Inducible and tissue-specific gene targeting to cholinergic neurons

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ABSTRACT

We have devised a strategy for cell type-specific and regulatable targeted expression of genes to cholinergic neurons. A 2336 base pair-long fragment from the rat choline acetyltransferase (ChAT) gene regulatory region, which we previously showed to direct expression to cholinergic neurons in transgenic mice, was cloned in front of the cytomegalovirus (CMV) and herpes simplex virus thymidine kinase (TK) promoters to control the expression of the rtTA chimeric transcriptional transactivator. Addition of the tetracycline analogue doxycycline transforms rtTA in a potent transactivator protein, capable of triggering high level of expression from reporter constructs containing a minimal promoter and binding sites from the tetracycline operon. Inducible expression of the luciferase reporter gene was seen in the cholinergic SN6 cell line upon doxycycline treatment. No induction above background was seen in FR3T3 fibroblasts transfected with the same constructs. The induction by doxycycline of reporter gene expression in SN6 cells was dose-dependent and saturable. Transgenic mice were generated with the ChAT-TK-rtTA construct. One of these lines showed predominant expression of rtTA mRNA in the spinal cord, one of the richest sources in cholinergic cell bodies in the nervous system. These animals will be of great value for developmental and functional studies on cholinergic neurons.

INTRODUCTION

An important population of neurons in the nervous system are the cholinergic neurons, mainly located in the ventral horn of the spinal cord, cranial nuclei, and in the basal forebrain. Cholinergic cell bodies are also found in cortex, striatum and the olfactory bulb. The cholinergic systems are involved in motor and cognitive functions and are affected in Alzheimer's disease. Choline acetyltransferase (ChAT) is the enzyme responsible for the synthesis of acetylcholine, and, together with the vesicular acetylcholine transporter (VAChT), is the best available marker for cholinergic neurons (Arvidsson et al., 1997). The VAChT gene has been shown to be contained within an intron of the ChAT gene, and some of its mRNAs are transcribed from the ChAT promoter (Bejanin et al., 1994; Erickson et al., 1994). The structure of the ChAT gene has been extensively studied and it has been shown to have several promoters in both rat, mice and man (Chireux et al., 1994; Hahn et al., 1992; Ibáñez and Persson, 1991; Kengaku et al., 1993; Misawa et al., 1992). We have previously isolated a 2336 base pair (bp) long region from the most upstream promoter of the rat ChAT gene that is able to confer cholinergic cell-specific expression of a reporter gene in transgenic mice (Lönnerberg et al., 1995). This transgene was also able to mimic the developmental regulation of the endogenous ChAT gene and showed similar regulation in a lesion model (Lönnerberg et al., 1995).

Gene targeting in transgenic mice is a powerful approach to overexpress or inhibit specific genes in a tissue-specific manner. A limitation of this approach, however, is that it is often difficult to separate the effects observed in the adult from abnormalities in the developmental history of the animal caused by the genetic manipulation. Several system have been developed to overcome this problem and to allow the investigator to induce gene expression by exogenous administration of small difusible molecules. One such system was developed by Bujard and colleagues (Gossen et al., 1995), and it is based in the use of a chimeric transcritptional activator which binding to specific DNA sequences is induced by tetracycline analogues. This strategy has recently been applied to target tissue-specific regulatable transgene expression in several systems (Hennighausen et al., 1995; Kistner et al., 1996; Yu et al., 1996). We have now utilized this aspproach to produce a line of trnasgenic mice that selectively expresses the chimeric tetracycline-dependent transactivator rtTA in cholinergic tissues.

MATERIALS AND METHODS

Plasmid constructions

The plasmids pUHD172-1neo, pUHD10-3 and pUHC13-6 were kindly provided by Dr. Hermann Bujard. pEH2.3TKrtTA expressing rtTA under control of the herpes simplex virus TK minimal promoter and a cholinergic-specific regulatory region was constructed by inserting an EcoRI-BamHI fragment of the rtTA gene from pUHD172-1neo into the pBLEH2.3CAT plasmid. pBLEH2.3CAT contains the 2336 bp long EcoRI-HindIII regulatory region from the rat ChAT gene (Ibáñez and Persson, 1991; Lönnerberg et al., 1995) subcloned by blunt-end ligation into the SalI site of pBLCAT$_2$ (Luckow and Schütz, 1987). In the second construct tested, pEH2.3CMVrtTA, a CMV minimal promoter was used instead of the TK promoter. This was done by inserting the 2336 bp EcoRI-HindIII regulatory region of the ChAT gene into the EcoRI site of pUHD10-3.

