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Controlled gene activation and inactivation in the mouse

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1. ABSTRACT

The emergence of techniques that allow fine manipulation of gene expression in the mouse have changed the way biomedically relevant processes are studied, as they allow their analysis in the living animal. In addition, this has opened the possibility to generate animal models for several human diseases, which are useful both for understanding the disease´s physiopathological mechanisms and for the eventual evaluation of novel therapeutic approaches. Many of the gene manipulation systems currently employed in the mouse are based on regulatory mechanisms normally operating in yeast and prokaryotic organisms. This has allowed specific experimental control with very limited unspecific interference with the normal physiology of the cell. Some of these systems use elements that permit transcriptional regulation of a particular gene or genes. Among them, I will discuss in detail the *tet*, *lac* and Gal4/*UAS* systems, which are among the most popular of these transcriptional systems because they can be used to achieve spatial and temporal control on the expression of a specific gene in a reversible fashion. The other major group of systems currently employed to manipulate gene expression in the mouse is based on site-specific recombination reactions. The Cre/*lox* and FLP/*FRT* systems are the most popular of these. I will discuss how these recombination systems are used in the mouse with special focus on their use to achieve specific gene activation.

2. INTRODUCTION

The analysis of complex basic and applied biomedical problems has been revolutionized by the emergence of methods that allow controlled manipulation of gene expression. Although gene expression can be modulated externally in a variety of vertebrates and invertebrates, the mouse is by far the most amenable animal, in which the most precise and sophisticated genetic manipulations are possible. Two main technological advances are at the center of this revolution. The first is the possibility to introduce into the mouse genome a chosen genetic cassette that allows the expression of any gene in a controlled fashion (1, 2). Although other methods of introduction exist (3, 4, 5), in most cases this is done by injection of the expression cassette into the pronucleus of a fertilized mouse oocyte (1, 2). It then becomes randomly integrated into the genome and is stably transmitted to the progeny of the resulting animals. Mice carrying these extra genetic elements are known as transgenic mice. The second technological advance is the possibility to create mice containing virtually any kind of custom-made modification in the genome, from point mutations to large deletions. The possibility to create these mice resulted mainly from the combination of two discoveries: the controlled introduction of modifications in the genome by homologous recombination (6, 7, 8) and the isolation of embryonic stem cells, which, when injected into mouse blastocysts, can contribute to any tissue of the resulting animals, including

the germ line (3, 9, 10). When embryonic stem cells containing specific gene modifications are used to generate new mice, the result is the production of mouse lines carrying those specific transformations in their genome (3, 11, 12).

The transgenic technology was initially used to express a specific gene in a target tissue, relying on the activity of a particular promoter. Although this basic design has proven very powerful and is still widely used, it soon became obvious that to address some questions a more precise spatial and temporal control on transgene expression would be required. Similarly, when gene targeting technologies achieved their initial goal of producing gene inactivation, more sophisticated gene manipulations were sought, which allowed precise spatial and temporal control on the activation or inactivation of the genomic loci (13, 14) or the specific deletion of large chromosomal areas (15).

The introduction of binary systems into the basic transgenic and gene targeting technologies has allowed refinements in the control of gene expression in living mice and these are being further improved constantly. In general, these systems are composed of two elements, a "target", which normally contains the gene whose activity one wants to modulate, and a "controller", which acts specifically on the "target" to control its activation status. The discussion of these systems, their uses and limitations, will be the main focus of this review. In general, binary systems are based on the ability of bacterial, phage and yeast genetic elements to function in higher eukaryotic cells, including living vertebrates, in a predictable fashion (16, 17, 18, 19, 20, 21) without major interference with normal cells physiology. Proper use of those elements thus allows the generation of genetic switches that can be subject to external control with minimal cross-reaction with endogenous control elements. There are two basic types of binary systems: those that rely on transcriptional control elements and those that make use of recombination systems.

3. TRANSCRIPTIONAL BINARY SYSTEMS FOR CONTROLLED GENE EXPRESSION

In general, the transcriptional binary systems rely on the specific interaction of a transcriptional regulator (the "controller"), either an activator or a repressor, and a target DNA sequence to which it binds. In the typical experimental design, the gene whose expression is to be controlled is placed downstream of a promoter that contains the target DNA sequence (the "target"); the transcriptional regulator is further provided with an expression cassette, in which the promoter defines the cell and/or tissue specificity. In a cell containing both elements, regulation of the target gene occurs through the binding of the transcriptional modulator to its target sequence. The specific architecture of the construct depends on whether the transcriptional modulator activates or represses transcription (for a schematic view, see Figures 1 and 2).

Ideally, the transcriptional regulator should be itself inactive in the genome of the un-manipulated host cell, and the DNA sequence to which it binds should be transcriptionally silent in the absence of the specific binding protein. It is therefore not surprising that the components of the most successful binary systems were typically borrowed from transcriptional control networks of prokaryotes and yeast (16, 19, 20, 21). Although other systems have also been used (22), I will discuss in detail the three systems that have either been most extensively used or might become increasingly used in the future in order to achieve interesting complementary combinations for genetic control *in vivo*.

3.1. The *tet* system

The *tet* system, based on the regulatory elements of the *E. coli* tetracycline resistance operon (23, 24), is by far the most popular of the systems currently used to control gene expression. In bacteria, transcription of the genes mediating resistance to tetracycline is under the control of the protein TetR (Tet repressor), which acts by binding to the *tetO* (operator) within the operon promoter to block transcription (23, 24). Upon exposure to tetracycline, the antibiotic binds TetR and thereby removes it from the operator to allow transcription (23, 24). Optimized tetracycline operators and several modifications of TetR have been widely used in mammalian cells to control gene expression, first in cultured cells and later in transgenic animals (16, 25). The general design of these systems (figure 1) consists in linking the target gene to a hybrid promoter that contains tandem repeats of *tetO* linked to a eukaryotic minimal promoter (known as P_{tet}) (16) and to express variations of TetR (16, 26, 27, 28, 29) using specific promoters that provide the spatial specificity. Activity of TetR (or of its derivatives), and thus transcription or not of the target gene, is then controlled by administration of tetracycline or a suitable analogue like doxycycline (30).

