The *lac* operator-repressor system is functional in the mouse

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We report the successful transfer of a fully functional *lac* operator-repressor gene regulatory system to the mouse. The key component is a *lac* repressor transgene that resembles a typical mammalian gene both in codon usage and structure and expresses functional levels of repressor protein in the animal. We used the repressor to regulate the expression of a mammalian reporter gene consisting of the tyrosinase promoter embedded with three short *lac* operator sequences and the tyrosinase coding sequence. Pigmentation of the mouse was controlled by the interaction of the *lac* repressor with the regulatable *Tyrosinase* transgene in a manner that was fully reversible by the lactose analog IPTG. Direct control of mammalian promoters by the *lac* repressor provides tight, reversible regulation, predictable levels of de-repressed expression, and the promise of reversible control of the endogenous genome.

[Key Words: *lac* repressor; gene regulation; transgenic mice; tyrosinase; pigmentation; albino]

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The mouse is the most widely used experimental animal to model mammalian development and disease. In recent years, technological advances in genetic manipulation of the mouse genome have made it even more valuable as a research tool, and a great deal of information has been learned from conventional knockout and transgenic experiments. Despite this, it can be difficult to draw definite conclusions from these studies. Embryonic lethality might preclude the possibility of analyzing adult phenotype, or activation of compensatory systems may confuse the analysis. Tight, reversible control of gene expression would greatly broaden the possible experimental questions that can be addressed. To gain such control, the ideal system would enable a target gene to be switched on and off repeatedly, without affecting the expression of nontargeted genes. With this ideal in mind, we have developed the *lac* repressor regulatory system for use in the mouse.

The *lac* operon of *Escherichia coli* consists of a set of genes coordinately regulated by lactose (Jacob and Monod 1961). The regulatory components of the system are the *lac* repressor and its DNA-binding sequence, the *lac* operator. In the absence of lactose, the *lac* repressor occupies the *lac* operators and prevents transcription. Lactose causes a conformational change in the repressor, and it vacates the operators, allowing RNA polymerase to gain access to the promoter and initiate transcription.

Hu and Davidson [1987] were the first to use *lac* elements to control reporter-gene expression reversibly in mammalian cells. Their results were extended by Figge et al. [1988], who demonstrated that the *lac* repressor was able to gain access to the mammalian chromosome to regulate a stably integrated reporter gene.

Our goal has been to adapt the *lac* regulatory system to control gene expression in the mouse. In an earlier paper, we reported that transgenes containing the bacterial-coding sequence for the *lac* repressor downstream of the β-actin promoter were heavily methylated and only transcribed in the testis of transgenic mice (Scrable and Stambrook 1997). Methylation and silencing in mice also was observed by Wyborski et al. when the bacterial *lac* repressor sequence was downstream of the F9–1 polyoma promoter [Wyborski et al. 1996]. To create an active transgene, we changed the primary DNA sequence of the bacterial *lacI* gene to resemble a mammalian coding sequence more closely and still code for the same amino-acid sequence. This *synlacI* gene was transcribed widely (Scrable and Stambrook 1997). However, we could not detect the *synlacI* protein product, either by Western blot or immunocytochemically, in the tissues of SynlacI transgenic mice or in cells transfected with the *synlacI* transgene. We also could not detect any effect of the repressor from *synlacI* on reporter-gene activity, either in embryonic mouse cells from transgenic animals, or in melanoma, neuronal, or kidney cell lines transfected with the *synlacI* transgene [data not shown].

In this paper, we describe how we changed the DNA sequence and gene structure of *lacI* so that it expresses functional *lac* repressor protein ubiquitously in mice. We show that the *lac* repressor can repress the activity of a reporter gene, which subsequently can be derepressed by IPTG in the drinking water of an adult or the mother of an embryo or nursing pup. The *lac* operator-repressor
system brings the temporal dimension of mammalian gene expression in the animal under experimental control that is both reliable and predictable, and should make it possible to introduce even lethal mutations into the mouse genome routinely and to study them at the organismal level.

