REVIEW

Changing colors in mice:
an inducible system that delivers

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Expression of exogenous genes in mammalian systems can be extremely informative for deciphering the function of an encoded protein or determining the role of a putative regulatory region. In many instances it is desirable or even essential to employ a system that has the ability to be controlled by the experimenter. For example, embryonic lethality that results from expression of an exogenous transgene or disruption of an endogenous gene can be problematic as it precludes examination of the phenotypic consequence in the organism under study. In these cases the use of an inducible gene expression system would be advantageous so that gene expression could be turned on or off at will. Inducible systems would not only be useful for analyzing the function of exogenous genes, but may also be implemented in gene disruption experiments or in gene therapy protocols in which a therapeutic gene is delivered. Many laboratories have designed systems that aim to achieve inducible gene expression. However, even the best laid plans often yield disappointing results in vivo. This is attributable to an incomplete understanding of regulatory mechanisms that orchestrate mammalian gene expression. Exacerbating this problem is the fact that bacterial or viral genes and regulatory elements that are often implemented in the scheme may be rendered nonfunctional in mammalian systems, further decreasing the chance of success and increasing the chance of frustration.

Features of the optimal inducible system

What features would the perfect inducible system have? The ideal inducible system should have several characteristics. First, gene expression should be tightly regulated such that “leaky” expression does not obscure experimental results or have deleterious effects on the organism under study. In other words, the basal level of gene expression should be low. Second, gene expression should be induced to sufficiently high levels to obtain a physiological response. This induction of gene expression should occur only when the inducer is present, and it should have absolute specificity for the target gene. In addition, the applied inducer should be nontoxic and should not alter the expression of endogenous genes in any way. Third, the system should be fully reversible, such that gene expression occurs at the will of the researcher. Fourth, results obtained with these systems in vitro should be recapitulated in in vivo systems within the desired target tissue. Thus, the optimal inducible system would provide high levels of tight, inducible, reversible gene expression in the appropriate tissue in vivo.

In this issue, Cronin and colleagues (2001) describe a two-component inducible system that achieves these standards. Their system uses regulatory elements from the bacterial lactose [lac] operon (Fig. 1). Regulatory elements from this operon, the lac operator and repressor, provide tight, reversible expression of genes involved in the uptake and utilization of lactose within the bacterial cell. In Escherichia coli, this expression is reversible—it is induced in the presence of lactose, and is rapidly shut down when lactose sources are depleted. The inducible and reversible gene expression that occurs with this bacterial system ensures that genes within the lac operon are expressed only when necessary. Cronin and colleagues (2001) used a modified version of the lac operator–repressor system to control expression of an exogenously introduced reporter gene in mice. In this custom-made system, expression of the reporter gene, Tyrosinase, is repressed by a synthetic version of the lac repressor. This repression is relieved in response to isopropyl beta-D-thiogalactoside (IPTG) treatment, and the Tyrosinase gene is turned on. This effect is fully reversible by withdrawing IPTG in both adult mice and in embryos that have been exposed to IPTG in utero. Tyrosinase encodes an enzyme that functions in the first step of melanin biosynthesis, and its presence imparts pigment to the hair (as well as to the inner ear and retina) that endows the mouse that expresses it with coat color. This study by Cronin and colleagues (2001) demonstrates the attributes of this inducible expression system by showing that coat color can be dramatically altered in response to IPTG. This system provides tight, inducible, reversible gene expression in vivo, and therefore it appears to fulfill the criteria for an optimal inducible system.
Inducible systems that are currently available

To fully appreciate this achievement, it is useful to highlight some of the features of other inducible systems that have been used by various laboratories during the last several years. This is not intended to be a thorough review of these inducible systems; rather, general principles of each approach and the limitations and advantages of the various strategies will be discussed.

Individual laboratories have developed a variety of different inducible regulatory systems for analyzing mammalian gene expression. These inducible systems can be classified based on the nature of the inducer that is used to regulate the system. Types of inducers that have been used include heavy metal ions, heat shock, antibiotics, steroid hormones, and IPTG. Some of these inducers are toxic to mammalian cells, and therefore have limited utility. Several approaches have been useful for regulating gene expression in mammalian cells.