Cell culture and transfection

FR3T3 fibroblasts and the cholinergic SN6 cells were grown in DMEM with 10% of Fetal Calf Serum supplemented with 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (all Gibco BRL) at 37ºC in a 5% CO2 atmosphere. Cells grown to approximately 70% confluency were transfected according to the calcium phosphate procedure. Doxycycline was added to the medium immediately after transfection. Forty eight hours later, the cells were harvested in lysis buffer containing 0.1 M potassium phosphate buffer pH 7.7, 1 % Triton X-100, 15 % glycerol and 2mM dithiotreithol. Luciferase assays were performed as previously described (de Wet et al., 1987).

Transgenic mice.

A 4.7 kb fragment containing the 2336 bp EcoRI-HindIII fragment from the regulatory region of the rat ChAT gene followed by the TK minimal promoter, the rtTA gene and polyadenylation sequences was excised from pEH2.3TKrtTA, purified on an agarose gel, and injected in pronuclei of fertilized mouse eggs to generate transgenic mice as previously described (Nilsson and Lendahl, 1993) (CBA x C57BL6)F1 mice were used as embryo donors, stud males, and pseudopregnant females. C57BL6 mature females were used for breeding. Preparation of high molecular weight DNA from tail biopsies was performed as previously described (Nilsson and Lendahl, 1993).

Tissue preparation, RNA extraction and RT-PCR analysis.

All tissues were immediately frozen on dry ice after dissection and stored at -70ºC until analysis. RNA was extracted by the acid-phenol method (Chomczynski and Sacci, 1987) and polyadenylated RNA isolated with Dynabeads (Dynal). The expression of rtTA mRNA was determined by reverse transcriptase-polymerase chain reaction (RT-PCR). 1 μg of RNA was reverse transcribed for 30 min at 60°C, then amplified with 5 cycles of 94°C for 45 s, 55°C for 1 min, 60°C for 1 min, and 18 cycles of 94°C for 45 s and 60°C for 1 min (AmpliTaq EZ rTth RNA PCR kit, Perkin-Elmer). The primers used were 5'-GCCGCCATTATTACGACAAGC-3' and 5'-GTCCTCGCCGTCTAAGTGGAG-3'. The products were run on an agarose gel, and photographed on a UV light table.

RESULTS AND DISCUSSION

We have previously characterized a fragment from the rat choline acetyltransferase gene regulatory region that directs tissue-specific expression of a reporter gene in cell

lines and transgenic mice (Ibáñez and Persson, 1991; Lönnerberg et al., 1995; Lönnerberg et al., 1996). With the aim of obtaining reversible and regulatable expression of genes targeted to cholinergic cells, we have combined the specificity of the enhancer region of the ChAT gene with the tetracycline inducible promoter system (Gossen et al., 1995).
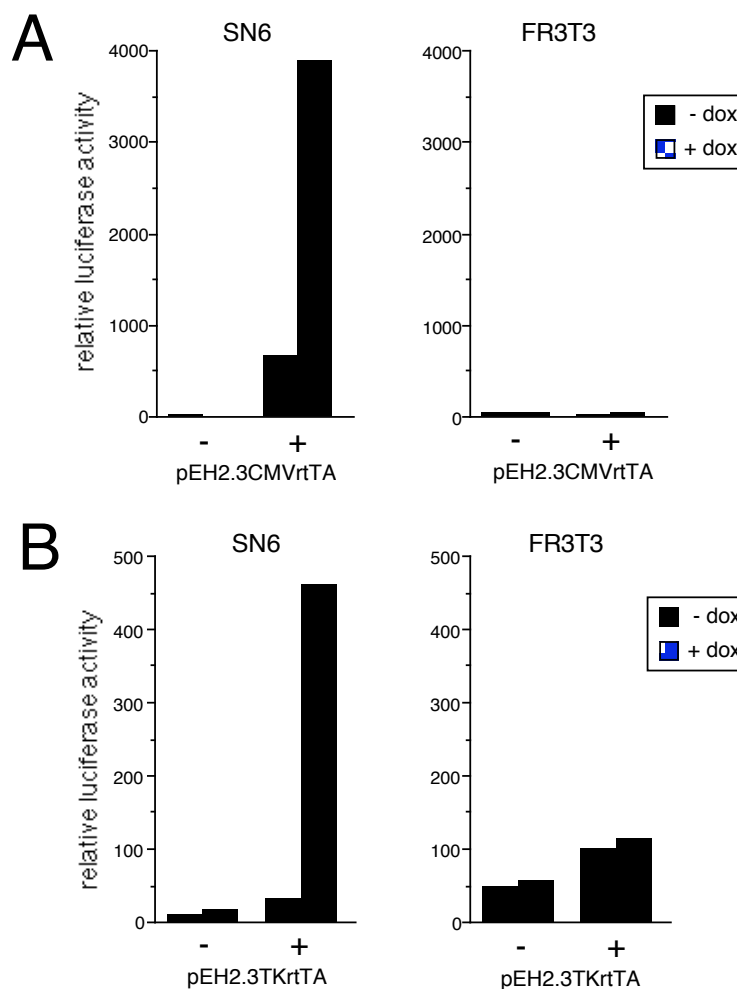
Figure 1. **Induction of luciferase activity by doxycycline in transfected cholinergic cells.** Cholinergic SN6 cells and FR3T3 fibroblasts were transiently transfected with pUHC13-6 alone (-) and together (+) with (A) pEH2.3CMVrtTA or (B) pEH2.3TKrtTA. 2.5 μg of each plasmid were used for a 35 mm well. Doxycycline was added to indicated wells immediately after transfection at a concentration of 1 μg/ml. Cells were harvested after 48 hours incubation followed by assay of luciferase activity.

