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Genetics

A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice

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ABSTRACT **A system for tetracycline-regulated inducible gene expression was described recently which relies on constitutive expression of a tetracycline-controlled transactivator (tTA) fusion protein combining the tetracycline repressor and the transcriptional activation domain of VP16 [Gossen, M. & Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* 89, 5547–5551]. This system yielded only low levels of transactivator protein, probably because tTA is toxic. To avoid this difficulty, we placed the tTA gene under the control of the inducible promoter to which tTA binds, making expression of tTA itself inducible and autoregulatory. When used to drive expression of the recombination activating genes 1 and 2 (RAG-1 and RAG-2), the autoregulatory system yielded both substantially higher levels of variable (diversity) joining [V(D)J] recombination activity (70-fold on average) and inducible expression in a much larger fraction of transfected cells (autoregulatory, 90%, vs. constitutive, 18%). In addition, this system allowed the creation of transgenic mice in which expression of a luciferase transgene was inducible tens to hundreds of times the basal levels in most tissues examined. Induced levels of expression were highest in thymus and lung and appear to be substantially higher than in previously reported inducible luciferase transgenic mice created with the constitutive system. With the modified system, inducible transactivator mRNA and protein were easily detected in cell lines by RNA and Western blotting, and transactivator mRNA was detected by RNA blotting in some tissues of transgenic mice. This autoregulatory system represents an improved strategy for tetracycline-regulated gene expression both in cultured cells and in transgenic animals.**

Systems for inducible mammalian gene expression have typically encountered limitations, such as basal leakiness, toxic or nonspecific effects of inducing agents or treatments, limited cell-type applicability, and low levels of expression (reviewed in ref. 1). Recently, a system was described (2) that overcomes many of these difficulties by placing target genes under the control of a regulatory sequence (*tetO*) from the tetracycline-resistance operon of Tn*10*. In bacteria, this short sequence is bound tightly by the tetracycline repressor protein (tetR), and binding is blocked by the antibiotic tetracycline (3). A hybrid fusion protein, the tetracycline-controlled transactivator (tTA), combines the tetR DNA-binding domain with the transcriptional activation domain of VP16, such that when tTA binds to a minimal promoter containing *tetO* sequences, transcription of the target gene is activated. Tetracycline binding to tTA prevents activation presumably by causing a conformational change in the tetR portion of tTA, which blocks binding of tTA to *tetO* (4); gene activation is achieved by removing tetracycline (2).

The primary limitation of this system is difficulty in expressing even moderate levels of the tTA protein [undetectable by Western blotting and barely detectable by gel electrophoresis mobility-shift assay (2)]. The authors speculated that this was due to transcriptional "squelching" (5) by the VP16 transactivator domain leading to death of cells expressing even modest levels of the tTA protein. These results, combined with the observation of an apparently low level of expression of an inducible luciferase transgene when using this system (6), suggest that inefficiencies in tTA expression may contribute to the difficulty.

By placing the tTA gene under the control of a promoter containing *tetO*, we have created an autoregulatory tTA expression vector that allows higher levels of tTA expression. We demonstrate here that our strategy permits the creation of highly inducible transfected cells with much greater efficiency than the constitutive system and, furthermore, allows the creation of transgenic mice in which expression of a luciferase reporter gene can be controlled by altering the concentration of tetracycline in the drinking water of the animals. The autoregulated expression of transactivator protein should make the tetracycline system applicable to a wider array of problems requiring inducible mammalian gene expression.

MATERIALS AND METHODS

Construction of Plasmids. Details of plasmid constructions are available upon request. The *Eco*RI–*Bam*HI fragment of pUHD15-1 (2) containing the tTA open reading frame was modified at its 5′ end by addition of a 24-bp oligonucleotide to provide an optimal context for translational initiation (7) and is hereafter referred to as tTAk. The tTAk fragment was inserted into pcDNA I/neo (Invitrogen) to yield pcDNA-tTAk, placing the tTAk gene under the transcriptional control of the enhancer and promoter sequences of the immediate early gene of human cytomegalovirus (hCMV). The plasmid pTet-Splice [with pBluescript II KS(+) (Stratagene) backbone] contains the *Xho* I–*Sal* I fragment of pUHC13-3 (2) (*tetP*, containing seven copies of the *tetO* upstream of a minimal promoter), separated from the simian virus 40 (SV40) small T antigen intervening sequence and the SV40 early polyadenylylation sequence from pHAV-CAT (8) by a multiple cloning site. The tTAk gene was cloned into pTet-Splice to yield pTet-tTAk, placing the start site of transcription 143 bases upstream of the tTAk AUG (see Fig. 1).