The most widely used versions of the *tet* system (figure 1) employ a TetR derivative that was converted to a transcriptional activator, by its fusion either with the Herpes Simplex Virus protein VP16 (16) or, more recently, with other transcriptional activator domains (27, 28, 29), which have fewer unwanted interactions with cellular transcription factors. This chimeric protein, generally known as tTA (for tet Transcriptional Activator), binds P_{tet} and activates transcription of any cDNA linked to it (16). In the presence of doxycycline tTA is removed from P_{tet} and transcription is thereby stopped. In this combination the system is known as "tet-off". Certain mutations in the TetR moiety of tTA resulted in a molecule (rtTA) with reversed regulation by tetracycline, i.e. it activates transcription from P_{tet} in the presence but not in the absence of the antibiotic (26). The *tet* system using rtTA is known as "tet-on".

In the typical experiment using the *tet* system to control gene expression in living mice two transgenic lines are produced, one containing the target gene downstream of P_{tet}, and another expressing either tTA or rtTA under the control of a suitable promoter (figure 1). Upon crossing the two mouse strains, expression of the target gene will become controlled by (r)tTA. The spatial specificity is provided by the promoter controlling (r)tTA expression and

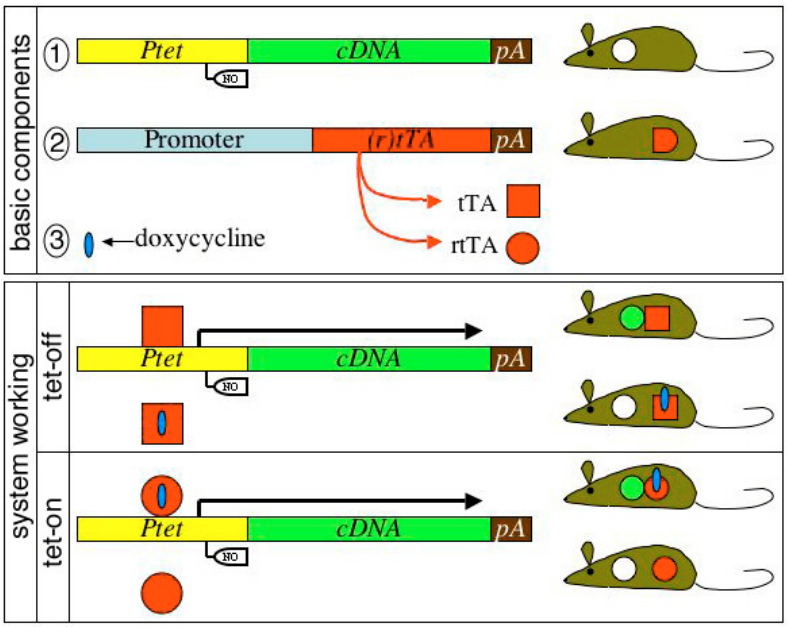

Figure 1. The use of the *tet* system in transgenic animals. The basic components of the system are: 1) an element containing the *tet* promoter (P_{tet}), which is composed of the tetracycline operator linked to a minimal eukaryotic promoter, positioned in front of a cDNA coding for the protein whose expression we want to control. A polyadenylation signal (*pA*) completes the first element. This element is by itself inactive (represented by the white circle in the mouse drawing); 2) another element contains an expression cassette to express a tet transactivator (tTA or rtTA) with a specific promoter (represented in red in the mouse drawing). The choice of the promoter defines the spatial domain of expression of the transactivator in the animal; 3) doxycycline is used to modulate the activity of the transactivator. When the system is working in the "tet-off" combination, component 2 synthesizes tTA. tTA binds *tetO* and activates transcription of the cDNA of interest (represented with the green circle in the mouse drawing). When doxycycline is added to the drinking water, the drug binds tTA and removes it from *tetO*. cDNA expression is thus stopped. In the "tet-on" combination, component 2 synthesizes rtTA. In the absence of doxycycine rtTA does not bind *tetO* and element 1 remains inactive (represented by the white circle in the mouse drawing). When doxycycline is added to the drinking water, rtTA binds *tetO* and cDNA expression from element 1 is activated (represented with the green circle in the mouse drawing).

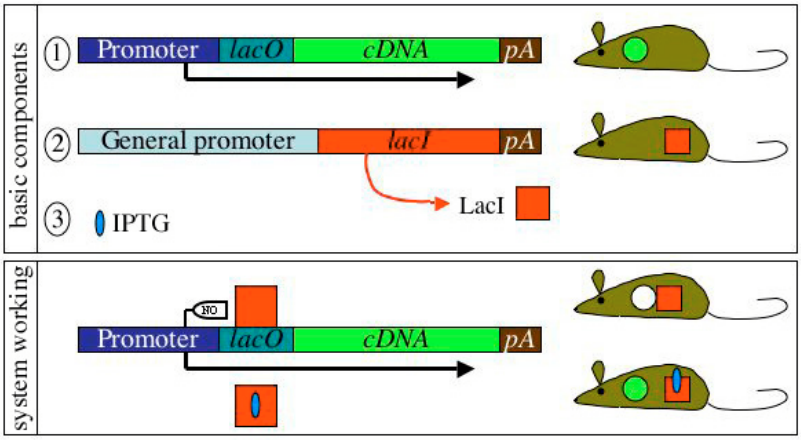

Figure 2. The use of the *lac* system in transgenic animals. The basic components of the system are: 1) an expression cassette that contains the *lac* operator (*lacO*) downstream of a specific promoter, positioned in front of a cDNA coding for the protein whose expression we want to control. A polyadenylation signal completes this construct. This element is transcriptionally active (represented with the green circle in the mouse drawing), being the spatial specificity defined by the promoter; 2) an expression cassette designed to drive expression of the lac repressor (*lacI*) ubiquitously in the animal (represented by the red square in the mouse drawing); 3) IPTG is used to modulate binding of LacI to *lacO*. When the system is set to work, LacI binds *lacO* and blocks transcription of the target cDNA (represented with a white circle in the mouse drawing). When IPTG is added to the drinking water, the drug binds LacI and removes it from *lacO*, thus activating cDNA expression from element 1 (represented with the green circle in the mouse drawing).

the temporal parameter is regulated by administering doxycycline or not. In more sophisticated designs, the effector construct contains a bidirectional P_{tet} promoter, which activates a reporter gene in one direction and the gene of interest in the other, thus allowing identification of the tissue where the target gene is activated (31, 32). When the luciferase gene is used as reporter, detection of (r)tTA activity can be performed in a non-invasive fashion during the experiment (33, 34).

