Lentiviral Vectors: Regulated Gene Expression

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Received for publication May 1, 2000, and accepted May 9, 2000

Lentiviral vectors can deliver and express genes in a wide variety of dividing and nondividing cells. These include terminally differentiated neurons, myotubes, hepatocytes, and hematopoietic stem cells. We now describe the generation of lentiviral vectors in which the expression of the transgene can be regulated. We have developed an inducible lentiviral vector system that contains the entire tetracycline (Tet)-regulated system developed by H. Bujard and colleagues. The novel vector expresses the GFP reporter gene and the tetracycline transactivator under the control of the tetracycline-inducible promoter and the human CMV promoter, respectively. In vitro transduction of human 293 cells resulted in a very low basal expression of GFP in the presence of the effector substance doxycycline. Withdrawal of doxycycline induced a more than 500-fold increase in transgene expression. Switching transgene expression "off and on" did not change either the kinetics or the magnitude of induction. Maximal suppression of GFP mRNA transcription was achieved within 24 h of addition of the drug; however, due to the slow turnover rate of GFP, green fluorescent cells could be detected up to 10 days following doxycycline treatment. Following transduction of rat brain with recombinant lentiviruses, doxycycline-regulated GFP expression could be observed in terminally differentiated neurons. Specifically, by adding or withdrawing doxycycline from the rats’ drinking water, induction and suppression of GFP expression could be regulated in vivo. These studies show that an inducible lentiviral vector can deliver and regulate transgene expression in vivo. We believe that regulated gene expression is an essential tool for successful gene therapy approaches.

INTRODUCTION

Lentiviral vectors can package and deliver large cDNAs to a variety of dividing and nondividing target cells. These vectors have proven to be effective in transducing brain, liver, muscle, and retina in vivo without toxicity or immune responses (1–4). The ability of the lentiviral vectors to efficiently transduce hematopoietic stem cells and maintain their repopulating ability further extends their utility (5). A significant improvement in the lentivirus-based vector system is the generation of an efficient packaging cell line that facilitates vector production and increases its safety (6). The safety of the lentiviral vectors has been further improved with the generation of self-inactivating vectors and a minimal packaging system (7, 8). These improvements, taken together with their ability to transduce a wide spectrum of tissues in vivo, argue strongly in favor of further development of lentiviral vectors for human gene therapy.

An important feature of all vectors for human gene therapy should be the ability to regulate the transgene expression in a clinical setting. Among the different regulatable transgene expression systems that have been developed (9–14), the tetracycline-based system has been best characterized (12, 15, 16). This system has been successfully employed to regulate transgene expression in vivo by incorporating it either into different viral vectors (17–19) or in the context of transgenic mice (20). Furthermore, recent developments in the “Tet system” allow selective induction and repression of different transgenes from a single inducible cassette (16, 21), which reduces the leakiness of the system and improves the ability to control transgene expression. Based on the advantages of the Tet system, we chose to incorporate it into the lentiviral vector system and investigate its ability to deliver and regulate transgene expression in vitro and in vivo.

We report that the entire Tet system can be incorporated into a single lentiviral vector. This novel, inducible lentiviral vector can be produced in high titers and when tested either on tissue culture cells or by direct injection into immunocompetent rat brain, exhibits doxycycline-regulated gene expression. We believe that such
inducible vectors will be beneficial not only for gene therapy applications, but also for basic neuroscience research.

**Materials and Methods**

**Plasmid construction.** The inducible vector pCL-CTIG (Fig. 1) was constructed by cloning an XbaI/Hpal fragment containing the tTA and the inducible promoter from pBStind into NheI/Eco473I sites of pCL-CG. To generate the pBSind plasmid we ligated a RamHl/NotI fragment containing the tTA gene into the RamHl/NotI site of pBSind (Bluescript-based plasmid containing the inducible promoter).