pTet-R1A/C contains the coding region of pR1A/C inserted into pTet-Splice. The coding region of pR1A/C is altered, compared with full-length mouse recombination activating genes (RAG)-1 by small N- and C-terminal deletions, which result in at least a two-fold increase in variable (diversity) joining [V(D)J] recombinase activity (C. McMahan, P.S., and D.G.S., unpublished results). pTet-R2A contains the coding region of pR2A-CDM8 inserted into pTet-Splice. The

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Abbreviations: RAG, recombination activating genes; tTA, tetracycline-controlled transactivator; RLU, relative light units; hCMV, human cytomegalovirus; Rn, percent recombination; PBMC, peripheral blood mononuclear cell; V(D)J, variable (diversity) joining.

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R2A coding region is altered relative to full-length mouse RAG-2 by a small C-terminal deletion which results in a small increase in V(D)J recombinase activity (C. McMahan and D.G.S., unpublished results). pTet-R1 and pTet-R2 contain the complete RAG-1 and RAG-2 open reading frames, respectively, inserted into pTet-Splice.

Cell Culture and Derivation of Transfected Cell Lines. Stable fibroblast cell transfectants of pcDNA-tTAk (including the *neo* gene) were generated essentially as described (9). Transfected cells were selected 48 h after transfection by adding 0.75 mg of G418 and 0.5 μg of tetracycline per ml to the culture medium. Single colonies were expanded in 0.5 mg of G418 and 0.5 μg of tetracycline per ml. Stable transfectants containing pTet-tTAk alone or pTet-tTAk, pTet-R1A/C, and pTet-R2A were generated as described above with cotransfection of pSV2-His (9), followed by histidinol selection and culture in the presence of 0.5 μg of tetracycline per ml.

Assay for V(D)J Recombinase Activity. NIH 3T3 fibroblast cell lines were transfected with 10 μg of pD243 (an extrachromosomal signal joint deletion substrate) (10) and, where indicated, 6 μg of pTet-R1A/C and 4.8 μg of pTet-R2A by the calcium phosphate/glycerol shock procedure. After transfection, cells were cultured in the presence (tet^+) or absence (tet^-) of 0.5 μg of tetracycline per ml. MC1061 bacteria were electroporated with a small aliquot of extrachromosomal plasmid molecules, harvested by rapid alkaline lysis of the cells 48 h after transfection. The electroporated bacteria were grown on LB agar plates containing either 100 μg of ampicillin (*A*) per ml or 11 μg of chloramphenicol and 100 μg of ampicillin (*CA*) per ml. The percent recombination (Rn) was calculated as the total number of *CA*-resistant colonies divided by the total number of *A*-resistant colonies, multiplied by 100. Over 99% of plasmids harvested from NIH 3T3 cells 48 h posttransfection have replicated at least once (data not shown and ref. 11).

RNA Blot Analysis. Total cell RNA was blotted to nylon membranes (Zetabind, Cuno, or Genescreen Plus, NEN) and hybridized with DNA probes prepared by using a random hexamer labeling kit (Boehringer Mannheim). Probes detecting RAG-1 and RAG-2 mRNA were fragments of the RAG-1 or RAG-2 coding regions (9, 12), respectively. The actin probe has been described previously (9).

Western Blot Analysis. Protein extracted from 1.5×10^7 cells per lane was subjected to SDS/8% PAGE and electroblotted onto a 0.2-μm pore size polyvinylidene difluoride membrane (Bio-Rad). Membranes blocked in a solution of 1% bovine serum albumin (BSA)/0.5% gelatin in Tris-buffered saline (TBS; 50 mM Tris/150 mM NaCl)/0.1% Tween 20 (TTBS) were probed with a monoclonal anti-tetR antibody (9F10)-containing hybridoma supernatant (S. Freundlieb and H. Bujard, Heidelberg) diluted 1:4 in 1% BSA in TTBS. tTA protein was detected by using an ECL Western blotting kit (Amersham).

