Tetracycline-controlled transcriptional regulation systems: countermeasures to eliminate basal transgene leaks in Tet-based systems*

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Abstract To analyze the function of any given transgene(s) accurately in transgenic mice, and to produce credible transgenic animal models of various human diseases (precisely and realistically mimicking disease states), it is critical to be able to control gene expression in the animals conditionally. The ability to switch gene expression "on" or "off" in the restricted cells or tissue(s) at specific time(s) allows unprecedented flexibility for exploring gene function(s) in both the health and the disease. Pioneering work on inducible transgene expression has led to the development of a wide variety of controlled gene expression systems that meet this criterion. Among them, the tetracycline-inducible systems (e.g. Tet-off and Tet-on) have been widely, frequently and successfully employed *in vitro* and *in vivo*. These systems, however, are not always tight but leaky; sometimes the leakage is significant. In some circumstances, the resulting leak is acceptable, but in others, it is more problematic. Though these systems face this disadvantage, i.e. basal transgene leakage *in vitro* and *in vivo*, several approaches, including using improved versions (e.g. rtTA2^S-M2 and rtTA2^S-S2) of rtTA, tetracycline-controlled transcriptional silencer (tTS), an "ideal" minimal promoter in responsive components or combinations thereof, have been developed to avoid this limitation effectively. In this review we discuss the countermeasures available to eliminate basal transgene leakage from Tet-based systems.

Keywords: tetracycline inducible system, transgenic mice, leaky expression, transcriptional silencer.

Conditional transgenic mouse models are becoming increasingly popular for controlling gene expression precisely in a spatiotemporal fashion. The ideal conditional overexpression system should allow the researches to turn transgene expression on and off, rapidly and reversibly, exclusively in the desired cell or tissue type at any time point during development or postnatal life. These criteria are fully met by the tetracycline-controlled transcriptional regulation systems[1,2]. There are two basic variants: the tTA ("Tet-off") system[1] and the rtTA ("Tet-on") system[2]. These systems, especially "Tet-on" system, have been most widely, most frequently and most successfully used in transgenic mouse modeling[3—7].

The tetracycline-controlled expression systems have been used successfully in many laboratories around the world to define the development-dependent and development-independent biological and pathological processes[4—7]. Despite these impressive studies and the intriguing properties of these systems, certain limitations of tetracycline (Tet)-based externally regulatable systems are increasingly being recognized, particularly in transgenic animals, such as leaky expression, cellular toxicity, instability and insensitivity to doxycycline (Dox) of reverse tetracycline-controlled transactivator (reverse tTA, rtTA) in certain tissues[8,9].

This review will focus on the countermeasures available to suppress or eliminate basal transgene leaks in Tet-based systems in transgenic mouse modeling. Because of editorial limitations, the article is not intended to be comprehensive, and the readers are encouraged to explore other reviews for a basic knowledge of tetracycline-controlled expression systems[4—9].

1 Leaky expression in Tet-based systems

Ideally, the Tet-on system should give negligible levels of transgene expression when no tetracycline

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(Tc) analogue has been administered or high levels after Dox induction. Unfortunately, this ideal is not always realized. Depending on the experimental conditions employed and the site(s) of integration of the transgenic construct(s), enhancer sequence(s) near the target gene(s) have been repeatedly demonstrated to increase basal expression of the given gene, thereby compromising the expected tight regulation[4—9]. This promoter leakiness, which can be caused by promoter-dependent or integration site-dependent effects, has been discussed in detail[10].

More importantly, however, detailed analyses of Tet-based systems have revealed intrinsic weaknesses that strictly limit their range of application under some conditions[5,6,9,11]. One major weakness is attributed to the fact that the regulatory protein rtTA retains some affinity for *tetO* sequences even in the absence of Dox. This is evident from the detectable expression levels of transgene activation and phenotype induction in animals or cells which are not receiving Dox[5,6,9,11]. It may not be acceptable (e.g. expression of toxins) and is certainly not acceptable in the regulatable transgenic mouse model for hepatitis[12](see below). The second recurring disadvantage of Tet systems is the undesired residual activity of the *tetO*-CMV responder even when an effector, e.g. active tTA or active rtTA without, is absent[5,6,9,11].

