CHARACTERIZATION OF A NEURAL-SPECIFIC INDUCIBLE GENETIC SYSTEM IN TRANSGENIC MICE

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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ABSTRACT

Gene function during mammalian development is often studied by making irreversible changes to the genome. This approach has a major drawback in that the function of the gene in question must be deduced from the phenotype of animals which have been deficient for the product of the disrupted gene throughout ontogeny. Compensation for the loss of the gene product could yield an apparently unaltered phenotype. Alternatively, the changes in the regulation of other genes could yield a misleading phenotype. If the genetic manipulation results in embryonic or neonatal lethality, gene function at later stages of development cannot be analyzed. It would thus be highly advantageous if the expression of a particular gene could be restricted both temporally and spatially through the use of an inducible genetic system. This paper describes an attempt to utilize a recently developed system based upon prokaryotic control elements, the tetracycline transactivator system, to direct inducible expression of reporter gene sequences specifically in the nervous system of transgenic mice. The transactivator protein, tTA was placed under the control of the human neurofilament light chain gene promoter (pNFL) to give neural-specific expression. Although tTA mRNA expression was detectable by reverse transcriptase-polymerase chain reaction in the brains of pNFL-tTA transgenic mice, no reporter gene expression was observed when these mice were crossed to tet-O-βgalactosidase transgenics. The most likely explanation for the lack of function is that the pNFL-tTA transgene sequences integrated into regions of the genome where proper transgene expression was prevented. Recent experiments from other laboratories have also suggested that an optimal level of tTA expression must be attained for proper functioning of the system. This is most conveniently done by choosing an appropriate promoter to drive tTA. It is possible that the human NFL promoter does not give this optimal level of tTA expression. Our laboratory is currently utilizing other neural-specific promoters to enable function of the tetracycline inducible system in neural tissue of transgenic mice.
My sincere thanks to my supervisor, Dr. John Roder, for providing me with the opportunity to broaden my scientific horizons, and for his continued support and encouragement.

I would also like to thank the members of my supervisory committee, Dr. Janet Rossant and Dr. Andrew Spence, for their invaluable guidance and helpful criticism.

I would like to send special thanks to all the members of the Roder lab, both past and present, for their own help and encouragement, and for contributing to a dynamic and exciting place in which to work.

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<thead>
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<th>Description</th>
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<tr>
<td>αCaMKII</td>
<td>alpha-type calcium dependent calmodulin kinase</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl phosphate</td>
</tr>
<tr>
<td>β-gal</td>
<td>β-galactosidase</td>
</tr>
<tr>
<td>β-geo</td>
<td>β-galactosidase / neomycin resistance fusion gene sequence</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s minimal essential medium</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem</td>
</tr>
<tr>
<td>hCMV</td>
<td>human cytomegalovirus</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>kb</td>
<td>kilobases</td>
</tr>
<tr>
<td>LTP</td>
<td>long term potentiation</td>
</tr>
<tr>
<td>MMTV-LTR</td>
<td>mouse mammary tumour virus - long terminal repeat</td>
</tr>
<tr>
<td>NBT</td>
<td>nitro blue tetrazolium chloride</td>
</tr>
<tr>
<td>NFL</td>
<td>neurofilament light chain sequence</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription - polymerase chain reaction</td>
</tr>
<tr>
<td>rtTA</td>
<td>reverse tetracycline transactivator</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Tc</td>
<td>tetracycline</td>
</tr>
<tr>
<td>tet-O-</td>
<td>tetracycline operator (tet operators + minimal hCMV promoter)</td>
</tr>
<tr>
<td>tTA</td>
<td>tetracycline transactivator</td>
</tr>
</tbody>
</table>
1. INDUCIBLE SYSTEMS: ADVANTAGES AND APPLICATIONS

Precise experimental control of gene expression is an invaluable tool in studying complex physiological processes. Studies of gene function in cultured cells and during mammalian development would benefit from the ability to regulate gene expression in a temporal and quantitative manner. Many studies in cultured cells make use of transient or stable transfection of plasmids and the isolation of constitutively expressing cells using a drug selectable marker. This approach, while very reliable, has a disadvantage in that obtaining cell populations with equivalent expression characteristics can be difficult to achieve because of variability in absolute expression levels. Constitutive expression of a given gene may also prove cytotoxic, and does not allow comparative analysis of the effects of this expression on the phenotype of an individual cell clone. Studies of genes involved in mouse development typically utilize irreversible genetic changes, such as the inactivation, ectopic or over-expression of a particular gene in transgenic animals from the onset of embryogenesis. These approaches have a major drawback. The function of a gene product must be deduced from the phenotype of animals that have aberrant amounts of the product of the altered gene throughout ontogeny, either in all cells or in certain cell types (Kühn et al., 1995). A mutant organism may compensate for the loss of a gene product so that no obvious phenotypic deviation is observed, or the organism may react to the mutation to give a complex, secondary phenotype. Moreover, if the complete loss of a gene product results in embryonic lethality, gene function at later stages of development cannot be analyzed. It would thus be highly advantageous if the expression of a particular gene could be restricted both temporally and spatially through the use of an inducible genetic expression system. A number of regulatory systems that allow the control of gene expression through the use of specific effectors permit this approach in bacteria and yeast, providing valuable insights into gene function in these organisms. Transcription control systems based on regulatory elements such as the lactose operon in *E. coli* and Gal4 in *Saccharomyces cerevisiae* allow tight control of the gene of interest. However, inducible
genetic expression systems developed for use in mammalian cells tend to be more complex
and problematic (Gossen et al., 1993). The various systems and their applications are
outlined below, and a comparative summary is presented in Table 1.

1.1 Heat Shock

An inducible system based upon the heat shock response was first developed for use in
mouse cell lines. One early application of the system was the inducible over-production of
the mouse c-myc protein (Wurm et al., 1986). These experiments led to the development
of a generic heat-shock inducible eukaryotic expression vector for use in mammalian cells.
This construct, containing a Drosophila melanogaster hsp70 promoter, relies on host-
supplied heat-shock transcription factors (HSTFs) for activity (Schweinfest et al., 1988).
In transient transfections of human H9 T-cells, the transactivator of transcription (Tat)
protein of the human AIDS virus HIV-1 was functionally expressed in response to heat
shock (shift in temperature from 37°C to 42°C). The promoter was very tightly regulated
in that no expression was detectable at 37°C. Expression was highly and rapidly (within
minutes) induced upon heat shock, lasted more than 4 hr, and subsided with a return to
normal temperature. Another system, based on a Caenorhabditis elegans heat shock gene
pair, further attested to the evolutionary conservation of heat shock response elements and
their possible utility in diverse organisms (Kay et al., 1986). The C. elegans gene pair was
shown to be transcriptionally inactive when introduced into mouse fibroblasts under
normal growth conditions, and it was strongly induced by heat shock (at 42.5°C) or
treatment with sodium arsenite, a stress agent. This demonstrated that the efficiency as
well as the inducibility of heat shock gene promoters can be faithfully reproduced in
distantly related host cells. A heat shock inducible system was also developed for use in
transgenic mice (Kothary et al., 1989). Mice were generated that expressed the E. coli β-
galactosidase (β-gal) gene under the control of the promoter from the mouse heat shock
gene, hsp68. The hsp68-lacZ hybrid gene was used to direct stress-induced expression of
β-gal in adult and various fetal tissues. Tail biopsies from adult mice subjected to a heat
stress at 42°C showed high levels of lacZ expression. Analysis of lacZ expression at fetal
<table>
<thead>
<tr>
<th>System</th>
<th>Induction ratio</th>
<th>Kinetics</th>
<th>Leakiness</th>
<th>Pleiotropic effects</th>
<th>In vitro</th>
<th>In vivo</th>
<th>Comments</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat shock</td>
<td>~4- to 10-fold</td>
<td>fast (1 hr)</td>
<td>low</td>
<td>many</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Koth</td>
</tr>
<tr>
<td>Heavy metal ions</td>
<td>5- to 10-fold</td>
<td>fast (16 hr)</td>
<td>high</td>
<td>many</td>
<td>+</td>
<td>-</td>
<td>inducers (Cd²⁺, Zn²⁺) toxic, in vitro use only</td>
<td>Filmu</td>
</tr>
<tr>
<td>Interferon</td>
<td>2- to 50-fold</td>
<td>slow (3-4 d)</td>
<td>low</td>
<td>many</td>
<td>-</td>
<td>+</td>
<td>function dependent on tissue or cell type kinetics measured by Cre recombinase activity</td>
<td>Kühn</td>
</tr>
<tr>
<td>FK506 dimer</td>
<td>up to 1.5-fold</td>
<td>fast (16 hr)</td>
<td>very low</td>
<td>few</td>
<td>+</td>
<td>-</td>
<td>used to activate endogenous signal transduction pathways</td>
<td>Belsh</td>
</tr>
<tr>
<td>Glucocorticoid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steroid hormone</td>
<td>up to 200-fold</td>
<td>fast (24 hr)</td>
<td>high</td>
<td>many</td>
<td>+</td>
<td>-</td>
<td></td>
<td>Kuo</td>
</tr>
<tr>
<td>Gal4-ER</td>
<td>up to 100-fold</td>
<td>fast (1-2 hr)</td>
<td>very low</td>
<td>none</td>
<td>+</td>
<td>-</td>
<td></td>
<td>Brase</td>
</tr>
<tr>
<td>Progesterone antagonist</td>
<td>10- to 50-fold</td>
<td>fast (~10 hr in vitro)</td>
<td>low</td>
<td>none</td>
<td>+</td>
<td>(+)</td>
<td>Ex vivo transplantation of stably transfected cells intramuscularly into rats and induction of target gene expression by RU486 administration demonstrated potential in vivo utility</td>
<td>Wang</td>
</tr>
<tr>
<td>Mutant estrogen receptor</td>
<td>-</td>
<td>slow (3-4 d)</td>
<td>low</td>
<td>none</td>
<td>+</td>
<td>-</td>
<td>used to express Cre recombinase in ES cells, mosaicism observed work currently underway to develop this system for use in transgenic mice</td>
<td>Zhan</td>
</tr>
<tr>
<td>Ecdysone</td>
<td>up to 10⁴-fold</td>
<td>fast (20 hr)</td>
<td>very low</td>
<td>none</td>
<td>+</td>
<td>+</td>
<td>direct comparison of this system with tet system in vitro showed lower basal activity</td>
<td>No e</td>
</tr>
<tr>
<td>Lac</td>
<td>lacR: 5- to 60-fold</td>
<td>lacR-VP16: slow (24-72 hr)</td>
<td>high</td>
<td>cytotoxic effects</td>
<td>+</td>
<td>(+)</td>
<td>inducer (IPTG) is cytotoxic at concentrations necessary for good control of the system lacR-O system used to attenuate gene expression in transgenic mice with some success</td>
<td>Baim</td>
</tr>
<tr>
<td>Tet</td>
<td>original: 10⁴-fold</td>
<td>fast (12-24 hr)</td>
<td>very low</td>
<td>none (possible tTA toxicity)</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Goss</td>
</tr>
<tr>
<td></td>
<td>rtTA: up to 1000-fold</td>
<td>auto-reg: ~1000-fold</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. A summary table outlining the general characteristics of the various inducible genetic expression systems developed for use in mammalian cells.
stress conditions in vitro. Considerable variation in expression was observed from tissue to tissue, indicating that activation of the gene was not uniform. This was probably partly due to tissue variation in ability to respond to heat stress and partly due to inadequate exposure to the stress stimulus (especially for inaccessible internal structures). However, the *hsp68* promoter showed a distinct advantage in that its activity was very stringently regulated, since expression was absent in all uninduced tissues and widely distributed in induced tissues. It was hoped that heat shock or arsenite treatment could be used to activate target genes in tissues of mouse embryos at distinct times during development. Unfortunately, it has not yet proved possible to induce promoter activity without subjecting embryos to stresses that may be deleterious to subsequent normal development. Nonetheless, the system could be useful for short-term studies where deleterious effects are not manifest.

1.2 Heavy Metal Ion

The 5' regulatory regions of the metallothionein (MT) gene family have been extensively used as inducible promoters in a limited number of mammalian cell lines. The best inducers for these promoters are heavy metal ions, particularly Cd$^{2+}$ and Zn$^{2+}$. Induction is mediated by metal-activated factors (MAFs) which recognize metal responsive elements (MREs) located within the MT promoters. Unfortunately, these promoters also contain elements which are recognized by transcription factors that are constitutive and generate a significant basal level of gene expression. As a consequence, the uninduced level of expression of vectors that use MT promoters is generally high and the induction ratio poor, usually no greater than 5- to 10-fold. The MT promoters can also be induced by other agents such as glucocorticoids, progesterone, interleukin-6, and interferon, limiting their usefulness further. A more successful approach has been to combine the MT promoters with other inducible elements to increase their utility. (Filmus et al., 1992) For example, a synthetic promoter was created by introducing additional glucocorticoid response elements (GREs) into a human metallothionein IIA (hMT-IIA)
activation of transcription was demonstrated in cells containing a CAT gene driven by the synthetic promoter under CdCl₂ and/or dexamethasone treatment. Induced expression levels of the modified promoters were significantly higher than those obtained with wild-type hMT-IIA or with the MMTV-LTR promoter. Since the increase in transcription was not accompanied by a concomitant increase in basal levels, the inducibility of the modified MT promoters was up to 6-fold higher. When CdCl₂ and dexamethasone were used for simultaneous induction, a synergistic effect resulted in an increase in CAT activity up to 65-fold above basal levels. Addition of only one kind of inducer could be used for an intermediate level of gene expression. Despite these improvements, the rather modest levels of induction, the leakiness of the promoter, and the toxicity associated with administration of heavy metal ions limits the usefulness of MT-inducible systems in cells, and prevents application of the system in transgenic animals (Gossen et al., 1993).

