Inducible Gene Expression and Gene Modification in Transgenic Mice

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Abstract. Animal transgenesis has proven to be useful for physiologic as well as pathophysiologic studies. Animal models with conditional expression of a transgene of interest or with a conditional gene mutation can be generated. This permits spatial and temporal control of the expression of the transgene or of gene mutations previously introduced by gene targeting. These approaches allow the generation of models suitable for physiologic analysis or models mimicking disease states.

In vivo analysis of gene function is often based on the production of animal models with specific genetic modifications. These approaches have been successfully applied to several areas in the renal field (1). Such modifications can result in a gain or loss of function. Two strategies are used to achieve these goals (2). The first is additive transgenesis, which consists of the introduction into the genome of a transgene, i.e., a fusion gene containing a promoter sequence and a cDNA of interest. The promoter confers widespread or tissue-specific expression of the cDNA. The cDNA of interest can encode a wild-type protein or a mutated protein acting as a negative-dominant protein or mimicking genetic mutations observed in human diseases. The second approach is gene targeting, which consists of the introduction of specific mutations into a gene of interest by homologous recombination using embryonic stem cell technology (3,4). In most cases, the mutation results in the generation of a null allele i.e., knockout, allowing gene inactivation after breeding of the animals as homozygotes. Truncated or mutated proteins can also be produced using gene targeting. Finally, using a similar technique (i.e., knockin), the expression of a protein of interest can be placed under the control of the endogenous regulatory sequences of a different gene (3,4).

In all of these cases, the expression of the wild-type or mutated protein, as well as the site-directed gene modification, is constitutive. The gene mutation is present in the first cell of the embryo and thereafter in all cells of the adult animal. Spatiotemporal restriction of the expression of the protein of interest is linked to the properties of the promoter used to make the fusion gene (in the case of additive transgenesis) or by the expression pattern of the gene that has been mutated (in the case of the knockin technique). Therefore, the protein is expressed when the promoter used in the transgenic construct is turned on, and its expression pattern follows the full expression pattern of the promoter used.

For more precise analysis of the function of a gene product, it may be disadvantageous that the transgene or the mutation is expressed early during development or in several organs, rather than one of interest. For example, toxic effects or lethality related to early expression of the transgene during embryonic development may impair analysis of the phenotype. Moreover, as often observed in knockout experiments, redundancy among related gene products can severely reduce the usefulness of such animal models. To overcome these limitations, several strategies have been developed to allow spatiotemporal control of the expression of proteins or gene mutations. Among these approaches, several have been proven to be functional in vivo in transgenic animals, allowing investigators to predict and control where and when transgenes or gene mutations are expressed.

To date, two major systems have been successfully used in transgenic mice, i.e., the tetracycline-inducible system and the Cre/loxP recombinase system (either constitutive or inducible). Other inducible systems have been described and used in transgenic mice (5). Because of editorial limitations, they are not detailed in this short review.

Both systems [tetracycline-controlled transactivator (tTA)/reverse tTA (rtTA) and Cre/loxP] can be used in cell culture models transfected with the appropriate constructs. To use these systems in vivo, it is necessary to generate two sets of transgenic animals. One mouse line expresses the activator (tTA, rtTA, or Cre recombinase) under the control of a selected tissue-specific promoter. Another set of transgenic animals express the “acceptor” construct, in which the expression of the transgene of interest (or the gene to be modified) is under the control of the target sequence for the tTA/rtTA transactivators (or is flanked by loxP sequences). Mating the two strains of mice allows spatiotemporal control of transgene expression or the desired gene alteration.

Tetracycline-Inducible Systems

The tetracycline-dependent regulatory systems (tet systems) developed in the laboratory of Herman Bujard permit stringent...
control of gene expression in a wide range of cells in culture, as well as in transgenic animals (6–9). The tet systems rely on two components, i.e., a tetracycline-controlled transactivator (tTA or rtTA) and a tTA/rtTA-dependent promoter that controls expression of a downstream cDNA, in a tetracycline-dependent manner (Figure 1A). tTA is a fusion protein containing the repressor of the Tn10 tetracycline-resistance operon of *Escherichia coli* and a carboxyl-terminal portion of protein 16 of herpes simplex virus (VP16). The tTA-dependent promoter consists of a minimal RNA polymerase II promoter fused to tet operator (tetO) sequences (an array of seven cognate operator sequences). This fusion converts the t repressor into a strong transcriptional activator in eukaryotic cells.

