REVIEW

Conditional transgenic technologies

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Abstract

Transgenic technology has been revolutionised by the development of techniques that allow temporo-spatial control of gene deletion or expression in transgenic animals. The ability to switch gene expression 'on' or 'off' in restricted tissues at specific times allows unprecedented flexibility for exploring gene function in both health and

disease. As use of these techniques grows in all areas of biomedical research, an understanding of this topic is essential. In this review we examine the theory, application and limitations of these strategies, with particular reference to endocrine research.

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Introduction

Conventional transgenic technologies (gene overexpression or knockouts) are invaluable for modelling genetic disorders and answering specific questions in relation to developmental biology. However, this 'all or nothing' approach is inflexible and cannot be used to answer more subtle questions about gene function. Frequently, a genetic change has developmental consequences that either preclude or complicate studies on adult animals (e.g. embryonic lethality). Furthermore, conventional knockout strategies affect every cell in an animal, so that it is often impossible to distinguish primary and secondary changes in a complex phenotype. In order to tease out more precise information about the role of a gene in a specific cell type at a critical stage of disease or development, a more sophisticated approach is required. Building on conventional transgenic techniques, conditional technologies allow flexible temporo-spatial control of gene expression. In these systems the switching 'on' or 'off' of a particular gene is conditional on a specific stimulus. Such techniques are increasingly applied to endocrine problems, providing elegant models to study physiology and disease. An appreciation of the principles involved is essential for researchers in this field.

This review will concentrate on the theory, application and problems of established conditional transgenic strategies. A basic knowledge of conventional transgenesis is assumed, and a good review of this is provided by Bronson & Smithies (1994).

Conditional overexpression

Tetracycline responsive system

The ideal conditional overexpression system should allow the investigator to switch transgene expression on and off, rapidly, reversibly, at any point during development or postnatal life, and only in the desired cell type. Many systems have been developed harnessing the inherent responsiveness of specific promoters to various stimuli, such as heat shock and steroid hormones (Gossen *et al.* 1993). However, in most cases the effects of the stimulus are too profound and non-specific to offer any practical *in vivo* application.

Pioneering work by Bujard and Gossen has established the tetracycline transactivator system as a reliable tool for regulated transgene expression. This exploits pathways that control the expression of a tetracycline resistance gene in *E. coli*. This gene is constitutively repressed by tetracycline repressor (tetR), a protein that binds specifically to tetracycline operator (*tetO*) sequences within the promoter, rendering the gene transcriptionally silent. Repression is relieved by tetracycline, which avidly binds tetR (Fig. 1a and b). In this way, tetracycline resistance is controlled in a simple on/off manner by tetracycline itself (Gossen *et al.* 1993).

Because tetR and *tetO* interact specifically with each other and do not overlap with eukaryotic systems, they avoid many of the problems that have beset previous attempts to regulate transgene expression. Two modifications have been made to suit transgenic purposes (Gossen & Bujard 1992). First, tetR has been converted

Figure 1 (a and b) Regulation of the tetracycline resistance operon. (a) tetR dimers bind tetO sequences upstream of the tetracycline resistance gene preventing transcription initiation by RNA polymerase. (b) Binding of tetR by tetracycline relieves transcriptional inhibition, leading to expression of the tetracycline resistance gene. Blue ellipses, tetR dimer; dark grey bar, tetO sequences; green bar, tetracycline resistance gene; orange, RNA polymerase; red star, tetracycline. (c and d) Tetracycline-regulated transgene expression. (c) Tetracycline absent. tTA binds the tetO-CMV promoter turning on transgene transcription. In contrast, rtTA is inactive and there is no transgene expression. (d) Tetracycline present. Tetracycline has opposite effects on tTA and rtTA: tetracycline inhibits binding of tTA at the promoter, turning off expression (tet-off system). In the presence of tetracycline, rtTA induces transgene expression (tet-on system). It should be noted that doxycycline is used in practice for regulating the rtTA system. Blue circles, tTA; yellow, rtTA; black and red bars, tetO-CMV promoter; green, transgene; orange, transcription complex; tet^r, tetracycline resistance gene.

into a transcriptional activator by fusing it with the activation domains of the herpes simplex virus VP16 protein. VP16 is a virally encoded factor that recruits cellular transcription factors and potentially activates viral transcription (Herr 1998). The hybrid molecule is termed the tetracycline transcriptional activator (tTA). The second modification is the use of a cytomegalovirus (CMV)-derived minimal promoter, fused with tetO sequences to control transgene expression. This chimaeric promoter is transcriptionally silent in basal conditions, and is induced

in the presence of tTA and tetracycline (Fig. 1c). Tetracycline inactivates tTA and causes transgene down-regulation (Fig. 1d).