Transgenic Mice and Assays for Luciferase. Mice transgenic for pTet-tTAk (*Xho* I–*Not* I fragment) and pUHC13-3 (*Xho* I–*Ase* I fragment) were created by comicroinjection of gel-purified DNA (in the presence of 0.5 μg of tetracycline per ml) into fertilized F_1 (C57BL/6 × C3H) eggs, which were then implanted into the uterus of pseudopregnant females, and provided with water containing 100 μg of tetracycline per ml and 5% (wt/vol) sucrose. Progeny were screened, and transgene copy number was estimated by probing Southern blots of tail DNA or control DNA with tTA (761-bp *Xba* I–*Sal* I) or luciferase (1365-bp *Hin*dIII–*Eco*RV) fragments labeled with [α-^{32}P]dCTP.

Luciferase activity in tissues of transgenic mice was measured by using a commercial assay system (E1500; Promega). Peripheral blood mononuclear cells (PBMCs) ($0.1–1.0 \times 10^6$ cells) were lysed in 50 μl of lysis buffer for 15 min at room temperature, insoluble material was pelleted by centrifugation for 5 sec at 14,000 rpm, 20 μl of the supernatant was mixed with 100 μl of luciferin reagent, and the light produced in 10 sec was measured in a luminometer (Lumat LB9501; Berthold, Wildbad, Germany). The number of cell equivalents of lysate in the assay was used to normalize luciferase activity between samples. Other tissues, harvested and quick frozen in liquid nitrogen, were ground to a powder with a cold mortar and pestle, placed in 100–200 μl of luciferase lysis buffer, and incubated at room temperature for 15 min. Cell debris was pelleted for 10 sec at 14,000 rpm, and supernatant was stored at −70°C until analysis, as above. For normalization, total protein concentration in tissue lysates was determined by using a Bradford protein assay (Bio-Rad). Sample activity was measured within the linear range of the assay, and activity varied only by ≈2-fold as lysates were diluted. Firefly luciferase protein standard (Sigma) added to extracts from a variety of tissues from wild-type mice showed no variation in activity.

RESULTS

The inducible tetracycline expression system described recently (2) relies on constitutive expression of the tTA gene from a fully functional hCMV promoter, and a luciferase reporter gene under the control of the inducible promoter *tetP*. In this system, tetracycline prevents the activation of luciferase gene expression, but does not prevent the tTA protein from exerting potentially deleterious effects on cells (5).

In the autoregulatory plasmid pTet-tTAk, we placed a modified tTA gene tTAk (see *Materials and Methods*) under the control of Tetp (Fig. 1). Tetracycline prevents tTA from binding to *tetP*, preventing expression of both tTA and luciferase. This negative feedback cycle ensures that little or no tTA is produced in the presence of tetracycline, thereby reducing or eliminating possible toxic effects. When tetracycline is removed, however, this strategy predicts that tiny amounts of

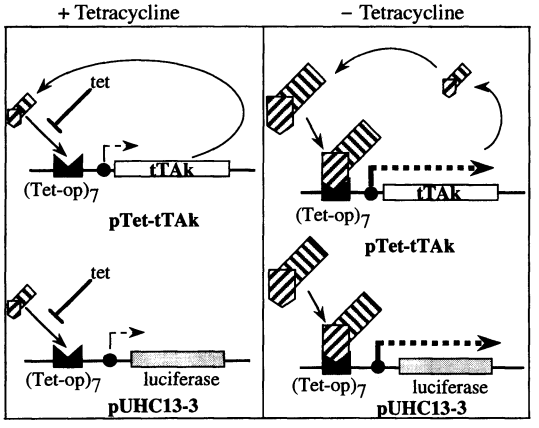

FIG. 1. Autoregulatory strategy for inducible gene expression. Autoregulatory expression of tTA is accomplished in pTet-tTAk by placing the tTAk gene (white box) under the control of *tetP* consisting of seven copies of the *tetO* sequence (Tet-op; darkly shaded box) upstream of the minimal hCMV promoter region containing a TATA box and transcription start site (black circle). The luciferase reporter gene (lightly shaded box) of pUHC13-3 is also controlled by *tetP*. The tTA protein is shown as two adjoining striped boxes to represent the two domains of the protein (for DNA binding and transactivation). In the presence of tetracycline (*Left*), the basal activity of the minimal hCMV promoter results in expression of very low levels of the tTA protein (represented as a small tTA icon), and any tTA protein produced is blocked from binding to Tet-op. Both luciferase and tTA expression are therefore maintained at low levels (thin, short dashed lines). When tetracycline is removed (*Right*), the small amounts of tTA present bind Tet-op, stimulating expression of the tTA gene. Higher levels of the tTA protein now stimulate higher levels of tTA and, thus, luciferase expression (heavy, long dashed lines).

tTA protein (which may result from the leakiness of the minimal promoter), will bind to *tetO* and stimulate expression of the tTAk gene. A positive feedforward loop is initiated which in turn leads to higher levels of expression of tTA and, thus, luciferase (Fig. 1). For constitutive expression of tTA, we placed the tTAk gene under the control of the hCMV promoter and followed it with additional sequences to direct tTA mRNA splicing and polyadenylylation.

