Use of the Tetracycline-controlled Transcriptional Silencer (tTS) to Eliminate Transgene Leak in Inducible Overexpression Transgenic Mice*

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The doxycycline-inducible reverse tetracycline transactivator (rtTA) is frequently used to overexpress transgenes in a temporally regulated fashion in vitro. These systems are, however, often limited by the levels of transgene expression in the absence of dox administration. The tetracycline-controlled transcriptional silencer (tTS), a fusion protein containing the tet repressor and the KRAB-AB domain of the kid-1 transcriptional repressor, is inhibited by doxycycline. We hypothesized that tTS would tighten control of transgene expression in rtTA-based systems. To test this hypothesis we generated mice in which the CC10 promoter targeted tTS to the lung, bred these mice with CC10-rtTA-interleukin 13 (IL-13) mice in which IL-13 was overexpressed in an inducible lung-specific fashion, and compared the IL-13 production and phenotypes of parental mice and the triple transgenic CC10-rtTA/tTS-IL-13 progeny of these crosses. In the CC10-rtTA-IL-13 mice, IL-13, mucus metaplasia, inflammation, alveolar enlargement, and enhanced lung volumes were noted at base line and increased greatly after doxycycline administration. In the triple transgenic tTS animals, IL-13 and the IL-13-induced phenotype could not be appreciated without doxycycline. In contrast, tTS did not alter the induction of IL-13 or the generation of the IL-13 phenotype by doxycycline. Thus, tTS effectively eliminated the baseline leak without altering the inducibility of rtTA-regulated transgenes in vivo. Optimal "off/on" regulation of transgene expression can be accomplished with the combined use of tTS and rtTA.

Overexpression transgenic modeling is being used with increasing frequency to investigate the processes involved in tissue homeostasis and disease pathogenesis. This is nicely illustrated in the lung, where the Clara cell 10-kDa protein (CC10)1 or surfactant apoprotein-C promoters can be used to selectively target genes of interest to the lung parenchyma and/or airway (1). In early studies, these promoters were used to directly drive the expression of transgenes in the lung. These studies provided impressive insights into the chronic respiratory effector functions of inflammatory mediators and the pathogenesis of asthma, adult respiratory distress syndrome, lung development, and pulmonary fibrosis (2–9). In these systems, the transgene is activated in utero and expressed in a constitutive fashion thereafter. As a result, these modeling systems are limited in their ability to accurately model waxing and waning disease processes such as asthma and are unable to differentiate transgene-induced phenotypic alterations that are due to alterations in lung development from alterations that would otherwise be induced in an adult/mature lung. These systems are also unable to appropriately study genes whose products are toxic in early life and cannot be used to define the natural history and/or reversibility of transgene-induced phenotypic alterations (1, 10, 11).

To deal with the limitations inherent in standard overexpression modeling, a number of investigators have established transgenic systems in which the expression of the transgene can be externally regulated. Although a variety of approaches have been utilized, tetracycline-controlled expression systems have been employed most frequently (10–17). Studies from our laboratory were the first to establish the utility of this approach in the lung (10). This system is based on the generation of transgenic mice with two molecular constructs. In the first, the promoter of choice (CC10) drives the expression of the reverse tetracycline transactivator (rtTA), a fusion protein made up of the herpesvirus VP-16 transactivator and a mutant Tet repressor from Escherichia coli. This transactivating fusion protein requires doxycycline (dox) (a tetracycline derivative) for specific DNA binding. The second construct contains multimers of the tetracycline operator (tet-O), a minimal cytomegalovirus promoter, and the gene of interest. In the absence of dox, the transactivator does not recognize or weakly recognizes its specific target sequence (tet-O), and target gene transcription occurs at low levels or not at all. The addition of dox allows the transactivator to bind in trans to the tet-O, activating the transgene of interest. This system has been used successfully in our laboratory and others to define the development-dependent and -independent processes that cause alveolar enlargement in the lung, inflammatory events that may contribute to the pathogenesis of pulmonary emphysema, cytokine-mediated loproteinase; MCP, monocyte chemoattractant protein; BAL, bronchoalveolar lavage; PAS, periodic acid-Schiff with diastase; RT, reverse transcription; PCR, polymerase chain reaction.
TABLE I