Many variants of the tTA system have been designed with specific properties: these are summarised in Table 1. Reverse tTA (rtTA) is a mutagenised version which binds tetO in the presence of doxycycline and activates transcription (Gossen *et al.* 1995) (Fig. 1c and d). In other words, the effects of doxycycline are the opposite of tTA. The major advantage of this is that gene induction occurs

Table 1 Tetracycline-regulated expression systems. Activity is enhanced (+) or suppressed (–) by doxycycline

	Activity	Response to doxycycline	Reference
Type			
tTA	Activator	–	Gossen & Bujard (1992)
rtTA	Activator	+	Gossen <i>et al.</i> (1995)
RtTA ^{2S} -M2	Activator	+	Urlinger <i>et al.</i> (2000a)
rtTE	Activator	+	Akagi <i>et al.</i> (2001)
tTS ^{kid}	Repressor	–	Freundlieb <i>et al.</i> (1995)
tTR	Repressor	–	Zhu <i>et al.</i> (2001)

rapidly because the low levels of doxycycline required for transcriptional activation are readily achieved. In contrast, the kinetics of gene induction by tTA are somewhat slower, since clearance of doxycycline can take days in animals. However, a limitation is that rtTA retains some affinity for *tetO* sequences even in the absence of doxycycline: this leads to low level basal expression, which may not be acceptable (e.g. expression of toxins) (Keyvani *et al.* 1999, Imhof *et al.* 2000). To overcome this problem, tet-repressors have been designed. Two constructs, tTS^{kid} and tTR, are available in which the repressor domain of tetR has been substituted with a mammalian equivalent. These repress transcriptional activity at *tetO* sequences in the absence of tetracycline (Freundlieb *et al.* 1995, Rossi *et al.* 1998, Sander *et al.* 2000). Used in combination with rtTA, complete suppression of transgene expression can be achieved *in vivo* without compromising inducibility (Zhu *et al.* 2001). An obvious disadvantage of this approach is that triple transgenic animals are required. An alternative method has been to engineer improvements in rtTA by a combination of random mutagenesis, use of minimal transactivating domains and codon optimisation for mammalian cells. The variant RtTA2^s-M2 was generated in this manner. This has virtually no background activity, enhanced doxycycline sensitivity and improved transcript stability (Urlinger *et al.* 2000a,b). However, evidence of improved performance in transgenic mice is still preliminary. Finally, substitution of the VP16 moiety of rtTA with the transactivation domain of the mammalian transcription factor E2F4 appears to be tolerated better by mammalian cells (Akagi *et al.* 2001).

The tetracycline analogues, doxycycline and anhydrotetracycline, tend to be used in preference to tetracycline itself due to higher tTA binding affinities and lower toxicities (Gossen & Bujard 1993, Efrat *et al.* 1995, A-Mohammadi *et al.* 1997).

Tet-regulated transgenic mice Use of this system in mice requires the generation of two independent transgenic strains: one carrying the transgene of interest under the control of the *tetO*-CMV promoter, and the other an (r)tTA transgene. Crossing the lines generates a proportion of progeny with both transgenes, allowing regulation of the gene of interest through the administration of tetracycline (Fig. 2).

Initial studies in mice transgenic for tTA or rtTA crossed with luciferase or β -galactosidase reporter strains demonstrated both the practicality and problems of this approach (Furth *et al.* 1994, Kistner *et al.* 1996). Between different transgenic lines, reporter expression varied, probably reflecting variation in chromosomal integration sites and transgene copy number. In many lines, significant leaky expression was found. However, in selected lines basal expression levels were close to the limits of detection. Expression could be induced up to 100 000-fold in certain tissues and, in rtTA transgenics, kinetic studies showed

Figure 2 tTA transgenic mice. Mice transgenic for tTA crossed to mice transgenic for a tet-regulated gene produce progeny in which tetracycline suppresses transgene expression. Withdrawal of tetracycline leads to induction of transgene expression (green). Tissue specificity is conferred by the promoter driving tTA expression.

significant induction within hours of oral doxycycline administration. With tTA, transgenic changes in expression occurred over days, due to the reasons mentioned above. Transgene expression could be switched on and off repeatedly without adverse effect. Histological analysis revealed mosaic expression of β -galactosidase within tissues when tTA was under the control of a CMV promoter (Furth *et al.* 1994), but this problem has not been seen using a strong tissue-specific promoter (Kistner *et al.* 1996).

Tissue-specific promoters allow expression of the transactivator to be restricted to a particular tissue or cell type. Defining the site of expression of the transactivator determines the site of transgene expression, because the minimal promoter itself confers no tissue specificity. It should be noted that the expression pattern of a promoter can be strongly influenced by the chromosomal context in which it integrates. Therefore, it is important to screen for ectopic expression. This can be done by screening for expression of the transgene itself, or using transgenic reporter lines (e.g. β -galactosidase) which allow visualisation of the expression pattern. An alternative approach is to use a bi-directional minimal promoter to drive expression of both the transgene and the reporter in the same animal (Baron *et al.* 1995, Yamamoto *et al.* 2000). This avoids the need to cross to additional lines, and gives an indirect measure of transgene expression.