Ideally, the P_{tet} -cDNA construct should be silent by itself. Leaky expression can be a problem (25, 35, 36), particularly in cases where the molecule under control has a strong dominant phenotype. Leakiness normally results from the activity of the basal transcriptional machinery on the minimal promoter included in the construct or from residual activity of rtTA on P_{tet} in the absence of doxycycline (37), but mechanisms involving P_{tet} -mediated activation by specific cellular transcription factors have also been reported (38). The levels and spatial/temporal characteristics of this leakiness usually depend on the site of transgene insertion and therefore they can vary from line to line, even for the same construct. One way to solve these problems is to test several transgenic mouse strains and then use one without basal expression. In cases when even very low amounts of cDNA expression can produce strong perturbations, a combination of TetR-derived activators and repressors can be used. In particular, when a "tet-on" design is used, the P_{tet} -cDNA construct can be kept silent with tTS (the TetR molecule linked to the KRAB repressor domain of Kox1 (39)), in the absence of doxycycline. When doxycycline is administered, tTS is removed from *tetO* and its position is taken by rtTA to activate transcription in a controlled fashion. This strategy has proven useful in transgenic mice using localized tTS expression (40) and after electrotransfer into mouse muscle (41). Interestingly, concomitant use of tTS and rtTA can even improve the performance of rtTA (42). The general use of this approach would be facilitated by the availability of mouse strains with ubiquitous expression of tTS (43). In particular cases, in which even very low basal levels of expression from the P_{tet} construct cannot be tolerated, the line constitutively expressing tTS could serve as the basis to produce the "target" (P_{tet} linked to cDNAs) transgenics.

Another practical problem that has been found with the *tet* system in living animals is that, while the tTA molecule seems to respond easily to doxycycline at non toxic levels, activation of rtTA apparently requires concentrations of doxycycline close to toxic levels (37). This makes its activation very inefficient (or impossible) in some tissues and in embryos. New mutant variants of rtTA have been developed that are more stable, have lower basic activity and respond to much lower doxycycline concentrations (37) (e.g. rtTA^{S}-M2 has about 10 times less basic activity and needs 100 times lower doxycycline concentrations than the original rtTA). It is expected that those molecules will facilitate the use of the "tet-on" system in transgenics. Indeed, transgenic lines for tissue-specific inducible gene expression based on this molecule have already been described and used with success (34, 44, 45).

The "tet-on" and "tet-off" systems have been extensively used to study a variety of biological problems in mice. Given the specific properties of the system it is best suited for the analysis of the temporal requirements of dominant gene functions, mostly in the generation of disease. As some of these dominant functions result in early phenotypes (including lethality) that hinder the analysis of later processes, the activatable *tet* system offered a practical solution. In this way the role of Glycogen Synthase Kinase-3-beta in Alzheimer´s disease could be evaluated (32), and a model for hyperproliferative retinopathy dependent on retinal vascular endothelial growht factor (VEGF) expression was generated (46). In the same way, conditional cell lineage ablation was achieved by activating expression of the diphtheria toxin (DTA) gene using the "tet-off" system based on two transgenes. In those experiments, one transgene consisted of the DTA gene downstream of P_{tet} and the other contained a transcription unit to express *tTA* under the myosin heavy chain promoter (47). DTA expression was induced by removal of doxycycline from the drinking water.

While induction of gene expression bypassing deleterious early effects is an important application of the *tet* system, the most interesting of all its possible uses stems from the reversibility of the induction process, which offers unique opportunities for the study of biomedical processes. For instance, the use of this system was instrumental in establishing that many oncogenes are not only required for tumour induction, but also for tumour progression. Neoplasias that had been induced by oncogene expression using the *tet* system frequently regressed and often their cells died by apoptosis when the system was inactivated. This seemed to be true for tumours and hyperplasias generated by a variety of oncogenes, like the hyperplasia of stratified epithelia produced by *ErbB2* expression (48), the hyperplasia of the submandibular gland produced by the SV40 T antigen (TAg) acting for up to 4 months (49), T-cell lymphomas and acute myeloid leukemias produced by *Myc* (50, 51), *Neu*-induced invasive mammary carcinomas and their metastasis (52) or acute B-cell leukemia induced by *BCR–ABL1* (53). However, the same kind of studies showed that this is not a universal principle in tumour biology. For instance, the hyperplasia of the submandibular gland produced by the SV40 TAg was not reversed when the TAg was removed after 7 months (49), and the metastasis of mammary carcinomas that had apparently regressed after inactivation of *Neu* expression became independent of the oncogene at some point and produced *Neu*-independent tumours (52). Experiments using a similar design were used to evaluate the importance of the persistence of a given antigen in specific autoimmune processes, and this could have relevance to understanding diseases as important as diabetes or systemic lupus erythematosus (54, 55).

The study of genetic diseases that result from dominant activity of mutant alleles has also benefited from the use of this system. For instance, experiments *in vivo* using the "tet-off" system revealed that the neuropathological symptoms of Huntington disease, which depend on the dominant activity of a variant of the

Huntingtin protein containing expanded polyQ stretches, requires constant production of this molecule (56, 57). In these experiments neuropathological symptoms of Huntington disease were generated by expression of the mutant form of *Huntingtin* in the central nervous system induced by the synthesis of tTA from a transgene using the *CamKIIa* promoter. Deactivation of the system with doxycycline resulted in disappearance of intranuclear agregates and amelioration of the disease.

The *tet* system has also been used to study physiological or developmental processes, most particularly those for which the temporal component could be of importance. Several experimental designs that use the *tet* system have been employed for this purpose. One possibility is to break a biochemical balance by forced expression of one component or by blocking an activity with the expression of a specific inhibitor and then revert the conditions to normal. This strategy was used to analyze the requirements of complementary kinase and phosphatase systems in the genesis and maintenance of synaptic long term potentiation (58, 59). While some of these studies could also be done using a normal transgenic strategy, the use of the *tet* inducible system strongly facilitated the study of how this molecular system influences dynamic processes like memory storage and retrieval (58, 59).