Comparison of the Constitutive and Autoregulatory Inducible Expression Systems in Cultured Cells. After confirming that pcDNA-tTAk and pTet-tTAk could direct inducible expression of luciferase activity in transfected fibroblast cell lines (data not shown), the functional properties of these plasmids were compared in a more stringent assay: the ability to express high levels of the proteins encoded by RAG-1 and RAG-2 (9, 12). During lymphoid development, RAG-1 and RAG-2 participate in the assembly of functional immunoglobulin and T-cell receptor genes from component gene segments by V(D)J recombination. Most important for the experiments described here, RAG-1 and RAG-2 are necessary and sufficient to activate the V(D)J recombinase in nonlymphoid cells (reviewed in ref. 13), and the activity of the V(D)J recombinase can be quantitatively assayed by using extrachromosomal recombination substrates (14).

Extensive efforts to express RAG-1 and RAG-2 in NIH 3T3 fibroblast cells by using various promoters have revealed that it is difficult to achieve an Rn of greater than a few percent, as assayed with standard extrachromosomal recombination substrates (data not shown). Only high titer RAG retroviruses developed by others have reproducibly shown the ability to achieve an Rn as high as 10% (15). What is clear, however, is that Rn correlates strongly with RAG expression levels over at least three orders of magnitude (Rn from 0.01% to well above 10%; ref. 16 and data not shown). Thus, the ability to express the RAG proteins, as measured by V(D)J recombinase activity, is an appropriate test of an inducible expression system, both because of the difficulties that have been encountered in expressing the proteins and because of the sensitivity and range of the assay.

NIH 3T3 fibroblast clones stably transfected with pcDNA-tTAk (17 clones) or with pTet-tTAk (10 clones) were tested for their ability to perform V(D)J recombination after transient transfection with a recombination substrate and *tet*P-regulated RAG-1 and RAG-2 (Fig. 2). Each clone was assayed in parallel in the presence (uninduced state) or absence (induced state) of tetracycline, and the results were compared to control transfections either lacking RAG-1 or RAG-2 (first sample in Fig. 2 *A* and *B*) or containing highly active, constitutive RAG expression constructs (in which RAG expression is driven by the hCMV promoter; second sample in Fig. 2 *A* and *B*). In addition, two NIH 3T3 clones stably transfected with pTet-tTAk and the *tetP*-regulated RAG expression vectors were assayed by transient transfection of the recombination substrate in the presence or absence of tetracycline (last two samples in Fig. 2*B*).

The autoregulatory expression system (pTet-tTAk) represents a substantial improvement over the constitutive expression system (pcDNA-tTAk). Only 3 of 17 (18%) pcDNA-tTAk transfectants had clearly detectable levels of V(D)J recombination (Fig. 2*A*), with the highest levels of recombination (in