Ecdysone-based inducible systems

Systems using the insect steroid hormone ecdysone as an inducer of gene expression in mammalian cells have been reported by several laboratories (Wang et al. 1998; Guo and Vishwanatha 2000; Luers et al. 2000; Yu et al. 2000). In some instances, these systems are able to function in vivo (No et al. 1996; Albanese et al. 2000; Hoppe et al. 2000). The first published report of an ecdysone-regulated system that functions both in mammalian cells and in transgenic mice used a mutated form of the ecdysone receptor that had altered DNA binding specificity such that it did not bind to endogenous response elements in mammalian cells (No et al. 1996). This receptor domain was ligated to the herpes simplex virus activation domain VP16 to generate a chimeric protein. This protein was able to induce reporter gene expression about 1000-fold in response to ecdysone treatment. To bind its response element (positioned upstream of a transgene, for example), the fusion protein must heterodimerize with ultraspiricle, the insect homolog of the retinoid X receptor in mammals. Because of the involvement of the retinoid receptor, one criticism of this approach is that pleiotropic effects may result from activation of endogenous genes.

Several groups have performed studies that aim to enhance the utility of this inducible system by eliminating the ability of the ecdysone inducible system to affect the expression of endogenous genes (Hoppe et al. 2000; Saez et al. 2000). The Evans laboratory screened natural plant compounds for their ability to induce the ecdysone system, but that were otherwise inert within mammalian cells. They found that ponasterone A was a potent inducer of gene expression in vitro and in vivo (Saez et al. 2000). Hoppe et al. (2000) used another strategy to bypass the potential problem of activation of endogenous genes by generating an inducible system that uses a fusion between the Drosophila and the Bombyx ecdysone receptors (Hoppe et al. 2000). This hybrid receptor retained the ability to bind to a modified ecdysone promoter but did not require the retinoid X receptor.

Estrogen-based inducible systems

A tripartite fusion was generated to achieve inducible gene expression that could be regulated by estrogen (Brausmann et al. 1993). This chimeric molecule contained the estrogen receptor, the yeast GAL4 DNA binding do-
main, and the VP16 activation domain. In response to estrogen, the chimeric molecule dimerizes, binds to GAL4-responsive promoters, and leads to activation of target gene expression. Although this system has been reported to achieve a 100-fold induction of gene expression in vitro, a disadvantage of using estrogen-based systems is that endogenous genes containing sequence elements similar to the consensus estrogen response element may also be modulated in response to estrogen treatment. For example the c-jun promoter contains such a sequence, suggesting a transcriptional mechanism for the increased c-jun mRNA levels found in cells during hormone-induced proliferation [Hyder et al. 1995]. Other endogenous genes have also been reported to contain estrogen-regulated sequences (Hyder et al. 2000). Therefore, the utility of estrogen-based inducible systems may be limited due to modulation of endogenous genes. A second drawback of using the estrogen-based system is that endogenous estrogen may activate basal transgene expression, resulting in leaky expression.

**Progesterone-based inducible systems**

Both the estrogen- and progesterone-based inducible systems use nuclear hormone receptors that are able to respond to ligand binding by dissociating from their inactivating complexes, forming dimers, binding DNA, and activating transcription [Evans 1988; Carson-Jurica et al. 1990, Pratt and Toft 1997]. The progesterone-based system uses a chimeric regulator, GLVP. This is a hybrid protein consisting of the GAL4 DNA binding domain, the herpes simplex virus transcriptional activation domain VP16, and a truncated form of the human progesterone receptor that retains the ability to bind ligand [Wang et al. 1994]. In response to treatment with a progesterone antagonist, RU486, GLVP forms a functional dimer that binds to promoters containing GAL4 upstream activator sequences and activates gene transcription. An advantage of this system is that RU486 can induce gene expression at a concentration that does not affect endogenous anti-progesterone or anti-glucocorticoid activity. This inducible system has been used to drive tissue-specific expression of a transgene [Wang et al. 1999], to regulate tissue-specific Cre-mediated gene disruption [Kellendonk et al. 1999], and to rescue a lethal phenotype in a knockout model characterized previously [Pierson et al. 2000].