1.3 Interferon

Interferon-inducible promoters have been used in studies of viral pathogenesis and immune responses in cultured cells and in animals. A particularly interesting example of the usefulness of this system was demonstrated in transgenic mice that express the intracellular anti-influenza virus protein Mx1 under control of an interferon-responsive regulatory element (Arnheiter et al., 1990). Mice could be rendered resistant to a virulent infection by “intracellular immunization” achieved through germline transformation. The Mx1 gene of mice is a host gene that controls viral infection. Mice with the autosomal dominant Mx1⁺ allele resist infection with influenza A and B viruses, whereas mice with Mx1⁻ alleles succumb to infection. The Mx1⁺ allele is inducible by interferons α and β and codes for the Mx1 protein. Early attempts to obtain transgenic mice constitutively synthesizing Mx1 protein in amounts sufficient for protection against influenza virus failed, possibly because constitutive expression of this protein may be deleterious. Subsequently, Mx1 expression was subjected to control by the infecting virus by using the Mx1 promoter itself as the viral-responsive element, since it is induced not only by interferon but also by
rapidly in transgenic Mx1 mice by injection of interferon, double-stranded RNA, or virus, and rendered the mice resistant to influenza virus infection. Injection of dsRNA or virus activates the Mx1 promoter directly as well as via induction of endogenous interferon. Maximal activation of transcription occurred 3 to 6 hours after induction in many tissues, with the exception of thymus and skeletal muscle. Induced Mx1 protein levels were approximately half of those observed in naturally Mx1+ mice. In addition, transgenic mice were resistant to infection by influenza virus, if expression of the Mx1 protein was turned on early enough and to a sufficiently high level. Arnheiter et al. (1990) noted that application of the Mx1 promoter in an inducible system would have certain advantages, such as the high levels of both rapid and transient induction in vivo in a variety of tissues. However, because the promoter is inducible by interferon, and since interferons are found naturally at low titers in mice and are induced during viral infections and other illnesses, the baseline activity of the transgene could vary from mouse to mouse, even within the same transgenic line. In addition, constitutive high level synthesis of interferons, if at all tolerated, may not be desirable in transgenic animals, because interferons have many different effects on an animal's physiology.

More recently, the first demonstration of inducible gene targeting in mice used the Mx1 interferon responsive promoter to control the expression of Cre recombinase, which was used to delete a segment of the DNA polymerase B gene flanked by loxP recombinase recognition sites (Kühn et al., 1995). Deletion was complete in liver and nearly complete in lymphocytes within a few days, whereas partial deletion was obtained in other tissues. Mice harbouring a target gene flanked ("floxed") by loxP and a Mx-cre transgene acquired an inactivating mutation of the target gene upon treatment of the mice with interferon or dsRNA. The extent of induced deletion was variable in different tissues, ranging from 94% in spleen to 8% in brain, with complete deletion found in liver and no deletion found in testis. This heterogeneity may reflect differing proportions of interferon-responsive cells or interferon availability in various tissues. Background recombination in nontreated mice was undetectable in some tissues but was as much as 10% in spleen, possibly because of the endogenous production of interferon in the mice, which were maintained in a
conventional facility. In addition to their antiviral activity, interferon-α/β and dsRNA exert transient antiproliferative and immunomodulatory effects in vitro and in vivo. Indeed, the number of thymocytes in spleen, known to be reduced by stress, was diminished by 50% in dsRNA treated mice and by 25% in interferon-treated mice. Although treatment of mice with interferon-α/β or dsRNA had side effects, it was believed that these effects would be limited to the brief exposure of the mice to the inducing agent. These results demonstrated that gene inactivation can be induced with high efficiency in mice within a few days and that Cre-mediated deletion is achievable in organs composed mainly of resting cells. Both alleles of a loxP-modified gene can be deleted in vivo with the same high efficiency as a single loxP-flanked allele in heterozygous mice; this result shows the feasibility of a conditional gene targeting strategy since both alleles of a target gene must be inactivated to investigate gene function in a mutant. At present, Cre-mediated gene deletion in Mx-cre mice cannot be restricted to certain tissues. Gene inactivation could be confined to certain cell types if Mx-cre mice were crossed to interferon-α/β receptor-deficient mice with an interferon-α/β receptor gene controlled by a cell-specific promoter.

1.4 FK506 Dimer

Recently, a novel system was developed for the inducible control of signal transduction with synthetic ligands in cultured cells (Spencer et al., 1993). Cell permeable ligands were devised that can be used to control the intracellular oligomerization of specific proteins. To demonstrate their utility, these ligands were used to induce the intracellular oligomerization of cell surface receptors that lacked their transmembrane and extracellular regions but contained intracellular signalling domains. Addition of these ligands to cells in culture resulted in signal transmission and specific target gene activation, while monomeric forms of the ligands were found to block the pathway. This method of ligand-regulated activity of cell signalling could potentially be applied wherever tight control of a signal transduction pathway is desired. Initially, signalling domains of receptors were intracellularly expressed as fusion proteins with a specific immunophilin-based
ligands linked together was used to initiate aggregation of an artificial receptor. The receptor was designed to propagate an intracellular signal through protein-protein interactions, activating a specific subset of transcription factors, whose actions were detected with a reporter gene assay. The ideal characteristics for a molecular matchmaker in mammalian cells and transgenic animals include lipid solubility, lack of untoward cellular actions leading to toxicity, high affinity binding to its target receptor, and metabolic stability (particularly if oral administration is desired). The FK506 molecule was considered as a precursor to such a molecule. It readily crosses cell membranes and binds to the immunophilin FKBP12 with high affinity. The FKBP12-FK506 complex binds to and inactivates calcineurin, a Ca^{2+} calmodulin-dependent protein phosphatase. The inactivation of calcineurin results in impaired signalling of the T cell antigen receptor (TCR) and subsequent immunosuppression. In addition, inactivation of calcineurin underlies the toxicity of FK506. To convert FK506 into a nonimmunosuppressive and nontoxic matchmaker, the molecule was engineered to be impaired in its ability to interact with calcineurin and allow for the linking of two (or more) FK506 molecules without interfering with FKBP12 binding. Several dimeric FK506 variants termed FK1012s were synthesized and examined for their ability to inhibit signal transmission in an FK506-sensitive T cell line. Inhibition of signal transduction was measured as a reduction in secreted alkaline phosphatase (SEAP) under the control of a nuclear factor of activated T cells (NF-AT)-responsive promoter element. (Figure 1) A chimeric signalling receptor was designed that could be activated by using FK1012s to aggregate the receptor. The activity of the NF-AT-responsive promoter could be regulated by several orders of magnitude by the use of FK1012 concentrations that also spanned several orders of magnitude. Signal transduction induced by intracellular crosslinking reflected signalling induced by extracellular crosslinking of the receptor using antibodies. FK1012s could also oligomerize and activate other receptors, as shown by the ability of FK1012 to induce signalling through a component of the B cell antigen receptor complex. Thus, although this approach can be used to investigate pathway regulation, the system cannot easily be coupled with a specific target gene because the membrane receptor-activated kinase
Figure 1. Intracellular activation of signalling cascade and downstream gene expression by FK1012s in T lymphocytes. Abbreviations are: PLC, phospholipase C; DAG, diacyl glycerol PKC, protein kinase C; MAPK, MAP kinase; and NF-AT, cytoplasmic and nuclear components of NF-AT transcription factor, respectively. Adapted from Spencer et al., 1993.
has proved useful in activating multiple signalling cascades through an artificial receptor in cultured cells, Spencer et al. (1993) claim that other synthetic molecules could be similarly designed to link endogenous proteins both in vitro and in transgenic mice. The synthesis of derivatives of FK1012s with substituents that interfere with binding to endogenous FKBP s and the construction of chimeric receptors with FKBP domains that have compensatory mutations could also result in reducing the activation of endogenous signalling cascades. Recently, this system was used to control selective association and subcellular localization of two exogenous proteins transiently transfected in cultured cells (Belshaw et al., 1996).

1.5 Glucocorticoid:

1.5.1 Steroid Hormone

Some of the earliest developed inducible systems designed for use in mammalian cells involved the use of various promoters containing glucocorticoid-responsive elements (GREs) (reviewed in Friedman et al., 1989). Most commonly, the mouse mammary tumour virus long terminal repeat (MMTV-LTR), which contains a GRE, was used as the inducible promoter in transfected cell lines treated directly with natural or synthetic glucocorticoids. Initially, this system was used to evaluate the regulatory mechanisms of the MMTV-LTR, to regulate expression of various oncogenes, and to induce the expression of genes whose products were otherwise cytotoxic. One of the limitations of the system was that the cells used for experimentation had to express endogenous glucocorticoid receptors (GRs) or be engineered to express GR transgenes (Israel and Kaufman, 1989). In general, the system had a high basal level of activity even in the non-induced state, as well as a propensity to evoke pleiotropic effects (Gossen et al., 1993, Kuo et al., 1994). Improvements made to the original system include: (i) the use of auto-inducible/positive feedback vectors to increase inducible expression from the MMTV-LTR without augmenting basal expression levels (Ko et al., 1989); (ii) the addition of cell-type
(iii) combination of GREs with metal-responsive promoter elements and the use of multiple GREs to elicit synergistic induction (Films et al., 1992, Mader and White, 1993). Strategies using modified ligands, receptors, and promoter elements based on components of the glucocorticoid-inducible system have been developed, and various successful attempts to control the activity of a protein by directly fusing it with the hormone-binding domain of steroid receptors have been made. Several transcription factors, as well as RAF kinase and the recombinases FLP and Cre, have been rendered functionally hormone-dependent by the latter strategy, but tend to exhibit high basal activity (Logie and Stewart, 1995; Metzger et al., 1995).

1.5.2 Gal4-ER

An estrogen-dependent inducible system using a specially designed yeast Gal4-mammalian estrogen receptor (Gal-ER) fusion protein has been described (Braselmann et al., 1993). Initially, the Gal-ER protein consisted of the DNA-binding domain of the yeast Gal4 protein linked to the hormone-binding domain of the human estrogen receptor. Induction using this system was shown to be highly selective, since most mammalian culture cells express neither endogenous estrogen receptor nor any Gal4-like activity. Subsequently, optimal Gal4-responsive promoters with low basal activity and high inducibility were constructed. The transactivation potential of Gal-ER was increased and rendered cell-type-independent by the incorporation of the strong transactivation domain of the herpes simplex virus VP16 protein. Gal-ER was used to conditionally express a Gal4-responsive c-fos gene in rat fibroblasts, leading to estrogen-dependent transformation of these cells. Basal levels of expression of c-fos from the modified Gal4-responsive promoter in the absence of estrogen were undetectable. A modest induction ratio (maximum 100-fold) but fast induction kinetics were observed, with maximal induction levels reached in 1-2 hrs.
Recently, a C-terminal deletion mutant of the human progesterone receptor which fails to bind to progesterone but can bind RU486 (Mifepristone) and other progesterone antagonists was discovered. This mutant receptor was used to activate transcription of a target gene containing a progesterone responsive element in the presence of these antagonists (Wang et al., 1994). The chimeric regulator was constructed by fusing the ligand-binding domain of the mutant human progesterone receptor to the yeast transcriptional activator Gal4 DNA-binding domain and the herpes simplex virus protein VP16 activation domain. This chimeric regulator was shown to activate target genes containing Gal4-binding sites in transient transfection assays in response to RU486. An approximately 50-fold induction was observed in transient transfection assays, and a 10- to 20-fold induction in a stably transfected rat fibroblast cell line. Reporter gene expression was detected within 2 hr after RU486 administration and reached maximum at approximately 10 hr. Ex vivo intramuscular transplantation of the cell line containing both the regulator and a reporter gene into rats and induction of target gene expression up to 10-fold by oral administration of RU486 demonstrated the potential utility of the system in vivo. The dosage of RU486 used was significantly lower than that required for antagonizing progesterone action, and the animals exhibited no deleterious effects. The fast kinetics of this system may have certain advantages, but the low induction ratios observed limit its general applicability.