| Moiety   | Sense primer                  | Anti-sense primer                  | Anneal temperature | Product size |
|----------|-------------------------------|------------------------------------|--------------------|--------------|
| IL-13    | AGACGAGACTCCCTCTGTGCA         | TGGGTCTTATGAGTGACATGG             | 60                 | 123          |
| tet      | GAGTGCCGAGCTTTCTTCC           | GAGCAGACGCCATCTGTCC              | 60                 | 472          |
| Estatin  | CCACCTGTCCTCCCTCCACATGG      | ATCCACATCTCCCTTCTCCGGCC          | 56                 | 546          |
| MCP-1    | ACCAGCAGCCTTGGAGGAGG          | CAGAGGCTGGGCACTGTGTTGG           | 60                 | 463          |
| MMP-12   | AAGAGACCTGAGGGCAGGACTC        | TGGTGACAGAAATGGTGTCG             | 57                 | 631          |
| Cathepsin| GGGAGACATGAGGAGCTGAGAAGGATT  | TGCTCTTTGACGGCTTCTTGG            | 57                 | 481          |
| β-Actin  | GTGGGCCGCTCTTAGGCGACCA       | TGCCCCATTGGTCCTGAGGCGGG         | 60                 | 241          |

bp, base pairs.

Moisturizing events in oxygen toxicity, and crucial windows in lung development that define cytokine effector profiles (4, 10, 18–20). Detailed analyses of these and other rtTA-based systems have, however, revealed some properties that restrict its application. One limitation stems from the fact that rtTA can exhibit a degree of residual affinity to tet-O in the absence of dox. This manifests as definable levels of transgene activation and phenotype induction in animals (or cells) that are not receiving dox (11, 16, 18, 21–25). Approaches that can be used to eliminate basal transgene leak in vivo have not been well characterized.

The tet-controlled transcriptional silencer (tTS) is a fusion protein made up of a mutant Tet repressor and the KRAB-AB domain of the Kid-1 protein, a powerful transcriptional repressor (24, 26). It binds to tet-O only in the absence of dox. Therefore, in systems containing tTS and tet-O-driven transgenes, tTS binds to the tet-O in the absence of dox and inhibits the expression of the gene of interest. As dox is added to the culture medium, the tTS dissociates from tet-O, relieving the transcriptional suppression. At sufficient concentrations, the dox also interacts with rtTA, allowing it to bind to tet-O and activate the gene of interest. The tTS system has been shown to confer exquisite regulating ability on transiently transfected and stably transfected rtTA-regulated reporter genes in cells in culture (24, 25, 27). Surprisingly, the feasibility and efficacy of tTS in regulating basal gene expression in vivo in transgenic animals has not been investigated.

We postulated that the principles involved in tTS inhibition of basal gene expression in vitro are also applicable to the in vivo state. To test this hypothesis, we generated transgenic mice in which the CC10 promoter targeted tTS to the lung and bred these mice with CC10-rtTA-IL-13 mice in which CC10 and rtTA were used to express IL-13 in the lung/airway in an externally regulatable fashion. These mice had been previously generated in our laboratory and shown to have a quantifiable level of transgene leak in the absence of dox administration (18). We then compared the IL-13 production and phenotypes of the dual transgenic CC10-tet-O-IL-13 and the triple transgenic CC10-tet-O/rtTA/IL-13 mice. These studies demonstrate that tTS totally abrogates basal transgene leak and phenotype induction in our transgenic system. They also demonstrate that tTS mediates this inhibition without decreasing the ability of dox to stimulate the rtTA-regulated transgene or the ability of the transgene to induce a tissue phenotype.

