 tTA/rtTA responsive mouse lines

Genes under Tet control	tTA/rtTA-responsive promoter		Reference(s)
	P_{tet}–1	P_{tet}bi	
Reporter genes			
Luciferase (L7)[a]	+		Kistner et al. 1996
LacZ, nls/luciferase (nZL-2)[a]		+	Kistner et al. 1996b
LacZ	+		Mayford et al. 1996
LacZ nls	+		Sarao et al. 1998
GFP and *lacZ*		+	Krestel et al. 2001
Luciferase/Cre recombinase (LC-1)		+	Schoenig et al. 2002
Cre recombinase	+		Saam et al. 1999; Lindeberg et al. 2002; Radomska et al. 2002; Perl et al. 2002
Cre-IRES-tau/*lacZ*	+		Gogos et al. 2000
Genes of interest			
Adenylylcyclase type VI	+		Gao et al. 2002
AML1-ETO fusion	+		Rhoades et al. 2000
Axin	+		Hsu et al. 2001
BCR-ABL1 fusion	+		Huettner et al. 2000
BOB.1-OBF.1/*luc*		+	Hess et al. 2001
α-CaMKII-Asp^{286}	+		Mayford et al. 1996
Calcineurin autoinhibitory domain	+		Malleret et al. 2001
Calcineurin (mutant)	+		Mansuy et al. 1998
cAMP response protein (CREB)	+		Chen et al. 1998; Sakai et al. 2002
cAMP response protein (CREB)-VP16 fusion	+		Barco et al. 2002
cAMP response protein (CREB) (mutant)	+		Pittenger et al. 2002
β-Catenin/eGFP		+	Cheon et al. 2002
Chemokine KC/lacZ		+	Wiekowski et al. 2001
Colony stimulation factor-1 (CSF-1)	+		van Nguyen et al. 2002
CYP1B1	+		Hwang et al. 2001
Diphtheria toxin A chain (DTA)	+		Lee et al. 1998
Diphteria toxin A chain (mutant)	+		Gogos et al. 2000

Table 2 (continued)

Genes under Tet control	tTA/rtTA-responsive promoter		Reference(s)
	P_{tet}–1	P_{tet}bi	
Dystrophin	+		Ahmad et al. 2000
Endothelin receptor B (EDNRB)	+		Shin et al. 1999
Epidermal growth factor receptor (EGFR) (truncated)	+		Roh et al. 2001
ErbB2 receptor tyrosine kinase	+		Xie et al. 1999
Estrogen receptor α	+		Hruska et al. 2002
FGF-receptor 2 (soluble dn mutant)	+		Hokuto et al. 2002
Fibroblast growth factor-7 (FGF 7)	+		Tichelaar et al. 2000
Fibroblast growth factor-10 (FGF 10)	+		Clark et al. 2001
Fibroblast growth factor-18 (FGF 18)	+		Whitsett et al. 2002
Forkhead transcription factor (FKHR) (truncated)	+		Leenders et al. 2000
ΔFosB[a]	+		Chen et al. 1998
Acid α-glucosidase (GAA)	+		Raben et al. 2001
GATA 6	+		Liu et al. 2002
GluR-A (eGFP-tagged)/lacZ		+	Mack et al. 2001
Glycogen synthase kinase-3β/*lacZ*		+	Lucas et al. 2001
Hormone-sensitive lipase (HSL)	+		Suzuki et al. 2001
Hox A10	+		Bjornsson et al. 2001
Huntingtin fragment and *lacZ*		+	Yamamoto et al. 2000
Id1	+		Passman et al. 1994
Idx-1 Hammerhead ribozyme	+		Thomas et al. 2001
α-Integrin/luciferase		+	Valencik et al. 2001
Lymphocyte specific protein tyrosine kinase $p56^{lck}$	+		Legname et al. 2000
MHC class I H-$2K^b$	+		Nagaraju et al. 2000
MHC class II Eα	+		Witherden et al. 2000
Met receptor	+		Wang et al. 2001
Mineralocorticoid receptor antisense RNA	+		Beggah et al. 2002
c-myc	+		Felsher et al. 1999; D'Cruz et al. 2001
Nogo A/lacZ		+	Pot et al. 2002
Inducible NO-synthetase (iNOS)/eGFP		+	Mungrue et al. 2002

Table 2 (continued)