In some circumstances, the leak is acceptable. In others, however, the leakage is more problematic. For example, if the product of the transgene under investigation is toxic or unwanted, even a low level of expression may be detrimental to embryos, preventing any further analysis of the potential phenotype during late antenatal development or in the infancy and adult. In addition, a few years ago, transgenic mice that express hepatitis B virus (HBV) envelope proteins and hepatitis C virus (HCV) core protein in a Tet-inducible manner (Tet-off system) were produced in Chisari's lab. Although the transgene expression in these transgenic mice was undetectable in utero or during early postnatal life, the animals remained tolerant to HBV envelope proteins and HCV core protein when they were immunized as adults (Chisari, 2003, personal communication). Therefore, Chisari assumed that the system leaks very slightly; the amount of leakage was not enough to detect biochemically but sufficient to induce immune tolerance. Furthermore, transgenic mice expressing HCV core protein (HCV-C) in a liver-specific and time-dependent manner mediated by the Tet-on system were also generated in Chen's laboratory at Sun Yat-sen University[13]. Our current data clearly demonstrate leaky HCV-C transgene expression in both TRE-HCV-C single transgenic mice and apoE-rtTA/TRE-HCV-C bi-transgenic mice when the inducer Dox is absent; after exposure to Dox, the significant pathological changes normally found in the liver were not observed in the double transgenic mice (unpublished observations from Chen's laboratory at Sun Yat-sen University). Moreover, no liver damage was detected in transgenic mice in which the HCV-C, E1, E2 and NS3 genes were expressed under the control of the heavy metal-inducible MT-1 promoter, because this promoter shows high basal activity in the absence of induction[14,15]. The transgenic animals for both HBV and HCV were derived from non-tight gene expression regulatory systems (see above; Table 1), and HBV or HCV transgenic mice produced from constitutive gene expression systems (Table 1) are not immunocompetent for the transgene product(s), e.g. viral antigen(s). Therefore, the immune system of the organism after birth cannot recognize the xenobiotic nature of these viral antigen(s). Actually, the immune system plays rather important roles in the pathogenesis of hepatitis. It is clear that such "leaky" systems are not suitable for modeling complicated disease processes (such as hepatitis) accurately and realistically or for assessing the effects of a gene product after the disease process is initiated[12].

Table 1. Hepatitis C virus transgenic mice

Promoter/ System	Transgene	Characteristics	Reference
Alb	C-terminally truncated E2	No pathological changes in the liver	[16]
Alb and MUP	Core, E1, E2	No liver damage	[17]
ApoE	NS5A	No histological change	[18]
MT-1	Core, E1, E2	No liver damage	[14,15]
MT-1	NS3	No liver damage	[14,15]
MT-1	Core, E1, E2, NS3	No liver damage	[14,15]
MSG	NS3	No abnormal phenotype	[12]
MUP	Core	No liver damage	[16]
Regulatory element from HBV	E1, E2	No evidence of liver tissue pathology	[19]
Cre/*lox* P system	Core, E1, E2, NS2	A powerful tool for investigating the immune responses and pathogenesis of HCV infection	[20]

Alb, the mouse albumin promoter; ApoE, human liver-specific apolipoprotein E promoter; MSG, an dexamethasone-inducible promoter; MT-1, mouse metallothionein-1 promoter; MUP, the mouse major urinary protein promoter

Though these systems and other rtTA-based systems face this disadvantage, i. e. basal transgene leakage *in vitro* and *in vivo*, several approaches have been developed to avoid this limitation effectively (see below). Many new variants of the Tet-based regulatory and responsive elements that can minimize or eliminate leaky transgene expression have been designed with specific properties and tested successfully, especially *in vitro* (see below).