Results

Reversion of four bases to the wt lacI sequence corrects a cryptic splice site in synlacI

To determine why no protein was observed from the synlacI transgenes, we analyzed the RNA transcripts for evidence of processing errors. We consistently observed a single transcript on Northern blots of RNA from animals transgenic for the bacterial, or wt lacI [W] construct, but a doublet in RNA from animals with the re-encoded, or synlacI [S] transgene [Scrable and Stambrook 1997; Fig. 3, below]. The sizes of the two transcripts with synlacI suggested that the upper band represented an unspliced transcript still containing an intron from the human β-actin promoter, and the lower band, the spliced transcript. On careful examination of synlacI and wt lacI transcripts, we found that the processed synlacI transcript was actually slightly smaller than the wt lacI transcript. Incorrect splicing at a cryptic splice acceptor site downstream of the start codon could explain both the smaller size of the RNA and the absence of protein product. We developed an RT-PCR assay, with primers that flank the region encompassing the intron in the β-actin promoter and the translation start site in the lac repressor coding region [Fig. 1A, arrows]. The difference in size between the correctly spliced W product and the correctly spliced S product (513bp vs. 499 bp) is due to a difference in the size of the region linking the promoter to the coding region [see Materials and Methods]. As can be seen in Figure 2A, the predominant product in RNA from the W animal is the correctly spliced product, whereas the predominant product in the S animal is smaller. This suggests that an aberrantly large region is spliced out of the synlacI transcripts.

We prepared a series of chimeric repressors made by exchanging the 5′ region of the wt lacI or synlacI constructs, as represented schematically in Figure 1A [5′C1–5′C4] and analyzed the splicing patterns of the constructs in transfected cells. Only one of these [5′C1] produced the correctly spliced product as the predominant PCR product [Fig. 2A]. We correlated the splicing pattern with the presence of specific regions from W and S, and we found that the first 36 bp of the synlacI coding sequence was the only region consistently associated with incorrect splicing. Within these first 36 bp of coding sequence, the wt lacI and synlacI constructs differ by only four bases, as shown in Figure 2B.

Analysis of potential splice sites was performed using the Walkers sequence analysis program [Rogan et al. 1998]. The only potential donor site in the transgenes is the splice donor site in the β-actin promoter. For the splice acceptor, in addition to the site in the β-actin promoter, there is a potential site just downstream of the four bases that differ between W and S in the lacI coding region [Fig. 2B]. Based on the size of the smaller product identified by our RT-PCR assay, this potential site is the acceptor site utilized in synlacI. We cannot explain why this acceptor site is used rather than the splice-acceptor site in β-actin, which by current models should be stronger.

Reversion of a 3′ region of the coding sequence to wt lacI corrects a translational block in synlacI

To test whether the constructs coded for functional lac repressor protein, we introduced each into Rat2 fibro-
Data are expressed as a percentage of the baseline struct in combination with the activity in Rat2 cells transfected with the regulatable SVOZ constructs. Graphs of (\text{lacI}) constructs containing the beginning of the coding region from synlacI and\textit{C1} and \textit{C2}/H11032, indicates the proposed (incorrectly utilized) splice acceptor site in the\textit{C1} and \textit{C2}/H11032 constructs other than W to produce predominantly the correctly spliced product like S, does code for functional repressor, although the level of repression is not as tight as with W (31% vs. 7%). These results indicated that, in addition to splicing, there was a second problem with the synlacI coding region that affected the expression of functional repressor.

We made a second set of chimeras in which 3’ regions of the W and S coding sequences were exchanged. These constructs are represented schematically in Figure 1A (3’C1–3’C4). As represented graphically in Figure 2D, both 3’C2 and 3’C4 had repressor activity, whereas 3’C1 and 3’C3 did not. These data indicate that the problematic region in synlacI is in the 150 bp between the 5’ EcoRV site and the 3’ PvuII site. Within this region, W and S differ by only 16 bases. We could find no coding error, cloning artifact, or other mutation in this region that could account for the lack of repressor function. We used site-directed mutagenesis to change all 16 bases in synlacI, in groups of two to five to the\textit{wtlacI} sequence. None of these smaller changes could restore repressor function on their own (data not shown).