1.5.4 Mutant Estrogen Receptor

The first demonstration of inducible site-directed recombination in mouse embryonic stem cells used a fusion protein consisting of Cre recombinase and a mutated hormone-binding domain of the murine estrogen receptor to activate a stably integrated lacZ reporter gene (Zhang et al., 1996). This work was based upon earlier findings which showed that FLP or Cre recombinase activity in mammalian cells could be rendered ligand dependent. Conditional FLP or Cre activity was achieved by expressing the recombinases
and Stewart, 1995; Metzger et al., 1995). These systems were found to work very efficiently in cultured cells, but the presence of endogenous estrogen in animals prevented direct application of the systems to transgenic mice. It was suggested that use of mutated LBDs, which would respond to synthetic ligands but not endogenous ligands, or the use of LBDs of other nuclear receptors, such as the ecdysone receptor, would provide feasible solutions. Zhang et al. (1996) adopted the former strategy; the mutant estrogen receptor portion of the protein still allowed binding of the anti-estrogen drug tamoxifen, but not 17β-estradiol. In embryonic stem cells expressing this fusion protein, tamoxifen was shown to efficiently induce Cre-mediated recombination, activating a lacZ reporter gene disrupted by a translational stop signal flanked by loxP sites. In the presence of tamoxifen or 4-hydroxy-tamoxifen, recombination of the lacZ gene was complete within 3-4 days. Using the initial fusion protein, consisting of a single tamoxifen binding domain (TBD) fused to Cre recombinase, an optimum of reporter activity was reached at a relatively high dose of tamoxifen; higher concentrations of tamoxifen were found to be toxic to cells and resulted in declining reporter activity. Reporter activity reached a plateau after 75 hr. In addition, uninduced cells exhibited some detectable basal activity, and after prolonged culture (8-10 weeks) reporter activity increased spontaneously to a significant level. To control the Cre enzyme more tightly, a fusion protein was constructed in which a TBD was appended to both ends of the Cre enzyme. This surprisingly resulted in lower basal levels of Cre expression, with no increase in the background level of activity in prolonged culture. Work is currently underway to use this system in transgenic mice, with tissue specific promoters driving expression of the Cre fusion protein. Since the Cre fusion protein is an efficient recombinase, the deletion or activation of a gene in transgenic mice should require only a short exposure to tamoxifen. Any potential side effects of tamoxifen in mice are expected to be relatively mild and should rapidly disappear upon withdrawal of the drug.
An ecdysone-inducible gene expression system for use in mammalian cells and transgenic mice has been recently described (No et al., 1996). The insect steroid hormone ecdysone triggers a cascade of morphological changes in *Drosophila melanogaster* via the ecdysone receptor, a member of the nuclear receptor superfamily. The ecdysone receptor (EcR) is by itself incapable of high-affinity DNA binding or transcriptional activation; instead, these activities are dependent on heterodimer formation with ultraspiracle (USP), the insect homologue of the vertebrate retinoid X receptor (RXR) (Yao et al., 1993). In mammalian cells, retinoid X receptor can substitute for *Drosophila* USP in dimerizing with EcR to form high-affinity DNA-binding complexes with several vertebrate nuclear receptors. Insect hormone responsiveness was transferred to mammalian cells by the stable expression of a modified ecdysone receptor that regulates an optimized ecdysone responsive promoter. Induction levels reaching four orders of magnitude were achieved upon treatment with ecdysone or muristerone A (murA), a synthetic steroid hormone that acts as a potent ecdysone agonist. Transgenic mice expressing the modified ecdysone receptor can activate an integrated ecdysone responsive promoter upon administration of hormone. Preliminary experiments showed that long-term exposure of transgenic mice to muristerone A had no apparent side effects. Ecdysteroids were found to be neither toxic, teratogenic, nor able to affect mammalian physiology appreciably. Further advantages for ecdysteroid use include the lipophilic nature of the compounds for efficient penetration into all tissues including the brain, short half-lives which allow for precise and potent inductions, and favourable pharmacokinetics that prevent storage and expedite clearance (No et al., 1996). To develop the system, a β-galactosidase reporter gene was placed under the control of ΔHSP, a minimal promoter derived from the *Drosophila* heat shock promoter with its enhancers deleted, and a series of tandemly arranged ecdysone response elements (EcREs). Initially, mammalian cells stably transfected with the reporter construct and cotransfected with EcR and USP produced a 3-fold induction upon treatment with muristerone. To maximize the sensitivity of the system, modifications of EcR were made. Experiments involving several modified versions of EcR showed improvement in the
an N-terminal truncation of EcR attached to the VP16 activation domain was observed to result in a 212-fold induction. Unlike most other nuclear receptor/VP16 fusion proteins, which exhibit high constitutive activity, VpEcR generates ligand-dependent superinduction while maintaining a very low basal activity. The receptor vector was also modified by inserting consensus binding sites for the ubiquitous transcription factor Sp1 between the minimal promoter and the EcREs. The inclusion of Sp1 sites in the ecdysone responsive promoter increased absolute activity 5-fold. Although no mammalian transcription factors have been shown to have a natural recognition site like the EcRE, it is difficult to preclude such a possibility. To circumvent this potential problem, the DNA binding specificity of VpEcR was altered to mimic that of GR. This new hybrid receptor, VgEcR, binds to a hybrid responsive element (E/GRE) which contains two different half sites, derived from those of the GRE and the type II nuclear receptors. The E/GRE was not activated by GR nor does VgEcR activate a dexamethasone responsive promoter. In stable cell lines containing VpEcR or VgEcR, RXR, and the modified ecdysone-inducible reporter, treatment with muristerone for 24 hr resulted in very rapid and potent induction. Inductions of 100-fold in 3 hr, 1000-fold in 8 hr, and 20 000-fold after 20 hr of treatment were observed. Also, transgenic mice harbouring both receptor and reporter constructs showed activation of the ecdysone responsive promoter upon administration of muristerone. A direct comparison of the tetracycline (see "The Tetracycline Transactivator System" below) and ecdysone inducible systems in cell culture demonstrated that the ecdysone system has both lower basal activity and higher inducibility than the tetracycline system (No et al., 1996).

1.6 Lac Operator/Repressor-Based Inducible Systems

The commonly used endogenous systems for inducible gene expression in mammalian cells tend to suffer from one or both of the following problems: (i) the inducer (such as heavy metal ions, steroid hormones, or heat shock) evokes pleiotropic effects that complicate the analysis of the resulting phenotype; and (ii) many promoter systems have
study and results in modest induction factors (Gossen et al., 1993). However, highly specific control of gene activity in higher eukaryotic cells has also been achieved through the use of prokaryotic regulatory elements. This is possible because the action of prokaryotic regulatory proteins can be modulated by effectors that are essentially inert to the physiology of the organism or cell and therefore do not elicit pleiotropic effects. Functional inducible systems based on two well-characterized regulatory elements from *Escherichia coli*, the *lac* and *tet-R* operons, have both been shown to act as true monospecific regulatory circuits in mammalian cells and transgenic animals.

The activity of the promoter controlling transcription of the *E. coli* *lac* operon can be repressed several hundred-fold by the *lac* repressor (lacR) which, upon binding its operator (lacO), prevents the formation of an initiation-competent complex between RNA polymerase and the promoter. This repression can be specifically relieved by the synthetic and non-metabolizable inducer isopropyl-β-D-thiogalactopyranoside (IPTG). The high specificity of the lacR-lacO-IPTG interactions made the *lac* system an obvious candidate for controlling transcription in higher eukaryotic cells. The most straightforward approach followed the prokaryotic paradigm: *lac* operator sequences were placed near the TATA-box or the start site of mammalian promoters. It was anticipated that a lacR-O complex within that region of a promoter would directly interfere with initiation of transcription. Several mammalian promoters such as the SV40 early promoter were modified accordingly, and lacR was transiently or stably expressed in a variety of cell lines at relatively high concentrations. Transcription from promoters containing lacO sequences could indeed be regulated in mammalian cells by IPTG (Hu and Davidson, 1987, Brown et al., 1987). However, using chloramphenicol acetyltransferase as a reporter enzyme, a rather modest regulatory potential was observed which never exceeded a factor of 60 (Figge et al., 1988). These experiments also revealed that insertion of lacO sequences could affect intrinsic promoter properties; the unrepressed activity of a lacO-modified SV40 early promoter was found to be reduced by up to 90% compared with the authentic viral promoter (Hu and Davidson, 1987). By contrast, efficient repression was obtained when specialized RNA polymerase systems, such as those found in coliphage T3 or in
Vaccinia virus, were controlled by the lac R-O complex in eukaryotic cells (Deuschle et al., 1989, Giordano et al., 1989). However, only in the vaccinia system have valuable studies been reported.

After experiments revealed that the lac R-O complex efficiently terminates transcription by E. coli RNA polymerase, an alternative strategy to control mammalian transcription through blocking the elongation process was pursued. Using this principle, the activity of any promoter was anticipated to be controlled by terminating transcription at a downstream lac R-O complex. Moreover, the efficiency of termination was expected to be increased by inserting multiple operator sequences, without affecting the function and specificity of the promoter. Such a system is indeed functional: mammalian transcription can be terminated at lac R-O complexes in vivo and in vitro (Deuschle et al., 1990). Interestingly, regulation factors were almost independent of the number of operators involved and never exceeded values of five to fifteen. The observed low efficiency of elongation interference was likely due to elongation factors that allow the transcriptional machinery to bypass "roadblocks" like the lac R-O complex. This property of lacO insertion was used to advantage in transgenic mice to attenuate an immunoglobulin promoter/enhancer sequence driving the lymphokine IL-4 gene (Tepper et al., 1990).

In a third strategy, lacR was used as a carrier for the transcriptional activation domain of the herpes simplex virus protein VP16. By properly linking multiple lacO sites upstream of a minimal promoter, the activity of such a promoter should depend on the binding of the lacR-VP16 fusion protein, which in turn would depend on IPTG. After transient transfection of suitable reporter constructs into lacR-VP16-positive cells, remarkable regulation factors (~1000-fold induction) were demonstrated (Labow et al., 1990). Moreover, the IPTG-controlled expression of a mutant p53 protein in rodent cells provided a practical example for the basic applicability of this principle (Zambetti et al., 1992). Nonetheless, because the effector molecule (IPTG) must be present continuously to keep the regulatory unit "silent" and has to be removed for induction of transcription, the precision required in some applications may not be attained if the removal process is too slow. To circumvent these problems, a lacR-VP16 fusion was constructed which exhibited a reverse DNA-binding phenotype, i.e. it actually requires IPTG for binding to
the presence of IPTG was necessary only during activation (Baim et al., 1991). However, the lacR moiety of the "reverse" transactivator was found to bind lacO in a temperature-dependent manner; unfortunately, to exploit the full regulatory potential of the system, cultures had to be kept at elevated temperatures. This would tend to evoke temperature-related pleiotropic effects, limiting the general usefulness of this system.

More recently, further attempts to improve the lac system for use in mammalian cells have been made. Cells expressing constructs of lacR fused at different positions to a nuclear localization sequence (NLS) from either the SV40 large T antigen or the adenovirus E1a exhibited tighter repression, efficient nuclear accumulation of repressor, and greater sensitivity to IPTG induction (Hu and Davidson, 1991). An NLS-modified lac repressor was subsequently shown to efficiently repress the expression of an Ha-ras transgene in NIH 3T3 cells (Liu et al., 1992). Activation of the transgene upon administration of IPTG led to rapid production of high levels of Ha-ras mRNA and cells took on the characteristics of an Ha-ras-transformed cell line. However, while repression was significant, it was not absolute; some leakiness of the transgene was observed. Besides the specific shortcomings of the various lacR-O systems described, there are two intrinsic problems that presently limit their wider application. First, to affect lacR-O binding, high IPTG concentrations, which approach cytotoxic levels are required (Figge et al., 1988). Second, despite high inducer concentrations in the medium, the kinetics of IPTG action are slow and the levels of induction are often far from 100% (when compared with the repressor-free situation). These phenomena do not seem to depend on the kinetics of IPTG uptake by the cell (which are fast), or on its intracellular stability (which is high) (Wyborski and Short, 1991, Fieck et al., 1992). It is possible that IPTG is cleared from cells before it can function, or that it is less effective at interacting with lacR bound to mammalian DNA.
The antibiotic tetracycline (Tc) inhibits prokaryotic translation at low concentrations (>0.30 μg/ml). Operons designed to mount a resistance response against the antibiotic are therefore optimized to sense minute subinhibitory concentrations of Tc. Consequently, the binding constant between Tc and the repressor (tetR) of the Tn10-derived Tc-resistance operon of E. coli is high. (Figure 2) In addition, tetR binds its operator (tetO) with exceptional specificity (Chopra, 1985). These properties, along with the well-studied and favourable pharmacological characteristics of tetracyclines, suggested that tetR, tetO, and Tc could be used as a regulatory system in higher eukaryotic cells (Gossen et al., 1993). Although tetR functions in HeLa and rodent cells as a repressor whose action can be fully inhibited by Tc, it proved difficult to obtain the high concentrations of tetR required for tight repression in stable cell lines. By contrast, fusion of tetR to the acidic transcriptional activating domain of herpes simplex virus VP16 protein produced a highly efficient tetracycline-controlled transactivator (tTA). tTA is able to bind to tetO in the absence of Tc to strongly activate transcription from a downstream promoter. When HeLa cells that constitutively produce tTA were stably transfected with a transcription unit containing the firefly luciferase gene under the control of a minimal promoter, derived from the human cytomegalovirus (hCMV) immediate early (IE) promoter fused downstream of a tandem array of seven tetO sequences, luciferase activity could be regulated over a range of up to five orders of magnitude in a Tc-dependent manner (Gossen and Bujard, 1992). Estimates with double-stable HeLa cells indicated that in the presence of 0.1 μg/ml Tc in the culture media luciferase expression was virtually undetectable, while upon removal of Tc, expression was induced over several orders of magnitude within a few hours. This activity could be limited to predetermined values by properly adjusting the Tc concentration in the medium. It was also shown that the properties of the minimal promoter used were critical for low background expression and high induction levels and could not be predicted for different cell lines, necessitating careful selection of clones with optimal properties.

