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Commentary

Diverse strategies for tetracycline-regulated inducible gene expression

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The recent development of tetracycline (tet)-regulated transactivation systems for inducible gene expression has dramatically enhanced the tools available for the temporal and quantitative control of exogenous genes in mammalian cells and transgenic mice and plants. Such systems have applications in many areas of biology and medicine, including the study of gene regulation and function in developmental systems; the role and biochemistry of particular genes in various biological processes; and the safe, controlled, administration of gene therapy. These systems have two central components: transcriptional transactivators that interact specifically with bacterial cis regulatory elements and antibiotics that modulate the binding of the transactivators at low, nontoxic doses. The consequence is a substantial reduction of nonspecific pleiotropic effects observed with earlier systems. Here we summarize the current status of the use of tet-regulated transactivation systems for the control of gene expression, including the contribution by Hoffman *et al.* in this issue of the *Proceedings* (ref. 1).

The first tet-regulated gene expression system for use in mammalian cells, developed by Gossen and Bujard (2), involved constitutive expression of the tet transactivator protein (tTA) with the human cytomegalovirus (CMV) immediate early (IE) promoter/enhancer. tTA is a fusion protein composed of the tet repressor of *Escherichia coli* and the transcriptional activation domain of the VP16 protein of herpes simplex virus. In the absence of tet, the tet repressor portion of tTA mediates high affinity, specific binding to sequences from the tet resistance operator of Tn10 (*tetO*). In the presence of tet, however, a conformational change in tet repressor prevents tTA from binding to its operator (3). Genes to be regulated by tTA (e.g., luciferase) were placed under the control of a hybrid, inducible promoter (hereafter referred to as tetP) which consists of a human CMV IE minimal promoter preceded by seven copies of *tetO*. In this initial study, performed in HeLa cells stably expressing tTA, expression of luciferase was very low in the presence of ng/ml quantities of tet, and removal of tet resulted in as much as a 100,000-fold increase in luciferase levels. Luciferase levels could be varied by titrating the amount of tet in the growth media, and maximal, steady-state levels of activity were achieved in about 24 h. Somewhat surprisingly, tTA was undetectable in the HeLa cells by Western blotting (although it was detected with a sensitive gel mobility shift assay), an observation consistent with toxicity of the tTA protein. This was speculated to be a consequence of transcriptional squelching, in which tTA would act as a sink for the general transcriptional machinery of the cell, resulting in the death of cells expressing moderate to high levels of tTA. Several technical and practical reviews of this system and its advantages over other inducible expression systems have appeared recently (4–6).

This basic system, since its description, has been used extensively in tissue culture for the expression of a variety of different genes. HeLa cells stably expressing tTA have been used to study the consequences of tet-regulated, tetP-controlled, stable expression of a number of different proteins involved in various areas of cell biology. These proteins include some that regulate the dynamics of proteosome subunit assembly, a viral protein that inhibits peptide transport across the endoplasmic reticulum membrane, and a mutant dynamin protein whose overexpression has consequences for endocytic-coated vesicle formation (6–9). This system has also been used to identify the targets of a viral transcriptional transactivator, to examine the phenotype of cells overexpressing a tyrosine kinase that regulates c-*src*, and to determine the consequences of disregulated expression of various cell cycle regulators both in HeLa cells and rat fibroblasts (10–14).

The basic tTA system has also been used to produce transgenic mice reversibly expressing luciferase or β-galactosidase in a variety of fetal and adult tissues (15). Expression was consistently highest in thigh muscle and tongue and was heterogeneous within these tissues. It was speculated that this heterogeneity might be inherent in the CMV IE promoter used to drive tTA or the was result of heterogeneous patterns of transgene methylation. These mice were created by breeding mice expressing tTA to mice expressing the reporter transgenes.