**Vector production.** The inducible pCL-CTIG lentiviral vector was generated by a transient three-plasmid transfection as previously described. Briefly, 293T cells were transfected with 7.5 µg of the pMDG envelope plasmid, 15 µg of the ΔRnr packaging plasmid (1), and 15 µg of the pCL-CTIG lentiviral vector plasmid. Vector particles were collected from conditioned medium 60 h posttransfection. The vector was further concentrated by two rounds of ultracentrifugation as previously described (3). Vector titers were determined by serial dilution on 293T cells. Absence of replication-competent particles was determined by three independent methods: marker transfer, vector rescue, and p24 ELISAs as described (1).

**FACS analysis.** Trypsinized cells were resuspended in 5 ml culture medium, washed in PBS, and fixed in 3 ml of 5% paraformaldehyde. The cells were washed twice in PBS and diluted in PBS to a concentration of 10^6 cells/ml. Fluorescence-activated cell sorter (FACS) analysis for cellular GFP was performed by FACSscan analysis (Becton-Dickinson) with the CellQuest program (version 3.0.1; Becton–Dickinson).

**RNA analysis.** Total RNA was extracted using the Qiagen Rneasy kit. Ten micrograms of total RNA was electrophoresed on 1.2% agarose-formaldehyde gel, transferred to Hybond-N+ (Amersham) membrane, and hybridized at 68°C to a 32P-labeled DNA probe. The membrane was washed twice for 15 min in a buffer containing 0.1% SDS and 2× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate) at 68°C and once in buffer containing 0.1 M SDS and 0.1 M SSC at 68°C for 15 min. The washed membranes were exposed to Kodak X-Omat Blue XB-1 film for 7 days.

**Regulation of transgene expression in brain of adult immunocompetent rats.** Adult female Fisher 344 rats (n = 12) were anesthetized (80 mg ketamine/ml with 5% sucrose in drinking water) was initiated in six animals. Two and a half weeks after initiation of treatment we sacrificed three of the treated rats and three of the doxycycline-free rats. In addition, doxycycline treatment was switched: We withdrew doxycycline from the previously treated animals and initiated doxycycline treatment in the previously untreated rats. After 2 1/2 weeks, the rats were anesthetized and perfused intracardially with 100 ml of saline followed by 200 ml of 4% paraformaldehyde. Brains were postfixed for 24 h and stored in 30% phosphate-buffered sucrose at 4°C until sectioning. Brains were frozen and sectioned on a sliding microtome into 40-µm slices. Sections were washed and blocked in TBS with 3% donkey serum and 0.3% Triton X-100 (TBS-plus). Primary antibodies raised in two different species were pooled in TBS-plus and incubated for 48 h at 4°C. The antibody for Neu-N (mouse monoclonal antibody (a generous gift of R. J. Mullin), 1:250) was combined with anti-body for GFAP (gliarial fibrillary acidic protein) [guinea pig polyclonal antibody (Advanced Immunochemicals), 1:500]. Corresponding secondary antibodies (donkey anti-mouse Texas red and donkey anti-guinea pig Cy5, 1:250) were pooled, and sections were incubated for 4 h at room temperature following washing in TBS-plus. Every 12th section was mounted and coverslipped with DABCO-PVA. Sections were analyzed by confocal scanning laser microscopy (Bio-Rad).

**Results**

**Construction of the Inducible Lentiviral Vector**

We based the construction of the inducible lentiviral vector on the previously described CL-CG (7). The CL-CG is a first-generation HIV-1-based vector (containing intact 3′ LTR) from which the GFP reporter gene is constitutively expressed under the control of the CMV promoter. To convert this vector into an inducible vector, we inserted an inducible cassette between the constitutively active CMV promoter and the GFP reporter gene (Fig. 1). The inducible cassette contains the tetracycline-dependent transactivator (tTA) at its 5′ end and an inducible promoter at its 3′ end. As a result, expression of the GFP gene is controlled by the inducible promoter, which contains a minimal promoter (mp) and seven copies of Tet operon (tetO). The tTA gene, which encodes a fusion protein of the tet repressor (tetR) and the herpes simplex virus transactivation domain (VP16), is constitutively expressed under the control of the CMV promoter. Following transduction, the constitutively expressed tTA (tetRVP16) binds to tetO-mp and induces the transcription of a high level of GFP mRNA. In contrast, binding of doxycycline to the tTA induces conformational changes that prevent it from binding to the tet(O)-minimal promoter and consequently leads to little or no GFP expression.