In the absence of tetracycline or its derivatives (such as doxycycline), tTA binds to the tetO sequences, allowing transcriptional activation of the tTA-dependent promoter. However, in the presence of doxycycline, tTA cannot interact with its target and transcription does not occur. The tet system that uses tTA is termed tet-OFF, because tetracycline or doxycycline allows transcriptional down-regulation (6,7). Because tetracycline and its derivatives are not usually present in living animals, exogenous administration of tetracycline or its derivatives allows temporal control of transgene expression in vivo. A mutant form of tTA, termed rtTA, has been isolated using random mutagenesis (8,9). In contrast to tTA, rtTA is not functional in the absence of doxycycline but requires the presence of the ligand for transactivation. This tet system is therefore termed tet-ON (Figure 1B).

The two systems function as mirror images and are functionally equivalent when transferred into mice (Figure 1C). It should be noted that tet-ON requires higher doxycycline concentrations to be active, compared with the concentration tet-OFF requires to be inactive (9). This may be of importance when the systems are used in vivo, because the doxycycline may differ among tissues. The advantages of tet-ON, compared with tet-OFF, are that the transgene is not expressed until doxycycline is given to the animals and that upregulation in vivo is faster than downregulation. Hallmarks of the tet systems are the tightness of control, the ability to regulate gene activity in a tissue-specific manner, the doxycycline dose-dependent responses, and the ability to return to a control situation by simply discontinuing doxycycline administration. However, the major disadvantage of the tet systems is that control of the expression of the acceptor construct is often leaky, because of strong positional effects on the tetO minimal promoter. This requires the generation of several acceptor mouse strains to identify those that express the transgene not constitutively but in an inducible manner. Several recently published reports described improvements of the tet systems (transactivators with less toxicity or different ligand sensitivities, bidirectional tetO minimal promoters, and reduced leakiness) (10–14).

The tet systems have been used in vivo for the inducible expression of several transgenes, encoding, for example, reporter genes, oncogenes, or proteins involved in the signaling cascade. These models are listed in Table 1. The tTA and rtTA systems are both active in the kidney. In whole-kidney extracts, 1,000- to 10,000-fold activation of luciferase activity was observed (6,9). In that case, expression of the tTA and rtTA transactivators was under the control of a strong promoter (cytomegalovirus IE). We are currently analyzing, in such mice, the cell-specific expression of the tTA and rtTA tran-
activators, as well as the inducible expression of a reporter gene along the nephron (S. Puttini, A. Beggah, and F. Jaisser, manuscript in preparation). This will permit the specific use of these existing transgenic mice for the inducible expression of transgenes of interest in renal pathophysiologic models. To obtain more specific expression of the tet systems in the kidney, we are currently generating transgenic mice in which expression of the rtTA transactivator is restricted to the collecting duct.

**Cre/lox System**

The Cre/lox system uses the Cre recombinase isolated from the P1 bacteriophage (3,15). The Cre recombinase catalyzes site-specific recombination by crossover between two distant Cre recognition sequences, i.e., loxP sites (Figure 2A). The loxP sites include two 13-bp inverted repeats separated by an 8-bp spacer sequence. Any DNA sequence introduced between the two 34-bp loxP sequences (termed “floxed” DNA) is excised because of Cre-mediated recombination. Therefore, control of Cre expression in a transgenic animal, using either spatial control (with a tissue- or cell-specific promoter) or temporal control (with an inducible system), results in the spatial or temporal control of DNA excision between the two loxP sites. In addition to conditional gene inactivation (conditional knockout), this approach can be applied to protein overexpression. In that case, a floxed stop codon is inserted be-

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**Table 1. Inducible gene expression using the tetracycline-dependent system**