We hypothesized that loss of repressor function was the result of a synlacI sequence element located between the EcoRV and PvuII restriction sites that blocked RNA transcripts from being translated. This hypothesis is supported by our finding that a synlacI construct that splices correctly (5’C1) is expressed strongly at the RNA level, but is only barely detectable at the protein level (data not shown). Two mechanisms that could account for this translational block are: (1) nuclear retention of the RNA such that it does not reach the cytoplasm to be translated, or (2) tight secondary structure of the RNA such that the translation machinery cannot access the primary sequence. The first hypothesis was ruled out by finding that equal levels of\textit{lacI} message were seen in the total and cytoplasmic fractions of both\textit{wtlacI} and...
synlacI RNA extracts on Northern blot (data not shown). The second hypothesis was ruled out by analyzing the secondary structure predicted for each RNA sequence (Zuker et al. 1991) in which no significant differences were found (data not shown). Consequently, we can conclude that the expression of functional lac repressor activity is blocked by the region of the synlacI sequence between the EcoRV and PvuII sites, but the mechanism responsible for this block is not known.

Restructuring optimizes transcription of CpG-rich lacI transgenes in the mouse

Having identified a modified synlacI coding sequence that was correctly spliced and translated in transfected cells (3’C4), we derived transgenic mice with this construct. We analyzed lacI expression by Northern blot, and the results are shown in Figure 3. We found that 3’C4 transgenes were expressed only in testis, resembling transgenes composed entirely of bacterial coding sequence. (See Materials and Methods for details on the mouse lines for each transgene).

We knew from our previous studies that the content of CpG dinucleotides in the coding sequence was a major determinant of RNA expression in the animal (Scrable and Stambrook 1997). The bacterially derived W construct contains 97 CpG dinucleotides in the coding region and 5’ linker and is expressed only in the testis of transgenic mice. Removal of all but five of these CpG dinucleotides resulted in the S construct, which was expressed in all tissues [Scrable and Stambrook 1997]. To examine the arrangement of CpG dinucleotides in each transgene more closely, we created CpG-density maps of each one and then aligned them with the CpG-density map of the β-actin locus (Fig. 3). In the endogenous β-actin locus there is a CpG island, which extends from the proximal promoter into the β-actin coding region. The high CpG content of W downstream of the β-actin promoter results in recapitulation of the CpG island in the β-actin locus, but both S and 3’C4 are relatively devoid of CpG in this same region. However, S is expressed like β-actin and 3’C4 is expressed like W.

Next, we made two constructs in which either W or 3’C4 coding regions were flanked with noncoding regions derived from the rabbit β-globin gene (constructs M and R, respectively). This added CpG-poor sequences to the transgenes, and moved the respective lacI coding regions farther away from their promoters. As shown in Figure 3, the M line does have a more widespread expression pattern than the W line, although it is not ubiquitous. However, the combination of the relatively CpG-poor coding region of 3’C4 and the flanking globin sequences [R] results in global lacI transcription (Fig. 3).

One possible explanation for these different expression patterns is that an open structure that allows for transcriptional activity relies on a downstream element in the β-actin coding region that is free of CpG dinucleotides. There are several segments downstream of the promoter that are devoid of CpG. Two of these β-actin elements, and their analogous regions in each of the lacI constructs, are indicated by the dashed lines in Figure 3. In W, both of these elements are CpG-rich and transgene expression is restricted to testis. In S, the regions are CpG-poor, and expression is nearly ubiquitous. In 3’C4, replacement of the sequence between the EcoRV and PvuII sites in S with the corresponding sequence in W changed the distal element from one devoid of CpG to one that is CpG-rich, and the expression pattern from one that is ubiquitous to one restricted to the testis. Flanking the coding region of 3’C4 with the globin sequences shifted CpG-poor sequence into the two regions, and resulted in nearly ubiquitous expression, similar to the expression pattern of the endogenous β-actin gene.

The lac repressor is widely expressed and functional in the LacI8 transgenic mice

The key to using the lac repressor system in mice is expression of functional levels of repressor protein. We prepared tissue protein extracts and tissue sections from mice transgenic for the R transgene (lacI8) and analyzed the expression of the lac repressor by Western blot (Fig. 4A) and immunohistochemistry (Fig. 4B). There is no evidence of a translational block in any tissue tested (brain, thymus, heart, lung, liver, spleen, kidney, testis, muscle, and skin). The strong nuclear staining of the protein seen with immunohistochemistry is a result of a
nuclear localization signal sequence appended to the carboxy terminal of the lac repressor (See Materials and Methods).