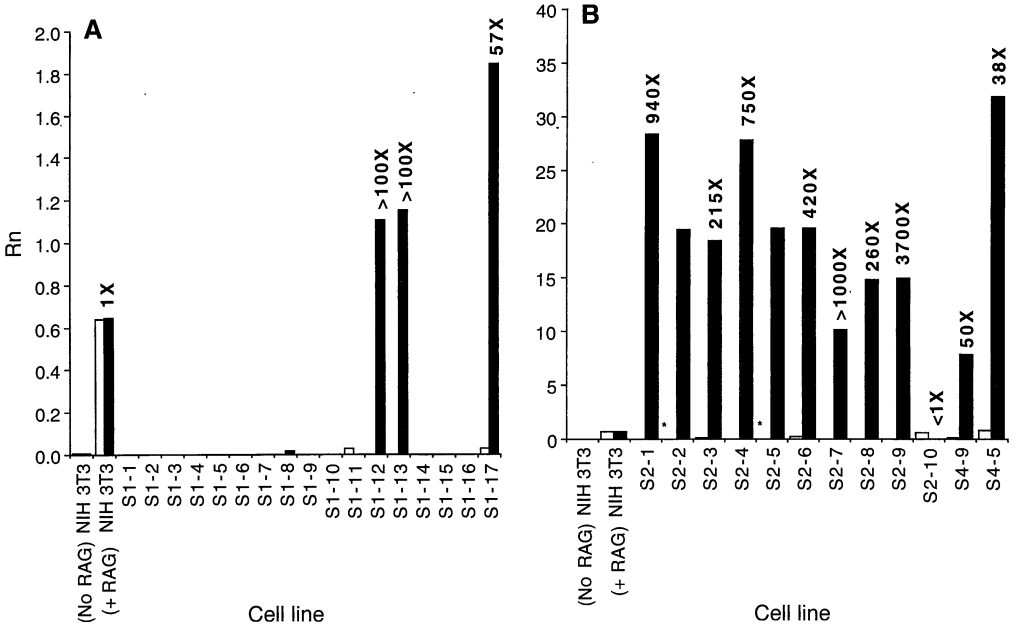

FIG. 2. Inducible V(D)J recombination in NIH 3T3 fibroblasts. (*A*) Analysis of clones containing pcDNA-tTAk (constitutive tTA expression). Seventeen stable transfectant clones (S1-1 to S1-17) were assayed for the ability to carry out V(D)J recombination by transient cotransfection with a V(D)J recombination substrate and *tetP*-controlled RAG-1 and RAG-2 expression vectors. Parallel transfections were performed in the presence (+ Tet; white bars) and absence (− Tet; black bars) of tetracycline in the growth media, and the V(D)J recombination frequency (expressed as a percent) was determined as described in *Materials and Methods.* For comparison, four control assays performed in NIH 3T3 cells are also shown (first two samples): transfection of the recombination substrate in the absence of RAG-1 and RAG-2, with and without tetracycline, and cotransfection of the recombination substrate with constitutive RAG expression vectors, with and without tetracycline. As expected, tetracycline had no effect on V(D)J recombination frequency when RAG-1 and RAG-2 were expressed from constitutive hCMV promoters. Fold induction achieved by removing tetracycline is indicated above the bars in cases where clearly detectable recombination was observed. (*B*) Analysis of clones containing pTet-tTAk (autoregulatory tTA expression). Ten stable transfectant clones (S2-1 to S2-10) and two clones containing pTet-tTAk, pTet-R1A/C, and pTet-R2A (S4-9 and S4-5) were assayed for the ability to carry out V(D)J recombination as described above. The first two samples are the same control samples described in *A*. Note the difference in the recombination frequency axis scale between *A* and *B*. Asterisks (*) mark two transfections (+ Tet) that yielded very small numbers of ampicillin-resistant colonies, making the calculated Rn unreliable. Consequently, the fold-inducibility for these clones is not shown. The number of ampicillin-resistant colonies was low in these experiments (range, 350–55,550). On the basis of additional assays on some of the cell lines, we estimate that the reported Rns are overestimated by as much as 2-fold in both *A* and *B*.

clone S1-17) being 3-fold that seen in the positive control with constitutively active RAG expression vectors (Fig. 2*A*, second sample). Removal of tetracycline induced recombination in these three clones (S1-12, S1-13, and S1-17) by greater than 50- to 100-fold. In contrast, 9 of 10 (90%) pTet-tTAk transfectants (Fig. 2*B*) showed high levels of recombination (note the difference in scale between Fig. 2 *A* and *B*), with the highest levels (28% in S2-1) being nearly 50-fold higher than the positive control (Fig. 2*B*, second sample). Inducibility in these nine clones was excellent, ranging from over 200-fold to 3700-fold. Equally high were the observed recombination frequencies achieved in clones stably transfected with pTet-tTAk and *tetP*-regulated RAG plasmids (S4-9 and S4-5; last two samples in Fig. 2*B*).

Further characterization of the pTet-tTAk transfectant S2-6 and the pTet-tTAk/pTet-R1A/C/pTet-R2A transfectant S4-9 demonstrated that the ability to induce high levels of V(D)J recombinase activity is reproducible and that recombination decreases by 67% with 0.01 μg of tetracycline per ml and by 95% with 0.1 μg of tetracycline per ml (data not shown). We have observed greater than 50% cell death (within 10 days) and a loss of detectable tTA protein (by 16 days) in S2-6 cells cultured in the absence of tetracycline (data not shown). We have not determined whether the inability to detect tTA results from the reduced expression of tTA or the selective death of cells expressing high levels of tTA. More experiments are necessary to determine the factors that influence tTA toxicity in this system.