2 Countermeasures to eliminate basal transgene leak in Tet-based systems

2.1 Improved versions of rtTA (rtTA2^S-M2 and rtTA2^S-S2)

One approach is to seek improved versions of rtTA by screening a series of tTA mutants to identify those with less (or even zero) basal activity and with enhanced sensitivity to the inducer Dox. This approach led to the identification of new rtTA mutants (e.g. rtTA2^S-M2 and rtTA2^S-S2)[21] that are more susceptible to Dox and showed improved transcript stability and a broader range of induction, with significantly lower basal activity than rtTA itself in stably transfected cell lines. Generally, cell lines carrying rtTA2^S-M2 or rtTA2^S-S2 demonstrate reduced basal activity in the absence of Dox and higher activation levels in its presence compared to an rtTA line[21—24]. In addition, rtTA2^S-S2 has lower background activity than rtTA2^S-M2 *in vitro*, but rtTA2^S-M2 is more responsive to Dox than rtTA2^S-S2[21,23,24]. After delivery of plasmids into the adult mouse quadriceps muscle by *in vivo* DNA electroinjection, both rtTA2^S-M2 and rtTA2^S-S2 showed considerably lower basal activity and higher windows of induction than rtTA *in vivo*[23]. A Dox-regulated lentiviral vector system with rtTA2^S-M2[25,26] and a Dox-controlled helper-dependent adenovirus vector with rtTA2^S-S2[27] also showed tight control of gene expression *in vitro*, *ev vivo* and *in vivo*. Stringent control of transgene expression using improved versions of rtTA, for example rtTA2^S-S2[28,29] and rtTA2^S-M2[30,31], has also been achieved in transgenic mice for Cre recombinase[28], ferritin H[29], pigment epithelium-derived factor (PEDF)[30] and lac Z[31]. Collectively, these results show that the phenotype of rtTA can be improved and designed by using appropriate screens. However, the utility of these new forms of rtTA in transgenic animals has yet to be demonstrated.

It should also be noted that rtTA2^S-S2 is toxic in mouse embryonic stem cells (ES cells) and that ES cells expressing rtTA2^S-S2 cannot grow in the presence of G418[32].

2.2 Tetracycline-controlled transcriptional silencer (tTS)

Another method is to generate an active transcriptional suppressor that can be used in combination with the rtTA system to suppress basal transgene leakage while allowing normal or high induction of the transgene by rtTA. To this end, Tet-repressors have been designed. Two constructs, tTR[11] and tTS^{kid}[33], are available in which the repressor domain of tetR has been substituted with a mammalian equivalent. These repress the transcriptional activity at the *tetO* sequences in the absence of Tc analogs[33,34]. This strategy appears to be more versatile in dealing with unwanted target gene expression than the promoter adaptation proposed below.

The tTS^{kid} construct is specially designed for use with the Tet-on system and prevents unregulated gene expression when Dox is absent[33,35]. The silencer tTS, a transcriptional repressor and a hybrid protein, is a fusion of TetR and the KRAB-AB domain of the Kid-1 protein[33,35]. Since the TetR portion of tTS is similar in function to that of tTA, tTS can recognize and subsequently bind to *tetO* sequences in the TRE in the absence of inducer. Because it has a silencing domain, tTS actively silences, instead of activating, transcription of the target gene when bound. Theoretically, therefore, no transgene product is produced without induction. When a sufficient concentration of Dox is present, tTS undergoes a conformational change and dissociates from *tetO*, relieving the transcriptional suppression and allowing rtTA to bind to *tetO* sequences, which then activate transcription of the gene under investigation.