The fine regulation of transgenes afforded by this system has been used to great advantage in numerous studies. This is best exemplified by experiments where neonatal lethality has been by-passed by delaying expression to later

time-points. For example, overexpression of the BCR-ABL1 oncogene (BCR-ABL) during development is lethal. Huettner *et al.* (2000) used the tTA system to induce BCR-ABL expression in B-lymphocytes in adult animals, resulting in acute leukaemia. Silencing transgene expression allowed complete resolution of the leukaemia, a process that could be reversed many times. Conditional activation of toxic transgenes has also been used to ablate specific cell populations: Lee *et al.* (1998) conditionally expressed diphtheria toxin A chain in cardiac cells leading to cell ablation and progressive cardiomyopathy. By contrast, Efrat *et al.* (1995) conditionally transformed islet β -cells with SV40 T antigen creating pancreatic tumours from which cell lines were derived.

Additional regulatory control can be built into experiments. For example, Redfern *et al.* (1999) created a modified kappa opioid receptor that only recognises a synthetic ligand, spiradoline. This modified G protein-coupled receptor activates G_i signalling in response to spiradoline. Using tissue-specific promoters, enhanced G_i signalling was targeted to the heart, liver and salivary gland in separate transgenic lines. In long-term experiments, chronic G_i signalling led to dilated cardiomyopathy which was partially reversible (Redfern *et al.* 2000). Bond *et al.* (2000) recently described a method of using the tTA system to control native gene expression. In this way, a reversible cell-specific 'virtual knockout' was generated. By homologous recombination, a tetracycline-based control cassette was placed in the 5' untranslated region of the SK3 gene (encoding a subunit of a Ca^{2+} -activated K^+ channel). Expression of tTA was under the control of the native regulatory elements of SK3. Expression of SK3 itself was in turn contingent upon expression of tTA, and could be abolished by administering doxycycline. However, disruption of the native locus led to elevated expression of the conditional allele in the brain in the basal state, although the pattern of expression was not altered. This allowed them to either augment or switch off SK3 expression.

Despite these impressive studies, certain limitations are increasingly recognised, such as leaky expression, cellular toxicity, unstable transcripts and insensitivity to doxycycline in certain tissues (Baron *et al.* 1997, Urlinger *et al.* 2000a). It is hoped that developments such as the R_tTA2^s-M2 variant will circumvent these problems. In addition, it should be noted that the genetic background of mice can influence the occurrence of such difficulties (Zhu *et al.* 2001). Another drawback is the need to generate two separate transgenic lines for each study. Schultze *et al.* (1996) have used a single construct containing all the necessary regulatory sequences and transgene on one plasmid. Theoretically, this approach is less time consuming and avoids the segregation of control elements during breeding. However, the use of double transgenic lines is more flexible if one wishes to make use of several different tissue-specific transactivator lines to study the effects of a single transgene.

Figure 3 Ecdysone-regulated transgene expression. (a) In the absence of ponasterone A the modified EcR does not bind the ERE. (b) Ponasterone A activates transgene expression by inducing dimerisation of EcR and the RXR, allowing binding to the ERE and recruitment of transcription factors. Red, modified EcR; black bar, ERE; yellow, ponasterone A; blue star, RXR; green, transgene; orange, transcription complex.

The wealth of transactivators and tetracycline analogues available with different properties theoretically allows independent regulation of more than one transgene in a single animal using combinations of the tetracycline system. The feasibility of this has been demonstrated in cell lines (Baron *et al.* 1999) although not *in vivo*.

It should also be noted that the various components of this system are now commercially available, making it highly accessible.

Ecdysone-inducible overexpression

Ecdysone is a steroid hormone that triggers metamorphosis in insects. It mediates pleiotropic effects via a heterodimeric receptor, comprising the ecdysone receptor (EcR) and the product of the ultraspiracle gene (USP) which together bind ecdysone response elements (ERE) to enhance transcription of genes required for metamorphosis. No *et al.* (1996) have adapted this system for mammalian cells in a manner analogous to the *tet* system described above. A novel transactivator was generated by fusion of a truncated EcR with transcriptional activation domains of the glucocorticoid receptor and the herpes simplex virus VP16. This hybrid retains specificity for binding ecdysone and ERE, but has enhanced transcriptional activity. In addition, a novel ecdysone-specific response element was constructed that allowed inducible gene expression without cross reactivity with endogenous mammalian nuclear hormone receptors. The retinoid X receptor (RXR) is the mammalian homologue of USP, and functions effectively with the hybrid EcR (Fig. 3).

Experiments in transfected cells and transgenic mice have demonstrated the potential use of this system. Mice transgenic for hybrid EcR and RXR under the control of T-lymphocyte promoters were crossed with reporter mice bearing an ecdysone-inducible β -galactosidase transgene. Basal reporter expression was minimal, but could be rapidly induced by intraperitoneal injection of a synthetic ecdysone analogue, muristerone A. Muristerone has not been found to exert any teratogenic or physiological effects in mice. More recently, a widely available natural plant steroid, ponasterone A, has been shown to be a potent and innocuous inducing agent (Saez *et al.* 2000). The use of synthetic RXR ligands synergises with muristerone induction (Saez *et al.* 2000), although the use of such compounds may have unwanted physiological effects. Hoppe *et al.* (2000a) have simplified the system using a chimaeric *Drosophila/Bombyx* ecdysone receptor which transactivates transgenes without the need for RXR at all.