The tet regulatory system has a number of advantages when compared with its lac-based counterpart. The high affinity of Tc for tetR and the low toxicity of Tc for
Figure 2. The transposon Tn10 of *Escherichia coli*. Vertical lines indicate ends of open reading frames. The regions which code for the tetracycline-inducible resistance protein, the autogenously controlled repressor protein, and transposase are also indicated. Horizontal arrows show the direction of transcription. P - promoter O - operator. Adapted from Chopra, 1985.
magnitude below those that affect growth rate or morphology of cells in culture. Among the many tetracyclines and their derivatives, there are compounds (the best example being anhydrotetracycline) with an increased affinity for tetR and high functional stability, which have proven to be superior effectors (Gossen and Bujard, 1993). In addition, the excellent pharmacokinetic properties of the tetracyclines (which include rapid uptake into cells and penetration of the placenta and the blood/brain barrier) allow the application of the tet regulatory system in transgenic animals (Boothe, 1985).

Theoretically, a regulatory system based on the principle of transcriptional activation has several advantages over one based upon repression (Gossen et al., 1993). In the most simple case, the activity of an otherwise "silent" promoter would depend entirely on the action of a transactivator which binds to one or more specific sites upstream of the TATA region. Depending on the synergistic effects and/or kinetic parameters of the assembly of a transcription-competent complex, a transactivator may have to occupy its binding site(s) only for a short period of time to stimulate the promoter. As in the case of the tetracycline-dependent transactivation system, this may activate transcription by several orders of magnitude. Unlike repressor-based systems, where a high repressor concentration is required to maintain tight control, a high intracellular concentration of activators is not strictly necessary. Optimal induction will occur if the activator concentration is above a critical threshold (which is dependent on the specificity of operator binding) and if overexpression of the transactivator does not elicit pleiotropic effects by titrating components of the transcription machinery ("squelching") (Gill and Ptashne, 1988). In addition, the regulation potential of activating systems can even be expanded by fusing multiple binding sites for the activator to a minimal promoter without changing its basic characteristics. This will ensure high occupancy of the upstream sites, even at varying intracellular concentrations of the transactivator. Finally, by driving the expression of a transcriptional activator such as tTA via tissue-specific promoters, specificity of the activating system will be established, since the function of a monospecific minimal promoter should be largely tissue-independent.
given gene activity and it allows the level of expression of a particular gene to be quantitatively controlled. The system provides the opportunity to address a variety of questions, including the determination of half-life time and turnover of specific mRNAs and proteins without affecting overall cell metabolism. The tet system can allow inducible production of proteins which would otherwise be cytotoxic if overexpressed conventionally. Moreover, by using the tet system to produce antisense RNAs, the activity of endogenous genes can be controlled by Tc. However, the clearance of Tc from cells may prove to be a limiting factor in this approach if rapid activation of transcription from a tTA-dependent promoter is required (Gossen et al., 1993). Several recent strategies designed to circumvent this problem are described below.

1.7.1 Applications Of Tetracycline-Inducible Gene Expression

Inducible systems designed for tetracycline-regulated gene expression in mammalian cells and transgenic animals 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 (Shockett and Schatz, 1996). The consequence is a substantial reduction of nonspecific pleiotropic effects observed with earlier inducible systems. The first tet-regulated gene expression system for use in mammalian cells involved constitutive expression of the tTA with the hCMV IE promoter/enhancer (Gossen and Bujard, 1992). In the absence of Tc, the tetR domain of tTA mediates high affinity, specific binding of tTA to tet operator sequences. In the presence of Tc, however, a conformational change in tet repressor prevents tTA from binding to its operator (Hinrichs et al., 1994). In this initial study, HeLa cells stably expressing tTA were transfected with a luciferase reporter gene under the control of a hybrid, inducible promoter/tet operator sequence. Expression of luciferase was very low in the presence of ng/ml quantities of Tc, and removal of Tc resulted in as much as a 100 000-fold increase in luciferase levels. Luciferase levels could be varied by titrating the amount of Tc in the growth media and maximal steady-state levels of activity were
achieved in about 24 hr. Somewhat surprisingly, tTA was undetectable in the HeLa cells by Western blotting, although it was detected using a sensitive gel mobility shift assay. The very low levels of tTA expression observed were consistent with either instability or toxicity of the tTA protein. Potential toxicity of tTA was speculated to be a consequence of transcriptional squelching, in which tTA would act as a sink for the transcriptional machinery of the cell, resulting in the death of cells expressing moderate to high levels of tTA (Gossen and Bujard, 1992, Damke et al., 1995).

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 Tc-regulated, tTA-controlled stable expression of a number of different proteins involved in various areas of cell biology. These proteins include those that regulate proteasome subunit assembly, a viral protein that inhibits peptide transport across the endoplasmic reticulum membrane, and a mutant dynamin protein whose overexpression affects endocytic-coated vesicle formation (Damke et al., 1994, Früh et al., 1994, Früh et al., 1995, Damke et al., 1995). The system has also been used to determine the consequences of deregulated expression of various cell cycle regulators both in HeLa cells and rat fibroblasts, where conventional overexpression of these proteins was found to be toxic (Bergman et al., 1995, Resnitzky et al., 1994, Wimmel et al., 1994, Shan and Lee, 1994). The tTA system has also been used to produce transgenic mice reversibly expressing luciferase or β-gal in a variety of fetal and adult tissues (Furth et al., 1994). These mice were created by breeding mice expressing tTA to mice carrying the reporter transgenes. 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 was the result of heterogeneous patterns of transgene methylation.

In an attempt to activate higher levels of target gene expression than those obtained with the basic system, and to prevent possible toxic effects of constitutive tTA expression, tTA was placed under the control of the tetO-minimal hCMV promoter, resulting in the autoactivation of tTA in the absence of Tc and suppression of tTA expression in the presence of Tc (Shockett et al., 1995). Basal transcription is thus essential for some
transactivator reaches a necessary threshold, the operator sequences are bound, further increasing transcription levels of the transactivator until a maximum tolerated level, at which point transactivator expression plateaus. 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. In addition, tTA could be readily detected on Western blots. Transgenic mice produced by coinjection of the autoregulatory tTA and a luciferase reporter transgene expressed luciferase inducibly in a variety of tissues with highest levels in thymus and lung. Induced luciferase levels were 1-2 orders of magnitude higher than those reported with the constitutive transgenic system, but the levels in the uninduced state were also greater. While the autoregulatory system has yielded high level expression in cultured cells, in comparison, the expression levels in transgenic animals are substantially lower. It is likely that further refinements of the system will be required for homogenous, high level expression in transgenic animals.

Gossen et al. (1995) have also described a modified system in which a reverse transactivator (rtTA) that binds tetO efficiently only in the presence of the Tc derivatives doxycycline or anhydrotetracycline was developed. The properties of this reverse system are particularly advantageous for in vivo studies requiring rapid activation of a target gene, because the kinetics of induction do not depend on the biological half-life of the effector. Using the CMV IE promoter to drive stable expression of rtTA in HeLa cells, luciferase could be induced by 3 orders of magnitude in 20 hr by the addition of Tc derivatives. 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, where long term exposure to Tc or its derivatives was undesirable or inconvenient (e.g. in gene therapy or transgenic animals), and where rapid induction was required.

Although the tTA and rtTA systems have been used successfully in many cell lines and to some extent in transgenic animals, some possible obstacles must be considered. The general efficacy of the systems in all cell or tissue types remains unpredictable. Furth et al. (1994) first showed that use of the original hCMV promoter-driven tTA system in
where high levels of reporter gene expression were observed (such as thymus), this expression tended to be heterogeneous. Because of the heterogeneity in gene expression that has been observed in some cases, it is anticipated that success in any given cell type or tissue might require different and specific minimal promoters and careful choice of constitutive or tissue-specific promoters for transactivator expression (Hennighausen et al., 1995). For example, in mice carrying lacZ reporter transgenes activated by tTA expressed from a mouse mammary tumour 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.

Another possible problem arises from the random nature of gene integration. Both in vitro and in vivo studies have shown great variability in target gene expression patterns between individual stably-transfected cell lines or transgenic mouse lines (Gossen and Bujard, 1992, Furth et al., 1994). Much of this variability can be attributed to the random genomic integration of either the tTA or tet-O-linked transgenes. Integration site-specific effects (such as constitutive activity or repression), which invariably occur when foreign DNA is stably introduced into a cell or the mouse germ line, might be overcome by surrounding individual transcription units with matrix attachment regions. These elements have been shown to insulate stably integrated vectors and transgenes from effects mediated by cis regulatory elements near their sites of integration (McKnight et al., 1992).

Several recent experiments have demonstrated tissue-specific expression of transactivator directed by tissue-specific promoters. Cardiac-specific expression of tTA and subsequently luciferase mRNA has been achieved using the rat α-myosin promoter in rats and mice (Fishman et al., 1994, Passman and Fishman, 1994). An interesting finding from these experiments was that reporter gene expression could be regulated by appropriate doses of tetracycline. This suggests that a wide range in the expression level of a given tet-O-linked target gene could be obtained simply by adjusting the dosage of tetracycline. Expression of SV40 large T antigen in pancreatic β cells in mice was achieved using a modification of the original tet system in which tTA was driven by the rat
expression of tTA to the epithelial cells of secretory organs and skin in transgenic mice. (Hennighausen et al., 1995)

The reported successes in using the tet system in transgenic mice have led to the development of more sophisticated strategies for manipulating target gene expression. Application of the tet system in the creation of conditional knockout mice using the Cre-loxP recombination system of bacteriophage P1 controlled either by general or tissue-specific promoters is currently being attempted. (Hasan et al., 1996) The tet system may also prove useful for the timed or pulsatile expression of a given target gene in a particular tissue (for example in gene therapy). Analysis of the quantitative regulation of target gene expression in transgenic mice indicates that control of the kinetics and level of activation can be achieved by altering the suppressive dose of Tc (Passman and Fishman, 1994). This feature would potentially allow the maintenance of target gene expression at intermediate levels, and would be particularly useful in studies of oncogenes or cytotoxic gene products.

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 (Shockett and Schatz, 1996). 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. Streamlined single vector expression systems for mammalian cells have been developed and provide advantages for certain applications. Several plasmids have been constructed that contain two minimal promoters in opposite orientations on either side of the heptamerized tet operator sequence, allowing the Tc-regulated expression of two genes in stoichiometric amounts from a single vector (Baron et al., 1995). This vector has been used to simultaneously express luciferase and β-gal in response to tTA (driven from a second construct). Such a vector may allow the co-regulation of two gene products as a prerequisite for the production of hetero-oligomeric proteins. Another plasmid that combines tTA (constitutively driven) and tetO-luciferase driven in the
plasmid exhibit basal and induced expression levels comparable to those in cells transfected with the two-plasmid system (Shockett and Schatz, 1996).