Genes under Tet control	tTA/rtTA-responsive promoter		Reference(s)
	P_{tet}–1	P_{tet}bi	
NR1/*lacZ*		+	Jerecic et al. 1999
NR1 (N598R)/*lacZ*		+	Jerecic et al. 2001
κ Opioid receptor (RASSL) /*lacZ*[a]	+		Redfern et al. 1999
Ornithine decarboxylase (ODC)	+		Guo et al. 1999
$p27^{KIP1}$/eGFP		+	Mitsuhashi et al. 2001
Parathyroid hormone related protein PThrP)	+		Dunbar et al. 2001
Pdx1	+		Holland et al. 2002
Peripheral myelin protein 22 (PMP22)	+		Perea et al. 2001
Prion (PrP^{C})	+		Tremblay et al. 1998
Protein kinase C-β	+		Bowman et al. 1997
Protein kinase C-ε	+		Choi et al. 2002
Protein phosphatase 1	+		Genoux et al. 2002
$H\text{-}Ras^{V12G}$	+		Chin et al. 1999
$K\text{-}Ras^{G12D}$	+		Fisher et al. 2001
Retinoblastoma	+		Nikitin et al. 2001
Serotonin 1A receptor	+		Ghavami et al. 1999; Gross et al. 2002
Serotonin 1B receptor	+		Ghavami et al. 1999
Surfactant protein D (SP-D)	+		Zhang et al. 2002
SV40 large T antigen	+		Efrat et al. 1995; Manickan et al. 2001
T cell receptor α OT1	+		Labrecque et al. 2001
TAC-β-integrin fusion/luciferase		+	Valencik et al. 2001
TGF-β	+		Liu et al. 2001
TrK B	+		Ghersa et al. 1998
Urokinase-like plasminogen activator (uPA)		+	Sisson et al. 2002
Utropin	+		Squire et al. 2002
Vascular chymase (RVCH)	+		Ju et al. 2001
Vascular endothelial growth factor (VEGF)	+		Ohno-Matsui et al. 2002; Dor et al. 2001

NIs, nuclear localization signal.

[a]Mouse line available at The Jackson Laboratory, Bar Harbor, ME 04609, USA.

Table 3 Mouse lines with cointegrated tTA/rtTA and P_{tet}-controlled genes

Promoter	Tissue	tTA/ rtTA	Gene under P_{tet} control	Reference
Clara cell 10 kd protein	Lung parenchyma	rtTA	Interferon-γ	Wang et al. 2001
Clara cell 10 kd protein	Lung parenchyma	rtTA	Interleukin-9	Temann et al. 2002
Clara cell 10 kd protein	Lung parenchyma	rtTA	Interleukin-11	Ray et al. 1997
Clara cell 10 kd protein	Lung parenchyma	rtTA	Interleukin-13	Zheng et al. 2000
Clara cell 10 kd protein	Lung parenchyma	rtTA	RANTES	Pan et al. 2000
Collagen type II	Chondrocytes	tTA	MMP-13	Neuhold et al. 2001
Insulin gene II	Pancreas	rtTA	TNF-α	Green et al. 2000
Insulin gene II	Pancreas	rtTA	pdx-1 Antisense RNA	Lottmann et al. 2001
LcK	T cells	rtTA	GATA3 (KRR)	Zhang et al. 1999
β-Lactoglobulin	Mammary gland	rtTA	α-Lactoglobulin	Soulier et al. 1999
Retinoblastoma (RB)	Thalamus, muscle, cerebrellum, eye	rtTA	Cre recombinase	Utomo et al. 1999
SK-channel 3 (SK3)	Brain	tTA	SK-channel 3 SK3	Bond et al. 2000
Whey acidic protein (WAP)	Mammary gland	rtTA	Cre recombinase	Utomo et al. 1999

3.1 Modeling Cancer in Mice

A new generation of mouse tumor models became reality with the advent of approaches that allowed for the control of the activity of oncogenes and tumor suppressor genes in a temporally defined and tissue-specific manner. Tumor initiation, progression, and maintenance could now be experimentally dissected with unprecedented precision. The most striking and exciting discovery made with these conditional tumor models is the complete regression of many, even highly invasive, tumors after turning down the activity of the initiating oncogene. Nearly all oncogenes examined to date, including *Myc, H-ras, K-ras, ErbB2, Bcr-Abl1, Fgf7*, and *SV40 Tag*, appear to be not only required for tumor initiation but also for tumor maintenance. Thus, as first shown for *Myc* (Felsher and Bishop 1999), inactivation of a single initiating oncogene can lead to complete regression of 90% of tumors in the hematopoietic system. Similarly, malignant melanomas which developed in an *Ink4a*- and *Arf*-tumor suppressor-deficient background underwent complete regression when the initiating oncogene H-*ras* was inactivated by Dox withdrawal (Chin et al. 1999). These and other groups' findings convey an important and unexpected message: Mutations accumulating in developing tumors do not necessarily introduce functional redundancies that stabilize tumor development. These findings imply that targeting the activity of a single oncogene could result in an effective therapy of a tumor. This reasoning is most impressively supported by a recent report (Jain et al. 2002) describing a conditional mouse model for *Myc*-induced tumors. The authors show that brief inactivation of *Myc* leads to sustained regression of osteogenic sarcoma and differentiation of osteogenic sarcoma cells into mature osteo-

cytes. Subsequent reactivation of *Myc* did not restore the malignant state of the cells, as would be predicted, but instead induced apoptosis.