In vitro comparison of the ecdysone and tetracycline induction systems demonstrated lower basal expression, greater inducibility and more rapid induction kinetics with ecdysone (No *et al.* 1996). In the uninduced state, there may even be transcriptional repression of the transgene which makes this system less leaky than *tet*-based ones (Rossant & McMahon 1999). However, direct *in vivo* comparison has not been carried out.

So far this technology has been used most extensively in cell culture systems and examples of ecdysone-based transgenics are sparse. Albanese *et al.* (2000) have successfully targeted expression of an ecdysone-responsive β -galactosidase transgene to the mammary gland. Similarly, Saez *et al.* (2000) directed inducible luciferase expression to the skin with a human keratin 5 promoter. Other *in vivo* work has been limited to adenoviral-mediated transfer of ecdysone-inducible constructs to the heart and carotid bodies (Hoppe *et al.* 2000b).

Until further studies are reported, the advantages of this system cannot be evaluated. However, the benefit of having more than one inducible system available is the potential for combination transgenics with multiple independently regulated transgenes.

Cytochrome P-450 induction system

An inherent drawback of the systems described above is the requirement for doubly-transgenic animals. Use of endogenous mammalian gene regulatory systems can avoid this complexity. For example, the mouse metallothionein promoter has been used for many years to regulate transgene expression (Palmiter *et al.* 1983). Transcription at this promoter is induced by heavy metal ions such as zinc and cadmium. However, there is invariably high-level constitutive expression in a broad range of tissues, and the degree of transgene induction is relatively poor (Joseph *et al.* 1999). For these reasons, this system will not be

Figure 4 *Cyp1a1* promoter/enhancer regulation. (a) In the absence of inducing agent *cyp1a1* is transcriptionally silent. (b) Aryl hydrocarbons such as I3C induce *cyp1a1* gene transcription by binding cytoplasmic AHR, translocating to the nucleus and complexing with ARNT. Here the complex binds aryl hydrocarbon responsive elements (ARE) within the enhancer region leading to alterations in chromatin structure around the promoter which enhance transcription factor recruitment. Blue star, AHR; red star, ARNT; yellow, I3C; black bar, ARE; red, promoter; green, transgene; orange, transcription complex.

discussed further. In contrast, the cytochrome P-450 system is highly inducible and provides more stringent control of gene expression.

Cytochrome P-450 mono-oxygenases are enzymes involved in metabolising and detoxifying lipophilic xenobiotics (Gonzalez & Nebert 1990). Expression of some P-450 enzymes is directly regulated by the availability of their substrate. In other words, exposure to a specific compound will induce expression of the cytochrome responsible for its metabolism. One such cytochrome, CYP1A1 (encoding aryl hydrocarbon (benzo[a]pyrene) hydroxylase (E.C.1.14.14.1)) is induced by aryl hydrocarbons and the enhancer/promoter sequences of this gene have been used successfully for conditional transgene expression.

CYP1A1 is not expressed constitutively and it is regulated entirely at a transcriptional level (Ryu *et al.* 1996). Induction requires binding of aryl hydrocarbon compounds to aryl hydrocarbon receptors (AHR) in the cytoplasm. AHR–ligand complexes shuttle to the nucleus where they bind to the aryl hydrocarbon nuclear translocator (ARNT), forming a heterodimer–ligand complex. Both AHR and ARNT are ligand-dependent basic helix–loop–helix transcription factors that bind aromatic hydrocarbon-responsive elements (AhREs) several hundred base pairs upstream of the *cyp1a1* promoter (Fig. 4). AhRE binding induces chromatin remodelling at

the promoter which favours transcription (Elferink & Whitlock 1990, Fujii-Kuriyama *et al.* 1995, Okino & Whitlock 1995). Chromatin structure is also important in maintaining transcriptional silence in the uninduced state (Okino & Whitlock 1995). AHR and ARNT are expressed predominantly in the liver (Dolwick *et al.* 1993, Li *et al.* 1994), and both are essential for *cyp1a1* induction (Ryu *et al.* 1996, Foussat *et al.* 1998, Zaher *et al.* 1998, Tomita *et al.* 2000).

This system has been characterised in transgenic mice and rats using various portions of the *cyp1a1* enhancer promoter. Small parts of the promoter region fail to control transgene expression adequately (Jones *et al.* 1991, Shachter *et al.* 1994, Smith *et al.* 1995a) whilst more extensive portions which include all the known enhancer/promoter sequences allow tightly regulated expression (Campbell *et al.* 1996, Kantachuvesiri *et al.* 2001). As for any transgene, the site of chromosomal integration has a profound effect on the level of baseline expression: many transgenic lines may have to be screened in order to identify a suitable one. Transgene expression can be induced in a dose-dependent manner as much as 10 000-fold (Jones *et al.* 1991). Expression is predominantly in the liver, but also adrenal, kidney, intestine, spleen, lung, pancreas, skin and the reproductive organs (Campbell *et al.* 1996, Kantachuvesiri *et al.* 2001). Within each tissue, expression is regulated in a cell-type specific manner (Campbell *et al.* 1996). The widespread expression reflects the tissue distribution of AHR and ARNT: this may be less than ideal for some applications.