**CID-based inducible systems**

This inducible system uses chemical inducers of dimerization (CIDs) to regulate gene expression. The system is based on the observation that rapamycin has the ability to induce dimerization of two cellular proteins, FKBP12 and FRAP. FKBP12 was fused to a DNA binding domain that has the ability to bind a unique DNA response element that is not bound by endogenous transcription factors. FRAP was tethered to the p65 subunit of NFκB. In response to rapamycin, the two different hybrid molecules dimerize, bind the DNA response element, and activate transcription of a downstream target gene. This system was able to induce expression of a reporter gene consisting of human growth hormone coupled to the DNA response element in a dose-dependent manner [Rivera 1996]. However, a disadvantage of using rapamycin to modulate gene expression is that it is involved in multiple signal transduction cascades that are likely to alter expression of endogenous genes.

For this reason, researchers have scrutinized the interaction between FKBP12 and different CIDs [Schultz 1998] and designed novel CIDs that do not activate the expression of endogenous genes [Keenan et al. 1998; Lamb and Jorgensen 1998; Lamb et al. 1999]. Using various modifications, CID-dependent gene regulation has been reported by several groups [Belshaw et al. 1996; Spencer et al. 1996; Fan et al. 1999; Shariat et al. 2001]. A drawback of this approach, however, is that genes encoding the two distinct chimeric proteins that are required for the formation of heterodimers must each be introduced into the system.

**Tetracyclin-based inducible systems**

Regulatory elements from the Tn10-encoded tetracyclin (tet) resistance operon from *E. coli* [Hillen and Berens 1994] have been implemented in gene expression systems that can be regulated by tet. One of these tet inducible systems utilizes a transactivator, tTA to regulate gene expression. This chimeric protein consists of a fusion between the herpes simplex virus transactivating domain VP16 and the tetracycline repressor from *E. coli* [Gossen and Bujard 1992, Gossen 1995]. tTA binds to the tet operator (tetO) when tet is absent. In the presence of tet, however, a conformational change in the tet repressor prevents the binding of tTA to tetO. When multiple tetO sequences are placed upstream of a reporter gene, continual expression of tTA results in activation of gene expression in the absence of tet. This system has been used in vitro [Fruh et al. 1994; Wimmel et al. 1994] and in vivo [Furth et al. 1994, Kistner et al. 1996]. However, a problem with this system is that tet must be present to repress gene expression, and tTA protein is toxic to mammalian cells [Gossen et al. 1993]. A second problem is that in several instances a high level of basal expression was observed [Furth et al. 1994; Howe et al. 1995; Kistner et al. 1996].

To eliminate the toxic effects of constitutive tTA expression in mammalian cells, an autoregulatory system was designed by inserting a tetO sequence within the tTA promoter. This autoregulatory system was able to achieve a higher level of reporter gene expression in transgenic mice as compared to the system in which tTA was constitutively expressed [Schockett et al. 1995].

Another version of the tet inducible system uses a reverse transactivator, rtTA, which binds tetO in the presence of the tetracycline derivative doxycycline. Using this system, target gene expression is induced in response to treatment with doxycycline [Gossen 1995]. However, a disadvantage of tet-regulated systems is that the effects
of long-term antibiotic treatment may have deleterious effects in vivo.

**IPTG-based inducible systems**

The virtue of the inducible system described in the work by Cronin et al. (2001) is that it calls on one of the earliest inducible systems to be characterized—the lactose operon described originally in *E. coli* by Jacob and Monod (1961). This bacterial operon consists of a set of genes that are regulated coordinately by lactose. The components of this system are a regulatory protein: the lac repressor (lacI), and a DNA sequence called the lac operator (lacO). This bipartite system ensures that lactose metabolism is regulated tightly based on the availability of lactose [Fig.1]. In the absence of lactose, lacO is occupied by lacI, thereby preventing transcription of genes involved in lactose utilization. With the addition of an inducer, such as IPTG, lacI undergoes a conformational change that decreases its affinity for lacO. This results in derepression, or release of the repressor from the operon, and subsequent transcription of genes that are needed for using lactose. This tight control of gene expression ensures that transcription is coupled to environmental factors, thereby preventing wastefulness. Therefore, within the context of the bacterial cell, the lac operator–repressor system fulfills several of the requirements for an optimal inducible system—it is both inducible and reversible.