An alternative method for delivery of Tc-regulated genes has been developed that adapts the single vector approach for use in a retrovirus (Paulus et al., 1996). The viral vectors contain tTA driven by either a viral or cell-type specific promoter and tet-O-luciferase driven in the opposite direction. The initial retroviral system was shown to be functional in cell culture, but unfortunately induction levels were significantly reduced as compared with the initial two-plasmid system. Hofmann et al. (1996) have recently developed a new system that combines the single vector retroviral approach with the autoregulatory tTA expression strategy. This vector encodes a bicistronic mRNA allowing expression of both β-gal and tTA from tetO-pCMV, 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 Tc-regulated elements. Another innovation introduced in the work of Hofmann et al. (1996) is the use of fluorescence-activated cell sorting (FACS) to isolate uniform populations of cells from culture that exhibit particularly low basal and high inducible levels of β-gal reporter expression. This greatly simplifies screening for clones which show optimal expression characteristics. The delivery of Tc-responsive 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 solve the problem of integration site-specific effects that are averaged in uncloned cell populations but which can become prominent in selected cell lines or clones. If the use of a retroviral vector is combined with FACS, only clones with optimal expression characteristics, and thus favourable integration site effects, would be selected. Another virus-based approach involves the testing of vectors derived from the autonomous parvovirus LuIII for their ability to deliver Tc-regulated gene expression units in vitro. These viruses, unlike retroviruses, are nonintegrating, a desirable feature for gene therapy strategies involving short-term delivery of a cytokine or toxin to certain cell types (for example in targeting cells for ablation in cancer therapy) (Shockett and Schatz, 1996).
of a Tc-regulated transgene is undesirable. Amplicon-based defective herpes simplex virus vectors have been used extensively for gene transfer into cultured neurons and the adult CNS. Newly developed herpes simplex virus vectors may allow the transfer of the tetracycline transactivator system into specific primary cell types, such as neurons, although the efficiency of infection by the herpes simplex viral vectors in vivo is rather low (Ho et al., 1996).

2. EXPERIMENTAL RATIONALE

Our laboratory is particularly interested in synaptic plasticity in the mammalian brain and the molecular mechanisms underlying the processes of learning and memory. Glutamate is the major excitatory neurotransmitter of the central nervous system. A certain class of glutamate receptors, the N-methyl D-aspartate (NMDA) receptors, have been implicated in the induction of an electrophysiological phenomenon termed long term potentiation (LTP). LTP is best defined as a long lasting strengthening of synaptic efficiency, and represents the best cellular correlate of learning and memory (Bliss and Collingridge, 1993). NMDA receptors are heteromeric; although the stoichiometry of these receptors has yet to be determined, all NMDA receptors consist of at least one NR1 subunit in combination with either NR2A, NR2B, NR2C, or NR2D subunits (Hollmann and Heinemann, 1994). Thus, the NR1 subunit is ubiquitously present in complete NMDA receptor complexes (Dani and Mayer, 1995). When the NR1 locus was knocked out in mice, it was found that complete lack of NR1 was neonatally lethal. All newborn NR1(-/-) homozygote mice die apparently of pulmonary failure by 48 hours after birth (Forrest et al., 1994, Li et al., 1994). It is likely that NR1 or functional NMDA receptor complexes is required for proper neural pathway development. Recently Kutsuwada et al. (1996) reported that NR2B knockout mice also die shortly after birth. Consequently, our laboratory designed a neural-specific inducible genetic system for use in transgenic mice to circumvent the problem of neonatal lethality so that LTP and behaviour of adult animals lacking particular NMDA receptor subunits could be studied.
control of the human neurofilament light chain (NFL) gene promoter. (Figure 3) Neurofilaments are abundant cytoskeletal components of neurons which appear to be a major determinant for the radial growth of axons. Neurofilaments are formed by the copolymerization of three neuron-specific proteins: NF-L, NF-M, and NF-H (light, medium and heavy chains). Initial expression of the neurofilament light chain gene in mice coincides with the appearance of postmitotic neurons at the onset of neuronal differentiation, an event which occurs at approximately E10 of embryonic development. NFL gene expression is restricted exclusively to neurons, with expression levels in mice peaking just before birth and continued expression at a low basal level throughout life. The human neurofilament light chain gene promoter has been shown to correctly direct the expression of the human neurofilament sequence and choline-acetyltransferase (CAT) and lacZ reporter constructs in transgenic mouse embryos (Beaudet et al., 1992). The temporal and spatial expression patterns of the NFL promoter during development and in adult mice suggested that it could be used to direct constitutive expression of tTA specifically in neuronal tissue. Transgenic mice carrying tTA under the control of the NFL promoter were constructed to form one half of a tetracycline-inducible gene expression system to allow manipulation of neuronal genes such as NMDAR1 in adult animals.

The tetracycline transactivator system was chosen because it has a number of advantages over other inducible systems developed for use in transgenic mice. Because the tetracycline system is based on prokaryotic elements, it was expected that the presence of these elements would cause minimal pleiotropic effects. The very low basal levels of expression, high induction ratios, tight regulation, and relatively fast induction kinetics of the system were also attractive features. In addition, the favourable pharmacological properties and low toxicity of tetracycline and its derivatives indicated the system was ideal for use in transgenic animals (Boothe, 1985, Gossen and Bujard, 1993). For my thesis project, I characterized several lines of mice carrying NFL-tTA transgenes for their potential utility as components of a neural-specific inducible genetic system.
Figure 3. The tetracycline transactivator system. Binding of tTA and transcription of the gene of interest occurs only in the absence of tetracycline. The system has been adapted to express tTA using the human neurofilament light chain gene promoter to achieve neural-specific function.
1. Plasmid constructs and mouse lines

Plasmids pUHD 15-1 (phCMV-tTA) and pUHD 10-3 (tet-O-phCMV) containing components of the tetracycline transactivator inducible system were provided by the laboratory of Dr. Hermann Bujard, University of Heidelberg. Plasmid pGCHNFL containing the human neurofilament light chain gene promoter region was provided by the laboratory of Dr. Jean-Pierre Julien, McGill University. The EcoRI/HindIII fragment containing the tTA sequence was released from pUHD 15-1 and blunt-end ligated into pGCHNFL to create plasmid TM1. A β-geo reporter gene (consisting of a fusion of β-galactosidase and neomycin resistance sequences; Friedrich and Soriano, 1991) was ligated into pUHD 10-3 to create plasmid TM3. Initial cloning was performed by Dr. Zheng Ping Jia in our laboratory. TM1 was linearized with KpnI/NotI, and TM3 was linearized with XhoI/HindIII. The two constructs were run in agarose gels to separate the linearized fragment from the plasmid backbone. The band containing linearized fragment was cut from the gel and put through a syringe to crush the gel slice. The fragment was phenol/chloroform extracted, ethanol precipitated, and resuspended in water.

The two linearized constructs were then separately microinjected into CD-1 or C57/B6 mouse zygotes to create transgenic animals. Potential transgenics were then screened by Southern blotting. Six mice were identified positive for the NFL-tTA transgene, and two identified positive for the tet-O-βgeo transgene. These founder animals were bred to CD-1 mates and progeny from these crosses were screened to determine germ-line transmission of the transgenes. Four NFL-tTA founders and one tet-O-βgeo founder showed transmission and were used in subsequent breeding.

2. Agarose gel electrophoresis

0.8% agarose gels prepared in Tris-Acetate EDTA (TAE) buffer were used to separate DNA fragments from restriction digests of genomic DNA extracted from mouse tail
products. Genomic DNA was extracted from tail biopsies by proteinase K digestion overnight at 65°C with shaking followed by chloroform extraction and ethanol precipitation. Extracted DNA was then resuspended in Tris-EDTA (TE) buffer pH 8.0. Genomic DNA was digested with BamH I and analyzed by Southern blotting.

3. Southern Blotting

Digested DNA separated on agarose gels was blotted overnight onto Amersham Hybond N+ nylon membranes using the capillary blotting technique described previously (Southern, 1975). DNA was then UV-crosslinked to the membrane using a UV Stratalinker 1800 (Stratagene) and placed in pre-hybridization solution (200 mM sodium phosphate, 1 mM EDTA, 1% essentially fatty acid free BSA, 15% formamide, 7% SDS). Membranes were pre-hybridized for 4 hours or overnight at 65°C without shaking.

4. Probes

tTA - The plasmid TM1 was cut with BamH I to release a 1.2 kb fragment containing a portion of the tTA coding sequence. The 1.2 kb fragment was resolved by agarose gel electrophoresis, and was purified from the gel, either by crushing the gel slice and phenol/chloroform extracting or by using a Gene Clean II kit (Bio 101, Inc.). The fragment was then radioactively labelled with α32P-dCTP using the Random Primed Labelling kit (Boehringer Mannheim). The probe was then purified on a G-50 Sephadex Nick column (Pharmacia) to separate unincorporated nucleotides from the labelled fragment. The radioactivity of the fragment was then determined by scintillation counting. Typically, probe was added to blots at 1 x 10⁶ CPM/ml of hybridization solution. The probe was then heat denatured and hybridized to blots of genomic DNA from NFL-tTA transgenic mice.
containing a portion of the βgeo reporter coding sequence and resolved by gel electrophoresis. The 3.1 kb fragment was purified from the gel, labelled as above and used as a probe for blots of DNA from tet-O-βgeo transgenic mice. DNA from double transgenic mice was screened using both tTA and βgeo probes.

Oligonucleotide probes - For RT-PCR experiments, oligonucleotide probes were designed to recognize PCR products amplified from tTA and mouse hypoxanthine phosphoribosyltransferase (hprt) mRNAs. Hprt was chosen as a positive control because of its relatively high expression level in brain (Thompson et al., 1989). Oligo jrg3 (sequence 5’-AGCGACTTGATGCTCTTGATC-3’) was designed as an internal probe for tTA, and oligo h330 (sequence 5’-GTTGAAGATCTAATTGACAC-3’) was designed as an internal probe for hprt. Both probes were end labelled using $^{32}$P-dATP and polynucleotide kinase according to the method described by Maniatis et al. (1989) and used in Southern blotting of RT-PCR products (see below). All oligonucleotides and PCR primers were prepared by R. Mak, Mount Sinai Hospital.

5. Hybridization

For screening of transgenic mice - Blots were hybridized overnight at 65°C in a shaking water bath with labelled probe(s) in pre-hybridization solution. Following hybridization, membranes were washed twice for 30 min at 65°C in a solution of 2X SSC and 0.1% SDS. Washed membranes were exposed in a Molecular Dynamics Phosphorimager cassette for 3-4 hr and the exposed image cassette was then scanned and computer analyzed. Blots were then also exposed to Kodak X-Omat film under an intensifying screen. Typically, films were developed after an overnight exposure.

For analyzing RT-PCR blots - Blots were hybridized overnight at 37°C as above. Following hybridization, membranes were washed once for 30 min at 37°C in 2X SSC and
exposed and analyzed as above.

6. Analysis of NFL-tTA transgenic mice

6.1 mRNA detection

(i) Northern blotting

Northern blotting analysis of tTA RNA extracted from brain tissue of transgene positive NFL-tTA mice was performed essentially as described by Maniatis et al. (1989). Mice were deeply anaesthetized with halothane and sacrificed by cervical dislocation. The brain was rapidly removed, cut in half, and either prepared immediately or flash-frozen in liquid nitrogen. Total RNA was extracted using guanidinium isothiocyanate and ultracentrifugation through CsCl. RNA samples resuspended in DEPC-ddH2O were quantified in a spectrophotometer, loaded and run in a 1.0% agarose/formaldehyde gel, and blotted overnight onto a nylon membrane. Northern blots were then probed using the 1.2 kb tTA DNA probe described above.

(ii) Reverse transcription - polymerase chain reaction (RT-PCR)

Primers - PCR primer sets were designed to amplify from tTA and hprt cDNA. Oligonucleotide primers jrg1 (5'-GAGCTGCTTAATGAGGTCGG) and jrg2 (GGCCGAATAAGAAGGCTGGC-3') were designed to amplify a 525 bp fragment from tTA, and primers h277 (5'-CCTGCTGGATTACATTAAAGCACTG) and h278 (CAAACAACACCTATACGGAACTG-3’) were designed to amplify a 750 bp fragment from hprt.

Total RNA was extracted from brain tissue of transgene positive mice using as above. Following RNA quantification, starting concentrations of RNA samples were adjusted to 1 μg/μl. Reverse transcription reactions were carried out at 37°C in the presence of RNase inhibitor (RNAsin, Promega) using the specific 3’ primers jrg2 and h278 for tTA and hprt, respectively and either Boehringer-Mannheim Mu-MMLV or Promega AMV reverse
transcriptase. For PCR, approximately 1 μg of cDNA template from the RT reaction was amplified using both the tTA (jrg1 and jrg2) and hprt (h277 and h278) specific primer sets, Taq polymerase (either Pharmacia or Promega), and standard reagents. PCR was performed as follows: 4 min hot start at 94°C; 1 min denaturation at 94°C; 1 min annealing at 55°C; 1.5 min extension at 72°C. Amplification was continued for 35 cycles. Negative controls included a sample containing no starting RNA template, and a sample that had no reverse transcriptase added. RT-PCR samples were run on 1.0% agarose gels and subsequently Southern blotted and probed using γ²P-dATP end labelled internal primers jrg3 (tTA) and h330 (hprt).