**Experimental Procedures**

*Generation of CC10-tet-O-IL-13 Mice—* In order to express tTS in a lung specific fashion, the construct CC10-tet-O-hGH was generated (Fig. 1A). The plasmid containing tTS, pTet-tTS, was obtained from Dr. Andrew Farmer (CLONTECH Inc., Palo Alto, CA). Oligonucleotide primers were synthesized that would introduce HindIII and BamHI restriction enzyme sites 5′ and 3′ of the tTS coding region respectively. Furthermore a silent mutation was introduced to disrupt the original BamHI site right before the stop codon. The primers were, tTSUP1: 5′-GTG AAG ACG TTTC ATGC GCC TGG AGA CG-3′ and tTSLO1: 5′-CTT ATG AGT GGA TCC ATT TAC CAG GGG GCC TCT CTG TG-3′. The tTS fragment was amplified by PCR and then inserted between CC10 promoter and hGH polyadenylation and intronic sequence in place of the rtTA sequences in construct CC10-rtTA-hGH that was previously described by our laboratory (10). After verifying the junction areas and tTS DNA by sequencing, the construct was isolated by electrophoresis. The DNA was then purified through an Elutip-D column following the manufacturer’s instructions (Schleicher and Schuell, Inc., Keene, NH) and dialyzed against microinjection buffer (25 mM Tris-HCl/0.5 mM EDTA, pH 7.5). Transgenic mice were generated in (CBA X C57BL/6) F2 eggs using standard pronuclear injection as previously described (6, 28).

The resulting animals and their progeny were genotyped using tail DNA and Southern blot analysis with 32P-labeled TTS DNA as a probe or PCR. For PCR reactions, primers were designed to cover the area that was unique to the TTS transgene (Table I). The primers were tTSUP2: 5′-GAG TTG GCA GCA GTT TCT CTG CC-3′ and tTSL02: 5′-GAG CAC AGG CAC ATC TTC AA-3′. The PCR protocol was 95 °C for 5 min; 30 cycles at 95 °C for 1 min; 60 °C for 1 min and 72 °C for 1 min and a final extension at 72 °C for 10 min. A product of 472 bp was expected and detected.

**Generation of CC10-tet-O/rtTA/IL-13 Triple Transgenic Mice—** CC10-tet-O/rtTA-IL-13 transgenic mice were generated as described by our laboratory (18). These dual transgenic mice express IL-13 in the lung in an externally regulatable fashion. The first construct in these mice, CC10-rtTA-hGH, contains the CC10 promoter, rtTA and human growth hormone (hGH) intronic and polyadenylation sequences (hGH) (10). The second construct, tet-O-IL-13-hGH, contains a tet-O and minimal cytomegalovirus promoter, murine IL-13 cDNA and hGH intronic and polyadenylation sequences. The constructs are illustrated in Fig. 1A.

**Bronchoalveolar Lavage (BAL)**—Total Cell Counts and Differentials—Mice were euthanized and a median sternotomy was performed. The trachea was then isolated via blunt dissection and small caliber tubing was inserted and secured in the airway. Three successive volumes of 0.75 ml of PBS with 0.1% bovine serum albumin were then instilled and secured in the airway. The BAL fluid aliquots were centrifuged and the supernatants were stored at −70 °C until utilized. The levels of IL-13 were determined immunologically using commercial ELISA kits as per the manufacturer’s instructions (R&D Systems, Minneapolis, MN). Selected samples were concentrated 10 X by volume using Microcon YM-10 following the manufacturer’s protocol (Millipore Corporation, Bedford, MA).

**Cytokine Quantification**—BAL fluid aliquots were centrifuged and the supernatants were stored at −70 °C until utilized. The levels of IL-13 were determined immunologically using commercial ELISA kits as per the manufacturer’s instructions (R&D Systems, Minneapolis, MN). Selected samples were concentrated 10 X by volume using Microcon YM-10 following the manufacturer’s protocol (Millipore Corporation, Bedford, MA).