To determine if the lac repressor in this line is functional, we prepared cultures of primary embryonic cells from R animals and transfected them with the regulatable SVOZ construct. The embryo-derived cells exhibited strong lac repressor activity, as there was very little β-galactosidase expression in the absence of IPTG in the culture media (Fig. 5). A titration curve of the amount of IPTG needed to derepress expression shows that as little as 50 µM IPTG in the culture media allows for full levels of expression (Fig. 5A), which correlates well with the level needed to derepress transcription at the bacterial lac operon (Cho et al. 1985). At 20 mM IPTG in the media, we observed some toxicity, indicated by decreasing numbers of β-galactosidase–positive cells (Fig. 5B). Although the level at which toxicity was observed agrees well with the findings of Figge et al. (1988), we found that full derepression was achieved at a much lower concentration of IPTG.

**Target gene activity is controlled by LacIR and IPTG in the mouse**

We tested the lac operator-repressor system in mice using a regulatable version of a well-characterized visible marker gene, tyrosinase. Tyrosinase is the protein product of the albino (c) locus (Kwon et al. 1987), and is the enzyme that catalyzes the first step in melanin biosynthesis. The target transgene consists of the wildtype murine tyrosinase cDNA under the control of the murine tyrosinase promoter modified to contain lac operator sequences. The major transcription start site in the tyrosinase promoter is 83 bp upstream of the start codon. To maintain the endogenous spacing of promoter elements in the critical region between the start of transcription and the start of translation, we used PCR-based, site-directed mutagenesis to change 25 bp of the endogenous sequence to create a primary lac operator centered at 59 bp upstream of the start of translation. Additional operators were inserted 176 bp and 526 bp upstream of the primary operator [Fig. 1B].

Mice containing this modified Tyrosinase transgene resemble pigmented animals previously described (Yokoyama et al. 1990; Methot et al. 1995) that had been microinjected with an unregulatable version of the same transgene. We established two lines of pigmented Tyrosinase transgenic mice with the regulatable transgene. The TyrlacO−25 line displays a himalayan pigmentation pattern (as shown in Figs. 6A and 7C), and the Tyr, LacIR double transgenic mouse. We crossed mice transgenic for the Tyrosinase transgene to mice transgenic for LacIR. In double transgenics, the lac repressor should bind to the operator sequences located in the tyrosinase promoter, block transcription of tyrosinase, and revert pigmented animals to albino. An example of a TyrlacO−25, LacIR double-transgenic mouse is shown in Figure 6A next to a mouse transgenic for TyrlacO−25 alone. The coat of the double transgenic is unpigmented and indistinguishable from that of a nontransgenic albino. Treatment of a double transgenic animal with 10 mM IPTG in the drinking water derepressed tyrosinase expression, resulting in a phenotype indistinguishable from that of the mouse transgenic for TyrlacO−25 alone [Fig. 6A].

The stringency of repression and derepression is illustrated by the pigmentation of the eye. Figure 6B shows dissected eyes, and Figure 6C shows sections through the eyes of a nontransgenic albino, a Tyr transgenic, a Tyr, LacIR double transgenic, and a Tyr, LacIR double transgenic mouse treated with IPTG. Repression of target transgene expression is accompanied by an absence of
melanin in the retinal pigment epithelium (RPE) of the double-transgenic animal (Fig. 6C). The entire RPE is devoid of melanin, as can be seen by the completely unpigmented appearance of the eye in whole mount (Fig. 6B). Derepression by IPTG is accompanied by a restoration of pigmentation in the RPE to levels indistinguishable from the nonrepressed state (Fig. 6B,C). We obtained similar results with both lines of regulatable Tyrosinase mice (data not shown for Tyr<sup>lacO-43</sup> adults, but see Fig. 7). This indicates that regulation is neither insertion site specific nor simply fortuitous, but controlled by the lac repressor acting specifically on a target gene with lac operator sequences in its promoter. The albino mutation is a single basepair change in the coding sequence of tyrosinase, which causes a single amino acid change in the protein. Because the mutant allele is both transcribed and translated, we have not been able to assay promoter activity quantitatively at the molecular level. Nevertheless, we can infer from its effect on pigmentation that the tyrosinase promoter is in fact regulated by the lac operator-repressor system tightly, in a biologically relevant manner.

These results also show that IPTG can be introduced into the drinking water and circulate in the mouse at a level sufficient to derepress target gene expression. This level appears to be completely nontoxic. At the time of this writing, we have had Tyr<sup>lacO-43</sup>, LacI<sup>R</sup> double-transgenic mice on 10 mM IPTG in their drinking water for up to 8 months with no deleterious effects.