In our laboratory, we have used the rat *cyp1a1* enhancer/promoter sequences to control mouse *Ren2* cDNA (encoding renin) expression in rats (Kantachuvesiri *et al.* 2001). This has created an inducible model of hypertension. Background expression levels are undetectable in non-induced rats. Blood pressure can be titrated in a dose-dependent manner to produce either malignant or chronic phase hypertension. Furthermore, the renin-angiotensin system and blood pressure return to normal in the absence of inducer, making this an extremely flexible model for studying hypertension, vascular injury and repair.

One prerequisite for a useful regulatory system is that the inducer must be innocuous, and that spillover activation of other loci is inconsequential. Most AHR-binding compounds have deleterious effects, but indole-3-carbinol (I3C) is an exception. This is a naturally occurring aryl hydrocarbon found in brassicae. It has anticarcinogenic, antioestrogenic, hypocholesterolaemic and immunomodulatory effects which are poorly understood (Dunn & LeBlanc 1994, Bradlow *et al.* 1999, Exon & South 2000). I3C itself is a weak inducer of *cyp1a1* but derivatives formed in the stomach are much more potent: up to 24 different active metabolites have been found in rats fed I3C (Stresser *et al.* 1995). Since it is readily absorbed and metabolised, induction of *cyp1a1* occurs within hours

(Stresser *et al.* 1995). It also induces other members of the aryl hydrocarbon battery including CYP1A2, CYP2B1/2 and CYP3A1/2, and inhibits both the activity and expression of flavin mono-oxygenase 1 (Katchamart *et al.* 2000): thus, metabolism of other xenobiotics is undoubtedly affected and this may interfere with pharmacological studies. Furthermore, *in vitro* studies on a hepatoma cell line indicated that induction of CYP1A1 with an I3C derivative can lead to oxidative DNA damage (Park *et al.* 1996). Despite these multiple effects, chronic oral administration in adult rats and mice has been shown to be safe.

The simplicity and robustness of this system make it eminently suitable for overexpression of secreted soluble proteins. However, limited tissue specificity restricts its broader application.

Conditional genetic deletion: somatic cell mutagenesis

Embryonic lethality in many conventional gene knockouts impedes attempts to study gene function in older animals. One way of avoiding this is to ablate specific genes at later stages of development or adulthood using recombinases. Two members of the α integrase family of site-specific recombinases, Cre and Flp, have proved invaluable for conditional transgenic use.

Cre (causes recombination) recombinase of the P1 bacteriophage directs recombination between *loxP* (locus of crossover) sites. Its function is to maintain phage-encoding plasmids as monomers. In a similar manner the Flp integrase of *Saccharomyces cerevisiae* mediates recombination between FRT (FLP recombination target) sites within yeast plasmids (Kilby *et al.* 1993). In each case, the only requirements for DNA rearrangement are the integrase and the recombination sites: no additional cellular factors are necessary. Both *loxP* and FRT sites are 34 bp DNA sequences comprising two 13 bp palindromes separated by an asymmetric 8 bp core. The recombinases catalyse DNA strand exchange between two aligned recombination sites, resulting in deletion, duplication, integration, inversion or translocation of sequences, according to the orientation of the recombination sites and the number of molecules involved (Fig. 5).

Application of these techniques in mice allows deletion of genetic material in selected cells at a specific time. In this way, one can avoid the complications of embryonic lethality and developmental compensatory changes. At its most simple, this requires incorporation of two recombination sites within a gene of interest, and then expression of a recombinase in only those cells in which one wishes deletion to occur. Because this technology relies on homologous recombination in embryonic stem cells, its use is restricted to mice.

Figure 5 Cre/Flp-mediated recombination reactions. Site-specific recombinases mediate inter- and intramolecular recombination reactions leading to deletion, duplication, inversion or integration events.

Cre-lox system

Modification of a specific gene with *loxP* sites flanking the region of interest is achieved using standard gene targeting vectors in embryonic stem (ES) cells. Such an allele is said to be 'floxed' (flanked by *loxP*) and depending on the exact position of the *loxP* sites and selection markers, may or may not be fully functional. Mice derived from these targeted ES cells can be bred to homozygosity for the floxed allele, and crossing with other mice transgenic for Cre recombinase under the control of a specific promoter allows time- or tissue-specific deletion of the floxed segment (Fig. 6). In this way, the activity of the gene can be modified in a limited range of cells at a particular developmental stage, whilst the genetic content of the rest of the animal is essentially unaltered. Crossing to a different Cre transgenic mouse line allows the generation of a different temporo-spatial pattern of recombination, and possibly phenotype (Fig. 6).