**mRNA Quantification**—mRNA levels were assessed using Northern analyses and/or RT-PCR as previously described by our laboratory (18, 21). In the RT-PCR experiments, total RNA from mouse lungs was prepared using Triol reagent (Life Technologies, Inc., Grand Island, NY). The PCR protocols for tTS were different as per the manufacturer's instructions (R&D Systems, Minneapolis, MN). Selected samples were concentrated 10 X by volume using Microcon YM-10 following the manufacturer’s protocol (Millipore Corporation, Bedford, MA).

**RT-PCR**—Selected samples were concentrated 10 X by volume using Microcon YM-10 following the manufacturer’s protocol (Millipore Corporation, Bedford, MA).
RESULTS

Generation of CC10-tTS-hGH Transgenic Mice—To characterize the effects of tTS in our transgenic system, we generated multiple lines of mice in which tTS was targeted to the lung using the CC10 promoter (Fig. 1A). The CC10-tTS-hGH construct was prepared, linearized and microinjected as previously described (6, 10). PCR using tail biopsy-derived DNA was used for genotyping and RT-PCR of whole lung RNA was used to evaluate tTS gene expression. Four transgene (+) founder animals were obtained from the microinjection (Fig. 1B). Each was bred onto a C57BL/6 background and independent lines were generated. In all cases, the transgene was propagated in a Mendelian fashion. tTS mRNA was readily detected in total lung RNA from transgene (+) littermate control mice (Fig. 1C) or (-) progeny mice (Fig. 1C). In contrast, tTS mRNA was not detected in BAL fluids from uninduced triple transgenic transgenic (CC10-rtTA/tTS-IL-13) mice. Single transgenic (CC10-tTS-hGH) and triple transgenic mice. To determine if tTS altered the levels of IL-13 production in the absence of dox administration, we compared the levels of BAL IL-13 protein and lung IL-13 mRNA in double transgenic (CC10-rtTA/tTS-IL-13) and triple transgenic (CC10-rtTA/tTS-IL-13) mice. Single transgenic (CC10-tTS-hGH) and transgenic (-) littermate control animals were also evaluated. IL-13 was not detected in the BAL fluid from the transgene (-) littermate controls or the CC10-tTS-hGH animals. IL-13 (50–110 pg/ml) was, however, readily appreciated in the BAL fluids from the dual transgenic (+) animals. The CC10-tTS-hGH mice were then crossed with CC10-rtTA-IL-13 double transgenic mice to generate CC10-rtTA/tTS-IL-13 triple transgenic mice.

Effect of tTS on IL-13 Production in the Absence of Dox Administration—To determine if tTS altered the levels of IL-13 that were produced in the absence of dox administration, we compared the levels of BAL IL-13 protein and lung IL-13 mRNA in double transgenic (CC10-rtTA-IL-13) and triple transgenic (CC10-rtTA/tTS-IL-13) mice. Single transgenic (CC10-tTS-hGH) and transgenic (-) littermate control animals were also evaluated. IL-13 was not detected in the BAL fluid from the transgene (-) littermate controls or the CC10-tTS-hGH animals. IL-13 (50–110 pg/ml) was, however, readily appreciated in the BAL fluids from the dual transgenic (+) animals. In striking contrast to this finding, IL-13 protein was undetectable in BAL fluids from uninduced triple transgenic animals containing the tTS construct (Fig. 2). After 10-fold BAL fluid concentration, IL-13 was still unable to be detected alter the histology, morphometry or compliance of the murine lung.