A different experimental design was used to determine the functional time window of gene activity, based on the recovery of a mutant phenotype through the expression of a cDNA for the gene to be functionally recovered using a "tet-on" or "tet-off" strategy. The temporal requirement for the Tyrosinase activity during the formation of optic nerve projections through the chiasm was analysed using such an approach (44). In this report, the constitutive *Tyrosinase* deficiency of albino mice (60) was rescued using a classical "tet-on" strategy built with two transgenics. One contained the *Tyrosinase* cDNA linked to P_{tet}. The other transgene was a cassette designed to express *rtTA* using the *Tyrosinase* promoter, which assured appropriate tissue distribution for rtTA expression. Phenotypic correction was observed in embryos upon doxycycline-mediated induction of *Tyrosinase* from their first week of pregnancy but not in uninduced animals (44) (see also Lavado and Montoliu in this special issue).

There are two main requirements for the general applicability of this system. The first is the existence of a mutant strain for the gene of interest. For many genes this is not a problem, as a large catalogue of mutants is available, which were built by conventional gene targetting methods or that were obtained by spontaneous or induced random mutagenesis approaches (61). The second requirement is the existence of a well characterized promoter that reproduces the expression pattern of the gene. This is essential in order to produce a transgenic line expressing tTA or rtTA in the appropriate spatial domain. For many genes this is not available, thus limiting the use of this strategy. A possible solution for this problem is to "knock-in" the tet-transactivators into the locus of interest. This is the basis of a related strategy that has been used to study the temporal requirements of *Ednrb* for the development of melanocytes and enteric neurons (62). In this experimental design (Figure 3) the inactivation of the endogenous *Ednrb* was a consequence of the construction of a "tet-on" or a "tet-off" expression system for controlled expression of an *Ednrb* cDNA within the endogenous *Ednrb* loci. For this, the two *Ednrb* alleles were modified by homologous recombination. In one of them the first exon and the proximal promoter area of the gene were replaced by an expression cassette containing P_{tet} followed by an *Ednrb* cDNA. This modification created an inactive *Endrb* locus that became responsive to tTA or rtTA. In the other allele either the *tTA* or *rtTA* gene was introduced in frame with the coding region of *Ednrb*, just downstream of the gene´s translational start ATG within the first exon. Under these conditions the *Ednrb* gene was also inactivated from this allele and the *tTA* or *rtTA* genes were expressed under the control of the *Ednrb* regulatory elements, thus reproducing *Ednrb´*s spatial and temporal expression pattern. In mice carrying both modified alleles, *Ednrb* expression was dependent on the *Ednrb* cDNA activation from the P_{tet}-modified locus by the *tTA* or *rtTA* produced from the other allele, which could be further modulated by the administration or depletion of doxycycline.

In principle, a similar strategy can be used with any other gene. The main difficulty is to obtain a strain in which the P_{tet}-cDNA "knock in" is silent, as it can be easily taken over by the gene´s control elements acting on the minimal promoter contained within P_{tet}.

A different approach has been recently developed that allows the analysis of temporal requirements for a gene's activity (43). In this strategy (Figure 4), the target gene is inactivated in a reversible fashion using the control elements of the *tet* system. In particular, 7 tandem copies of *tetO* (without the eukaryotic minimal promoter) were incorporated into the control area of the *Hoxa2* gene by homologous recombination (63). This produced a *Hoxa2* allele susceptible of negative regulation by tetR or a derivative more efficient in transcriptional repression, like tTS (39). *tTS* was provided in *trans* from a transgene expressing this gene ubiquitously (43). Binding of tTS to *tetO* resulted in the transcriptional inactivation of the *Hoxa2* gene, an effect that was reversed by the administration of doxycycline. The critical issue in this strategy is the introduction of *tetO* in a location that does not affect normal gene activity and still allows transcriptional regulation by TetR or its repressor derivatives. An obvious possibility is the gene´s 5´ untranslated region. An alternative might be to introduce *tetO* further upstream relative to the transcriptional initiation site or even downstream within an intron, provided that tTS (and not TetR) is used as repressor, because this molecule has been shown to block transcription from *tetO* sequences located as far as 3 kb from the transcriptional start site (39).

The biggest advantage of this strategy over other described methods is that it requires fewer manipulations to achieve gene inactivation and recovery. It also does not require a well characterized promoter for the specific gene to construct a recovery transgene. Moreover, the same tTS-

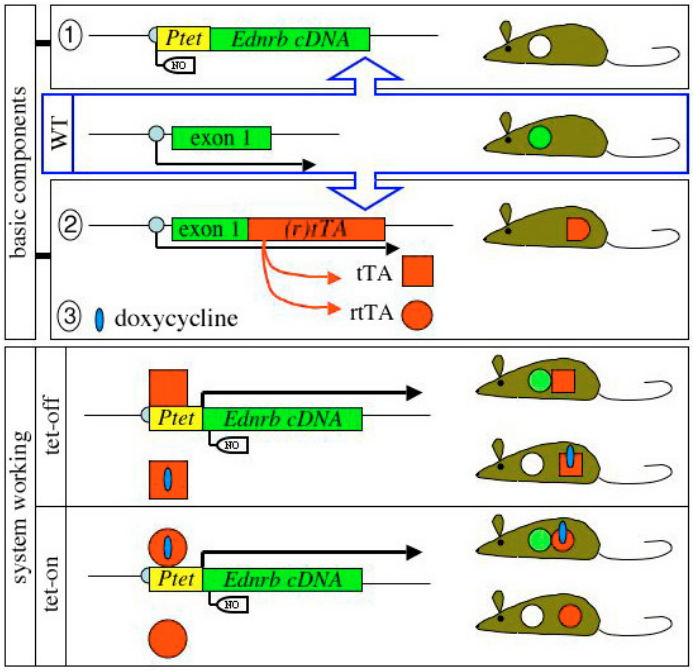

Figure 3. The use of the *tet* system for controlled gene expression in a null background, as used by Shin *et al* (62) for the temporal control of *Ednrb* expression. In the blue box it is depicted the 5´area of the wild type *Ednrb* locus, showing the first exon (green box) and the basic promoter element (grey circle). The basic components are obtained by modification of the wild type locus. 1) One of the modifications results in the replacement of the first exon and the basic promoter element of *Ednrb* by an element containing P_{tet} and a *Ednrb* cDNA, which is transcriptionally silent (represented with a white circle in the mouse drawing). 2) The other modification introduces *tTA* or *rtTA* into the *Ednrb* locus in a way that they become under the control of the normal regulatory elements of the *Ednrb* gene. 3) Doxycycline is used to modulate the activity of the transactivator.When the system is set to work, mice containing both *Ednrb*-modified alleles are not able to express the endogenous *Ednrb* gene (represented with a white circle in the mouse drawing). Expression of *Ednrb* thus becomes dependent of tTA or rtTA activity on P_{tet}, the first in the absence of doxycycline, the last in its presence, which activates expression of the *Ednrb* cDNA (represented with a green circle in the mouse drawing).