Fig. 3*A* demonstrates that mRNA corresponding to the tTAk, RAG-1, and RAG-2 genes is detected in induced cell lines stably expressing tTA and either stably or transiently expressing RAG-1 or RAG-2. Fig. 3*B* shows that tTA protein is easily detectable by Western blotting in S2-6 cells induced for 48 h by tetracycline removal.

The Creation of Inducible Transgenic Mice. The relevant portions of pTet-tTAk and pUHC13-3 were purified and comicroinjected into fertilized eggs, which were then implanted into pseudopregnant female mice. Five transgene-positive founders were screened for inducibility by measuring luciferase levels in PBMCs 3–18 days after removal of tetracycline. Three founders, nos. 17, 19, and 20, showed high levels of luciferase activity after induction, ranging from 70- to 900-fold that obtained in extracts of PBMCs from transgene-negative mice in the same experiments (data not shown). Expression in founder 11 was leaky, and founder 12 showed no inducible luciferase in PBMCs. We presume that variability in inducibility and leakiness of transgenes in different founders is a consequence of the site of integration or structure of the integrated transgenes. There was no obvious correlation between levels of luciferase expression or leakiness in PBMCs and the copy number of the transgenes. Particularly significant was the observation that when founder mouse 20 was given water containing tetracycline for 18 days after a previous 7 day induction in the absence of tetracycline, luciferase levels dropped essentially to background, demonstrating that transgene induction is reversible (data not shown). We achieved germ-line transmission of the transgenes from founders 17 and 20, but not from founder 19.

To analyze the inducibility of luciferase in a variety of tissues and organs in transgenic mice, second or third generation transgene-positive progeny of founder mice 17 and 20 (backcrossed to C57BL/6) were removed from tetracycline for 7 or 8 days and were compared with transgene-positive progeny maintained on tetracycline. As shown in Fig. 4 and Table 1, the progeny of mouse 51 (from founder 17) showed luciferase activity in all organs examined. Levels of luciferase activity varied substantially between tissues, with expression consistently high in thymus and lung and low in liver and kidney. Induction ranged from 2-fold in testes to 150-fold in thymus. Luciferase activity (10^5–4×10^6 RLU/mg of protein) was also detected in day 17 fetal brain and liver of transgene-positive mice conceived in the absence of tetracycline. Additionally, transgene-positive mice conceived and maintained from gestation through 3.5 months in the absence of tetracycline continued to express optimal levels of luciferase and appeared normal. Progeny from founder 20 also showed the highest levels of inducible luciferase activity in thymus and lung, although inducibility and tissue distribution of luciferase were more restricted than in the progeny of founder 17. Northern blotting demonstrated that tTA mRNA levels were clearly induced in the thymus and lung from progeny of mouse 51 after removal of tetracycline (Fig. 3*C*). Mice removed from tetra-

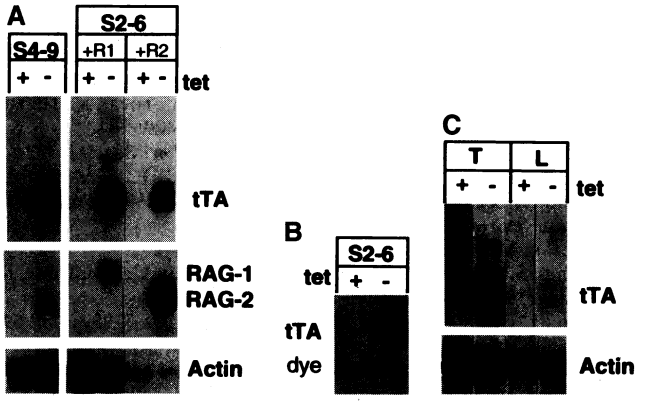

FIG. 3. Detection of mRNA and protein expression activated using the inducible autoregulatory system. (*A*) RNA blot of total cell RNA from S4-9 (stable cotransfectant containing pTet-tTAk, pTet-R1A/C, and pTet-R2A) cultured for 23 h in the presence or absence of tetracycline, and S2-6 (stable transfectant containing pTet-tTAk) transiently transfected with either pTet-R1 or pTet-R2 and cultured for 48 h in the presence or absence of tetracycline. Blots were sequentially hybridized with probes detecting tTAk, RAG-1 and/or RAG-2, and γ-actin mRNA. (*B*) Western blot of cell extracts from S2-6 cells cultured for 48 h in the presence or absence of tetracycline. The blot was probed with anti-tetR-containing hybridoma supernatant which detects the tTA protein. The dye front is indicated. (*C*) Blot of total cell RNA from thymus (T) and lung (L) of Tet-tTAk/Tet-luciferase transgenic mice maintained for 7 days in the presence or absence of tetracycline in their drinking water. Approximately 20 μg of RNA was loaded per lane.