Subsequently, Gossen *et al.* (16) described a modified system in which a reverse transactivator (rtTA, or rtTA-nls, which contains a nuclear localization signal at its 5′-end) was developed that binds *tetO* efficiently only in the presence of the tet derivatives doxycycline or anhydrotetracycline. Using the CMV IE promoter to drive stable expression of rtTA in HeLa cells, luciferase activity could be induced by 3 orders of magnitude in 20 h by the addition of the tet derivatives. The vectors encoding these transactivators also contain a *neo*^{r} cassette for selection in mammalian cells. It was proposed that this system would be especially useful in situations where cells or individuals were to be kept in the repressed state for long periods of time and where long term exposure to tet or its derivatives was undesirable or inconvenient (e.g., in gene therapy or transgenic animals), and in situations where one desired rapid induction, which might otherwise be limited by the rate of disappearance of tet from the system.

In an attempt to activate higher levels of gene expression than those obtained with the basic system, and to prevent possible toxic effects of constitutive tTA expression, we placed tTA under the control of tetP, resulting in the autoactivation of tTA in the absence of tet and suppression of tTA expression in the presence of tet (17). This autoregulatory system appeared to have two important advantages when compared with a system constitutively expressing tTA: it yielded substantially higher levels of target gene expression, and the frequency of inducible clones obtained was higher. We could readily detect tTA on Western blots, and optimal levels of the RAG proteins were detected after 12 h of induction by Western blotting (ref. 17, and unpublished data). Transgenic mice produced by coinjection of the autoregulatory tTA and a tetP-driven lucif-

Abbreviations: tet, tetracycline; tTA, tet transactivator protein; CMV, cytomegalovirus; IE, immediate early; LTR, long terminal repeat; SV40, simian virus 40; MMTV, mouse mammary tumor virus.

erase transgene expressed luciferase inducibly in a variety of tissues with highest levels in thymus and lung (17). Induced luciferase levels were 1–2 orders of magnitude higher than those reported with the constitutive transgenic system (15), but the levels in the uninduced state were also greater. We have not determined whether more efficient suppression would be achieved with a more potent tet derivative, tet pellet implants, or doses of tet greater than 170 μg/ml (15, 16, 18). Luciferase was expressed in fetal tissues and continued to be expressed in mice bred and maintained in the absence of tet to 3.5 months of age. We are currently analyzing the heterogeneity of gene expression in these mice and the potential for breeding them to mice harboring other tetP-driven transgenes for activation in trans. It is important to note, however, that while the autoregulatory system has yielded high level expression in cultured cells (with induced mRNAs easily detected by Northern blotting of total RNA), expression levels in transgenic animals are substantially lower. We estimated that in thymus, where the highest induced levels of luciferase were observed, that cells contained on average only 30 molecules of luciferase protein. It is likely that further refinements of the system will be required for homogeneous, high level expression in transgenic animals.

Although the systems described above have been used successfully in many cell lines and to some extent in transgenic animals, some possible obstacles must be considered when attempting to use these systems. Cautions have been raised regarding the general efficacy of the systems in all cell or tissue types (19, 20). Because of the heterogeneity in gene expression that has been observed in some cases, it is generally agreed that success in any given cell or tissue milieu might require alternative minimal promoters and careful choice of constitutive or tissue-specific promoters for transactivator expression (refs. 19, 21, and commentary, ref. 22). For example, in mice carrying *lacZ* reporter transgenes activated by tTA expressed from a mouse mammary tumor virus (MMTV)-LTR, relatively homogenous expression was observed in epithelial cells of the seminal vesicle and salivary gland, and in Leydig cells of the testis, but heterogeneous expression was observed in mammary epithelial cells and basal cells of the epidermis (22). Another possible problem comes from the random nature of gene integration. Integration site-specific effects (such as constitutive activity or repression), which are inherent when foreign DNA is stably introduced into a cell or the mouse germline, might be overcome by surrounding individual transcription units with matrix attachment regions, shown previously to insulate stably integrated vectors and transgenes from effects mediated by cis regulatory elements adjacent to their sites of integration (22–25).