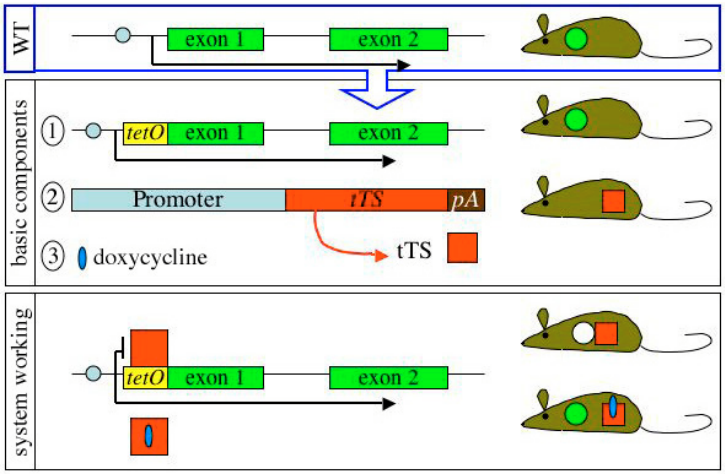

Figure 4. The use of the *tet* system for controlled and reversible gene inactivation. In the blue box it is depicted the genomic locus we intend to regulate, showing two exons (green boxes) and the basic promoter element (grey circle). The basic components include: 1) a modified locus in which tetO was introduced in the 5´area. Transcriptional activity from this locus should be normal (represented with a green circle in the mouse drawing); 2) an expression cassette designed to drive expression of a tet-derived repressor (tTS) either ubiquitously or in a localized area of the animal (represented by the red square in the mouse drawing); 3) doxycycline is used to modulate the activity of the transactivator. When the system is set to work, tTS binds *tetO* and blocks transcription from the locus (represented with a white circle in the mouse drawing). When doxycycline is added to the drinking water, tTS is removed from *tetO* and transcription from the locus resumed (represented with a green circle in the mouse drawing).

expressing transgenic lines can be used to modulate expression of different genes, provided that they have been *tetO*-modified. In addition, while transgenic lines ubiquitously expressing tTS can elicit global inactivation, tissue-restricted expression of tTS can produce reversible gene inactivation in a tissue-specific fashion. A desirable development of the technique, which will increase its versatility, is the development of effective rtTS molecules (i.e. activated by the addition of doxycycline), that respond at low doxycycline concentrations.

A possible limitation of this technique derives from the fact that reactivation of the silenced gene after revomal of tTS from *tetO* depends on the activity of the endogenous regulatory elements of the gene. While this would normally not pose a problem, if initiation and maintenance of a particular expression domain depends on different sets of factors, the reactivation process can be hindered if it happens when the initiation complex is not anymore available in the target cells.

3.2. The *lac* system

Although they have not been as widely used as those of the tetracycline operon, the regulatory elements of the *E coli lac* operon (64) were the first to be assayed in mammalian cells (19, 20). In the *lac* system (figure 4), the lac repressor (LacI, encoded by the *lacI* gene) was mostly used as a repressor to block either transcriptional initiation or elongation when binding to lac Operator (*lacO*) placed within a promoter (19, 20, 65). LacI has also been converted to an activator, which proved active in cell lines, but so far it has not been as extensively used as the tet-based activators (66, 67).

Initial attempts to use of the *lac* system in transgenic animals were not as successful as those with the *tet* system, mostly owing to specific inactivation of *lacI* by methylation of the transgene in the animal, which resulted in the loss of transgene expression (68, 69). This problem has recently been circumvented by "humanizing" the codons of the bacterial *lacI* gene, to avoid both methylation and cryptic splicing signals in the cDNA (70). The modified LacI repressor has been effectively used to block transcription from a *lacO*-modified *Tyrosinase* promoter both in adult animals and in embryos (70). In addition this transcriptional repression was completely reverted by isopropyl beta-D-thiogalactopyranoside (IPTG) when orally administered at non-toxic concentrations (figure 2). These results show the feasibility of the use of the *lac* system to effectively control gene expression from transgenes in living animals. In fact, it has been used to determine the functional time frame for the *Tyrosinase* gene in proper axonal pathfinding in the mouse visual system (71). In these experiments the inactive *Tyrosinase* gene of albino mice (60) was complemented using two transgenes designed to express the *Tyrosinase* gene in a controlled fashion. One of the transgenes contains the *Tyrosinase* cDNA downstream of a chimeric control region, which includes the *Tyrosinase* promoter (for proper tissue-specific expression) modified to include *lacO* to allow regulation by LacI. The other transgene expresses the *lacI* gene ubiquitously from the human *beta-actin* promoter. When the two transgenic lines were crossed in the albino mouse background, Tyrosinase recovery occurred in the absence of IPTG but was blocked when the drug was added to the drinking water.

The main difference between the use of the *tet* and *lac* systems in recovery experiments (44, 70) is that in the *tet* system the transcriptional modulator is usually an activator (either tTA or rtTA) and in the *lac* system a repressor. This implies that the design of the two transgenes is different (compare figures 1 and 2). In the *tet* system the cDNA for the gene that we want to restore is placed under P_{tet} control and is inactive until activated by (r)tTA, while in the *lac* system the effector is constantly expressed unless LacI binds *lacO* to repress transcription. This also implies that the proper control of the spatial and temporal expression domain is provided differently in the two systems, being associated to (r)tTA and to the "target" cDNA in the *tet* and *lac* systems respectively.