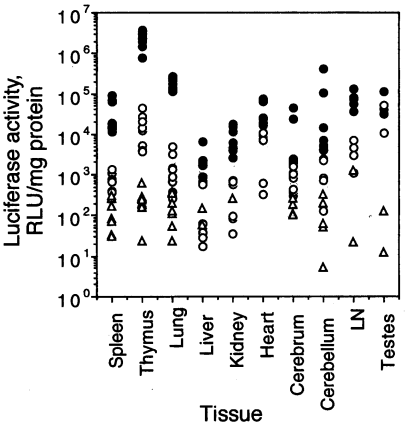

FIG. 4. Inducible luciferase activity in tissues of transgenic mice. Values represent the relative light units (RLU) (with lysis buffer background subtracted) per mg of protein in tissue lysates from 4- to 7-week-old mice maintained for 7 or 8 days in the presence or absence of tetracycline in their drinking water. △, transgene-negative mice; ○, uninduced transgene-positive mice; ●, induced transgene-positive mice. Mice were genetically identical with respect to the transgenes. Results are compiled from three separate experiments. LN, lymph node.

Table 1. Average luciferase activity and fold induction in tissues of transgenic mice

Tissue	Nontransgenic	Uninduced transgenic	Induced transgenic	Fold induction
Spleen	107 (9)	684 (8)	33,180 (10)	48
Thymus	220 (9)	16,243 (8)	2,448,580 (10)	151
Lung	138 (9)	1,617 (8)	169,538 (10)	105
Liver	69 (3)	214 (6)	2,022 (8)	9
Kidney	87 (3)	361 (6)	9,440 (8)	26
Heart	0 (3)	5,971 (6)	32,540 (8)	5
Cerebrum	94 (7)	754 (6)	9,836 (8)	13
Cerebellum	91 (7)	904 (6)	67,410 (8)	75
LN	617 (2)	3,892 (4)	74,449 (5)	19
Testes	71 (2)	30,398 (2)	60,911 (3)	2

The values shown are averages of data from the experiments shown in Fig. 4. The number of mice in each group is indicated in parentheses. The average fold induction for each tissue is shown. Values represent RLU/mg protein after subtraction of lysis buffer background (130–180 RLU). LN, lymph node.

cycline for up to 6 months appear healthy, indicating that induction of the tTA protein *in vivo* is not toxic or lethal. We have also observed mRNA hybridizing to a probe specific for the luciferase gene in thymus from induced mice.

DISCUSSION

The autoregulatory system (pTet-tTAk) described here represents a substantial improvement over a constitutive expression strategy (pcDNA-tTAk) in cultured cells, in all likelihood because it prevents toxic effects of the transactivator in the uninduced state and allows for higher levels of transactivator after induction. The constitutive expression strategy is less effective in two regards: a smaller fraction of clones produces any expression at all (18% vs. 90%) and induced V(D)J recombinase levels are much lower (by more than 98%, averaging over all clones). Stable transfection of pTet-tTAk should allow easy derivation of activator cell lines in which a variety of genes can be inducibly expressed by subsequent transient or stable transfection.

Previous attempts to create inducible transgenic mice by using genes activated by heavy-metal ions or aromatic hydrocarbons have been hampered by leakiness, relatively low levels of induction, restricted tissue specificity, and toxicity or carcinogenicity of inducing agents (refs. 8 and 17 and reviewed in ref. 1). The constitutive tetracycline system has been used to create inducible transgenic mice (6) and avoids some of the difficulties of these earlier approaches. Assuming that equally sensitive luciferase measurement procedures were employed, the autoregulatory system provides approximately two orders of magnitude more luciferase activity in thymus (1.1×10^4 RLU/mg protein maximum with the constitutive system vs. 2.5×10^6 RLU/mg protein with the autoregulatory system) and lung (1.5×10^3 RLU/mg protein maximum with constitutive system vs. 1.7×10^5 RLU/mg protein with autoregulatory system). Additional benefits of the modified system appear to be a greater induction of luciferase activity in the thymus (150-fold vs. 67-fold), and easily detectable levels of luciferase activity in tissues which show little or no activity in the unmodified system, such as lung, kidney, and brain. Additionally, since we detect activity in thymus, spleen, and lymph nodes, this system might be especially suited to studies of the immune system. As seen with the unmodified system, leakiness varies between tissues, though it is higher in the thymus with the autoregulatory system than with the constitutive system.