**Optimization of an inducible system from the past: the lactose paradigm**

Although the lac operator/repressor system performs optimally within the organism in which it has evolved to serve, the bacterial cell, it functions quite poorly in mammalian systems. The lac operon in bacteria functions by a repression mechanism; that is, binding of lacI to lacO prevents gene expression when lactose is absent. Another version of this bacterial system used a modified form of the repressor that was able to drive reporter gene expression in eukaryotic cells (Gossen et al. 1993). Removing its repressor function and fusing its remaining IPTG- and DNA-binding domains to the VP16 activation domain created a modified version of lacI. This version of lacI functions by an activation mechanism; it binds to lacO sequences when IPTG is present (Deuschle 1989; Baim et al. 1991). This chimeric regulator binds lacO-containing promoters in response to IPTG, thus inducing the expression of a downstream reporter gene. The success of both the lac- inducible systems relies on a high occupancy of lacO by lacI. One of the intrinsic difficulties of this system is the problem of generating and maintaining high intracellular concentrations of the repressor protein. Although this system was able to achieve a dramatic induction of reporter gene expression in response to IPTG treatment in mouse cells (Baim et al. 1991), inducible gene expression in vivo has been problematic.

The Stambrook laboratory opted to use the lac operator-repressor system that functioned by a repression mechanism to generate transgenic mice. However, they found that the transgene was silenced by methylation (Scrable and Stambrook 1997). Although expression of bacterial genes in mammalian systems is notoriously difficult to achieve in vivo, Cronin et al. (2001) seemingly thwarted this obstacle by painstakingly examining the cis-acting regulatory elements that modulate expression of LacI. Using a combination of chimeric transgenes and detailed sequence analysis coupled with transfection assays that culminate with in vivo analysis, they were able to generate a souped-up version of the lac repressor protein that has the ability to repress genes that contain promoter elements that have lac operator sequences embedded within them. During this process, these researchers were presented with several challenges.

**Problem 1: cryptic splicing**

Results of a study that used the β-actin promoter to drive expression of LacI revealed that the promoter was methylated heavily and that the transgene was transcribed only in testis (Scrable and Stambrook 1997). Although this model may be useful for some applications, its utility is limited. In an effort to modify the LacI transgene so that it would be expressed ubiquitously in the mouse, the gene sequence was altered in a manner that would more closely resemble that of a mammalian gene, i.e., the codon usage was changed without altering the amino acid sequence (Scrable and Stambrook 1997). This improved version of LacI, called SynlacI, proved to be transcribed more widely in mice as compared to the original bacterial version. But disappointingly, neither LacI protein nor activity could be detected in cells transfected with SynlacI constructs, or in tissues from SynlacI transgenic mice.

Cronin and coworkers diligently persevered by analyzing the structure of the SynlacI transgene. An RT–PCR strategy was used to evaluate splicing fidelity of both the LacI and SynlacI transgenes. A putative cryptic splice site was detected in the SynlacI construct and chimeric constructs containing different combinations of LacI and SynlacI transgenes were generated to assess the functionally relevant sequence within the S’ portion of the transgenes. When these constructs were used in transfection assays, it was found that one of these transgenes was very efficiently and correctly spliced. This version of LacI is transcribed correctly in a wide range of tissues.

**Problem 2: translational block**

These various versions of the LacI transgene were then assessed for their activity by measuring their ability to repress a reporter gene in cotransfection assays. The reporter gene consisted of an SV40 promoter containing a lacO sequence, driving expression of β-galactosidase (LacZ). Again, the results obtained demonstrated the complexity of gene expression—there was a posttran-
scriptional block that precluded functional lacI protein from being expressed even though correctly spliced LacI transcripts were plentiful. Again, by generating transgenes that were chimeras between functional and non-functional transgenes helped to pinpoint the problem. This analysis determined that there was a 16 nucleotide difference in the 3' end of the transgene that precluded efficient translation, but the mechanism responsible for this phenomenon remains elusive. Nonetheless, this work generated a chimeric transgene (3'C4) that was efficiently expressed and has the ability to repress the reporter construct.