Together, the results emerging from several laboratories studying the conditional activation/inactivation of various oncogenes shed new light on mechanisms governing induction and commitment of tumorigenesis. They suggest that many tumors are rather rigidly dependent on expression patterns that are established under the governance of the initiating oncogene whose continued function is required. This "oncogene addiction" apparently makes tumors vulnerable to interference with single oncogenes as shown by Felsher and coworkers, where a brief interruption of *Myc* expression caused the breakdown of the malignant state. On the other hand, when *Myc* is induced in mammary gland, only 30% of the tumors arising regress upon inactivation of the oncogene, whereas around 60% have acquired a *Myc*-independent but preferred secondary pathway via mutations in *K-ras* gene (D'Cruz et al. 2001). This finding would suggest that targeting of *Myc* and *K-ras* will result in the regression of many known tumors.

It is likely that numerous human cancers will be modeled in conditional mouse lines in the near future, yielding new insights into functions of genes which contribute to tumor development, thereby revealing new pharmacological targets.

3.2
Conditional Mouse Models for Neurodegenerative Diseases

The first impressive example for applying Tet control to brain functions stems from the laboratory of Eric Kandel. Placing the tTA gene under control of the αCamKII promoter yielded mouse lines which expressed the tTA gene in defined areas of the forebrain, particularly in the hippocampus, cortex, amygdala, and striatum. Crossing such mice with animals that contained a Ca^{2+}-independent version of the *αCamKII* gene under P_{tet} control resulted in double transgenic individuals suitable for the study of synaptic plasticity. In a first set of experiments, Mayford et al. (1996) demonstrated that expression of the dominant active gene product caused a loss of long-term potentiation in the hippocampus and a deficit in spatial learning, whereas repression of the transgene reversed both the physiological and memory deficit.

The same tTA mouse line was used by René Hen and colleagues to model the human Huntington's disease (HD) in the mouse. HD is an inherited progressive neurological disorder caused by an expansion of repeated CAG codons in the huntingtin gene. In the genetic model described by Yamamoto et al. (2000), a chimeric mouse/human huntingtin gene containing 94 CAG repeats was placed under P_{tet} control. When respective mice were crossed with animals expressing tTA via the αCamKII promoter, it was found that in double transgenic animals the expression of the huntingtin gene in the adult brain led to neuroanatomical abnormalities typical for HD. These neuropathological changes resulted in severe progressive motor dysfunction. Strikingly, however, in symptomatic adult

animals, the shut off of huntingtin gene expression via Dox could reverse the HD phenotype. Specifically, the nuclear and cytoplasmic aggregates disappeared and the behavioral phenotype approached again the one of control animals. In a further publication (Martin-Aparicio et al. 2001), it was shown that aggregate formation is dependent on the balance between huntingtin synthesis and its degradation via proteasomes. Interestingly, neither mutant huntingtin nor its aggregates are lethal to the cell, demonstrating that HD is due to neuronal dysfunction and not to cell death. These results have yielded significant insights as they show that (1) for progressive HD the continued expression of the huntingtin gene is required, (2) the plasticity of the brain is likely to be sufficient for recovery even from severe symptoms if one could influence the balance between huntingtin production and degradation and, thus, (3) pharmacological interventions may be successful even after symptoms have become visible.

Obviously, corresponding questions can be addressed to prion diseases which result from transformation of cellular prion protein (PrP^{c}) into a pathogenic scrapie isoform (PrP^{sc}). Indeed, Stanley Prusiner and his group described a mouse model in which PrP^{c} production was controlled via tTA in a *PrP*^{c}-deficient background. They found that high levels of PrP^{c} are lethal during embryogenesis and postnatal development, whereas in the adult animal neither induction nor repression of *PrP*^{c} was detrimental. By contrast, the development of the prion disease upon infection of the animals with low amounts of PrP^{sc} was strongly dependent on the expression level of the transgene encoding PrP^{c}. These results indicate that while PrP^{c} may not play an essential role in the brain of adult animals, its continued presence is a prerequisite for acquiring the disorder. It will be interesting to learn whether neurodegenerative symptoms can be halted or even reversed also in this disease by turning down the activity of the *PrP*^{c} gene. Mouse models as described by Tremblay et al. (1998) should, in principle, be suitable to examine such questions.