Characterization of CC10-tTS-hGH Mouse—To determine if tTS altered lung structure, we compared the hematoxylin and eosin, PAS and trichrome evaluations, alveolar morphometry and compliance of 1–3 month old CC10-tTS-hGH mice and wild type littermate controls. In all cases, differences could not be appreciated (data not shown). Thus, tTS expression did not

FIG. 1. Panel A: Schematic illustration of transgene constructs used to generate CC10-tTS-hGH mice and CC10-rtTA/tTS-IL-13 triple transgenic mice. CC10-tTS-hGH construct was prepared and microinjected as described under "Experimental Procedures." The genotype and organ-specificity of tTS expression in CC10-tTS-hGH mice were characterized. In panel B transgene (+) and transgene (-) littermate control mice were identified by PCR with tail biopsy-derived DNA and tTS primers. The results are compared with results obtained with DNA from a negative control wild type mouse (negative) and positive control CC10-tTS-hGH DNA (positive). In panel C RT-PCR was used to define the organ specificity of transgene expression in transgenic (+) animals. The CC10-tTS-hGH mice were then crossed with CC10-rtTA-IL-13 double transgenic mice to generate CC10-rtTA/tTS-IL-13 triple transgenic mice.
in BAL fluids from triple transgenic mice, whereas significant levels (0.9–1.5 ng/ml) of IL-13 were detected in similarly concentrated BAL fluids from dual transgene (+) animals (Fig. 2). These studies demonstrate that the tTS construct decreased the BAL IL-13 content of uninduced mice by at least 3 orders of magnitude. In accord with these observations, mRNA encoding IL-13 was readily detected via RT-PCR analysis in dual transgene (+) animals. In contrast, IL-13 mRNA was unable to be detected in lungs from triple transgenic tTS mice even after 35 cycles of RT-PCR analysis (Fig. 2). When viewed in combination, these studies demonstrate that the inclusion of the tTS construct totally eliminated the background leak in our dual transgenic rtTA-regulated animals.

Effects of tTS on Phenotype in the Absence of Dox Administration—Lungs from CC10-rtTA-IL-13 animals that did not receive dox were enlarged and had larger alveoli and enhanced compliance when compared with lungs from transgene (-) littermate controls (Fig. 3). They also manifest increased BAL cellularity, eosinophil, lymphocyte and macrophage rich BAL and tissue inflammation, mucus metaplasia and enhanced accumulation of eotaxin, MCP-1, MMP-12 and cathepsin K mRNA (Fig. 4, Table II and data not shown) (2, 18). In contrast to these findings, the lung volumes, alveolar size and pulmonary compliance of triple transgenic tTS containing animals were unable to be differentiated from the same parameters in transgene (-) littermate control animals or CC10-tTS-hGH mice (p, 0.01 comparing triple and dual transgene (+) animals for each parameter) (Fig. 3). In addition, the BAL abnormalities, mucus metaplasia and the eotaxin, MCP-1, MMP-12 and cathepsin K gene expression that were prominent features of the dual transgene (+) animals were totally abrogated in the triple transgene (+), tTS-containing animals (Figs. 4 and Table II). These studies demonstrate that the inclusion of the tTS construct totally abrogated the inflammatory, structural, mucus, physiologic and target gene alterations induced by the basal IL-13 leak in this murine transgenic system.

Effects of tTS on Dox-Induction of IL-13 and the Generation of the IL-13 Phenotype—For the CC10-tTS-hGH construct to have maximal utility, it needs to suppress transgene expression in the absence of dox without suppressing the induction of the transgene after dox administration. To determine if tTS had effects on dox induction, we compared the IL-13 elaboration and phenotype induction in dox-treated dual transgene (+) and triple transgene (+) animals. As previously reported, dox was a potent inducer of IL-13 protein production and mRNA accumulation in these dual transgenic animals (18). This induction was noted within 48 h and persisted with chronic dox administration. This inducibility was not significantly altered by tTS since virtually identical levels of BAL IL-13 protein were seen in comparably dox treated dual and triple transgenic animals (Fig. 5). In addition, comparable levels of IL-13 mRNA