**Problem 3: silencing by methylation**

Transgenic mice expressing the modified version of the SynlacI transgene, called 3'C4, were generated. Once again, the result was disappointing as the 3'C4 transgene was only expressed in testis. To investigate the possibility that CpG content in the transgene contributed to silencing via methylation, CpG density of 3'C4 was compared to versions of the LacI transgene that were expressed ubiquitously, to versions of the LacI transgene that were expressed only in testis, as well as to the β-actin gene. The structure of the 3'C4 transgene was then altered by flanking the transgene-coding region with noncoding sequence from the rabbit β-globin gene. This alteration added CpG-poor regions to the transgene and also altered the proximity of the coding region to the promoter. This modified version of the transgene, [LacI], was ubiquitously expressed in transgenic mice. Expression of the LacI transgene produced functional levels of lacI protein that efficiently repressed a reporter gene both in vitro and in vivo. Furthermore, IPTG-induced de-repression of Tyrosinase expression was observed in adult mice, and in embryos exposed to IPTG in utero.

The take-home messages from this study are as follows. First, the transgene must be correctly transcribed and processed. Second, the transgene message must be efficiently translated. Third, the transgene integration site must remain transcriptionally active in vivo.

**Future uses for IPTG inducible systems**

This inducible system will be useful for expressing exogenous genes and for assessing the consequence of tissue-specific gene inactivation. In addition, the system may prove to be useful for developing animal models that will be valuable for designing novel approaches for gene therapy regimens. Aside from these important uses, the reporter gene chosen to demonstrate the applicability of this system, Tyrosinase, may itself be very effective for facilitating mouse functional genomics. By using IPTG-inducible Tyrosinase expression, it may be possible to tag specific genomic alterations with a marker gene affecting coat color. For example, mutant alleles generated by gene-targeting approaches could be marked using this system. The inducible Tyrosinase transgene could also be implemented into chromosome engineering strategies that use Cre-loxP technology to generate large-scale chromosome rearrangements in the mouse genome such as deletions, duplications and inversions (Ramirez-Solis et al. 1995). Tagging particular genomic modifications with genes expressing the visible coat color markers Tyrosinase and Agouti has been reported previously (Zheng et al. 1999). Whereas the Agouti transgene implemented in the study by Zheng et al. (1999) proved to be very useful, the Tyrosinase transgene produced mice with variable coat color that was dependent on the position of integration. Perhaps the inducible Tyrosinase transgene described by Cronin et al. (2001) could be used to generate modified alleles that are stably tagged. This would be invaluable for strain maintenance because it would obviate the need for genotyping. But even more importantly, tagging precise rearrangements with coat color markers would greatly facilitate phenotype-based genetic screens (Justice et al. 1997).

Although this work is certainly an achievement that will undoubtedly impact our ability to express genes in an inducible fashion in vivo, there are several questions that remain before we can say that this is, in fact, the ideal inducible system. Will the inducibility demonstrated for Tyrosinase be attainable for the expression of other genes? Is the inducibility tissue-specific? Does induction lead to a graded response? What are the long-term effects of repeated IPTG treatment and withdrawal within mammalian systems? Are these exogenous regulatory elements eventually doomed to silencing by methylation? Does the site of transgene integration alter the inducibility of the system? Future experiments that use this inducible system for delivering other gene products, introducing the inducible Tyrosinase transgene at various genomic loci by gene targeting, and generating inducible gene-targeted knockouts within specific tissues, will address these and additional questions.

**Conclusions**

Although it is becoming increasingly clear that mammalian gene expression is regulated by multiple mechanisms that are extremely complex, especially in vivo, recent successes such as that described by Cronin et al. (2001) now make the goals of inducible gene expression within the context of an entire organism within our grasp. These systems provide an avenue for analyzing the effects of altered expression or ablation of a particular gene product, and for delivering therapeutic genes in an inducible fashion in gene therapy protocols. The work by Cronin and coworkers is an exercise in the analysis of gene expression that provides a valuable tool for exploring gene function. Although there is still much to learn in the game of regulating gene expression in mammalian systems, it is clear this is an inducible system that delivers.

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