<table>
<thead>
<tr>
<th>Tissue-Specific Promoter</th>
<th>Transactivator</th>
<th>Gene to be Expressed</th>
<th>Phenotypic Effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse mammary tumor virus long terminal repeat</td>
<td>tTA</td>
<td>Simian virus 40 T antigen</td>
<td>Salivary glands hyperplasia</td>
<td>(32)</td>
</tr>
<tr>
<td>α-Myosin heavy chain (heart)</td>
<td>tTA</td>
<td>LacZ</td>
<td>Myocardial LacZ expression</td>
<td>(33)</td>
</tr>
<tr>
<td>α-Myosin heavy chain (heart)</td>
<td>tTA</td>
<td>Diphtheria toxin A</td>
<td>Myocardial plasticity</td>
<td>(34)</td>
</tr>
<tr>
<td>α-Myosin heavy chain (heart)</td>
<td>tTA</td>
<td>Protein kinase C-β (mutant)</td>
<td>Heart remodeling</td>
<td>(35)</td>
</tr>
<tr>
<td>α-Myosin heavy chain (heart)</td>
<td>tTA</td>
<td>G₁-coupled receptor</td>
<td>Heart rate control</td>
<td>(36)</td>
</tr>
<tr>
<td>Muscle-specific creatine kinase (muscle)</td>
<td>tTA</td>
<td>TrkB/Fc</td>
<td>Serum TrkB/Fc secretion</td>
<td>(37)</td>
</tr>
<tr>
<td>Tie (endothelial cells)</td>
<td>tTA</td>
<td>LacZ</td>
<td>Endothelial β-galactosidase expression</td>
<td>(38)</td>
</tr>
<tr>
<td>Tek (endothelial cells)</td>
<td>tTA</td>
<td>LacZ</td>
<td>Endothelial β-galactosidase expression</td>
<td>(38)</td>
</tr>
<tr>
<td>Endothelin receptor B</td>
<td>tTA, rtTA</td>
<td>Endothelin receptor B</td>
<td>Conditional endothelin receptor B expression</td>
<td>(39)</td>
</tr>
<tr>
<td>Ca²⁺-calmodulin-dependent kinase II (hippocampus)</td>
<td>tTA</td>
<td>Ca²⁺-calmodulin-dependent kinase II (Asp286 mutant)</td>
<td>Deficiency in memory storage</td>
<td>(40)</td>
</tr>
<tr>
<td>Ca²⁺-calmodulin-dependent kinase II (hippocampus)</td>
<td>rtTA</td>
<td>Ca²⁺-calmodulin-dependent kinase II (Asp286 mutant)</td>
<td>Deficiency in memory storage</td>
<td>(41)</td>
</tr>
<tr>
<td>Ca²⁺-calmodulin-dependent kinase II (hippocampus)</td>
<td>tTA</td>
<td>Calcinurin (truncated mutant)</td>
<td>Deficiency in long-term memory</td>
<td>(42)</td>
</tr>
<tr>
<td>Prion protein promoter</td>
<td>tTA</td>
<td>Prion protein</td>
<td>Prion disease</td>
<td>(43)</td>
</tr>
<tr>
<td>Liver-enriched activator protein (liver)</td>
<td>tTA</td>
<td>LacZ</td>
<td>Hepatocyte β-galactosidase expression</td>
<td>(9)</td>
</tr>
<tr>
<td>Clara cell 10-kD protein (lung Clara cells)</td>
<td>rtTA</td>
<td>Interleukin-11</td>
<td>Lung remodeling</td>
<td>(44)</td>
</tr>
<tr>
<td>Mammary gland-specific β-lactoglobulin (mammary gland)</td>
<td>rtTA</td>
<td>α-Lactalbumin</td>
<td>Overexpression of lactalbumin in milk</td>
<td>(45)</td>
</tr>
<tr>
<td>Keratin 14 (keratinocytes)</td>
<td>rtTA</td>
<td>Erb2 oncogene</td>
<td>Skin hyperplasia</td>
<td>(46)</td>
</tr>
<tr>
<td>RB (neurons and myocytes)</td>
<td>rtTA</td>
<td>Cre recombinase</td>
<td>Cell-specific DNA recombination</td>
<td>(29)</td>
</tr>
<tr>
<td>Whey acidic protein (epithelial mammary cells)</td>
<td>rtTA</td>
<td>Cre recombinase</td>
<td>Cell-specific DNA recombination</td>
<td>(29)</td>
</tr>
<tr>
<td>Fatty acid binding protein (small and large intestine)</td>
<td>rtTA</td>
<td>LacZ</td>
<td>Intestinal β-galactosidase expression</td>
<td>(30)</td>
</tr>
<tr>
<td></td>
<td>rtTA</td>
<td>Cre recombinase</td>
<td>Intestinal cell DNA recombination</td>
<td>(30)</td>
</tr>
</tbody>
</table>