**Regulation of GFP Expression in Vitro**

To investigate the ability of the “Tet system” containing lentiviral vector to transfer and express the GFP reporter gene in a regulatable fashion, we generated high-titer vector stocks by transient three-plasmid transfection followed by ultracentrifugation, as described previously (3). Recombinant lentivirus titers (−10^9 IU/ml) and P24/gag/IU ratios were comparable to titers and ratios obtained with noninducible vectors. Human embryonic kidney (HEK) 293 cells were transduced with a m.o.i. of 0.5 and, following 2 weeks in culture in either the pres-
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Control non-transduced cell.

Days

0

1

3

5

8

10

12

Repression + Dox

Induction - Dox
ence or absence of doxycycline, the cells were evaluated for GFP expression by fluorescence-activated cell sorter (FACS) analysis. Although the expression of the GFP was not completely repressed by the presence of doxycycline, it was 500- to 1000-fold lower than its expression in the absence of doxycycline, as demonstrated in Fig. 2 (compare c and d). Encouraged by this result, we characterized the kinetics of the inducible system in response to doxycycline, which was either added to or withdrawn from the culture medium. Transduced cells were collected every 2 days and analyzed for cellular GFP by FACS (Fig. 2). Additionally, mRNA levels were measured by Northern blot analysis (Fig. 3). Induction of GFP mRNA is observed 3 days post doxycycline withdrawal and reaches its peak by day 8. Further increase in the intensity of GFP in the induced cells is likely due to intracellular accumulation of the protein.

It is clear from these results that the induction of GFP mRNA synthesis correlates with the increase in the intracellular levels of the GFP as demonstrated by Northern and FACS analysis, respectively (Figs. 3 and 2). In contrast, we found a discrepancy between the early repression of GFP mRNA, which was accomplished within 1 day of doxycycline addition (Fig. 3), and the slower decline in cellular GFP levels which continued for more than 10 days (Fig. 2). We attribute this lack of correlation between the kinetics of the FACS and RNA analysis to the long half-life of the GFP (over 120 h). The processes of repression and activation were found to be completely reversible. They were repeated several times in a 3-month period without any significant differences in the kinetics or magnitude of induction (data not shown).

**Regulation of Transgene Expression in Vivo**

Based on the *in vitro* results we investigated the ability of the inducible lentiviral vector to deliver and regulate gene expression *in vivo*. For this purpose, we injected 2 µl of the concentrated vector (10^9 IU/ml) into the striatum of 12 adult Fisher rats. Ten days postinjection we sacrificed groups A and B, and the brains were sectioned and analyzed by confocal microscopy for GFP expression. As seen in Fig. 4, we could detect the injection tract in all the brains, although there were very few positive cells in the brains of the doxycycline-treated ani-

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**FIG. 2.** FACS analysis of GFP levels. HEK 293 cells transduced with the inducible lentiviral vector were cultured for 2 weeks in either the presence or the absence of doxycycline. Induction or repression of GFP expression was initiated by withdrawal or addition of doxycycline to culture medium, respectively. GFP levels were analyzed by FACS analysis on days 1, 3, 5, 8, 10, and 12. Control HEK293 cells were not transduced by the vector. c (0, days in repression) indicates maximal GFP before addition of DOX, whereas d indicates least GFP expression before removal of DOX.

**FIG. 3.** Northern blot analysis of GFP mRNA. HEK 293 cells were treated as in Fig. 2 and total RNA extracted from each cell sample. Ethidium bromide-stained RNA samples were electrophoresed and transferred to positively charged membrane. Efficiency of transfer was determined by UV fluorescence (lower panels). The membranes were hybridized to 32P-labeled GFP probe and visualized by autoradiography. The expected migration of GFP mRNA is indicated with an arrow. The positions of 28 and 18S ribosomal RNAs are also marked.
mals (group B). Furthermore, most of the positive cells were only faintly green. In contrast to the doxycycline-treated animals, all nontreated animals demonstrated very high levels of GFP (group A). Positive cells could be detected as far as 2–3 mm from the injection site. Immunostaining and cell morphology indicated that most of the transduced cells were neurons.