A more stringently controlled regulatory system can be achieved by combining a trans-silencer such as tTS with rtTA or its new improved versions, e.g. rtTA2^S-M2 and rtTA2^S-S2. The two transregulators bind in a mutually exclusive manner. This efficiently reduces or completely eliminates background expression in yeast[36], in mammalian cells[8,22,25,35,37—43], in transgenic mice[44,45], and in mice in which tTS tightly controls transgene expression by delivering

combinations of the Dox-regulated target gene construct, rtTA/rtTA2^S-M2/rtTA2^S-S2, and tTS expression plasmids[46,47] or virus vectors[40,42,43,48,49] into tissues using *in vivo* plasmid and viral vector electrotransfer; the maximal expression level is affected only slightly[22] or not at all[33].

Combined tTS and rtTA systems have already been shown to confer precise regulation of target genes *in vitro* (see [8, 33, 35]), but they are not well characterized in transgenic mouse modeling (see above and below). Since tTS in combination with rtTA actively suppresses background expression or eliminates "leakiness" without imparing the inducibility of target gene *in vitro* (see [8, 33, 35]), providing a true "on/off" transgene switch, this approach might also be employed to control basal transgene leakage in transgene systems *in vivo*. Interestingly, Zhu's experiments in transgenic mice provided the first convincing evidence for tTS-mediated repression that completely suppresses the transcription of a given gene without compromising rtTA-mediated activation: tTS completely eliminated transgene leakiness in the mouse lung[45].

A detailed comparison of CC10-rtTA/tTS-IL-13 with CC10-rtTA-IL-13 mice showed that tTS completely suppresses basal expression of IL-13 and the IL-13-mediated phenotype in the absence of Dox induction, but does not alter the ability of Dox to increase IL-13 expression or induce a full-blown IL-13 phenotype. More importantly, in the presence of Dox, tTS suppression was fully relieved, allowing rtTA to activate IL-13 transcription and leading to an IL-13-induced phenotype in the lung[45]. It is therefore reasonable to believe that the triple transgene CC10-rtTA-tTS system can be used to define the natural history of *in vivo* injury and repair responses accurately and with a level of precision that has not previously been attainable. Further proof of the usefulness of tTS technology *in vivo* was obtained in Elias's lab (for more details, see Ref. [11]). In other words, in transgenic mouse modeling, the optimal "off/on" regulation of transgene expression can be achieved by the combined use of tTS and rtTA or its more advanced variants.

On the basis of the combined use of tTS and rtTA in transgenic mouse modeling in Elias's laboratory, the transgenic fragment of apoE-rtTA-tTS (apoE-rtTA-tTS: rtTA and tTS fragments amplified by PCR and cloned into the same vector of pLiv. 7 containing the apoE promoter to prepare the rtTA and tTS expressing vector, designated pApoE-rtTA-tTS) has been employed to generate apoE-rtTA-tTS transgenic mice (unpublished data from Chen's laboratory at Sun Yat-sen University), which will be anticipated to express rtTA-tTS protein actively suppressing the basal leakage of HCV-C in apoE-rtTA-tTS/TRE-HCV-C bi-transgenic mice, but whether the addition of tTS to the Tet system can completely eliminate both detectable and undetectable leakiness from Tet-based systems in apoE-rtTA-tTS/TRE-HCV-C transgenic mice remains to be elucidated.

Combined use of tTS and rtTA systems in transgenic mouse modeling requires the generation of another transgenic strain harboring the tTS transgene under the control of a ubiquitous promoter or a cell-type/tissue-specific promoter. tTS transgenic mice are mated with bi-transgenic mice (e.g. rtTA/target gene mice) to produce triple transgenic offspring, allowing tight, reversible, quantitative and spatiotemporal control of transgene expression *in vivo* through addition or non-addition of Dox via the food or drinking water. Although an obvious disadvantage of this approach is the need to generate triple transgenic animals, simultaneous microinjection of CC10-rtTA, CC10-tTS and tetO-TGF-b1 constructs successfully generated an inducible TGF-b1 mouse model in Elias's laboratory (for more details, see Ref. [11]). Moreover, there is the alternative of combining the two control elements (i.e. rtTA and tTS) in a single transgene under control of a ubiquitous promoter or a cell-type or tissue-specific promoter to produce rtTA-tTS transgenic mice, e.g. apoE-rtTA-tTS transgenic mice (unpublished data from Chen's laboratory at Sun Yat-sen University)], but whether this "two-in-one" system is functional in transgenic mice remains to be fully demonstrated. This "two-in-one" or "all-in-one" strategy has also been confirmed *in vitro*[25,37,40,43] (see 2.4, below, for more details).