Regulation is functional during embryogenesis, and reversible

We next wished to determine if lac elements could regulate pigmentation during embryogenesis and if IPTG could act transplacentally. Figure 7A and B shows the embryonic and newborn pigmentation of the Tyr<sup>lacO-43</sup> line. At E9, tyrosinase activity in the embryonic eye begins to deposit pigment in the developing retinal pigment epithelium. At E12.5, the mouse RPE clearly is pigmented (Drager 1985). As shown in Figure 7A, Tyr<sup>lacO-43</sup> transgenic mice recapitulate this developmental event. A distinct band of pigmentation surrounding the central lens is seen in the Tyrosinase transgenic embryo that is not seen in the nontransgenic albino (Fig. 7A). The lac repressor blocked pigmentation during embryogenesis in the double-transgenic embryo, but not when the mother was treated with IPTG during pregnancy (Fig. 7A). These results clearly demonstrate not only that lac regulatory sequences function well during embryogenesis, but also that IPTG can cross the placenta to alter the phenotype of developing animals.

Finally, we tested the reversibility of the system by switching the Tyrosinase transgene on after it had been off, or off after it had been on, in the same animal. In Figure 7B, the phenotypes of eyes of newborn mice are compared to the phenotypes of embryonic eyes of mice of the same genotype. The mother of the IPTG-treated Tyr<sup>lacO</sup>, LacI<sup>R</sup> double transgenic shown in Figure 7B was not started on IPTG in her drinking water until E12.5 of the pregnancy. As is shown in Figure 7A, this double-transgenic pup would have shown the albino phenotype at E12.5. The fully pigmented eyes seen at birth in this animal demonstrate that even after a period of silencing, derepression by IPTG was able to switch tyrosinase expression on. Figure 7C illustrates that reversibility is also possible in the opposite direction. The Tyr<sup>lacO-25</sup>, LacI<sup>R</sup> double-transgenic pup shown on the left at P8 was taken off IPTG at P9. Removal of IPTG caused reversion of the phenotype to albino, as shown in the picture of the same animal as an adult on the right. As expected, the eyes remain pigmented due to the low turnover of cells and melanosomes in the RPE.
Discussion

We have described modifications made to the lac operator-repressor system that have allowed it to be used to regulate gene expression in the mouse. We have expressed the lac repressor in mice at levels that provide tight and reversible experimental control of gene expression. We have inserted lac operators into the mammalian target promoter so that it is directly controlled by the lac repressor. These changes greatly improve the reliability and predictability of the lac system over other regulatory systems used in mice. This system is a technological advance that should increase significantly the kinds of experimental questions that can be addressed in a mammalian model system.

A re-encoded, restructured version of the bacterial lacI gene can transit embryogenesis without being silenced and can function like a normal mammalian gene

Experiments using the lac operator-repressor system in cultured mammalian cells (Brown et al. 1987;
that the target for the lac repressor is its DNA binding sequence, the lac operator. The lac operator is a short (~25 bp) sequence that easily can be incorporated into an existing promoter without compromising the efficacy of the promoter itself. In principle, this makes it possible to confer regulatability on virtually any mammalian gene simply by inserting operator sequences at appropriate sites in its promoter region. Our target promoter was a genomic fragment of DNA shown to be the promoter of the murine Tyrosinase gene (Kwon et al. 1987). It was modified at three sites, inserting a little more than 100 bp over 2.2 kb stretch of DNA, with lac operators positioned in a way that simulated the overall structure of the regulatable promoter in the lac operon. The pigmentation patterns of our two founder lines both closely resembled the patterns of pigmentation observed in animals that had been microinjected with the unmodified Tyrosinase transgene (Yokoyama et al. 1990; Methot et al. 1995). These results indicate that the modifications we made to the promoter when we inserted lac operators did not have a significant, nonspecific impact on promoter activity. Tyrosinase activity originating at the transgene locus, as determined physiologically by the deposition of melanin, followed a normal pattern during development in the absence of repressor, further supporting our contention that operator insertion only minimally affected unregulated promoter activity, if at all.