By comparison with luciferase protein standards, the luciferase activity that we observe in thymus corresponds to an average of approximately 30 molecules of luciferase per cell. However, we do not know what fraction of cells express luciferase activity or how expression levels vary between expressing cells. Since induction of tTA expression in this system depends upon a low level of leakiness of the tTA transgene, we expect that inducibility will vary with the transcriptional profiles of individual cell types and stages of differentiation. Therefore, per cell calculations of luciferase protein may underrepresent the actual levels induced in individual cells.

Our results demonstrate that highly inducible and reversible expression from a *tetP*-controlled reporter transgene can be obtained using the pTet-tTAk construct, and suggest that mice can develop normally in the presence of tetracycline and these transgenes and that induction by removal of tetracycline does not lead to any obvious ill effects on the mice, their ability to breed, or fetal development. Transgene expression appears to be tolerated and maintained for months. Therefore, the potential toxicity of the tTA protein *in vivo* may not be a serious difficulty.

The pTet-tTAk system should be widely applicable to the study of gene function in transfected cells and *in vivo*, to the creation of disease models for the testing of therapeutic agents, and to efforts to understand the development of mammalian organisms. It will be particularly useful in allowing regulated transgenic expression of genes otherwise too toxic to be tolerated by the organism during development.

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1. Yarrington, G. T. (1992) *Curr. Opin. Biotechnol.* **3,** 506–511.
2. Gossen, M. & Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 5547–5551.
3. Hillen, W. & Wissmann, A. (1989) in *Protein-Nucleic Acid Interaction: Topics in Molecular and Structural Biology*, eds. Saenger, W. & Heinemann, U. (Macmillan, London), pp. 143–162.
4. Hinrichs, W., Kisker, C., Düvel, M., Müller, A., Tovar, K., Hillen, W. & Saenger, W. (1994) *Science* **264,** 418–420.
5. Gill, G. & Ptashne, M. (1988) *Nature (London)* **334,** 721–724.
6. Furth, P. A., Stonge, L., Böger, H., Gruss, P., Gossen, M., Kistner, A., Bujard, H. & Hennighausen, L. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9302–9306.
7. Kozak, M. (1984) *Nucleic Acids Res.* **12,** 857–872.
8. Jones, S. N., Jones, P. G., Ibarguen, H., Caskey, C. T. & Craigen, W. J. (1991) *Nucleic Acids Res.* **19,** 6547–6551.
9. Schatz, D. G., Oettinger, M. A. & Baltimore, D. (1989) *Cell* **59,** 1035–1048.
10. Lewis, S. M. & Hesse, J. E. (1991) *EMBO J.* **10,** 3631–3639.
11. Lieber, M. R., Hesse, J. E., Mizuuchi, K. & Gellert, M. (1987) *Genes Dev.* **1,** 751–761.
12. Oettinger, M. A., Schatz, D. G., Gorka, C. & Baltimore, D. (1990) *Science* **248,** 1517–1523.
13. Schatz, D. G., Oettinger, M. A. & Schlissel, M. S. (1992) *Annu. Rev. Immunol.* **10,** 359–383.
14. Hesse, J. E., Lieber, M. R., Gellert, M. & Mizuuchi, K. (1987) *Cell* **49,** 775–783.
15. Silver, D. P., Spanopoulou, E., Mulligan, R. C. & Baltimore, D. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 6100–6104.
16. Oltz, E. M., Alt, F. W., Lin, W. C., Chen, J. Z., Taccioli, G., Desiderio, S. & Rathbun, G. (1993) *Mol. Cell. Biol.* **13,** 6223–6230.
17. Goodnow, C. C., Crosbie, J., Jorgensen, H., Brink, R. A. & Basten, A. (1989) *Nature (London)* **342,** 385–391.