Fig. 2. Effects of tTS on IL-13 gene expression in the absence of dox administration. Two month old wild type, CC10-tTS-hGH (tTS +), CC10-rtTA-IL-13 (IL-13/rtTA +) and CC10-rtTA/tTS-IL-13 (IL-13/rtTA +; tTS +) mice on normal water were sacrificed, BAL was performed and whole lung RNA was isolated. The levels of IL-13 in unconcentrated (A) and 10 X concentrated (B) BAL fluids were evaluated by ELISA. Each value represents the mean ± S.E. of a minimum of 4 mice. In all cases the levels of IL-13 in BAL fluids from CC10-rtTA/tTS-IL-13 mice were similar to those in the BAL fluids from wild type mice and at or below the limits of detection of the assay (−10 pg/ml). In panel C RT-PCR was used to characterize the levels of IL-13 mRNA in RNA from lungs of these mice.

Fig. 3. Effect of tTS on pulmonary phenotype in the absence of dox administration. Two month old wild type, CC10-tTS-hGH (tTS +), CC10-rtTA-IL-13 (IL-13/rtTA +) and CC10-rtTA/tTS-IL-13 (IL-13/rtTA +; tTS +) mice on normal water were sacrificed and their phenotypes were compared as described under “Experimental Procedures.” Lang volumes, alveolar chord length, and pulmonary compliance are illustrated in Panels A, B and C respectively. (*p < 0.01 compared with CC10-rtTA-IL-13 animals).
were also noted in these dox-treated dual and triple transgenic animals when assessed via RT-PCR or Northern analysis (Fig. 5 and data not shown). Thus, the presence of the tTS construct did not significantly diminish transgene inducibility in our transgenic system.

As noted above, modest increases in lung and alveolar volume, enhanced lung compliance, an eosinophil, lymphocyte and macrophage rich inflammatory response, mucus metaplasia and eotaxin, MCP-1, MMP-12 and cathepsin K gene expression were seen in dual transgenic mice on normal water. Administration of dox caused a further increase in IL-13 production and an increase in the intensity of all of these phenotypic features (Figs. 6 and 7, Table II and data not shown). In the triple transgenic mice, no phenotype was seen in the absence of dox administration. After dox administration, however, enhanced lung volumes, alveolar enlargement, enhanced pulmonary compliance, increased BAL cellularity, eosinophil, lymphocyte and macrophage rich tissue and BAL inflammation and mucus metaplasia were all readily appreciated. In addition, RT-PCR and Northern analysis demonstrated prominent eotaxin, MCP-1, MMP-12 and cathepsin K gene expression. In all cases, the magnitude of the alterations in each of these parameters in the dox-treated triple transgenic mice were comparable to those in dox-treated dual transgenic animals (Figs. 6 and 7, Table II and data not shown). Thus, the tTS construct, while inhibiting basal IL-13 production, did not diminish the ability of dox to induce IL-13 elaboration or the ability of IL-13 to induce its tissue phenotype.

**Table II**

| IL-13/rtTA | tTS | Dox | Cell recovery |
|------------|-----|-----|--------------|
| -          | +   |   -| 5.2 ± 0.82 |
| -          | +   | +  | 12.6 ± 1.42 |
| +          | +   | -  | 5.1 ± 1.25 |
| +          | -   | +  | 32.8 ± 13.3 |
| +          | +   | +  | 22.5 ± 2.4  |

*p < 0.05 versus CC10-rtTA-IL-13 mice on normal water.