The lac regulatory system in mice

Insertion of lac operators into a mammalian promoter make it directly regulatable by the lac repressor

The target for the lac repressor is its DNA binding sequence, the lac operator. The lac operator is a short (~25 bp) sequence that easily can be incorporated into an existing promoter without compromising the efficacy of the promoter itself. In principle, this makes it possible to confer regulatability on virtually any mammalian gene simply by inserting operator sequences at appropriate sites in its promoter region. Our target promoter was a genomic fragment of DNA shown to be the promoter of the murine Tyrosinase gene (Kwon et al. 1987). It was modified at three sites, inserting a little more than 100 bp over 2.2 kb stretch of DNA, with lac operators positioned in a way that simulated the overall structure of the regulatable promoter in the lac operon. The pigmentation patterns of our two founder lines both closely resembled the patterns of pigmentation observed in animals that had been microinjected with the unmodified Tyrosinase transgene (Yokoyama et al. 1990; Methot et al. 1995). These results indicate that the modifications we made to the promoter when we inserted lac operators did not have a significant, nonspecific impact on promoter activity. Tyrosinase activity originating at the transgene locus, as determined physiologically by the deposition of melanin, followed a normal pattern during development in the absence of repressor, further supporting our contention that operator insertion only minimally affected unregulated promoter activity, if at all.

Reversible regulation of pigmentation in the transgenic mouse by elements from the lac operon of E. coli is the first successful demonstration that bacterial sequences can be used to create trans-operons in mice that function analogously to their bacterial counterparts. The normal or “ground” state of the regulated promoter is repressed, or inactive. In the case of our Tyr<sup>lacO</sup>, LacI<sup>P</sup> double-transgenic mice, this results in a total lack of pigment and a phenotype indistinguishable from that of a nontransgenic albino. IPTG derepresses the regulated gene: tyrosinase is switched on and the phenotype of the derepressed transgenic animal becomes indistinguishable from that of the nonrepressed transgenic animal.

This all-or-none switch is achieved at a nontoxic dose of IPTG. We have seen full derepression in cells from the LacI<sup>P</sup> transgenic animals with as little as 50 µM IPTG, which agrees with the levels needed in E. coli for derepression of the endogenous lac operon (Cho et al. 1985). This is in contrast to a previous result suggesting that the levels of IPTG needed for full expression with the lac system are toxic (Figge et al. 1988).

The lac operator-repressor system has advantages over the currently available gene regulatory systems for the mouse

To overcome the problems of embryonic lethality and developmental compensation that hinder the utility of traditional knockout strategies, a system was developed that allows target genes to be excised from the genome in a tissue-specific and timing-controlled manner. The Cre/loxP recombination system (Gu et al. 1993, 1994) is based on the cre recombinase of bacteriophage P1 that catalyzes site-specific recombination between loxP sites. An advantage of cre-mediated excision is that the experimental manipulation can be performed on an endogenous locus. However, excision is a one-time event, which cannot be reversed. The lac system is fully reversible. In addition, by incorporating lac operators into a mammalian promoter by homologous recombination, it should be possible to regulate the expression of endog-
genic loci, as has already been done at the CDC2 locus in cultured cells [Itzhaki et al. 1997]. Thus, with the lac system, it should be possible to create reversible mouse models of disease and elucidate gene function in their natural context.

Reversible systems that use mammalian regulatory elements to control gene expression in cells and animals (such as heavy metal or hormone responsive systems) have been used for some time. They suffer from the fact that, by definition, the inducing agent also will have effects on endogenous genes. Regulatory systems based on elements from the bacterial tet and lac operons, on the other hand, are exquisitely specific for the gene of interest in the context of the mammalian genome.

To use the tet system of gene regulation in mammalian cells, Gossen and Bujard converted the tet repressor into the tet transactivator. This fusion of the tet repressor and the activating domain of the herpes simplex virus VP16 transcriptional activator, made it necessary to couple the tet operator to a viral promoter permanently [Gossen and Bujard 1992]. Binding of repressor to operator serves to align the VP16 fusion partner with its specific binding site in the viral promoter, and it is the binding of VP16 to the viral promoter that activates transcription. The tet transactivator [tTA] or reverse transactivator (rtTA) no longer functions as a repressor, and instead functions analogously to the typical mammalian transcription factor. Tissue-specific gene expression can be achieved by having a specific mammalian promoter drive expression of the tet transactivator (or rtTA).