Moreover, tTS, which specifically inhibits the *tetO*-modified allele and does not affect neighboring genes, can be used to control transcription tightly in a *tetO*-modified mouse gene *in vivo*; transcription is completely blocked in the absence of Dox but restored when the antibiotic is administered to the animal[44].

In summary, although the evidence for improved performance by combining the rtTA and tTs systems in transgenic mice is still preliminary, these studies demonstrate that the incorporation of tTS into rtTA-

based externally regulatable overexpression transgene systems greatly optimizes the regulation of transgene expression *in vivo*. By effectively eliminating the baseline transgene leak and phenotype induction without altering the Dox-inducibility of rtTA-regulated transgene(s), tTS may convert the rtTA-based system from one with low leaky expression and high levels of transgene expression to one with true "off" and "on" regulation. In transgenic mouse modeling, this "off/on" regulation will be very useful for studies in which toxic genes are expressed in a temporally restricted manner, and for studies in which critical windows of development, and HBV or HCV infection are being precisely defined.

2.3 Development of an "ideal" minimal promoter in responsive components

Promoter-dependent leakiness has been addressed by the use of alternative minimal promoters[1,50]. pTRE-Tight (http://www.bdbiosciences.com/clontech/techinfo/vectors/vectorsT-Z/pTRE-Tigh.shtml), a recently developed and improved response plasmid derived from pTRE that can be used to express a gene of interest (Gene X) in the Tet-on and Tet-off gene expression systems, contains a Tet-responsive P_{tight} promoter immediately upstream of MCS. P_{tight} contains a modified Tet-response element (TRE_{mod}), which consists of seven direct repeats of a 36-bp sequence containing the 19-bp *tetO* sequence. Consequently, P_{tight} is silent in the absence of binding of (r)tTA to the *tetO* sequences. The P_{tight} promoter was originally developed as the P_{tet-14} promoter in the laboratory of Dr. H. Bujard.

Gene expression can be tightly regulated in response to varying concentrations of Tc. Particularly useful applications of these response vectors include: (1) the inducible expression of proteins that are extremely potent or toxic to the host cells, such as tumor suppressors or apoptotic proteins; (2) generating HBV or HCV transgenic mice that are immunocompetent for the transgene product(s), e.g. hepatitis viral antigen(s). In these cases, where background expression is simply unacceptable, use of a pTRE-Tight vector ensures that basal gene expression is minimal or even zero. Increasing amounts of evidence show that the pTRE-Tight vector ensures remarkably low (or apparently zero) background expression or undetectable basal expression, and very high induction *in vitro*[51—54]. In conclusion, whichever system of gene regulation is chosen, use of a pTRE-Tight vector ensures that background expression is minimized. Thus, we finally ensure the two important qualities we require in a single Tet-response vector: reduced background and maximal induction. However, the utility of this new form of Tet-response vector in transgenic animal modeling has yet to be demonstrated.