6.2 Protein detection

(i) Western blotting

Western blotting analysis of tTA protein extracted from brain tissue of transgene positive NFL-tTA mice was performed essentially as described by Maniatis et al. (1989). Soluble protein was extracted from fresh or frozen brain tissue by homogenization and detergent lysis in the presence of protease inhibitors. As a control, protein was also extracted from cultured cells expressing tTA. Protein samples were quantitated using a Bradford colourimetric assay, denatured by boiling in sample buffer containing β-mercaptoethanol and SDS, run in a polyacrylamide gel, and blotted onto a Nytran membrane. Western blots were then probed using a rabbit monoclonal antibody raised against herpes simplex virus protein VP16, the transcriptional activator portion of the tTA protein. (Antibody provided by Dr. J. Ingles, University of Toronto) Bound primary antibody was then detected using an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody and treatment of blots with NBT and BCIP.
(i) Staining for β-galactosidase

Double transgenic NFL-tTA/tet-O-βgeo and control mice were deeply anesthetized with halothane, sacrificed by cervical dislocation, and nervous system tissue was dissected out and placed in cold PBS (w/o Ca²⁺ and Mg²⁺). Tissues were washed once in 0.1 M phosphate buffer (pH 7.3) and fixed in a solution of 0.2% glutaraldehyde, 5 mM EGTA (pH 7.3), and 2 mM MgCl₂ in phosphate buffer for 20 min at room temperature. Tissues were then washed three times (5 min each) at room temperature in wash buffer containing 0.01% deoxycholate, 0.02% Nonidet-P40, and 2 mM MgCl₂ in phosphate buffer. Samples were stained overnight at 37°C in a X-gal stain containing 1 mg/ml X-gal, potassium ferrocyanide (125 μM), and potassium ferricyanide (125 μM) in wash buffer. After staining, tissues were rinsed and then kept in wash buffer at 4°C for several hours to intensify staining. Samples were then photographed on a Leitz M10 microscope (without blue filter) using Kodak Elite 100 colour slide film.

8. Inducible expression of β-galactosidase in NG108 cells

Rat NG108 cells, a neuroblastoma-glioma hybrid cell line, are commonly used for in vitro studies because they exhibit neuron-like properties. For example, under certain conditions (such as serum starvation or the administration of nerve growth factor), NG108 cells will extend neurites. These cells also express neuron-specific markers such as the neurofilament proteins. Unlike primary neurons in culture, NG108 cells divide rapidly and can be easily transfected (Ng et al., 1996). NG108 cells were transiently transfected with plasmids constructed to drive expression of tTA under the control of various promoters, and with tet-O-βgeo plasmid as a reporter. These experiments were performed in collaboration with Dr. Jeff Henderson in our laboratory.

Liposome-mediated transfection was used to introduce plasmid DNA into NG108 cells. Cells were grown to 50% confluency on 4 cm tissue culture dishes in DMEM/10% fetal
Driving tTA and 3 μg of reporter plasmid (tet-O-βgeo) was prepared. DNA was mixed in OptiMEM serum-free medium (Gibco) to a final volume of 100 μl in a microfuge tube. In a second tube, 50 μl of Lipofectin (Gibco) was mixed with 50 μl of OptiMEM. The contents of the tubes were then mixed, and incubated for 15 minutes at room temperature. Meanwhile, medium was removed from the cells, and the cells were washed twice in OptiMEM. 0.8 ml of OptiMEM was then added to each plate (enough to cover). The 200 μl DNA/Lipofectin suspension was then added slowly to the plate and swirled into the medium. Cells were then incubated for 18-20 hrs. The medium was removed and replaced with DMEM with (+) or without (-) doxycycline (prepared fresh, added at a final concentration of 1 μg/ml). After 48 hr, cells were stained for lacZ expression with X-gal stain for 1 hr or overnight and photographed on a Leitz inverted microscope using Kodak 64T EPY colour slide film.

RESULTS

1. Plasmid constructs and mouse lines

The structures of plasmids TM1 and TM3 are shown in figures 4 and 5. The ~7.3 kb Kpn I/Not I fragment of plasmid TM1, containing the tTA transactivator under the control of the human neurofilament light chain gene promoter (pNFL), was gel purified and used for microinjection of mouse CD1 and C57/B6 zygotes. The ~4.5 kb XhoI/HindIII fragment of plasmid TM3, containing the tet-O, phCMV minimal promoter, βgeo fusion gene, and SV40 polyA signal, was gel purified and used for microinjection of mouse C57/B6 zygotes. Zygotes were then introduced into pseudopregnant foster mothers to be carried to term. The pNFL promoter fragment from plasmid TM1 included intragenic cis-acting regulatory elements which have been shown to be essential for appropriate neural-specific expression using the promoter (Beaudet et al., 1992). It was expected that the NFL promoter would drive high level expression of tTA specifically in neurons of NFL-tTA transgenic mice, and that the presence of tTA by itself would have no phenotypic
Figure 4. Plasmid TM1, showing ~7.3 kb Kpn I/Not I fragment used for microinjection containing the tTA transactivator under the control of the hNFL promoter.
Figure 5. Plasmid TM3, showing ~4.5 kb Xho I/Hind III fragment used for microinjection containing the tet-O, phCMV minimal promoter, βgeo fusion gene, and SV40 polyA signal.
2. Southern blot screening of NFL-tTA and βgeo mouse lines

Four founder NFL-tTA mice showed germline transmission through Southern blot analysis of DNA extracted from tail biopsies of their progeny. These initial Southern blots indicated that two lines (designated 17 and Kpn1) probably carried multiple copies of the NFL-tTA transgene, whereas the other two lines (designated 16 and 18) presumably carried few or a single copy of the transgene based on band intensity. The inheritance pattern of the transgene in all four lines was consistent with transgene integration at random autosomal loci. A sample blot of positive NFL-tTA transgenics from each of the four lines is shown in Figure 6a, with tTA being detected as a 1.6 kb band. Subsequently, a single founder tet-O-βgeo mouse also showed germline transmission by Southern blot analysis of DNA extracted from tail biopsies of progeny. A sample blot for a positive tet-O-βgeo transgenic is shown in Figure 6b, with βgeo being detected as a 3.1 kb band. As expected, the presence of the tTA transgene in NFL-tTA mice appeared to have no overt phenotypic consequences. Tet-O-βgeo mice also had a normal phenotype. Animals from each of the four NFL-tTA lines and the tet-O-βgeo line were eventually used to establish breeding pairs to produce double transgenic NFL-tTA/tet-O-βgeo progeny.

3. Analysis of NFL-tTA transgenic mice

3.1 mRNA detection

(i) Northern blotting

Several attempts at Northern blotting total RNA extracted from brain tissue of NFL-tTA mice were unsuccessful in detecting tTA RNA. Simultaneous probing of RNA blots with DNA probes for tTA and β-actin (as a positive control) led to strong detection of β-
Figure 6A. Sample Southern blot performed on genomic DNA from mice of each of the four NFL-tTA transgenic lines. DNA was digested with BamHI, run on an agarose gel, blotted, and probed using the tTA probe described in Materials and Methods. B. Sample Southern blot performed on genomic DNA from a tet-O-βgeo transgenic mouse. DNA was digested with BamHI, run on an agarose gel, blotted, and probed using the βgeo probe described in Materials and Methods.
This was not entirely unexpected, as other investigators using the standard two-plasmid tTA system had also been unable to detect tTA RNA by Northern blotting of RNA extracted from appropriate tissues of transgenic animals and from transfected cells in culture. The inability to detect tTA RNA may in part be due to the very short half life of tTA RNA or as a result of tTA toxicity. This may be due to the prokaryotic nature of the tTA sequence, which may be recognized as foreign to the cell and actively destroyed or selected against. Because of the inability to detect tTA RNA by Northern blotting (or protein by Western blotting, see below), a sensitive RT-PCR protocol was designed to ensure that tTA was definitely being expressed in the brains of NFL-tTA transgenic mice.

(ii) RT-PCR

When initial RT-PCR experiments were performed on total RNA extracted from the brains of NFL-tTA mice, it was not possible to visualize RT-PCR products in agarose gels using ultraviolet transillumination. It was impossible to tell from these results whether tTA RNA was being expressed at all in the transgenic mice, since positive control RT-PCR products (derived from RNA extracted from Cos-7 cells transiently transfected with hCMV-tTA) were not visible either. PCR directly off limiting quantities of hCMV tTA plasmid, in contrast, gave a very intense band of the expected size (525 bp). It was not clear from these results whether the reverse transcription or the PCR reaction were actually working on extracted RNA. Subsequently, a more complicated RT-PCR protocol was employed, using primers for hprt (because of its relatively high expression in brain) as internal positive controls for each sample. Again, none of the RT-PCR products could be visualized on agarose gels. For greater sensitivity, the RT-PCR gels were Southern blotted and then probed for both tTA and hprt. Southern blotting using tTA probe revealed that tTA RNA was indeed produced in the brains of mice from all four NFL-tTA lines. (Figure 7) Probing of the same blot using hprt confirmed that the reverse transcription reaction was working and that there was no DNA cross-contamination in the samples. In addition, when the RT-PCR products were used as templates for a second
Figure 7. Reverse transcription - polymerase chain reaction experimental results. A. Photograph of a UV-transilluminated gel loaded with RT-PCR samples. No bands are visible. B. Southern blot from this gel probed with a tTA probe. A 525 bp band is clearly recognized in samples obtained from all four NFL-tTA transgenic lines. C. As an internal control, the same blot was probed with an hprt probe. A 720 bp band is recognized in both experimental and control samples. D. Photograph of a UV-transilluminated gel loaded with RT-PCR samples re-amplified using tTA primers. In this case, the expected 525 bp band is visible in samples derived from all four NFL-tTA transgenic lines.
3.2 Protein detection

(i) Western blotting

Western blotting was performed on total soluble protein extracts from brain tissue, with blots probed using an antibody against herpes simplex virus protein VP16, the transcriptional activator portion of tTA. The antibody detected a protein with a molecular weight of ~37 kD, corresponding to the calculated molecular weight of the tTA protein. This 37 kD protein was also identical in size to tTA protein extracted from stably transfected GH4 cells and transiently transfected Cos-7 cells. However, a 37 kD protein was also detected by the VP16 antibody in total soluble protein extracted from brain tissue of negative control mice. Sample blots are shown in Figure 8, with the 37 kD protein marked by an arrow. It seems that the antibody cross-reacts with an endogenous mouse protein present in brain tissue. tTA protein expressed using the standard two-plasmid tTA system either in vitro or in vivo has also been found by other investigators to be undetectable by Western blotting, by using antibodies directed against either the VP16 or the tet repressor domains of tTA. The initial experiments by Gossen and Bujard (1992) in HeLa cells showed that tTA protein was barely detectable only by a gel mobility shift assay, based upon tTA binding to the tet operator sequence (Gossen and Bujard, 1992).

4. Functional analysis of double transgenic NFL-tTA/tet-O-βgeo mice

In general, the detection of tTA expression both in cells and in transgenic animals has proved to be very difficult. Other investigators have found that the only reliable test for the tetracycline transactivator system is a functional assay, in which tTA is required to activate a reporter construct (usually β-galactosidase or luciferase). To this end, all four
Figure 8. Western blot analysis of tTA protein expression *in vivo* and *in vitro*. Total protein extracted from brain tissue or cells was run on an acrylamide gel, blotted to a nylon membrane, and probed using a primary antibody against the VP16 transcriptional activating domain of tTA. Blots were then probed with a secondary antibody conjugated to alkaline phosphatase and developed. The ~37 kD band indicated by the arrows corresponds to the calculated molecular weight of the tTA protein, but the band also appears in protein extracted from brain tissue of non-transgenic mice.
minimal hCMV promoter upstream of a βgeo transgene consisting of a fusion between β-galactosidase and neomycin resistance sequences. Expression of βgeo has been shown to parallel that of βgal reporters, and can easily be detected in appropriate tissues of transgenic animals by staining for β-galactosidase activity (Friedrich and Soriano, 1991). Double transgenic NFL-tTA/tet-O-βgeo were screened by Southern blot analysis and age-matched individuals were selected to be sacrificed. Mice ranged in age from one week to three months. Whole brain, trigeminal, optic, and facial nerves, ulnar nerve, and sectioned spinal cord were dissected out, rinsed, fixed, washed, and stained for lacZ expression. Unfortunately, as shown in Figure 9, none of these tissues exhibited staining. Nonspecific staining is evident particularly in exposed bone tissue of both transgenic and control samples, in a pattern typical of spurious “background” staining. The failure of pNFL-driven tTA to activate the reporter construct suggested that the insertion site of the reporter transgene was not optimal for proper expression. It was learned from other investigators using the tet system in transgenic mice that the frequency of obtaining an optimally functional reporter line, having low basal expression but high inducibility, was rather low. Probably because of insertion site effects, at best only approximately 1 out of every 3 reporter lines tested show proper function (Hennighausen et al., 1995; Mayford et al., 1996a). At this point, the construction of additional reporter lines was curtailed in favour of obtaining a reporter line already known to be functional from the laboratory of Dr. Lothar Hennighausen, National Institutes of Health. However, crossing of this reporter line to all four NFL-tTA lines and staining of neural tissue from double transgenic progeny for β-galactosidase activity was also unsuccessful in showing reporter expression. A transgenic line containing tTA under the control of the mouse mammary tumour virus (MMTV) promoter was also obtained. (Hennighausen, N.I.H.) This line supposedly showed high levels of β-galactosidase expression in brain when crossed to the known reporter line; however, when the cross was performed in our laboratory, this observation was not reproducible. Again, in all neural tissues, no blue staining above background was seen, although control tissues (salivary gland, skin, lung, and liver) stained blue. The reason for this discrepancy is unclear.
Figure 9A. Nervous system tissue of control (L) and double transgenic NFL-tTA/bgeo (R) transgenic mice stained for β-galactosidase expression. In the double transgenic samples, no staining above background levels is evident.
samples, no staining above background is evident.