Cholinergic cell-type specific activation of a reporter gene by doxyclin.

Two different promoters were tested for their efficiency at directing rtTA expression. Plasmids expressing rtTA under control of the 2336 bp cholinergic-specific regulatory region followed by the CMV (plasmid pEH2.3CMVrtTA) or TK (plasmid pEH2.3TKrtTA) promoters were transiently co-transfected together with a plasmid expressing luciferase under control of heptamer tetop rtTA binding sites and the TK

minimal promoter (plasmid pUHC13-6) into the cholinergic neuroblastoma cell line SN6 or into the rat fibroblast line FR3T3. For each plasmid pair, 1 μg/ml of doxycycline was added to one of two duplicate wells immediately after transfection. Forty eight hours later, the cells were collected and processed for luciferase assays. Doxycycline induced 7- and 13-fold activation of the reporter gene in cholinergic SN6 cells that carried the pEH2.3CMVrtTA and pEH2.3TKrtTA constructs, respectively. Neither promoter showed any induction in non-cholinergic FR3T3 fibroblast cells (Fig. 1). The CMV promoter exhibited higher absolute expression levels than the TK promoter when induced by doxycycline in SN6 cells, but displayed a significant background luciferase activity in the absence of doxycycline. No measurable background was detected with the pEH2.3TKrtTA construct (Fig. 1). Thus, cholinergic-specific inducibility was higher with the TK promoter construct, even though this promoter had weaker overall activity than CMV.
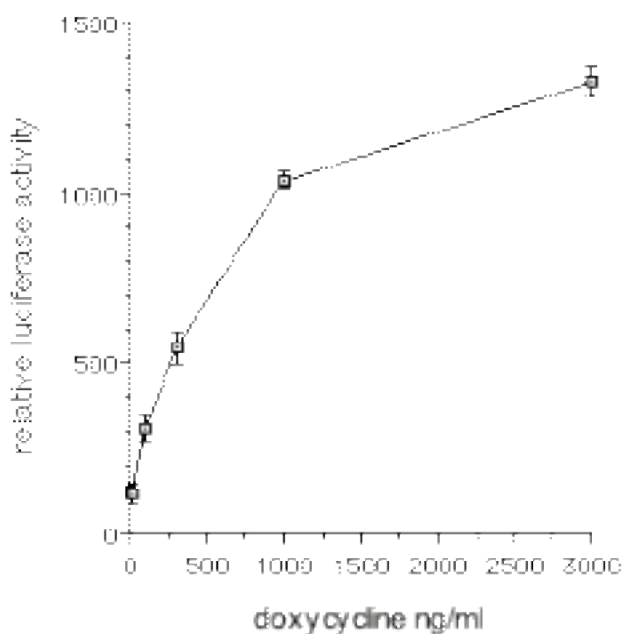

Figure 2. **Dose-dependent induction of luciferase activity by doxycycline in transfected SN6 cells.** SN6 cells were transfected with 2.5 μg each of pUHC13-6 and pEH2.3TKrtTA. Immediately after transfection the indicated concentrations of doxycycline were added to the medium. Cells were grown for 48 hours, harvested, and processed for luciferase assays. Values shown represent mean +/- standard error (n=3).

Dose-dependent induction of the luciferase reporter gene

SN6 cells were transiently co-transfected with equal amounts of pEH2.3TKrtTA and pUHC13-6, and grown in the presence of various concentrations of doxycycline. Cell extracts were made after 48 hours, and luciferase enzyme activity was measured. A dose-dependent induction of the reporter gene was observed; maximal effects were seen at a doxycycline concentration of 3000 ng/ml (Fig. 2).