2.4 Assorted uses of improved rtTA variants, tTS/tTR and pTRE-Tight vector

Theoretically, when the improved rtTA variants, tTS/tTR and an "ideal" minimal promoter in the responsive component are used in combination, more stringent conditional control of transgene overexpression is readily achievable *in vivo* and *in vitro*; and this has been shown to be the case in practice (see [22,26,37,40,43,48]). Tighter positive regulation of transgene expression by a single adenovirus vector containing $rtTA2^S$-S2 and tTS expression cassettes was achieved both *in vitro* and *in vivo*[40]. Combination of $rtTA2^S$-M2 or $rtTA2^S$-S2 with the transrepressor tTR showed that basal expression was actively repressed while the expression level of the transiently transfected reporter gene was not affected; whereas if the target gene is also chromosomally integrated, tTR leads to a further reduction of basal expression[22]. Tight control of expression of the secreted alkaline phosphatase gene was also easily achieved *in vitro* and *in vivo* by a helper-dependent adenovirus vector carrying $rtTA2^S$-M2 and tTS[43]. In addition, a simple "all-in-one" vector, containing the elements of the Dox-inducible Tet-on system in their most advanced variants ($rtTA2^S$-M2 and tTS), can be used to control transgene expression efficiently in long-term tissue culture and in the mouse hematopoietic system following bone marrow transplantation[25]. Bornkamm et al.[37] achieved more stringent Dox-dependent control of gene activities *in vitro* using an episomal one-vector system (pRTS-1, a simple "all-in-one" vector), which carries all the elements (including $rtTA2^S$-M2, tTS and the bidirectional promoter P(tet)bi-1 in the same transgene construct), for conditional expression of target gene(s) via Tet-regulation. These results demonstrate that various regulatory windows can be achieved using different transregulators or combinations thereof. We suppose that these properties would also allow the generation of transgenic mice with preselected expression windows.

It is important to point out that, besides tTS, other approaches to controlling basal transgene leakage have been described. These include the use of insulators such as those characterized in *Drosophila* chromatin[55] and novel rtTA mutants that expand the range and sensitivity of the rtTA system[21]. Homogeneity and long-term stability of tetracycline-regulated gene expression with low basal activity were attained by using the rtTA2^S-M2 and insulator-flanked reporter vectors[56]; Dox-inducible lentivirus vectors bearing insulators have been proved useful for applications demanding the lowest levels of basal leaky expression[52]. In contrast to tTS, the utility of these approaches in transgenic mice has not been established. If subsequent studies demonstrate that they work in transgenic systems *in vivo*, it will be pretty important to compare them with the tTS systems to identify the relative merits and limitations of each.

2.5 Others

Leakiness in the control of expression of the acceptor construct may also result from strong positional effects on the *tetO* minimal promoter. Moreover, the degree of expression and level of leakiness associated with rtTA, rtTA2^S-M2, tTS/rtTA or tTS/rtTA2^S-M2 depend on both the promoter and the cell type utilized[57]. Specifically, CMV-driven tTS/rtTA2^S-M2 showed the highest level of inducibility in HEK293 cells (about 1000 fold) versus the dopaminergic cell line, MN9D (about 70 fold); in contrast, tyrosine hydroxylase (TH)-driven rtTA2^S-M2 demonstrated the highest level of expression with the least background expression in dopaminergic cell types versus HEK293 cells. Integration site-dependent leakiness has been attributed to enhancers located close to the integration site of the target gene construct[9]. Thus, the cross-talk between the minimal promoter in the target construct and nearby enhancers would be minimized by the ideal integration site, and subsequently fully maintain Dox inducibility. Initial studies in transgenic mice for tTA or rtTA crossed with Luc or Lac Z reporter strains illustrated both the practicality and the problems of this approach[4—7]. In many cell lines, significant leaky expression was observed, but basal expression levels in selected lines were close to the limits of detection[6]. This requires the generation of several acceptor mouse strains and the identification of those that express the transgene not constitutively but in an inducible manner. In theory, this type of integration event can be achieved if large numbers of transgenic microinjections (for *in vivo* studies) are undertaken, but in practice, this can be a very extensive and sometimes impracticable undertaking. An alternative approach is to increase the yield of functional dual transgenic offspring using microinjection approaches in which both transgenic constructs are transferred simultaneously (see above), and concurrently to insert constructs that shield the tet-controlled transgenic unit from extraneous activation (see [58]). Besides screening additional clones until one with the desired properties is found, the problem has been approached by insulating Ptet-1 from external activating signals by inserting a chicken lysozyme matrix attachment region just upstream of Ptet-1[59] or by flanking the target gene expression unit with either chicken β-globin insulators[60] or SCS and SCS boundary elements from *Drosophila*[61]. Moreover, tTS provides a powerful shield for and tightens the regulation of *tetO* constructs that are not integrated in an "ideal" location (see above)[44,45]. Finally, unregulated basal transcription can be observed frequently whenever the integration of the given gene(s) driven by promoter responsive to (r)tTA does not occur at the suitable chromosome site(s).