While the experiments described above utilize transactivator driven by ubiquitously active promoters, tet-regulated gene expression also holds much promise for experiments that require tissue-specific expression. Several recent experiments have demonstrated tissue-specific expression of transactivator directed by tissue-specific promoters. Cardiac-specific expression of tTA and subsequently luciferase protein or Id1 mRNA has been achieved using the rat α myosin promoter in rats or mice, respectively (see below) (26, 27). Expression of SV40 large T antigen in pancreatic β cells in mice was achieved using the original tTA system modified such that tTA was driven by the rat insulin promoter (RIP) (28). In induced mice, β-cell tumors were evident by 5–6 months of age, and transformed cell lines were derived that, upon restoration of tet, stopped proliferating, and that, upon implantation, were able to reverse hyperglycemia in diabetic mice. Inducible expression of CIITA, a transcription factor that regulates major histocompatibility class II expression, has also been achieved in mouse pancreas in which the rat insulin promoter drives tTA expression (C-H. Chang and R. A. Flavell, personal communication).

In some instances, for example during gene therapy, there is a need for timed or pulsatile expression of a given target gene in a particular tissue. The temporal requirement for viral oncogene expression for cellular transformation in the submandibular gland has recently been investigated in mice producing tTA under MMTV-LTR control and SV40 large T antigen driven by tetP (L. Hennighausen, personal communication). The ability to silence large T antigen expression at desired time points uncovered a time dependence of oncogene expression for persistent cellular transformation and underscores the utility of the system for controlling the timing of gene activation.

The quantitative regulation of gene expression by modulation of tet levels has not been analyzed as comprehensively in mice as in cell lines. However, experiments involving the transfection of skeletal muscle or heart tissue by the direct injection of DNA have addressed this issue. Oral, tet dose-dependent suppression of luciferase activity driven by the basic two plasmid system has been achieved in injected mouse skeletal muscle with maximal expression after tet removal achieved by 48 h (29). Expression of luciferase driven by tTA expressed under the control of the cardiac-specific rat α myosin promoter injected into cardiac tissue in rats also exhibited tet dose dependence (26). Additionally, transgenic mice expressing the cardiac specific tTA showed a more rapid and greater induction of Id1 mRNA expression in the heart upon removal of tet from mice bred and maintained on sub optimal doses of tet (0.1 mg/ml) compared with mice bred and maintained on higher doses (1 mg/ml), demonstrating that control of the kinetics and level of activation might be achieved by modulating the suppressive dose of oral tet (27).

Most reported studies of mammalian cell lines and transgenic mice made using either the constitutive or autoregulatory tTA systems have introduced transactivator and target genes on separate plasmids or transgenes. In stable cell lines, DNA is introduced by transfection of individual plasmids consecutively or by cotransfection. Transgenic mice have been derived by breeding mice expressing transactivator to mice carrying the target gene and also by coinjection of transactivator and target DNA. An elegant study of transgenic tobacco plants by Weinmann *et al.* (30) demonstrated the feasibility of placing the tTA and reporter genes in opposing orientations on a single vector. tTA expression was controlled by either a plant-specific virus promoter or a structure-specific promoter and the β-glucuronidase (*gus*) reporter gene was controlled by a minimal promoter with upstream tet operator sites. Tight regulation of expression by tet allowed the measurement of *gus* mRNA and protein half-lives.

Streamlined single vector expression systems for mammalian cells have also recently been developed and provide advantages for certain applications. Baron *et al.* (31) constructed a series of plasmids that contain two minimal promoters in opposite orientations on either side of the heptamerized tet operon allowing the tet-regulated expression of two genes in stoichiometric amounts from a single vector. Vectors co-expressing luciferase and β-galactosidase were described, as were vectors that allow mixing and matching between other genes and luciferase or β-galactosidase. It was suggested that such a plasmid, if modified to contain two different minimal promoters, might allow two genes to be co-regulated at different efficiencies. Another plasmid has recently been described that combines tTA (driven by a CMV promoter plus the SV40 late promoter) and the luciferase gene driven in the opposite orientation by tetP (32). COS cells transiently transfected with this plasmid (which also includes a *neo*^{r} gene for selection in mammalian cells) expressed luciferase at levels comparable with, and with the same degree of leakiness as, those transfected with the initial two-plasmid system. Trans-

genic mice made with this vector express luciferase inducibly and reversibly with highest levels seen in thigh and abdominal muscles.