The ground state of the minimal viral promoter is one that is less active than the induced state, and binding of the transcription factor increases the activity of a promoter that is already in the active configuration. This has made the tet system particularly suited for those applications in the mouse in which the phenotype to be controlled depends on the relative levels of an active transgene. For example, hyperplasia [Guo et al. 1999; Xie et al. 1999], prion disease [Tremblay et al. 1998], cardiomyopathy [Redfern et al. 1999], and Huntington’s disease [Yamamoto et al. 2000] all have been successfully modeled in mice using the tet-responsive minimal CMV promoter and the tTA or rtTA. The tet system also has been used to study the molecular mechanisms of memory formation [Mayford et al. 1996, Mansuy et al. 1998a,b] and the role of FGF7 in lung development and injury repair in the adult mouse [Tichelaar et al. 2000].

The drawbacks of using an activator-responsive promoter, however, become apparent in situations where it is desirable to switch the promoter between an inactive state and an active state that results in physiological levels of tissue-specific expression. The endogenous endothelin receptor-B locus was replaced with tet-regulated elements to determine the role of the receptor during embryogenesis [Shin et al. 1999]. In addition to the enormous effort such an undertaking must have required, the use of the tet system to control gene expression in this way was not entirely predictable and induction was incomplete.

Tet relies on viral promoter elements, and in the mouse, nonmammalian promoters very frequently lead to erratic expression of downstream coding sequences [Furth et al. 1991]. Low-level leakiness [Shockett et al. 1995] and heterogeneous expression [Redfern et al. 1999] have been problems with use of the minimal CMV promoter, and the VP16 activating domain has been found to be toxic to cells [Baron et al. 1997]. Our approach of directly targeting the lac repressor to operator sequences incorporated into mammalian promoters completely eliminates the necessity of using viral promoters or viral DNA-binding proteins in conjunction with a prokaryotic-based regulatory system. A target gene made up entirely of mammalian elements is more likely both to maintain functionality after transiting embryogenesis and to be expressed in the appropriate tissue at the appropriate level. This lends the system a particularly strong element of predictability that other prokaryotic-based systems cannot match.

In summary, by modifying both the target promoter and the gene encoding the lac repressor, we have been able to adapt a regulatory system used in bacteria to control the transcription of genes so that it can function analogously in the complex environment of the mouse. The LacI<sup>B</sup> mouse described in this report expresses the lac repressor ubiquitously, so it can be used in the future to regulate other promoters with the same degree of control we have demonstrated for our regulatable Tyrosinase transgene. Targeting endogenous promoters should move the system to the next level, where endogenous loci can be switched on and off repeatedly to create reversible models of human disease and normal development in the mouse.

**Materials and methods**

**Construction of lac repressor genes**

The lac repressor constructs W, S, 5'C1, 5'C2, 5'C4, 3'C1, 3'C2, 3'C3, and 3'C4 are driven by a 4.3-kb promoter region from the human β-actin gene up to the Alu site at −7 [Leavitt et al. 1984]. Followed by a short linker of either 44 bp [aactcgagctctgacccc agactgtata cacccgttgg] in W, 5'C1, 5'C4, 3'C1, 3'C2, 3'C3, and 3'C4, or 30 bp [aactcgagctctgacccc ggttttcttgc] in S and 5'C2. 5'C3 contains the 4.3-kb promoter up to the start of translation with no polylinker. All of the above constructs contain the polyadenylation signal sequence from the bovine growth hormone gene [Woychik et al. 1982] connected to the 3’ end of the construct by a BamHI and EcoRI linker region [taggttctgc] connected to the 5’ end of the construct by a BamHI and EcoRI linker region [taggttctgc] connected to the 5’ end of the construct by a BamHI and EcoRI linker region [taggttctgc].

Coding regions for the original wtlacI (W) and synlacI (S) constructs are as previously described [Scrable and Stambrook 1997]. 5'C1 and 5'C2 were made by switching the linker region and the first 36 bp of the coding region between wtlacI and synlacI using the BsrFI site shared by both constructs. 5'C1 contains the wtlacI linker and first 36 bp of the coding region, and then the synlacI coding region. The nuclear localization signal sequence (NLS) that had been attached to the synlacI coding sequence was removed by PCR mutagenesis, so that 5'C1 codes for a protein identical in amino-acid sequence to the endogenous lac repressor. 5'C2 contains the synlacI linker and first 36 bp of the coding region, and then the 3’ wtlacI coding
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RT-PCR assay for splice site use in lac repressor transcripts