In addition, the genetic background of the mice can influence the occurrence of such difficulties[45]. The confounding effects of transgene leakage can readily be appreciated in the context of the CC10-rtTA-IL-13 mice generated in Elias's laboratory. When these mice were initially generated on a mixed CBA/C57BL/6 genetic background, very low levels of BAL IL-13 and marginally detectable phenotypes were obtained[62]. However, as breeding on to pure murine genetic backgrounds was accomplished, basal levels of IL-13 increased and a more impressive phenotype was observed.

In summary, improvements and additions to the Tet system, including the regulatory components, are still possible and necessary. Promoter development has not yet received the same degree of attention as the transregulators[9]. The number of *tetO* elements and their spacing have not yet been optimized[63], nor has the linker sequence separating the operators[64]. It remains to be seen whether an "ideal" minimal promoter with no intrinsic leakiness supporting very high-level activation can be identified or designed[21].

Several new variants of the Tet-based regulatory elements that can minimize or eliminate leaky trans-

gene expression have been successfully examined in vitro, but to date, approaches to eliminating basal transgene leakage *in vivo* have not been fully characterized. If investigators expect to use Tet-based systems and their new vartiants to produce credible transgenic animal disease models (precisely and realistically mimicking disease states) for such human diseases as hepatitis B and C, detectable and undetectable levels of transgene leakage in the absence of Dox must be completely avoided, because a very slight leakiness of the system, too low to detect biochemically, is sufficient to induce immune tolerance against the transgene product(s)[12]. Tetracycline-controlled transcriptional activation systems, which are the most widely employed inducible regulation systems and are continuously being improved, are reliable tools allowing "relatively stringent", reversible ("on" and "off"), quantitative, temporal and spatial control of transgene expression *in vitro* and *in vivo*[1,2]. However, data from our experiments and from Chisari's laboratory appear to demonstrate that they are not suitable for modeling human hepatitis pathogenesis in transgenic mice that are not immunocompetent for the transgene product(s), because leaky transgene expression often results from the intrinsic limitations of Tet-based systems and from extrinsic factors (such as positional effects). On the other hand, the Cre/*lox* P switching expression system (Table 1) seems to be an alternative to tetracycline-inducible systems because it shows true "off" and "on" regulation, avoiding the defects of the Tet-based systems described above. The point has already been successfully confirmed by Wakita et al.[20]. To examine the immune response to HCV structural proteins, Wakita et al.[20] used the Cre/*lox* P system to express the core, E1 and E2 proteins efficiently and conditionally in transgenic mice (e.g. CN2 mice), providing a useful animal model with which to investigate the host immune response against HCV infection and the pathogenesis of this infection[20,65,66]. Unfortunately, the "off" and "on" regulation mediated by the Cre/*lox* P switching expression system is not reversible.

3 Conclusion and prospects

The Tet regulatory systems are currently the most widely used regulatory systems for conditional gene expression. Tetracycline-controlled expression systems, however, are leaky; sometimes this leakage is substantial. New variants of the Tet-based regulatory elements that can minimize or eliminate leaky transgene expression have been tested successfully *in vitro* and/or *in vivo*. Ongoing improvements of the existing components and the continuous addition of new components to expand its range of applicability will make the Tet regulatory systems tight, versatile and flexible. Particular applications will include modeling the complex regulatory setups required to analyze sophisticated and multifactorial biological processes in development and disease, consequently not only improving our understanding of living organisms, but also demonstrating some novel and innovative strategies and approaches to the treatment of various maladies.

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