In an alternative method for delivery of tet-regulated genes, Paulus *et al.* (33) have adapted the one-vector approach for use in a retrovirus. The virus vectors contain tTA driven by either the SV40 promoter or a glial cell-specific promoter and either one or two copies of a luciferase gene driven in the opposite direction by tetP (33). These vectors also provide a gene for puromycin resistance. Induced luciferase activity (1–2 orders of magnitude) in infected cells was detected with these vectors upon removal of tet, although activity was not strictly glial-specific with the glial-specific promoter. It was proposed that in the vectors harboring a single luciferase gene (positioned along with the tTA gene between the viral LTRs) antisense inhibition of basal (but not induced) luciferase gene expression occurred as a result of transcription from the 5′-LTR.

Hoffman *et al.* (1) have now developed a system that merges the one-vector retroviral approach with the autoregulatory tTA expression strategy. This vector encodes a bicistronic mRNA allowing expression of both β-galactosidase and tTA from tetP, with tTA translation being initiated at an internal ribosome entry site. The virus self-inactivates during replication by deleting critical transcriptional control elements from the 5′-LTR, which prevents LTR interference with tet-regulated elements. Infection of primary myoblasts with this vector in the absence of tet resulted within days in β-galactosidase-expressing cells with a frequency expected for the viral titer used. The expression of β-galactosidase allowed for successive fluorescence-activated cell sorting and the subsequent enrichment of a population of cells with low basal and highest induced activity. Upon analysis of these selected cell populations, maximal activation of gene expression (1–2 orders of magnitude) upon removal of tet occurred by 48 h and was resuppressed 50% by 8 h. The authors discuss the need to include an additional fluorescence-activated cell sorting-detectable marker in the vector for the use of this strategy with target genes whose products cannot themselves be detected by fluorescence-activated cell sorting analysis. A bicistronic expression cassette encoding a tet-responsive target gene and a downstream alkaline phosphatase gene has also been used to screen stably transfected cells for low basal and high induced activity (34). The delivery of tet-regulated transgenes by retroviral infection is promising for some cell lines or primary cells that are difficult to transfect and possibly for gene therapy. Additionally, this method can theoretically eliminate integration site-specific effects that are averaged in uncloned populations of cells but which can become prominent in selected cell lines or clones.

Finally, another interesting virus-based approach involves the testing of vectors derived from the autonomous parvovirus, LuIII, for their ability to deliver tet-regulated gene expression units in tissue culture (35). These viruses, unlike retroviruses, are nonintegrating, a desirable feature for those contemplating gene therapy strategies involving short-term delivery of a cytokine or toxin to certain cell types (for example in targeting suicide genes for cellular ablation in cancer therapy). These viruses have no pathological effects in humans, and they have a related mouse counterpart. In NB324K cells, transiently transfected with a vector driving tTA expression from the CMV IE promoter, expression of a tetP-driven luciferase gene introduced on a LuIII-derived vector was up to 200-fold higher in the absence of tet, compared with in the presence of tet. Titration of tet levels resulted in a dose-dependent increase in luciferase activity. These vectors will potentially be useful in situations where long-term persistence of a tet-regulated transduced gene is undesirable.

These early tet-regulated transactivation systems have already proven useful for addressing several basic experimental questions. Biologists and clinicians are actively working to apply these systems to a broader array of biological problems (e.g., the creation of conditional knockout mice using the Cre-loxP recombination system of bacteriophage P1) (36–38). With the continued cooperation between the many labs using the existing systems and the ongoing development of novel vectors, it seems likely that tet-regulated expression systems will play an important role in biological research and perhaps clinical medicine in the future.

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