This strategy has been used extensively since Lakso *et al.* (1992) first described the Cre-mediated activation of an SV40 T antigen transgene in lens cells leading to tumour formation. Tissue specificity is determined by the choice of promoter driving Cre recombinase expression. Many tissue-specific Cre transgenic and floxed mice strains are

becoming available, and a database cataloguing such animals is available at <http://www.mshri.on.ca/nagy/cre.htm>: from this it can be seen that many important endocrine tissues can be selectively targeted, although the list is far from complete. Tissue-specific Cre-lox techniques have yielded unexpected insights into endocrine physiology in recent years. Table 2 gives published examples of Cre-loxP-mediated gene deletion in endocrine tissues or target tissues. Cre-lox strategies can also facilitate cell fate-mapping studies. Using Cre recombination to genetically tag the progeny of pancreatic stem cells, Herrera (2000) demonstrated separate developmental lineages for α and β cells.

Ideally one would wish to control not only the site of recombination but also the timing. Several strategies have been designed to address this, using inducible forms of Cre recombinase. Fusion of the ligand-binding domain (LBD) of a mutated oestrogen receptor to Cre results in a hybrid that is active in the presence of tamoxifen, but not endogenous steroids (Metzger *et al.* 1995). The LBD allows sequestration of hybrid Cre in the cytosol by heat-shock proteins. Tamoxifen liberates the recombinase, allowing recombination to proceed at a specified time in tissues expressing Cre. Similar hybrids have been developed using modified progesterone and glucocorticoid

Figure 6 Tissue-specific gene ablation using Cre-lox. Mouse lines expressing Cre (blue) in a tissue-restricted manner, such as the brain or liver, can be crossed to a line containing a floxed allele. Cre-mediated recombination results in allele deletion in the brain or liver alone, leading to altered function (red).

receptor LBDs, responsive to RU486 and dexamethasone respectively (Kellendonk *et al.* 1996, Brocard *et al.* 1998). Validation of ligand-activated Cre in transgenic mice demonstrated no detectable recombination in the absence of tamoxifen (Feil *et al.* 1996) but almost 100% recombination in Cre-expressing cells after 3 days of exposure (Brocard *et al.* 1997). However, in some cases incomplete and ligand-independent recombination have been observed (Schwenk *et al.* 1998, Kellendonk *et al.* 1999). New forms of tamoxifen and RU486-inducible Cre are now available with enhanced ligand sensitivity and low background activity (Indra *et al.* 1999, Kuhbandner *et al.* 2000, Imai *et al.* 2001, Wunderlich *et al.* 2001). Thus ligand-activated Cre shows great promise for controlling

the timing of recombination. Since tamoxifen is only given for a brief time, no lasting complications arise, and it has even been used with pregnant mice (Danielian *et al.* 1998).

An alternative strategy for inducing Cre activity is to use an inducible promoter. Tetracycline-regulated expression of Cre has been used by several groups. For example, Shin *et al.* (1999) successfully ablated endothelin B receptors (EDNRB) in mice at various time-points and were able to define a critical developmental period in which EDNRB is essential. Tetracycline-inducible tissue-specific knockouts are also possible (Saam & Gordon 1999, Utomo *et al.* 1999). Kuhn *et al.* (1995) have used an interferon-inducible promoter to control Cre expression,

Table 2 Studies using Cre-loxP-mediated gene deletion in endocrine tissues or target tissues

	Tissue-specificity	Phenotype	Reference
Disrupted gene			
<i>C/ebpa</i>	Liver	Impaired gluconeogenesis and bilirubin metabolism	Lee <i>et al.</i> (1997)
Glucocorticoid receptor	Glia	Altered HPA regulation Reduced anxiety behaviour	Tronche <i>et al.</i> (1999)
Glucokinase	Liver	Defective glycogen synthesis Impaired insulin secretion	Postic <i>et al.</i> (1999)
	Pancreatic β cells	Fatal hyperglycaemia	
<i>GLUT4</i>	Adipose tissue	Hepatic and skeletal muscle insulin resistance	Abel <i>et al.</i> (2001)
<i>IGF1</i>	Liver	Skeletal muscle insulin resistance Normal postnatal growth	Yakar <i>et al.</i> (2001) Yakar <i>et al.</i> (1999)
Insulin receptor	Pancreatic β cells	Impaired insulin release: impaired glucose tolerance	Kulkarni <i>et al.</i> (1999)
Insulin receptor	Skeletal muscle	Altered fat metabolism Preserved glucose tolerance	Brüning <i>et al.</i> (1998)
<i>PTHrP</i>	Global	Perinatal lethality: aberrant endochondral bone formation	He <i>et al.</i> (2001)
<i>RXRα</i>	Adipose tissue	Obesity resistance Impaired lipolysis	Imai <i>et al.</i> (2001)
SF1	Anterior pituitary	Hypogonadotrophic hypogonadism	Zhao <i>et al.</i> (2001)

and this has been used successfully to delete the low density lipoprotein receptor-related protein in hepatocytes (Rohlmann *et al.* 1998). Adenoviral delivery of Cre can also be used to determine the timing of recombination in certain tissues: after systemic delivery most recombination occurs within the liver, none in the central nervous system, and intermediate levels in other tissues (Wang *et al.* 1996, Akagi *et al.* 1997). However, immune responses to adenovirus limit the permanence of Cre expression (Akagi *et al.* 1997).