* tTA, tetracycline-controlled transactivator; rtTA, reverse tTA.
ligand that is introduced at a chosen time by researchers. The inducible Cre recombinases are fusion proteins containing the original Cre recombinase and a specific ligand-binding domain (Figure 2C). The functional activity of the Cre recombinase is dependent on an external ligand that is able to bind to this specific domain in the fusion protein. Binding of the ligand is thought to produce conformational changes of the fusion protein and/or changes in the intracellular localization associated with targeting of the recombinase to the nucleus (15,19).

Initially, fusion proteins containing the Cre recombinase and the hormone-binding domain of nuclear receptors, such as receptors for glucocorticoids, estrogens, or progesterone, were generated. To avoid activation of the engineered Cre recombinases by endogenous ligands, such as circulating glucocorticoids, estrogens, or progesterone, mutated hormone ligand-binding domains have been used. In such cases, synthetic ligands bind the modified Cre recombinases but endogenous ligands cannot.

Metzger et al. (20) focused their interest on Cre recombinase fusion proteins with the estrogen ligand-binding domain. A mutated form, which was previously demonstrated to bind synthetic antagonists (such as tamoxifen or its derivative 4-hydroxy-tamoxifen) but not circulating estrogens, was used. The so-called Cre-ERT recombinase has been demonstrated to be functional \textit{in vivo}, allowing widespread time-dependent recombination when Cre-ERT expression is placed under the control of a strong promoter, such as the cytomegalovirus IE promoter (21). Tissue-specific recombination was recently obtained by placing the expression of another inducible Cre recombinase under the control of tissue-specific promoters. Vasioukhin et al. (22) designed a Cre recombinase similar to Cre-ERT, using a mutant form of the mouse estrogen receptor-binding domain (termed Cre-ERTM). Expression of this inducible Cre recombinase was placed under the control of a human keratin 14 promoter, allowing restriction of inducible Cre recombinase expression to keratinocytes (22). In an elegant experiment, those authors demonstrated that recombination could occur after a 4-d intraperitoneal administration of 1 mg of tamoxifen and also, in an even more restricted way, after topical application of tamoxifen directly to a limited area of the skin of transgenic mice. Using a similar Cre recombinase, Danielian et al. (23) were able to induce conditional DNA recombination in developing mouse embryos. After injection of tamoxifen into pregnant mothers, the authors observed DNA recombination in organs expressing the Cre recombinase. Despite adequate recombination events (observed in embryos), abortion often occurs, probably because of the toxic effects of tamoxifen (23). Indeed, one major limitation of using tamoxifen \textit{in vivo} may be its toxicity. To minimize this problem, Indra et al. (24) recently developed a second generation of inducible Cre recombinase (termed Cre-ERT2), which is approximately 10 times more sensitive to 4-hydroxy-tamoxifen \textit{in vivo} than is the original Cre-ERT. Another disadvantage of the system is that it may be somewhat leaky, resulting in constitutive rather than inducible activation. To obtain spatial and temporal restriction of the recombination process, the activity of the engineered Cre recombinase should be strictly dependent on the presence of the