Targeted expression of rtTA in cholinergic cells of transgenic mice

Having demonstrated the cholinergic-specificity and tetracycline-inducibility of pEH2.3TKrtTA and pEH2.3CMVrtTA constructs *in vitro*, we set out to generate lines of transgenic mice with targeted expression of rtTA in cholinergic cells. Siince our goal was to develop a system for reversible and regulatable gene targeting with minimal developmental effects, we chose the construct based on the TK promoter as this is the one that showed higher inducibility with neglible background activity, despite its overall lower activity compared to the CMV construct. The pEH2.3TKrtTA construct has the rtTA coding region replacing the chloramphenicol acetyltransferase reporter gene used in our earlier study of cholinergic-specific gene targeting in transgenic mice (Lönnerberg et al., 1995), and it was therefore expected to behave in a similar fashion.
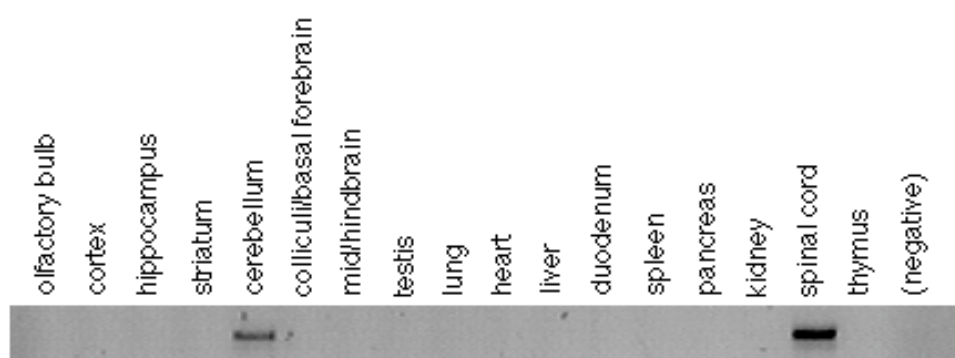

Figure 3. **Targeted expression of rtTA in cholinergic cells of transgenic mice.** Total RNA was extracted from various tissues of an adult male of transgenic line 4, and RT-PCR was performed on 1 μg of total RNA for 31 cycles. The amplified products were run on an agarose gel, stained with ethidium bromide and photographed on a UV light table. No additional bands were detected after further 8 cycles of amplification.

Several lines of transgenic mice were obtained carrying the EH2.3TKrtTA transgene. Eight adult males each from a different line were sacrificed and various tissues dissected for RNA extraction. Expression of rtTA mRNA was investigated by analyziing alliquots of a RT-PCR reaction taken at different times of amplification. rtTA mRNA expression was detected in the spinal cord, which is rich in cholinergic motorneuron cell bodies, in most of the lines (data not shown). One animal (transgenic line 4), expressing the highest level of rtTA mRNA in the spinal cord, was selected for further analysis. Fig. 3 shows the result of the survey of several tissues from this animal after 31 cycles of amplification and demonstrates the predominant expression of rtTA mRNA in the spinal cord. Similar to several transgenic lines generated in our previous study (Lönnerberg et al., 1995), line 4 also showed transgene expression in the cerebellum, despite no cholinergic cell bodies have been reported in this region. Further studies will be required to establish the cellular origin of rtTA expression in this line using reporter genes suitable to histological analysis such as the *E. coli* gene product beta-galactosidase.

In conclusion, we have documented *in vitro* in cell culture experiments a system for regulatable cholinergic cell-specific expression of a reporter gene. We have furthermore established several transgenic mice lines, one of which shows predominant expression of the rtTA transactivator in the spinal cord, which is the richest source of cholinergic cell bodies in the nervous system. These mice will be useful tools for developmental and functional studies on cholinergic neurons. For example, targeted and regulatable expression of the CRE recombinase to cholinergic neurons would allow for specific gene-deletion in cholinergic cells. The function of several important genes, such as neurotrophic factor receptors TrkA, TrkB, TrkC and c-Ret, may thus be studied in adult cholinergic neurons. Targeted, dose-dependent and reversible overexpression of neurotrophic factors in cholinergic neurons will also be possible using these mice. This should allow the investigation of acute effects of neurotrophic factors in brain repair and learning without the complication of developmental effects. Finally, this apporach may also be useful to reversible and dose-dependent targeting to the cholinergic system of specific toxins. This may prove a novel and elegant approach to generate animal models of cholinergic degeneration, which could be very useful to study the role of the cholinergic system in cognition and behaviour, as well as to model neurodegenerative diseases of the cholinergic system, such as amyotrophic lateral sclerosis (ALS) and Alzheimer's disease.

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