Cre-*lox* has been used most extensively for small-scale DNA rearrangements affecting single loci. However, it can also orchestrate chromosomal rearrangements, such as deletion of large regions, or translocations (Smith *et al.* 1995b, Clerc & Avner 1998, Buchholz *et al.* 2000, Collins *et al.* 2000, Su *et al.* 2000, Zheng *et al.* 2000). Deletion between *loxP* sites in *cis* can extend up to at least 60 centimorgan (cM) in ES cells *in vitro*. *In vivo*, a cardiac-specific 2 cM deletion has been demonstrated (Zheng *et al.* 2000). Such manipulations are very attractive for loss of heterozygosity studies and screens for tumour suppressor genes. Both deletion and duplication of regions within the *Hox* gene cluster has been achieved by inducing unequal exchanges between homologous chromosomes at *loxP* sites (Herauld *et al.* 1998). Such exchanges can be engineered to cause whole chromosome loss by generating acentric/dicentric chromosomes (Lewandoski & Martin 1997). Anti-parallel *loxP* sites have been used to invert regulatory elements within the *HoxD* complex, resulting in dysregulation of limb and gut development (Kmita *et al.* 2000).

It is clear from most Cre-*lox* mouse experiments that there are several potential pitfalls. First, expression of Cre within a particular cell type or tissue is rarely uniform, leading to mosaic recombination. In certain instances this may be desirable or at least inconsequential, in so far as it models random somatic mutagenesis (e.g. carcinogenesis). However, for some physiological studies, even remnant levels of wild-type product may not be acceptable. Determining the extent of recombination is essential for interpreting experiments. In tissues of heterogeneous cell type, Southern blotting may be an inadequate method of analysis because it does not indicate the cell type in which recombination has occurred. *In situ* PCR for the floxed allele or immunohistochemistry for Cre expression are alternatives that give some indication of cell specificity (Selbert *et al.* 1998). Reporter strains for Cre activity such as ROSA26- β *geo* (Mao *et al.* 1999, Soriano 1999) in which an activatable floxed β *geo* has been knocked-in to the ubiquitously expressed ROSA26 locus, give a clear indication of the sites and extent of recombination for a particular Cre transgenic. Alternative reporter strains with activatable enhanced green fluorescent protein (GFP) are also available (Kawamoto *et al.* 2000, Mao *et al.* 2001). 'Double-reporter' lines have been developed that constitutively express *LacZ* throughout development and adult-

hood: Cre-mediated recombination inactivates the *LacZ* transgene whilst activating a second reporter such as GFP or alkaline phosphatase (Lobe *et al.* 1999, Novak *et al.* 2000). A major assumption of reporter strains is that they accurately reflect recombination for other floxed loci. However, recombination efficiency is known to vary between different target loci (Holzenberger *et al.* 2000a) so results obtained in this manner can only be a guide. One solution is to design the initial targeting vector in such a way that recombination leads to activation of a reporter gene (Kmita *et al.* 2000, Moon & Cappechi 2000). This has the advantage of giving a direct read-out of recombination efficiency within the animal under study.

A second problem is that of transcriptional interference by vector-derived sequences, such as the *neo* selection cassette, or even the *loxP* sites. Tronche *et al.* (1999) found that the floxed glucocorticoid allele gave rise to levels of expression twice as high as wild type, whilst others have found underexpression (Fiering *et al.* 1995, Olson *et al.* 1996, McDevitt *et al.* 1997, Nagy *et al.* 1998, Holzenberger *et al.* 2000a,b). Such alleles are referred to as 'knockdown' or 'hypomorphic'. Intermediate expression levels may be informative (Meyers *et al.* 1998, Holzenberger *et al.* 2000b), but this is unpredictable. Cryptic splicing of plasmid sequences can also create abnormal transcripts (Yu *et al.* 1998) which may have unexpected effects (Meyers *et al.* 1998, Forlino *et al.* 1999). Vectors can be designed to allow the removal of unwanted plasmid sequences from cultured ES cells prior to blastocyst injection (Xu *et al.* 1999). However, the extended period of culture required may theoretically interfere with ES cell pluripotency. Alternatively, mice expressing Cre in the testes have been used successfully for excision of floxed plasmid sequence in germline chimaeras (O'Gorman *et al.* 1997, Blume-Jensen *et al.* 2000, Holzenberger *et al.* 2000a).