As for the *tet* system, the general use of this strategy to evaluate the time requirements for a specific gene function is limited by requiring the existence of both a mutant background and a well characterized promoter for the gene to be used to build the recovery transgenes. It is possible that the use of *lacO* and *lacI* to generate a reversible inactivation strategy similar to that described for the *tet* system (43) could simplify the use of the *lac* system to achieve conditional, temporal and reversible gene inactivation. In any case, the success of the *lac* system *in vivo* (70) opens new possibilities for finer designs for the control of gene expression, most particularly if combined with the *tet* system. For instance, a *tetO* and *lacO* doubly modified gene could be controlled in two different tissues using specific combinations of *lacI* and *(r)tTA* or *(r)tTS* under the control of two tissue-specific promoters; addition or removal of doxycycline and IPTG would then determine the specific activation status in each of the tissues in a reversible fashion. A transgenic model using this system and a luciferase reporter that allows the evaluation of the dynamics of gene expression in the living animal has recently been described (72).

Other possibilities that should also be worth exploring with the *lac* system include the possible feasibility of using the LacI as a transcriptional activator *in vivo* and the possibility of obtaining mutants in the *lacI* gene that result in reversed *lacO*-binding properties when combined with IPTG (i.e. that bind *lacO* when the drug is administered). The existence of such systems would allow designing experiments with better control on the induction process as it will rely on IPTG administration, which is normally kinetically more favourable than the drug´s removal because the latter depends on IPTG´s clearance from the tissues.

3.3. The Gal4/*UAS* system with temporal control

Another binary system that can be used to control gene transcription in living animals is the so-called Gal4/*UAS* system. In this system the *Saccharomyces cerevisiae* gene *Gal4* is used to activate transcription of DNA sequences linked to the Gal4 DNA binding sites, the so-called *UAS* (upstream activating sequences).

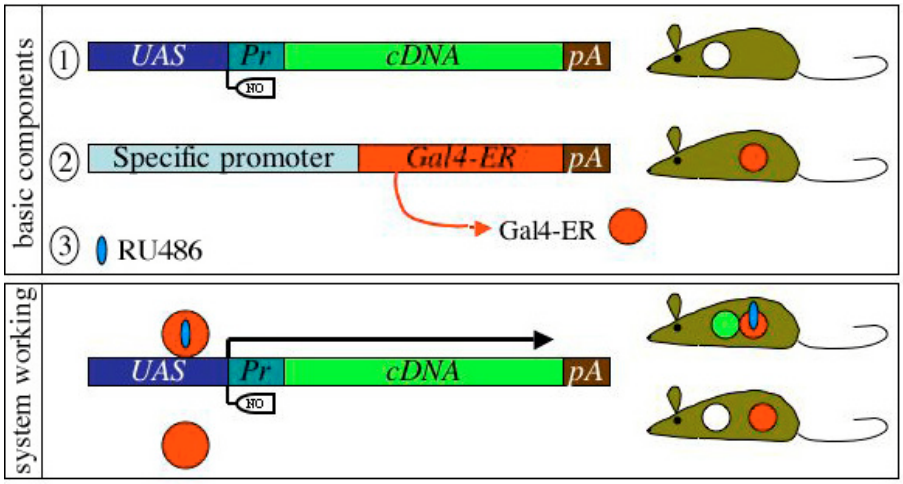

Figure 5. The use of the "Gene-switch" system in transgenic animals. The basic components of the system are: 1) an element that contains the *UAS* linked to a minimal promoter (*Pr*), which is positioned in front of a cDNA coding for the protein whose expression we want to control. A polyadenylation signal completes the first element. This element is by itself inactive (represented with a white circle in the mouse drawing); 2) an expression cassette to drive expression of GLVP or GLp65 (generically represented as Gal4-ER) under the control of a specific promoter. The choice of the promoter defines the spatial domain of expression of the transactivator in the animal; 3) RU684 is used to modulate the activity of the transactivator. When the system is set to work, Gal4-ER is sequestered by Hsp90 rendering it unable to bind *UAS*. Under these conditions element 1 remains inactive (represented with a white circle in the mouse drawing). When RU684 is administered Gal4-ER is liberated from Hsp90, binds *UAS* and activates transcription of the cDNA of interest (represented with a green circle in the mouse drawing).

Although this was the first binary system to be used in transgenic mice (21), it never became as popular among the mammalian community as it is in the Drosophila field, where it is by far the most used approach to control gene expression *in vivo* (73). This system is a pure transactivator system in which one module contains the gene to be expressed downstream of *UAS* sequences and Gal4 is provided from another expression cassette under the control of a specific promoter. A further development of the system, known as "Gene-Switch", allows temporal control of the Gal4 transcriptional activity (74), which is active in transgenic mice (75) (Figure 5). The "Gene Switch" uses a chimeric transactivator (there are two versions, GLVPc and GLp65) (74, 76) composed of the Gal4 DNA binding domain, a transcriptional activator domain (respectively, from the herpes simplex virus VP16 or from NF-kappa-B) and a truncated form of the ligand binding domain of the progesterone receptor, which binds antiprogestins (like RU486) but not endogenous steroids (77). In the absence of antiprogestins, GLVPc and GLp65 are retained in the cytoplasm by the heat shock protein (hsp) 90 and is therefore transcriptionally inactive (78). Upon administration of the antiprogestin, the chimeric molecule is released from the hsp90, translocates to the nucleus and activates transcription from *UAS*.

As for the other binary transactivator systems, its use *in vivo* typically requires the genesis of two transgenic lines, one containing the gene of interest downstream of the *UAS* sequences and another expressing the transactivator under the control of a tissue-specific promoter (figure 5). The introduction of the binary system within a single construct has also been described (76).

As previously discussed for other binary systems, the "Gene-Switch" has been used to address the effect of temporal and reversible expression of dominant functions on disease and to recover a specific gene function (which had been previously inactivated using an independent strategy) in a controlled fashion (79, 80, 81). However, contrary to other binary systems, the use of the Gene-Switch has been so far restricted to studies in adult mice, maybe because of the abortogenic properties of antiprogestins.

Interestingly, the use of a steroid analogue to activate the system made it particularly useful for studies in the skin because RU486 can be administered topically to produce localized gene activation (79, 81). This same property has also opened the possibility to use the skin as a "bioreactor" to produce proteins that can be delivered systemically using the Gene-Switch system (82).

