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Doxycyline regulation in a single retroviral vector by an autoregulatory loop facilitates controlled gene expression in liver cells

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ABSTRACT

The tetracycline system has limitations in liver cells, such as toxic effects and low controllability. We generated different retroviral vectors for controlled gene expression in liver cells, in which the regulatory elements were arranged in different patterns. Only the organization of the tetracycline system in an autoregulatory loop in the sense orientation results in high retroviral titres and in tight regulation of gene expression in highly differentiated hepatoma cells. Because of the toxicity of the transactivator tTA, it was impossible to establish doxycycline-dependent stable HepG2 cell lines. To avoid sequelching-related toxicity in liver cells, we replaced tTA with new non-toxic transactivators. By using tTA2, tTA3 and tTA4, we observed tight doxycycline-dependent gene expression in 23, 49 and 45% of the isolated clones. The tTA4 vector was used to transduce hepatocytes of mice *in vivo*. Tight doxycycline-controllable gene regulation was also observed in the liver of mice, confirming our hypothesis that retroviral vectors with autoregulatory loops of the tetracycline system facilitate inducible gene expression in the liver *in vivo*. Our new retroviral vector system allows rapid isolation of controllable clones in a very high yield and should make the tetracycline system more applicable to liver-derived cells and in liver gene therapy *in vivo*.

INTRODUCTION

Controlled gene expression systems serve as fundamental tools for investigating gene function in cell lines and tissues. Fine tuning of heterologous gene expression reflects more the real *in vivo* situation and therefore gives more reliable data about the function of gene products than the introduction of uncontrolled numbers of plasmids with uncontrolled gene expression. However, not only for experimental investigations in molecular or cellular biology, but also for many gene therapy approaches, regulation of gene expression through exogenous stimuli is essential. Therefore, the development of non-toxic systems for the specific and tight temporal and quantitative control of exogenous gene expression in mammalian cells is an important challenge in molecular biology.

Inducible gene expression systems have typically encountered limitations, such as basal leakiness, toxic or non-specific effects of inducing agents, limited cell type applicability and low levels of expression. One of the most promising exogenous gene regulation systems is the tetracycline-controlled gene expression system (tet system) (1,2). In one component of this system, the gene of interest is placed downstream of a minimal cytomegalovirus (CMV) promoter linked to seven tandem copies of the tetR-binding site ($TetO_7$). The second component, the regulatory unit, encodes a hybrid tTA protein composed of the tetracycline repressor (tetR) fused to the herpes simplex virus (HSV) transactivation domain VP16. In the presence of tetracycline or tetracyline derivates, a conformational change in the tet repressor prevents tTA from binding to its operator (1). Also, a modified system has been described in which a reverse transactivator (rtTA) was developed that binds tetO only in the presence of tetracycline derivates, such as doxycycline (2). Both systems have been used in different cell lines and transgenic animals for the expression of a variety of different genes (3,4).

To regulate the expression of a heterologous gene by the tet system, the two described components have to be introduced into the cell of interest. Since stable plasmid transfection is ineffective, a major advance in broadening the utility of the tet system was the use of retroviruses. However, obtaining well regulated cell clones by using two different retroviruses is sometimes cumbersome, labour intensive and often low in yield of inducible clones. To overcome these problems, several attempts have been made to develop modified versions of the tet system within one retroviral vector (5–8). However, these 'all-in-one' systems also have some limitations such as the lack of selection genes, low retroviral titres or ineffective control of gene expression.

Some of the problems with the tet system may be related to the toxicity of the tTA or rtTA transactivator. It has been demonstrated that over-expression of the VP16 domain will result in squelching, as a consequence of titrating components

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of the transcriptional machinery from their intracellular pools. It has been shown that the efficacy of tetracycline-controlled gene expression is influenced by cell type, and in some cells stable expression of tTA results in morphological changes, growth rate attenuation and alterations in cell cycle distribution (9). To avoid sequelching-related toxicity, a panel of novel tetracycline-controlled transactivators has been developed which contain VP16-derived minimal activation domains (10,11). The new tet-dependent transactivators tTA2, tTA3 and tTA4 possess a graded transactivation potential spanning more than three orders of magnitude and are tolerated in cells at higher concentrations compared with the original tTA (10).

We have developed several versions of the tet system for use within one retroviral vector. Our results demonstrate that only the organization of the tet components in a sense-orientated autoregulatory unit results in high retroviral titres and in tight regulation of gene expression in almost all transduced cells. The use of modified non-toxic tetracycline transactivators within this autoregulatory loop allows rapid isolation of controllable clones in high yields and improves the applicability to a variety of cultured hepatoma cells and in gene therapy of liver diseases.