To confirm the reversibility of the GFP induction we switched the doxycycline treatment in the remaining two groups. We withdrew doxycycline from the drinking water of group C and initiated doxycycline treatment with group D. Two and a half weeks after switching doxycycline treatment the animals were sacrificed and the brains analyzed for GFP expression. The results of these experiments demonstrated that induction and repression of the Tet system containing lentiviral vector are reversible. The number of positive cells and the intensity of green fluorescence in the brain of group C (doxycycline treated during the first 2.5 weeks) were equal to those observed in the brains of group A, which were never exposed to doxycycline. Very few positive cells could be detected in the brains of the doxycycline-treated rats (group D), which were originally not treated with doxycycline. These results indicate that we can efficiently and reproducibly modulate the activation of the transgene in vivo. Furthermore, we could not detect cell death, local inflammation, or other pathology that could indicate the toxicity of the Tet system to the target cells.

**DISCUSSION**

We describe here the first inducible lentiviral vector that is based on the first-generation lentiviral vectors (7). The novel vector contains the entire “Tet-inducible system” and can be produced in titers equivalent to those obtained with first-generation lentiviral vectors (>10^9 IU/ml). Our study demonstrates the ability of this vector to deliver the entire Tet system and to efficiently regulate expression of a reporter transgene in vitro and in vivo.
The fact that the size of the minimal Tet-inducible cassette (containing the Tet-inducible promoter, a constitutive promoter, and the tTA transactivator) is no more than 2 kb allows the lentiviral vectors to package relatively large DNA sequences and is thus a more versatile vector. Unlike the AAV-based vector system (22), the inducible lentivector system can stably transfer relatively large cDNAs without the need to separate the Tet system into two separate vectors.

Induction of the inducible cassette by doxycycline withdrawal resulted in a more than 500-fold increase in cellular GFP levels, although a basal level of GFP production was still detectable. This basal GFP expression can be attributed to the leakiness of the inducible promoter, which may have been further enhanced by either the neighboring CMV promoter or the HIV LTRs. We believe that an inducible self-inactivating HIV vector that lacks the U3 HIV enhancer will show lower basal activity. Incorporating an inducible repressor into the lentiviral vector (21) may increase the complexity of the vector on one hand but may significantly reduce the leakiness of the inducible promoter on the other hand. This approach is currently attractive in the context of lentiviral vectors, as these vectors have the capacity to accommodate an additional repressor gene.

Although the cellular tTA level was sufficient to induce a more than 500-fold increase in GFP levels, Northern blot analysis revealed minimal amounts of ITA-specific mRNA. It is not clear whether this is a result of in vitro selection against cells producing high levels of the tTA mRNA or whether it is the vector structure that allows minimal transcription from the ITA gene. The fact that the number of positive neurons in the rat brain did not decrease with time combined with the lack of inflammation at the injection site does not support the possibility of tTA toxicity explanation; however, more in vivo experiments using different target organs are required to rule this out.

In summary, this newly developed lentiviral vector can transfer and maintain long-term regulatable gene expression in adult rat brains without toxicity. We believe that these vectors will be very useful in basic biomedical research that will require controlled expression of growth factors, neuropeptide, and other signaling molecules. Clearly, the future of the inducible vectors will depend on possible side effects of the inducible elements, such as toxicity, immunogenicity, etc. It is our belief that use of regulatable vectors in human gene therapy will be highly desirable.

ACKNOWLEDGMENTS

We thank members of the Verma and Gage laboratories for their support. We are grateful for the continued support of the Christopher Reeve Paralysis Foundation, the Lookout Fund, the Parkinson’s Disease Foundation, and the NINDS (NS28121) and NIA (AG10435). Dr. Kafri was supported by the Cystic Fibrosis Foundation. Dr. Verma is an American Cancer Society Professor of Molecular Biology. His work is supported by the March of Dimes, the Wynn and Gladys Valley Foundation, and the H. N. and Frances C. Berger Foundation. This work was also supported by the National Institutes of Health. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

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