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**Figure 2. Diagram of the Cre/lox system and its use as an inducible expression system.** (A) The Cre recombinase has been isolated from the P1 phage of \textit{E. coli}. Cre induces recombination between two 34-bp, site-specific recognition sequences (loxP), allowing excision of the DNA flanked by these loxP sites (floxed DNA). In transgenic mice, expression of Cre recombinase is under the control of ubiquitous or tissue-specific promoters (P) or follows transient infection with a Cre adeno virus. This allows conditional gene targeting when two loxP sites have been introduced into the genome by homologous recombination. (B) Conditional gene expression can be achieved using Cre recombinase. In this case, a floxed stop sequence is placed between the promoter and the cDNA to be expressed. In double-transgenic mice, the cDNA cannot be expressed in tissues or cells in which Cre recombinase is not expressed. However, Cre recombinase expression results in excision of the floxed stop sequence and allows the cDNA to be expressed. (C) Temporal control of Cre recombinase activity can be added to the spatial control described in B. Chimeric recombinases with the ligand-binding domain of several steroid hormone receptors have been produced. The activity of the chimeric fusion protein is dependent on ligand binding to the ligand-binding domain. Mutated ligand-binding domains have been used to avoid Cre recombinase activation by endogenous steroids rather than exogenous ligands such as tamoxifen (a synthetic glucocorticoid agonist) or RU486 (a synthetic antiprogestosterone).
Conclusions and Perspectives

In recent years, several strategies have been developed to gain more precise spatial and temporal control of gene expression and/or gene modification. These strategies rely on the use of site-specific recombinases and/or inducible systems. The two major systems currently in use, i.e., the Cre/lox and tet systems, have been described in this short review. Several new approaches are under investigation and are expected to refine these powerful tools in the near future. It should be noted that these sophisticated systems are difficult to handle and to develop. However, they are very effective for the elucidation of specialized gene functions or the creation of specific animal models for fundamental research or study of the pathogenesis of human diseases.

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References


exogenous ligand. Zhang *et al.* (25) reported a modified Cre recombinase that included two mutated murine estrogen-binding domains, which were located at the amino and carboxyl termini of Cre (termed MerCreMer). Those authors demonstrated that the control of the recombination process was more stringent, compared with other inducible Cre recombinases (25,26).

Various nuclear hormone-binding domains have been used to produce inducible Cre recombinases. The progesterone-binding domain has been used in a variant of inducible Cre recombinase. The progesterone-binding domain of the human progesterone receptor has been mutated to one that binds the synthetic steroid RU486 (a well known glucocorticoid receptor antagonist) but not endogenous progesterone or glucocorticoids (27). The generation of transgenic mice that express that inducible Cre recombinase specifically in the brain, using either calmodulin-dependent kinase II or Thy-1 promoters to direct expression of the recombinase, allows conditional knockout of the glucocorticoid receptor, in a space- and time-dependent manner (27).

Another way to control the functional activity of the Cre recombinase is to control its expression level. In that case, Cre-mediated recombination is dependent on the spatiotemporal control of Cre recombinase expression. Two strategies have been reported to date, namely the use of an inducible promoter that directs expression of the Cre recombinase and the use of a bi-génic inducible system in which expression of the Cre recombinase can be induced in a ligand-dependent manner. In the first approach, the expression of the Cre recombinase is placed under the control of the Mx1 promoter, whose activity is inducible by interferon α or β, as well as by pI-pC (an interferon inducer). Cre recombination was observed *in vivo* after pI-pC administration (28). However, although deletion of the floxed gene was almost complete in liver and spleen, DNA recombination reached only 20 to 70% in other tissues, reflecting either poor bioavailability of the inducer into such organs or restricted tissue-dependent activity of the Mx1 promoter (28). In the second approach, the expression of the Cre recombinase is under the control of a minimal promoter whose activity is inducible by dexamethasone, as well as by pI-pC (an interferon inducer). Cre recombination was observed *in vivo* after pI-pC administration (28). However, although deletion of the floxed gene was almost complete in liver and spleen, DNA recombination reached only 20 to 70% in other tissues, reflecting either poor bioavailability of the inducer into such organs or restricted tissue-dependent activity of the Mx1 promoter (28).