4. RECOMBINATION BINARY SYSTEMS

The finding that site-specific recombination systems normally operating in phages and yeast are also functional in mammalian cells opened new possibilities for conditional gene manipulation in the mouse (17, 18, 83). The most widely used systems are the so called Cre/*lox* and FLP/*FRT* systems. They both work in a similar way: a recombinase (Cre from the P1 phage or FLP from *Saccharomyces cerevisiae*) binds and cleaves a specific DNA target site (*loxP* for Cre and *FRT* for FLP) and ligates it to another copy of a similar target (84). If the two copies of the *loxP* or *FRT* sites are on the same DNA molecule and with the same orientation, recombination results in the

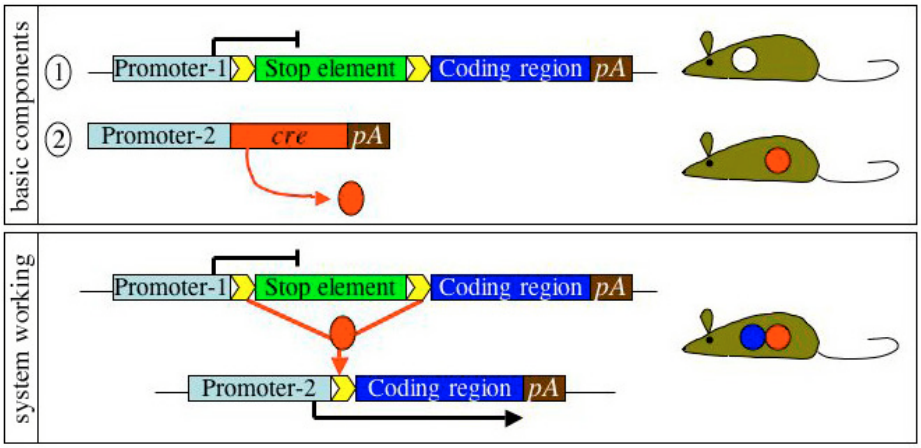

Figure 6. The use of the Cre/*lox* system to activate gene expression. The two basic components of the system include: 1) a transcription unit (which can be a transgenic construct or an endogenous gene locus) modified to introduce a terminator element flanked by *LoxP* sites (yellow arrows) between the promoter and the gene´s coding region. This renders the transcription unit inactive (represented with a white circle in the mouse drawing); 2) a transcription unit expressing the *cre* recombinase under a specific promoter. When the system is set to work, the Cre recombinase produces recombination between the two *LoxP* sites and removes the terminator element. The promoter then activates expression of the coding region (represented with a blue circle in the mouse drawing). This activation occurs only in the areas where cre is expressed, which is defined by the activity of the promoter used in the *cre*-expressing cassette.

removal of the DNA fragment located between the target sites (Figure 6) (84). If they have opposite orientation, the intervening sequence is inverted upon recombination between the sites. Independently of the physiological roles that these simple reactions have in their natural environment, their usefulness as a genetic tool in mammalian cells derives mainly from the possibility to delete specific DNA sequences that have been flanked by the specific target sites. In addition, as these sites are 34 bp long (84), the probability that they are present within a normal genome is very low, thus reducing the possibility of unwanted reactions to levels that normally would not interfere with the experiments. Since the use of the two systems is essentially the same and, for historical reasons, the Cre/*lox* system has been more frequently used than the FLP/*FRT*, I will center the discussion on the first, but keeping in mind that both can be used similarly.

The most popular use of the recombinase binary systems is to produce tissue or cell-specific gene inactivation (13). As genes are often involved in different processes, there are situations in which early functions of the gene result in phenotypes that make it impossible to analyse the role the gene plays in later processes. In these cases, the gene locus can be modified to introduce *loxP* sites in places that do not affect the normal gene function (typically within introns). The resulting modified alleles, usually called "floxed" (flanked by *loxP*) alleles, are fully functional. In the presence of Cre recombinase, the "floxed" area is deleted and the gene inactivated (13). In this way, provided that the *cre* recombinase gene is properly targeted to the tissue of interest, gene deletion can be achieved in a tissue-specific fashion, and thus other phenotypes resulting from the inactivation of the gene in a different tissue will not interfere with the objective of the study. This application of the recombination-based binary systems will be discussed in detail in the accompanying review by Garcia-Otin and Guillou.

Tissue-specific gene activation can also be achieved with recombinase binary systems (figure 6). Actually, in the first reports of the use of these systems in living animals, the goal was the tissue-specific activation of a transgene (85). The use of the recombination systems to activate gene expression is in many ways similar to what I have described for the transcriptional binary systems or, in some cases, to a classical transgene. In fact, both transcriptional and recombination binary systems have been employed to answer the same kind of question, even with very similar conceptual experimental designs. The major difference resides in the fact that the recombination reactions are permanent, and thus do not require the constant administration of a particular inducer, and that the transcriptional binary systems are reversible, thus allowing the analysis of the need or not of the constant input of a particular gene to achieve a specific function or phenotype.

Gene activation using recombination binary systems (figure 6) requires the modification of a transcription unit to keep it transcriptionally inactive in a way that can be reversed by Cre- (or FLP)-mediated recombination. Typically, this is done by the introduction, between the promoter and the cDNA we intend to regulate, of "floxed" transcriptional terminator elements or another transcription unit also flanked by *loxP* (or *FRT*) sites (17, 85, 86). Under this configuration the transcription unit is maintained in a transcriptionally inactive state, which can be reverted by removing the terminator elements by Cre- (or FLP)-mediated recombination.