Total RNA was extracted from testis of W and S transgenic animals, or Rat 2 fibroblasts transfected by calcium phosphate with the indicated lacI construct using TRI Reagent (Molecular Research Products, Inc.). RNA was DNase treated with RQ1 DNase [Promega] and 1 µg reverse transcribed with AMV-RT. cDNA was subjected to 30 rounds of amplification (95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min.) using a primer in the β-actin promoter [5’acagacgtctcgctttg3’) and a primer in the lacI coding sequence [5’tgacgtcgattcaca3’]. PCR products were run on a 4% polyacrylamide gel in 1X TBE and transferred to Hybond -N+ membrane (Amersham) by semi-dry electrophoresis in NAQ transfer solution (0.08 M Tris-HCl, 0.118 M Borate, 2.4 mM EDTA, pH8.3) at 220 mA for 1 h. The resultant Southern blot was UV crosslinked and then prehybridized and hybridized according to the methods described in Scoble and Stambrook (1997). 3’C1 is identical to 5’C1 up to the EcoRI site, then identical to W downstream. 3’C3 is identical to 5’C1 up to the PvuII site at +950 from the start of translation, then identical to W downstream. 3’C4 is identical to W upstream of the PvuII site, then identical to 3’C1 downstream.

RT-PCR assay for lac repressor function

Rat 2 fibroblasts were transfected with 2.5 µg pSV10 DNA and 2.5 µg of the indicated lac repressor construct DNA [or pBSSK carrier DNA] per 3 × 10^5 cells by standard calcium phosphate-mediated transfection. Growth media was DMEM, 0.1 units/ml penicillin, 0.1 µg/ml streptomycin [Life Technologies], 5% FCS [Hyclone], with 20 mM IPTG, if indicated. Two days after transfection, cells were fixed in 0.5% gluteraldehyde, incubated with X-gal containing solution (0.5mg X-gal in 1X PBS) overnight at 4°C, labeled with peroxidase (ABC reagent, Vector) and visualized with DAB.

Preparation of primary mouse embryo cells

Pregnant females were euthanized on day E13.5 (where E0.5 was the day a vaginal plug was observed). The embryos were dissected out and a small section frozen for genotyping. Embryonic tissue was minced and placed in 2-ml dissociation solution (2 mg/mL Collagenase B, 2 U/mL RQ1 DNase in RPMI 1640 media [GIBCO]) at 37°C for 2 h, triturating the solution after 1 h. Cells were spun at 175g, washed one time with Hank’s BSS, plated with growth media [DMEM, 0.1 units/mL penicillin, 0.1 µg/mL streptomycin (GIBCO), 10% FCS (Hyclone)], and transfected by calcium phosphate.

Construction of the regulatable Tyrosinase transgene (Tyr^lacO)

The regulatable Tyr^lacO transgene is based on the construct TYBS described in Yokoyama et al. (1990). The first lac operator was created by site-directed mutagenesis [ExSite, Stratagene]. 25 bp of the endogenous promoter sequence [from −72 to −48] was changed to make a 29 bp operator centered at −59, identical in sequence to the primary operator of the lac operon [gtggaatt gtgccgattcaaccttc (ggatccagatc)].
into the BsrGI site at -203 and the EcoRV site at -548 of the promoter.

Production of transgenic mice

Production of the W and S lines is described in Scrable and Stambrook (1997). The rest of the transgenic lines described were produced by microinjection into the outbred ICR line (Harlan) using standard procedures. We made two transgenic founders for the 3′C4 transgene, both showed the testis-only expression pattern shown in Figure 3. One founder line was established for the M construct. Three founders were transgenic for R; two (lines 1 and 3) exhibited the ubiquitous expression shown in Figure 3, with (line 13) had more limited expression that ranged from low to moderate in various tissues. Eight founders were transgenic for Tyr<sup>neo</sup>, an F1 generation was produced from all eight, and two of those established pigmented transgenic lines (lines 25 and 43). Of the animals indicated as Tyrosinase transgenic, those in Figure 6A were homozygous for Tyr<sup>neo</sup>, and all others were hemizygous for Tyr<sup>neo</sup>. All lac transgenic mice described were hemizygous for lacI.

Analysis of eye pigmentation and IPTG treatment of mice

For adult eyes, mice were given a lethal dose of Nembutol so- drano, and M. Burton for helpful comments. This work was

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The *lac* operator-repressor system is functional in the mouse

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