3.3 Modeling Cardiac Diseases

Several routes have been followed in the quest of modeling cardiac pathologies. A number of laboratories have made use of Fishman's mouse line expressing the tTA gene under the control of the αMHC promoter, highly specifically in heart muscle (Passman et al. 1994). Cardiomyopathies were, for example, induced by limited cell ablation via tTA-controlled diphtheria toxin induction (Lee et al. 1998), by conditional expression of specifically designed G_i-coupled receptors (Redfern et al. 1999), or by controlling the expression of the mineralocorticoid receptor (MR). Here, we would like to briefly discuss the recent work of Frédéric Jaisser and colleagues (Beggah et al. 2002), a particularly interesting example representative of the approach. MR, a ligand-dependent transcription factor of the steroid receptor family, is a target for hypertension therapy. It is produced in the kidney, where it is involved in sodium reabsorption and potassium excretion. It is also present in the heart, where its physiological role is not

clear. To study MR function exclusively in the heart, the production of antisense mRNA directed towards the murine transcript of the *MR* gene was placed under heart muscle-specific tTA control. The resulting mouse model allowed for reversibly inhibited expression of *MR* in cardiomyocytes. Inhibition resulted in dramatic cardiac hypertrophy, ventricular dysfunction, fibrosis, and heart failure. However, when expression of MR-specific antisense RNA was turned off by Dox administration, heart failure and cardiac remodeling were reversed and heart/body weight ratios returned to control values within one month. Most impressively, interstitial cardiac fibrosis regressed as well, indicating that the pathological extracellular matrix depositions were reversible, in contrast to previous observations (Redfern et al. 2000).

The different conditional mouse models for cardiac diseases will continue to yield novel and complementing insights into complex pathological pathways. Some of these models may develop into useful tools for drug efficacy studies in not too distant future.

4 Concluding Remarks

The disease models briefly discussed herein just give a glimpse into a rapidly growing area of biomedical research, where advances in the genetic manipulation of the mouse are being exploited and begin to yield novel insights into pathways and dynamics of pathological conditions. This is also underlined by work compiled in Table 1–3, in which numerous other pathologies were modeled in the mouse including the induction of autoimmune myositis (Nagaraju et al. 2000), the study of autoimmunity in diabetes type I (Green and Flavell 2000; Christen et al. 2001), lung development (Tichelaar et al. 2000), airway remodeling in asthma (Zhu et al. 2001b), osteoarthritis (Neuhold et al. 2001), and Alzheimer's disease (Lucas et al. 2001) to mention a few. In all these models, the temporal program of pathological processes and its potential reversibility was examined, and in an unexpected high number of cases, pathological states could, despite severe symptoms, be abrogated by repressing the disease-causing gene. Thus, already some of the first truly conditional disease models, which demonstrated the reversibility of even advanced pathological conditions, have changed our views on respective diseases, sparking at the same time new ideas for pharmacological interventions.

Obviously, the mouse is by far the genetically most accessible mammalian system with significant physiological similarities to humans and has become the experimental animal of choice for studying gene functions in vivo and for uncovering molecular mechanisms underlying pathological conditions. Indeed, we can expect a wealth of new information on in vivo gene functions in the near future. On the other hand, one has to keep in mind the physiological differences between humans and mice, which limit the value of mouse models. Future efforts have, therefore, to be directed towards "humanizing" the mouse, and mak-

ing other suitable mammalian systems such as the rat more readily accessible for defined genetic alterations.

Progress is also required in methods and technologies, which allow us to control from outside individual genes in vivo with high precision. Despite the great potential of various experimental approaches available today, many problems remain. Thus, as long as we know little about the influence of chromatin structure on gene expression and its sensitivity towards perturbations caused, e.g., by recombination events, reliable experimental designs where cell type specificity is maintained and position effect variegation is prevented will remain difficult. Precise and minimal disturbance, gene targeting, as well as the application of the BAC technology may ameliorate some of these shortcomings in future. On the other hand, approaches that leave the gene of interest totally untouched in its genomic context would be highly valuable and may be available soon. For example, controlling custom-made zinc finger-based transcription factors recognizing specific sequences within the promoter of a target gene or regulated synthesis of RNAs interfering with the expression of mRNA or polypeptides that attack specific gene products may develop into systems of choice. Finally, many biological processes are governed by kinetics too fast to be resolved by methods currently available. Progress in the development of noninvasive monitoring systems will overcome some of the present limitations. Obviously, in our strive towards a better understanding of in vivo gene function, there is a broad spectrum of problems waiting for good ideas.

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