**Figure 9B.** Nervous system tissue of control (C) and double transgenic NIH-TAβ/βεεο

(II) Ulnar nerve
An in vitro assay was performed in order to assess the ability of various promoters driving tTA to activate expression from a tet-O-βgeo reporter plasmid in NG108 cells. Four promoter constructs were tested: phCMV-tTA, pβ-actin-tTA, pNFH, and pNFL. Test plasmids were co-transfected with a tet-O-βgeo plasmid, and cells were incubated in the absence or presence of doxycycline to turn reporter expression on or off. Cells were then stained for lacZ activity, and the approximate number of blue cells per plate was counted. The results are shown in Table 2. All of the promoters (with the exception of pNFH) were able to activate reporter expression in a doxycycline-dependent manner. phCMV-tTA gave the highest amount of activation, pβ-actin-tTA gave approximately 20% of this level, pNFL 5%, and pNFH 1%. Reporter activation was not completely blocked by doxycycline administration (at 1 μg/ml) for all promoters tested; various levels of background lacZ expression were observed. The tet-O-βgeo plasmid alone had no activity. These experiments demonstrated that the neurofilament light chain gene promoter can drive expression of tTA to levels sufficient to activate tet-O-βgeo reporter expression in a neuron-like cell type.

**DISCUSSION**

The above results indicate that the tetracycline inducible genetic system described here, based upon pNFL-driven expression of the tTA transactivator, does not drive expression of a β-galactosidase reporter gene in the nervous system of transgenic mice. The original two plasmid tetracycline system utilized the hCMV IE promoter to express tTA. When this promoter was used to drive tTA expression in transgenic mice, inducible expression of β-galactosidase and luciferase reporter genes in the brain was negligible (Furth et al., 1994). Consequently, the human neurofilament light chain gene promoter, a promoter known to direct constitutive transgene expression exclusively in the nervous system of transgenic mice (Beaudet, 1992) was employed for neural-specific expression of tTA.
Table 2. In vitro assay of the ability of tTA expressed from various promoters to activate expression of a tet-O-βgeo reporter plasmid in transiently transfected NG108 cells. Positive cells expressing the βgeo reporter were stained blue following a 1 hr treatment with gal stain. Cell counts are included as mean values ± standard error of the mean. Relative expression levels are normalized to the expression level observed for phCMV-tTA (100%). "Leaky" expression was estimated by comparing the number of cells in the unrepessed (nodoxycycline) and repressed (+ 1 μg/ml doxycycline) states.

<table>
<thead>
<tr>
<th>Transfected plasmids</th>
<th>Number of +ve cells/plate (- dox)</th>
<th>Relative expression level (- dox)</th>
<th>Number of +ve cells/plate (+ dox)</th>
<th>Percentage of “leaky expression (+ dox)</th>
</tr>
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<tbody>
<tr>
<td>phCMV-tTA + tet-O-βgeo</td>
<td>&gt;1000</td>
<td>100%</td>
<td>186 ± 5</td>
<td>19%</td>
</tr>
<tr>
<td>pβactin + tet-O-βgeo</td>
<td>200 ± 5</td>
<td>20%</td>
<td>19 ± 3</td>
<td>10%</td>
</tr>
<tr>
<td>pNFL-tTA + tet-O-βgeo</td>
<td>53 ± 3</td>
<td>5%</td>
<td>34 ± 3</td>
<td>64%</td>
</tr>
<tr>
<td>pNFH-tTA + tet-O-βgeo</td>
<td>(14 ± 2)</td>
<td>(1%)</td>
<td>(12 ± 1)</td>
<td>(86%)</td>
</tr>
<tr>
<td>tet-O-βgeo alone</td>
<td>0</td>
<td>0%</td>
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Once the components of the system had been constructed, DNA fragments encoding pNFL-tTA and tet-O-βgal were microinjected into mouse zygotes to create transgenics. Positive transgenic mice were then screened by Southern blotting, and attempts were made to determine the level of tTA expression in the brains of NFL-tTA mice. Northern and Western blotting performed on RNA and protein extracts did not conclusively show that tTA was being expressed. However, by using RT-PCR, it was demonstrated that tTA RNA was being produced in the brains of NFL-tTA mice. This is, to our knowledge, the first direct demonstration of tTA expression in vivo - other investigators have tried Northern and Western blotting without success. In the original report by Gossen and Bujard, a sensitive gel-mobility shift assay was used to assess tTA expression in HeLa cells based upon binding of the tTA protein to the tet-O-reporter DNA sequence (Gossen and Bujard, 1992).

It is unclear why tTA expression is so difficult to detect. Despite the lack of other overt pleiotropic effects caused by the presence of tTA, the observation that tTA is undetectable in HeLa cells by Western blotting is consistent with toxicity of the tTA protein. This may 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 (Gill and Ptashne, 1988). The presence of elements of the tTA system in mammalian cells may not be tolerated or may be selected against. In myogenic cell lines, either one or both of the tTA or the tet-O-regulatable plasmids ceased to be expressed or was eventually eliminated over time (Hofmann et al., 1996). Other stable cell lines (including the original HeLa cells) also have a tendency to lose expression after prolonged culture, necessitating repeated testing of expression levels or replacement of cells every one to two months (Damke et al., 1995). Characterization of the autoregulatory tTA system in fibroblasts revealed that in some transfected lines, greater than 50% cell death occurred within 10 days following transfection, with complete loss of detectable tTA protein by 16 days. (Shockett et al., 1995)

Several investigations performed in cultured cells have reported that cell confluency is apparently an important factor influencing the system, with optimal expression of tet-O-regulated transgenes occurring at low cell density. This effect may reflect possible
although it is not clear why cell confluency should affect tTA or tet-O-regulated transgene expression. If tTA is toxic to mammalian cells in some way, it is possible that spontaneous loss or elimination of tTA and/or reporter expression could occur very rapidly in a small population of cells derived by clonal propagation from a single founder cell. However, since the finding has not been observed in all cases, the apparent dependence of regulation on cell density may result from differences in the integration site or in the gene of interest. In addition, use of the autoregulatory tetracycline inducible system in cultured cells showed that induced tTA mRNA and protein could be easily detected by Northern and Western blotting of total RNA and protein, but expression levels in transgenic animals (based on induced levels of luciferase) were substantially lower. This suggests that over-expression of tTA in vivo is somehow deleterious (Shockett and Schatz, 1996). The half-lives of tTA mRNA and tTA protein either in vitro or in vivo have not been determined, but the kinetics of the system suggest that both mRNA and protein are rapidly cleared from positively expressing cells (Gossen et al., 1995). If tTA is indeed toxic, it is possible that tTA is recognized as foreign to the cell and is actively sequestered and/or destroyed, perhaps by lysosomes.

Because of the prokaryotic nature of the regulatory elements involved and codon usage differences between bacteria and mammalian cells, the tTA sequence may be inefficiently transcribed and translated. Transcription by RNA polymerase II in eukaryotes produces a primary mRNA transcript that is processed: the 5' end of the mRNA is capped, and splicing occurs. Some mRNAs contain an open reading frame flanked by untranslated regions. These untranslated regions can act as binding sites for proteins necessary for efficient translation, or in stabilization of an mRNA. In prokaryotes, mRNAs can be translated as transcription is proceeding, since transcription occurs in the cytoplasm. In eukaryotes however, mRNA must be properly transported to the cytoplasm and localized to ribosomes. Once the mRNA is translated, the resulting polypeptide chain must be properly folded, modified, and transported. It is possible that the tTA sequence, although engineered for expression in mammalian cells, may not be efficiently translated because it is improperly processed or lacks appropriate untranslated regions that would act to
cells before it can be transported to ribosomes and translated. Similarly, inefficient folding, modification, or transport of tTA protein in mammalian cells may also limit the production of functional tTA. There are efforts underway to alter the tTA sequence so that it will be comprised of codons used by mammalian cells (Mayford et al., 1996b).

In total RNA extracted from brain tissue of mice from four transgenic NFL-tTA lines, tTA RNA was detected by RT-PCR. Despite this result, tTA transactivator driven by the human neurofilament light chain gene promoter fails to activate tet-O-βgal reporter transgenes in double transgenic NFL-tTA/tet-O-βgal mice. It is worth considering that attempts by other investigators to apply the original or modified tetracycline inducible systems, especially in transgenic mice, have met with limited success. There are several possible obstacles that appear to prevent full function of the systems. The system may function better in some cell or tissue types than in others. Because of the heterogeneity in gene expression that has been observed in some cases, success in any given cell or tissue milieu will probably depend upon careful choice of constitutive or tissue-specific promoters for transactivator expression (Shockett and Schatz, 1996). For example, in mice carrying lacZ reporter transgenes activated by tTA expressed from a mouse mammary tumour virus (MMTV)-LTR, relatively homogeneous expression was observed in epithelial cells of the seminal vesicle and salivary gland, and in the Leydig cells of the testis, but heterogeneous expression was observed in mammary epithelial cells and basal cells of the epidermis (Hennighausen et al., 1995). There is limited evidence that, at least in vitro, the level of tTA expression from a given promoter must be within a defined "window" for proper tet-O-regulated transgene activation (Damke et al., 1995). Levels of tTA expression outside this optimal "window" may prevent proper activation. Titrating the concentration of tetracycline in the media of transfected HeLa cells presumably results in variation in the level of tTA able to bind to the tet operator sequence. Even in the presence of high levels of tetracycline, some individual cells show relatively high basal levels of expression of the tet-O-linked transgene. Similarly, other cells remain in an uninduced state even when tetracycline is absent (Damke et al., 1995). It is possible that tTA expression driven by the human NFL promoter in neurons fails to reach an adequate
promoter-driven tTA was shown to activate expression of a tet-O-βgeo reporter in NG108 cells, although the level of activation was quite low and the leakiness observed was substantial. This low activation level may reflect a low level of tTA expression, and may explain the inability of hNFL-tTA to function in vivo.

Very recently, several preliminary reports have shown successful use of the tetracycline inducible system by driving tTA using certain promoters that drive expression primarily in the brain. Using the α-type calcium-dependent calmodulin kinase II (αCamKII) promoter to drive expression of tTA, Mayford et al. (1996b) have demonstrated inducible expression of a mutant, constitutively active form of CamKII linked to the tet-O- sequence in transgenic mice. Hasan et al. (1996) have used the L7 promoter to drive expression of tTA specifically in neocortex and hippocampus, and have employed pL7-tTA to control the inducible expression of a tet-O-linked Cre recombinase transgene. This inducible Cre system was then used to activate a modified β-gal reporter gene containing loxP sites flanking a stop codon within the coding sequence. Storck et al. (1996) have targeted the tTA sequence to the promoter region of the NMDAR1 locus to evaluate the feasibility of using the NMDAR1 promoter to drive tTA expression. Interestingly, this group discovered that reporter transgene activation driven by expression of tTA from a NMDAR1 promoter fragment in transgenic mice was very low, an observation attributed to the presence of a potential splice acceptor site within the tet operator/minimal CMV promoter sequence of the reporter transgene. These preliminary studies indicate that the tetracycline inducible system can indeed function in nervous tissue, if an appropriate promoter can be utilized to drive expression of tTA. All of the promoters mentioned here would be expected to drive a lower level of expression than the human neurofilament promoter, suggesting that low level expression of tTA in neurons may be advantageous. These other promoters also contain cis-regulatory regions that tend to restrict expression of linked transgenes to discrete brain regions or cell types, and may ultimately prove better suited to manipulate specific gene expression in the brain.