One of the most common uses of the Cre-mediated gene activation systems is the characterization of

the tissue specificity of *cre*-expressing transgenic lines, which is central for the proper design of conditional mutagenesis experiments. There are two major series of reporters for Cre activity. One of them is based on modifications of the *ROSA26* locus (87), which provides ubiquitous expression and can be inactivated without apparent phenotypic effects. Insertion of a reporter gene (e.g. for *beta-galactosidase* or fluorescent proteins) in this locus results in its ubiquitous expression (86, 88, 89). The introduction of a "floxed" transcriptional unit with increased transcriptional termination activity between the promoter and the reporter´s coding region results in the constitutive silencing of the locus, which can be reactivated by Cre-mediated recombination (86, 88, 89). When *ROSA26-term* mice are crossed with a particular *cre*-expressing line, activation of the reporter becomes diagnostic for Cre activity. A mouse reporter line based on the *ROSA26* locus has also been described for the detection of FLP activity (90). The other reporter mouse series for Cre activity (4, 91) derives from a single copy transgene designed to activate ubiquitous expression of either of two different reporters: a first reporter, typically beta-galactosidase, is expressed from the transgene in the absence of Cre recombination; the second reporter (alkaline phosphatase or enhanced green fluorescent protein in the Z/AP and Z/EG lines respectively) is only activated upon Cre-mediated recombination, which removes the *beta-galactosidase* gene and leaves the second reporter under the control of the ubiquitous promoter (4, 91). In these mice, the areas negative for beta-galactosidase and positive for alkaline phosphatase or EGFP (enhanced green fluorescent protein) represent the domain of Cre activity.

Cre-mediated reporter activation is permanent, and thus becomes an indeleble marker of the cell where it occurred, which is transmitted to the cell´s progeny without dilution. For this reason, it became the method of choice for *in vivo* fate mapping studies in the mouse. This approach has been successfully used to study a variety of processes that required the unambiguous identificaton of the progeny of a specific group of cells (92, 93, 94, 95, 96, 97, 98, 99, 100, 101). Virtually any cell can be labelled with this method, provided that Cre is expressed specifically in that cell line, either by using a transgenic containing a specific promoter or "knocking in" the recombinase into a locus with specific expression in the target cell type.

Cre-mediated gene activation has also been used to produce specific cell ablations *in vivo*, which allowed the study of the role of particular tissues or cell types in developmental processes and to model particular human diseases that occur by specific cell loss (102, 103, 104). The common theme in these studies was the construction of a transgene or the modification of a genomic locus to introduce an open reading frame for a toxin (typically the dyphteria toxin DTA), which was kept transcriptionally inactive by the insertion of "floxed" elements that hamper transcription of the toxin´s gene. As for other applications, multiple transcriptional terminators or another reporter cassette have been successfully used to avoid toxin expression. Toxin expression, with subsequent cell death, is then achieved by Cre-mediated recombination. The tissue or cell specificity of toxin expression was typically provided by linking the silenced toxin gene to a cell-specific promoter, either as part of a transgene or by a "knock in" strategy. Activation of the toxin by tissue-specific *cre* expression has also been reported (105).

A similar strategy has also been used as an alternative to transcriptionally based regulation of gene expression to study the effects of particular genes or specific mutations in the genesis of disease (85, 106). Actually, the first *in vivo* demonstration of the feasibility to use the phage-derived Cre-*lox* system was performed in an experiment that achieved Cre-mediated tissue-specific activation of a transgene in which the trascription of the SV40 oncogenic *TAg* was kept silent by "floxed" terminator sequences just downstream of the promoter (85). More recently, a more sophisticated scheme was used to express a mutated allele of the *Keratin10* gene in the skin with experimental control on the timing of the activation (106). In those experiments the authors modified the *Keratin10* locus by homologous recombination to introduce a disease-associated point mutation and a "floxed" *neo* expression cassette in the first intron, which completely stopped *Keratin10* transcription from this allele. Transcriptional activity of this gene was recovered by removal of the *neo* cassette with Cre recombinase. In these experiments the Cre recombinase was expressed in the skin with the help of the keratinocyte-specific promoters (106). However, as control of the timing of Cre activity was also essential, a hybrid Cre recombinase that included a truncated steroid-binding domain of the progesterone receptor was used (107). As discussed for the GLVP and GLp65 molecules, Cre-ER only becomes active when an antiprogestin like RU486 is present. In the case of the skin this drug can be topically applied.

Reactivation of totally or partially silent alleles has also been used to determine the requirements of a particular gene function in a specific tissue or the activity of a mutated allele in a particular tissue (14, 106, 108, 109). This strategy can be used as a complement or an alternative to the tissue-specific gene inactivation, using essentially the same ES cell lines and derived mice. An intermediate step in the genesis of a "floxed" allele requires a homologous recombination that introduces a drug-resistance cassette that can be removed either by Cre or FLP recombination (110). Frequently, alleles containing these insertions are less active than their wild type counterparts, thus generating hypomorphic or even null alleles (14, 111, 112). Crossing mice carrying these alleles (which are sometimes also produced as an intermediate step in the conditional inactivation procedure), with a specific Cre or FLP expressing line results in the activation of the gene in a specific tissue on a gene-deficient background.

5. SUMMARY AND PERSPECTIVE

During the last years many techniques have been devised that allow fine manipulation of gene expression in the mouse. All these techniques can help in understanding the role genes play during normal cellular function and how they lead to disease when altered. In addition, these

techniques provide the means to obtain animal models for a variety of diseases, and they might even provide a reasonable basis for novel therapeutic approaches.

As I have discussed, the variety of techniques that are currently available is broad, and can involve the modification of endogenous alleles in a controlled fashion or the introduction of genetic units that can be regulated according to the researcher´s needs. It is expected that new developments will appear in the coming years. Among the major challenges is to find methods that allow control not only of the spatial and temporal components of gene activity, but also of the quantitative parameter, which is known to be central to many biological processes and that could play unexpected roles in certain cells, tissues or organs under normal and pathological conditions. Logically, some of these improvements will result from the continuous refinement of existing protocols, like the use of the *lac* system for reversible gene inactivation from endogenous genes. Others are expected to arise from combinations of several of the existing techniques, like the use of the *tet* and *lac* systems together to control expression in two different tissues independently. And, of course, it can be expected that completely new approaches will appear, which will increase the experimental possibilities for the solution of theoretical and practical questions. Assessment of what has been happening during the last few years indicates that RNAi-based technology (113) will soon be strongly stepping into the mouse field to produce inactivation or downregulation of specific genes. Also, it can be anticipated, that the use of custom-designed DNA-binding molecules to modulate transcription of endogenous genes (both as activators and repressors) (114) and, eventually, to produce other kinds of manipulations, like insertions and deletions, will find a place in the gene manipulation technologies in living animals. Finally, completely new approaches, not expected from our current knowledge, can still emerge and take the front stage in the way we manipulate gene activity in the mouse.

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