**DISCUSSION**

Tetracycline responsive regulatory systems have been shown to control gene expression in cultured cells and whole organisms including yeast, Drosophila, plants, mice and rats. A major requirement for these systems is a target transgene that is under tight “outside” control. Specifically, these systems should have negligible levels of transgene expression in the absence of tetracycline analogue administration and high levels of transgene expression after tetracycline/dox induction. Unfortunately, these goals are not always achievable. Depending on the experimental conditions that are employed and the site(s) of integration of the transgenic constructs, enhancer sequences near the target gene have been repeatedly demonstrated to increase basal target gene expression thereby compromising the tight regulation that is desired. In some circumstances, the leak that results is acceptable and does not negate the ability of the experimental system to appropriately address the hypothesis that is being investigated. In others, however, the leak is more problematic. The confounding effects of transgene leak can be easily appreciated in the context of the CC10-rtTA-IL-13 mice generated in our laboratory. When these mice were initially generated on a mixed C57BL/6 genetic background, very low levels of BAL IL-13 and marginally detectable phenotypes were appreciated. However, as breeding on to pure murine genetic backgrounds was accomplished, basal levels of IL-13 increased and a more impressive phenotype was appreciated. Since the tTS has been shown to eliminate/control basal leak in *in vitro* cell culture systems, we hypothesized that this approach could also be used to control basal transgene leak in *in vivo* transgenic systems. To test this hypothesis, we generated mice in which tTS was targeted to the lung, bred these mice with a dual transgenic CC10-rtTA-IL-13 mice and then compared the IL-13 production and the phenotypes of the resulting dual and triple transgenic mice before and after dox administration. These studies demonstrate that the incorporation of tTS in our transgenic system decreased basal transgene leak to undetectable levels and totally eliminated the IL-13-induced phenotype in the absence of dox induction. These studies also demonstrate that tTS did not alter the ability of dox to increase IL-13 elaboration and induce a full-blown IL-13 phenotype. When viewed in combination, these studies demonstrate that tTS converted our rtTA-based system from one with low and high levels of transgene expression to one that now has true off and on settings in the absence and presence of dox induction.

When transgenes are inserted into cells, or whole organisms like transgenic mice, the levels of basal transgene expression...
are regulated by a number of factors including the site of integration in the host genome and the number of copies of the integrated transgene. When rtTA-based regulatory systems are employed an ideal integration site would be one that minimizes cross talk between the minimal promoter in the target construct and nearby cis-acting enhancers while maintaining dox inducibility. In theory, this type of integration event can be obtained if large numbers of cellular transfections (for in vitro studies) or transgenic microinjections (for in vivo studies) are undertaken (11, 24). This can, however, be very large and, at times, unachievable undertaking. The alternative approach is to increase the yield of functional dual transgenic offspring using transfection or microinjection approaches in which both transgenic constructs are transferred simultaneously (see below) and concurrently insert constructs that shield the tet-controlled transgenic unit from extraneous activation. We chose to do this by simultaneously microinjecting the two transgenic constructs in our transgenic system and then adding TTS, a fusion protein made up of the Tet-R and a transcriptional silencing domain. This protein binds tet-O in the absence of dox, protecting it from outside activation. Importantly, it also releases in the presence of dox allowing rtTA to bind to tet-O and induce transgene activation. The Tet-R-based rtTA and TTS regulatory proteins both bind tet-O as homodimers. In theory, when they are co-expressed they can also form TTS/rtTA heterodimers that do not function appropriately and compromise the overall inducibility of this system (24, 30). To ensure that this did not occur, the TTS construct has been modified at its dimerization surface to prevent this rtTA interaction. Our studies show that TTS provides a powerful shield for and tightens the regulation of tet-O constructs that are not integrated in an “ideal” location. They also demonstrate that TTS does not

**FIG. 5.** Effect of TTS on dox induction of IL-13 gene expression. One month old wild type, CC10-tTS-hGH (tTS +), CC10-rtTA-IL-13 (IL-13/rtTA +) and CC10-rtTA/tTS-IL-13 (IL-13/rtTA +; tTS +) mice were placed on dox water for 1 month and sacrificed. BAL IL-13 levels were determined by ELISA (Panel A). RT-PCR was used to evaluate the levels of IL-13 mRNA in lungs from these animals (Panel B). (*p < 0.05 compared with wild type and CC10-tTS-hGH mice.)