Another possible problem affecting the tetracycline inducible system relates to the random nature of gene integration. Integration site-specific effects (such as constitutive
activity or repression), which are inherent when exogenous DNA is stably introduced into a cell or the mouse germline, arise from the genetic context of the integration site (Shockett and Schatz, 1996). The basic tTA system described in Gossen and Bujard's original paper, when adapted for use in transgenic mice, was observed to consistently activate high level expression of reporter transgenes in particular tissues. However, this expression was invariably heterogeneous to some extent, depending on the tissue type (Furth et al., 1994). It was speculated that this heterogeneity was directly caused by the hCMV IE promoter used to drive tTA or was the result of insertion of the tTA transgene into transcriptionally "silent" loci in the genome. Both the tTA and the tet-O- sequences are subject to this effect. As a result, integration site effects have necessitated the extensive screening of optimally functional cell lines or transgenics carrying both elements. If the transgenes become inserted into regions of transcriptionally inactive chromatin (for example, highly packaged tracts of heterochromatin), the activity of the inserted transgenes are likely to be repressed. Conversely, insertion into sites in the vicinity of other endogenous promoter or enhancer sequences may also affect the activity of the transgenes. The effect may result either in constitutive activation of the transgene or, through binding competition of transcription factors at the transgenic and endogenous promoter sites, repression of transgene activity. This seems to be especially true of the tet-O-/minimal CMV promoter linked transgene; reporter tet-O- constructs seem to have a highly variable amount of basal activity both in vitro and in vivo. The variable basal activity observed is perhaps due to an inherent susceptibility of the minimal CMV promoter to be affected by surrounding DNA elements. An additional possibility involves the insertion of either the tTA or tet-O-linked transgenes within or near endogenous loci which are regulated in a spatial or temporal manner. In this case, despite being integrated in the same location, at any time during development, the elements of the system may have full function in some tissues, but limited function in other tissues.

These speculations regarding integration site specific effects have been supported by the limited success reported from approaches either involving coinjection of the tTA and tet-O-linked transgenes, or the use of streamlined single-vectors that combine the tTA and tet-O-linked transgenes in a single vector (Taverna, 1996 unpublished data, Baron et al.,
The general failure of these approaches to improve inducible expression is likely due to insertion of the tTA and tet-O-linked transgenes in multiple copies and/or in close proximity. The interaction between the tTA and tet-O-linked elements of the system can lead to a loss of optimal function. Cotransfection of the transgenes most probably results in cointegration, and the minimal CMV promoter may then act as an enhancer trap, increasing tTA-independent background expression. In HeLa cells, the basal activity of the tet-O-/minimal CMV promoter increases significantly when it is located in proximity to the phCMV promoter driving tTA (Damke et al., 1995). Perhaps as a consequence of integration site-specific effects, other investigators utilizing the tetracycline inducible system in transgenic mice have reported that the frequency of obtaining an optimally functional tet-O-linked reporter line is quite low. According to published reports, at least three and as many as eighteen individual transgenic lines have been screened to find a single optimally functional reporter line that shows both proper spatial and temporal tTA-restricted expression. Since our laboratory was only able to obtain two tet-O-βgal reporter lines, it is possible that neither line contained the transgene at an appropriate insertion site, and thus when crossed to the four NFL-tTA lines, double transgenic mice derived from both reporter lines failed to show βgal expression in neurons. However, because of the blue staining observed in control tissues, the inability of the reportedly functional tet-O-βgal reporter line to drive βgal expression in the nervous system may be the result of tissue-specific effects.

The heterogeneous expression usually observed with the original tetracycline inducible system was also attributed to differential patterns of transgene methylation. In the first application of the tetracycline inducible system in animals, histological analyses for β-galactosidase activity in double transgenic phCMV-tTA/tet-O-βgal mice revealed mosaic expression in all tissues examined (Furth et al., 1994). This mosaicism could not be attributed to a property of the β-galactosidase reporter gene used, since the gene can be homogeneously expressed in a wide variety of tissues. It was speculated that stochastic methylation of the tTA transgene, the reporter transgene, or both in individual cells was responsible for the observed mosaicism. In our laboratory, experiments combining the use of methyl-sensitive restriction enzymes with assessment of reporter transgene transcription
extensively methylated (Wang, 1996 unpublished data). DNA extracted from mouse embryonic stem (ES) cells stably transfected with the phCMV-tTA and tet-O-βgal transgenes was digested with two restriction enzymes, *HpaII* and *MspI*, which have the same consensus sequence but differ in sensitivity to methylation; *MspI* is methyl-sensitive, and *HpaII* is methyl-resistant. Combining the results from the restriction digests with analysis of reporter transgene transcription levels showed a correlation between transgene methylation and transcriptional inactivity, suggesting that methylation was the cause of transgene inactivation. Experiments on ES cells using the nucleoside analogue 5-azacytidine, a potent differentiating agent, also revealed the importance of methylation in modulating the efficiency of the system (Wang, 1996 unpublished data). When 5-azacytidine is incorporated into DNA, it cannot be methylated, and it effectively counteracts the activity of cellular maintenance methylases. Consequently, 5-azacytidine containing DNA is undermethylated, and the expression of genes which are ordinarily repressed by methylation can be induced with prolonged treatment. When 5-azacytidine was added in very low doses (0.1 μM) to ES cells stably transfected with the phCMV-tTA and tet-O-βgal transgenes, the proportion of cells which stained positive for lacZ expression increased from approximately 30% (no drug) to nearly 90% (with drug). This result confirmed that methylation is an important factor influencing the activity of the tetracycline inducible system *in vitro*. Whether methylation also plays a role in limiting the expression of the tTA and/or tet-O-linked transgenes *in vivo* is difficult to answer. Since ES cells methylate genes to a greater extent than other cell types, methylation of the transgenes at the tissue level *in vivo* may not reflect that observed *in vitro*. However, it is conceivable that insertion of either one or both transgenes into regions of chromatin that are rendered transcriptionally inactive via constitutive methylation may also permanently inactivate the transgenes themselves. More speculatively, the prokaryotic origin of the transgenes may also predispose them to methylation as part of a cellular defense mechanism. (Richards and Huber, 1993)

Future use of the tetracycline inducible system, particularly in transgenic mice, will require the careful consideration of the obstacles that appear to prevent full function.
*in vivo*, it appears that promoter choice may be the most fundamental variable crucial to optimal functioning of the system. If proper control of tet-O-linked transgenes indeed requires tTA expression within a certain “window”, practical use of the system will necessitate the identification and employment of promoters giving an appropriate level of tTA expression. However, the use of well-characterized cell or tissue-specific promoters may continue to be hindered by problems relating to methylation and/or the transgene integration site(s). Potential problems involving methylation could be overcome by including signal sequences for the ubiquitous transcription factor Sp1 in individual transgenes to prevent their methylation (Brandeis et al., 1994, No et al., 1996), or (less plausibly) by treating whole animals with methylation inhibitor drugs such as 5-azacytidine (Richards and Huber, 1993, Pace et al., 1994, Kato et al., 1996). Integration site-specific effects could be avoided by surrounding transgenes with matrix attachment regions, which have been shown previously to insulate stably integrated vectors and transgenes from the effects mediated by cis regulatory elements in proximity to their sites of integration (McKnight et al., 1992). Alternatively, the tTA and/or tet-O-linked transgenes could be targeted into endogenous loci so that their patterns and levels of expression would be more predictable. This approach would unquestionably benefit from a determination of the optimal level of tTA expression for the cell type or tissue under consideration, and whether there are certain cell and tissue-specific threshold and tolerance levels. Unfortunately, it is still not clear whether or not tTA expression is toxic, and if so, through what mechanism this toxicity occurs. Advances in single vector retroviral approaches may facilitate the creation of functional transgenic lines and could reduce integration site-specific effects due to the very high frequency of transgene incorporation afforded by retroviral infection. In a simplistic way, the potential problems of promoter choice, preferential methylation, and integration site-specific effects may be effectively avoided indirectly by the creation of multiple transgenic lines so that the frequency with which the transgenes insert into transcriptionally favourable regions of the genome may be maximized. This appears to be, in fact, the method of choice among other investigators who are currently applying the system for use in transgenic mice.
Gene knockout of either the NMDAR1 or NMDAR2B glutamate receptor subtypes in mice is neonatally lethal (Forrest et al., 1994, Li et al., 1994, Kutsuwada et al., 1996). There has been considerable interest in designing an inducible, preferably neural-specific genetic system in transgenic mice to circumvent the problem of neonatal lethality so that LTP and behaviour of adult animals lacking particular NMDA receptor subunits could be studied. Recent work by Mayford et al. (1996b) and Tsien et al. (1996a; 1996b) has come close to accomplishing this goal. Expression of tTA was placed under the control of the α-type calcium-calmodulin-dependent kinase II (CaMKIIα) gene promoter. CaMKIIα is a serine-threonine protein kinase that is restricted to the forebrain. It is postnatally expressed in the neurons of the neocortex, the hippocampus, the amygdala, and the basal ganglia. An 8.5 kb genomic DNA fragment derived from the CaMKIIα promoter can confer this regional specificity to a downstream coding sequence in transgenic animals (Mayford et al., 1996b). The temporal and spatial expression patterns of the CaMKIIα promoter during development and in adult mice suggested that it could be used to direct high level expression of tTA in restricted regions of the brain. Transgenic mice carrying tTA under the control of the CaMKIIα promoter were constructed and crossed to a tet-O-βgal reporter line. Double transgenic progeny of this cross showed highly restricted, Tc-dependent expression of the reporter transgene in subregions of the forebrain. pCaMKIIα-tTA mice were then crossed to a line of transgenic mice with a tet-O-linked transgene encoding a mutated, Ca2+-independent form of CaMKIIα. Inducible expression of mutant CaMKIIα resulted in a loss of hippocampal LTP and a deficit in spatial memory. Suppression of transgene expression by administration of doxycycline to animals reversed both the physiological and behavioural phenotypes. Expression of the tTA transgene at high levels in the lateral amygdala and the striatum but not in other forebrain structures led to a deficit in fear conditioning that was also reversible (Mayford et al., 1996b).

pCaMKIIα was subsequently used by Tsien et al. (1996a) to achieve subregion- and cell type-restricted gene knockout of the NMDAR1 gene specifically in CA1 pyramidal
LbIIJ
UI
LI16
Ill~~UbUlII~U3.
LAplC331UII
VI
Lllb~IlCl~C,
II-Ub1IVC.U
WU3

placed
under
the control of
pCaMKIIα, restricting expression to various regions of the
forebrain in transgenic mice. Patterns of Cre recombinase expression were determined by
crossing pCaMKIIα-Cre mice to a reporter line carrying a lacZ transgene disrupted by a
translational stop signal flanked by loxP sites. One mouse line in particular showed
expression restricted exclusively to the CA1 pyramidal cells of the hippocampus. This line
was then crossed to a line of mice carrying a targeted NMDAR1 gene with two loxP
sequences flanking a region encoding the four transmembrane domains and the entire C-
terminus of the gene. Because of the temporally and spatially restricted
expression driven by pCaMKIIα, postnatal deletion of the NMDAR1 gene was achieved
in mice carrying both pCaMKIIα-Cre and the "floxed" NMDAR1 gene. In contrast to the
NMDAR1 knockout mice produced by conventional gene targeting, these mice grew to
adulthood without obvious abnormalities. Adult mice were shown to lack NMDA
receptor-mediated synaptic currents and LTP in CA1 synapses and exhibited impaired
spatial memory but unimpaired nonspatial learning. The results of Tsien et al. (1996b)
thus strongly suggest that NMDA receptor-mediated modifications of CA1 synapses play
an essential role in spatial learning and memory in mice.

Combination of the cell-type restricted knockout technology with an inducible system
such as the tetracycline transactivator system would allow temporal control of Cre
recombinase expression, and could be used to develop "inducible knockout" mice. Use of
this genetic technology to study particular candidate genes involved in learning and
memory should ultimately lead to an elucidation of the cellular and molecular signalling
pathways important for higher cognitive processes.

The tetracycline inducible system and its application in transgenic mice holds exciting
promise as a useful technique for addressing important biological and physiological
questions. The rapidity with which the system has been adopted for use in transgenic mice
and demonstration of its practical use both in vitro and in vivo attests to the advantages
offered by the system. In this thesis, the potential utility of a neural-specific tetracycline
inducible gene expression was investigated. Employing the human neurofilament light
chain gene promoter to drive tTA transactivator expression specifically in neurons of
transgenic mice did not result in any β-galactosidase reporter gene activity. This was in spite of the fact that pNFL-driven expression of tTA could be detected by RT-PCR on total RNA isolated from brain tissue. The most likely explanation for the lack of function observed is that either the pNFL-tTA or the tet-O-βgal transgenes inserted into sections of genomic DNA which partially or completely prevented their activity. Realistically, this problem could be avoided by expanding the limited number of transgenic lines available. Currently, our laboratory is creating additional transgenic lines carrying tTA or its “reverse” form, rtTA, driven by a variety of different promoters, including the CaMKIIα promoter, as well as a series of new tet-O-linked reporter lines. We have also constructed a series of tet-O-linked Cre recombinase transgenic lines, and are in the process of constructing a series of “floxed” target genes. Combination of these elements in a single transgenic line will allow the inducible knockout of the target gene. Possible strategies for achieving a reversible gene “knockout” would involve either introducing an inducible cDNA into a knockout background, or the overexpression of a mutated cDNA in a dominant-negative manner. The successful application of the tetracycline inducible system to delete candidate genes specifically in the nervous system of transgenic mice should provide an essential link between molecular genetics and conventional neuroscience.


containing multiple GRE's in CHO cells overexpressing the glucocorticoid receptor. Nucleic Acids Research 17: 4589-4604.