In most circumstances, mammalian cells appear surprisingly tolerant of Cre recombinase expression and DNA rearrangements. However, several studies have indicated that Cre can act efficiently at endogenous pseudo-*loxP* sites identified within mammalian genomes (Thyagarajan *et al.* 2000). Schmidt *et al.* (2000) found that mice expressing Cre in spermatids were sterile due to gross chromosomal rearrangements that may have been caused by this mechanism. Although this problem has not been reported in somatic cells its occurrence may have less dramatic consequences and it has therefore escaped attention. For this reason it would seem sensible to restrict Cre expression as far as possible: one approach has been to design self-excising Cre cassettes (Bunting *et al.* 1999).

Despite these caveats, Cre-*lox* technology has revolutionised genetic analysis of mice and is now well established as a routine genetic tool. It should be considered the first choice for conditional gene deletion experiments.

FLP recombinase

The uses of Flp mirror those already established for Cre; conditional gene knockouts, transgene activation, selection cassette removal, chromosomal rearrangement and mapping studies. The same cautions regarding mosaicism and ectopic expression are also pertinent. However, use of Flp in transgenic mice is at a less advanced stage than that of Cre and a direct comparison between the two is not possible at this time.

Dymecki (1996) has demonstrated dose-dependent Flp-mediated recombination in a FRT-*lacZ* reporter strain, and elegantly used *Wnt1* promoter-driven Flp to fate-map neuronal migrations in the developing brain (Dymecki & Tomasiewicz 1998, Rodriguez & Dymecki 2000). Exhaustive analysis of the pattern and extent of Flp expression/recombination events demonstrated high levels of recombination (>90%) in a highly restricted cell-type specific pattern (Rodriguez & Dymecki 2000). Vooijs *et al.* (1998) conditionally ablated the retinoblastoma gene in anterior pituitary cells, leading to adenoma formation. In this study, a modified form of Flp was used, and the efficiency of recombination was far less than 100%.

Concern that the recombination efficiency of Flp is inferior to that of Cre has been allayed somewhat since the development of an enhanced, mutant form (FLPe) (Buchholz *et al.* 1998). This exhibits fourfold greater activity than wild type, and maximally excises a target gene in a broadly expressing reporter line (Rodriguez *et al.* 2000). No ubiquitously expressing reporter lines for Flp are currently available, and this is another disadvantage compared with Cre.

As for Cre, an oestrogen-LBD-Flp hybrid has been developed to allow rapidly inducible recombination activity (Logie & Stewart 1995, Logie *et al.* 1998). However, activity of the hybrid is only 50% that of the wild-type enzyme.

Use of Flp and Cre in combination allows sophisticated manipulation of loci: for example, removal of unwanted plasmid sequences from a conditional allele at the ES cell stage with Flp/FRT, and subsequent deletion by Cre *in vivo* (Moon & Cappechi 2000). Meyers *et al.* (1998) ingeniously used both to create an allelic series at the *Fgf8* locus. A floxed hypomorphic allele could be knocked out completely using conditional Cre, or reverted back to wild type using Flp. One can easily envisage many more situations where multiple simultaneous or sequential conditional mutations are created using recombinase combinations, either at single or multiple loci. However, whilst Flp/FRT is an increasingly attractive system, it does not yet have the track record of Cre-*lox*.

Discussion and Conclusions

The phenotypic modification of monogenic disorders by the genetic background of inbred laboratory animals has

been recognised for many years. Under identical environmental conditions, such observations may be attributable to one or more 'modifier loci' that increase or decrease the severity of a phenotype (Nadeau 2001). Formal identification and analysis of modifiers may provide a novel insight into the biology of a disease, for which there are numerous examples in the literature (Kantachuvesiri *et al.* 1999, Nadeau 2001).

Genetic background is of great importance in conditional transgenic experiments because it may influence not only the primary pathology but also the efficiency of the conditional system itself. For example, evidence already suggests that the regulation of the rtTA system differs between mouse strains (Zhu *et al.* 2001). In a similar manner, one might anticipate that the efficiency of Cre recombination is strain dependent, although this has not been formally investigated. Complications of this nature may have unpredictable and misleading consequences, particularly when different conditional models on different inbred backgrounds are intercrossed (genetic multiplexing). For this reason every effort should be made to maintain pure genetic backgrounds.

The availability of reliable conditional transgenic technologies is set to revolutionise our understanding of physiology and disease. This comes at an opportune time, when advances in genome sequencing, array technology and miniaturised physiological assessment will provide both the raw material and the means for analysis. Conditional techniques should be seen as supplementing rather than supplanting conventional transgenesis, which remains an invaluable tool.

Theoretically one may choose to overexpress or delete genes in specified tissues at will. Choosing between the available systems depends very much on the goals of the experiment and available expertise. However, some systems have well-established track records and should probably be the first choice unless there are special experimental considerations. In practice, these experiments are a considerable investment of time and resources without guarantee of success. Problems still remain to be resolved, such as mosaic, ectopic and leaky expression, necessitating careful characterisation and quantification of transgene expression patterns (e.g. using reliable reporter lines or built-in reporters). With the growing availability of complementary transgenic lines (e.g. Cre/Flp, floxed/FRT, tet/ecdysones) convenient inter-crossing experiments of ever greater complexity will be possible.

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