**FIG. 6.** Effect of TTS on the dox-induced IL-13 phenotype. One month old wild type, CC10-tTS-hGH (tTS +), CC10-rtTA-IL-13 (IL-13/rtTA +) and CC10-rtTA/tTS-IL-13 (IL-13/rtTA +; tTS +) mice were placed on dox for one month and sacrificed. Lung volumes, alveolar chord length, and pulmonary compliance are illustrated in Panels A, B, and C, respectively. (*p < 0.05 compared with wild type and CC10-tTS-hGH animals only.)
were generated by breeding of single transgene (+) animals do not lend themselves readily to experiments in which these animals are bred to mice with targeted null mutations. This makes it difficult for investigator to define the contributions to the transgene-induced phenotype of genes that are regulated by the overexpressed transgene. It is well documented in studies from our laboratory and others that dual transgene-rTATA-based systems with acceptable levels of leak can be achieved using an approach in which both constructs are microinjected simultaneously (10, 11, 18, 21). The present studies also demonstrate that the tTS optimizes the regulation of these transgenes when significant basal leak is noted. Thus, it is reasonable to hypothesize that, in the future, inducible transgenic systems may be able to be generated most efficiently using an approach in which the tTS construct, rTATA construct and tet-O-transgene construct are all simultaneously microinjected or included in a single construct. Since transgenes tend to insert into the genome in a head to tail fashion, this would provide transgene (+) animals with tight transgene control in which all 3 constructs are passed to progeny as if they were a single gene. This would minimize the breeding and genotyping required for phenotypic characterization. It would also facilitate experiments in which mice with null mutations of downstream genes are used to define the role(s) that these genes play in mediating the transgene-induced phenotype.

IL-13 is a 12 kDa cytokine produced in large quantities by T helper (Th) 2 and lesser quantities by Th1 cells (32, 33). Studies from our laboratory and others have demonstrated that IL-13 plays a central role in the pathogenesis of airways disorders such as asthma and chronic obstructive pulmonary disease (COPD) and can confer protection in the setting of oxidant-induced lung injury (2, 18, 20, 34, 35). This is nicely illustrated in the CC10-rTATA-IL-13 animals in which dox-induction resulted in a phenotype that includes mucus metaplasia, eosinophil and lymphocyte-rich inflammation, lung and alveolar enlargement and an enhanced ability to tolerate the toxic effects of 100% O2 (18, 20). Since dox-induction of IL-13 caused an increase in alveolar size in adult mice, the CC10-rTATA system allowed us to demonstrate that IL-13 did not cause alveolar enlargement by blocking alveolar development. It also allowed us to define the roles of matrix metalloproteinases (MMPs) and cathepsin in the generation of this response and the mechanisms of IL-13-induced protection in hyperoxic acute lung injury (18, 20). Our knowledge of the pathogenesis of asthma, COPD, acute lung injury and virtually all other disorders is strikingly limited as regards the reversibility of the injuries involved in these disorders and the nature of the repair responses that they cause. This deficiency is due, in part, to a lack of modeling systems that allow defined injuries to be applied and then fully removed in an appropriately controlled fashion. The basal leak in the dual transgenic, CC10-rTATA system renders it similarly lacking in this regard. The triple transgenic CC10-rTATA-tTS system, however, can be used in a true “on/off” fashion. It is thus reasonable to believe that this system can be used to define the natural history of in vivo injury and repair responses with a level of precision that has not been previously achievable.

In summary, these studies demonstrate that the incorporation of tTS into rTATA-based externally regulatable overexpression transgenic systems optimizes the regulation of transgene expression in vivo. By eliminating basal transgene leak and phenotype induction without altering the dox-inducibility of rTATA-regulated transgenes, tTS converts the rTATA-based system from one with low and high levels of gene expression to one with true “off” and “on” regulation. The “off/on” nature of this regulation will be very useful in studies in which toxic genes...
are expressed in a temporally restricted fashion and studies in which critical windows of development and the natural history of injury and repair are being precisely defined. The tight control that is seen with the combined use of tTS and rtTA should now be the standard against which other inducible overexpression systems are judged.

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