MATERIALS AND METHODS

Construction of retroviral plasmids

For the vector backbone pRetro-ON (Clontech), a Moloney murine leukaemia virus-based retroviral vector bearing an extended packaging signal and all the elements of the tet system was used. In order that the puromycin resistance gene could be exchanged in further cloning steps, a ClaI site was introduced at position 1370 of pRetro-ON by site-directed mutagenesis with the primers sense: 5′-CGG CCG GAT AGC TTA CCA TCG ATG AGT ACA AGC CCA CGG TCG-3′ and antisense 5′-GCA CCG TGG GCT TGT ACT CAT CGA TGG TAA GCT ATC CGG CCG-3′. Site-directed mutagenesis was performed using the Quick Change kit (Stratagene) according to the manufacturer's instructions. The resulting plasmid of the retroviral vector was designated p617-SV40 rtTA. To evaluate doxycycline-regulated gene expression in p617-SV40rtTA, an enhanced green fluorescent protein (EGFP)-coding fragment was inserted between the BamHI sites downstream of the TO promoter.

To facilitate bicistronic expression of the selection gene and the tet transactivator in the p617-I-tTA-EGFP vector, the SV40 promoter rtTA element was replaced by an IRES-tTA, excised from the retroviral vector pBabe-SIN CMV 15-1, which was kindly provided by H. Blau, Stanford University, CA.

To obtain both retroviral vectors p616-T-EGFP-I-tTA and p617-T-EGFP-I-tTA_{AS}, an autoregulatory cassette containing TO-EGFP-IRES-tTA was inserted between BamHI and XhoI in the sense and antisense orientation.

Unique restriction sites of the four retroviral vectors are indicated in Figure 1. Further details of plasmid constructions are available upon request.

To obtain doxycycline-controllable retroviral vectors with different VP16-derived transactivation domains, the tTA transactivator of p617-T-EGFP-I-tTA was replaced by tTA2, tTA3 and tTA4 from ptTA2, ptTA3 and ptTA4 (Clontech). In p617-neo-T-EGFP-I-TA, p617-neo-T-EGFP-I-TA2, p617-neo-T-EGFP-I-TA3 and p617-neo-T-EGFP-I-TA4, the puromycin gene was replaced by a neomycin cDNA.

Cell culture

The human hepatoma cell line HepG2 was obtained from the American Type Culture Collection. The amphotopic retroviral packaging cell lines $Phoenix_{ampho}$ and $Phoenix_{eco}$ were a generous gift from Gary Nolan, Stanford University, CA. All the cells were maintained in growth medium [Dulbecco's modified Eagle's medium (DMEM)/Glutamax, Gibco-BRL, Gaithersburg, MD, supplemented with 10% heat-inactivated tet system-approved fetal bovine serum (Clontech), 100 U/ml of penicillin and 100 µg of streptomycin] at 37°C in 5% CO_2. The Phoenix cells were reselected every 2 months by the addition of hygromycin (200 µg/ml) and cholera toxin (2 µg/ml) for 7 days.

Transfections, infections and determination of retroviral titre and doxycycline-regulated gene expression

Amphotropic or ecotropic Phoenix cells were seeded at a density of 2×10^6 cells per 60 mm dish in 4 ml of DMEM supplemented with 10% fetal calf serum, glutamine and doxycycline in a concentration of 1 µg/ml. The following day, chloroquine was added to each plate to a final concentration of 25 µM and cells were then transfected with 10 µg of retroviral plasmids using the calcium phosphate 'bubble' precipitation method. First, a transfection cocktail was prepared in a 15 ml tube composed of 10 µg of DNA and H_2O in a final volume of 438 µl. A 62 µl aliquot of freshly prepared 2 M $CaCl_2$ was then added to the DNA/H_2O solution. Finally, 500 µl of 2× HBS (pH 7.0–7.1) were mixed with the transfection cocktail by bubbling with a syringe immediately (15 s) before the Phoenix cells were transfected by gently agitating the solution with the growth medium. Approximately 10 h after the transfection, the chloroquine/transfection cocktail-containing medium was replaced with fresh growth medium containing 1 µg/ml doxycycline. The retrovirus-containing supernatant was harvested 48 h after the transfection and was filtered through a 45 µm filter to remove living cells. The retroviral supernatant was either used immediately for the experiments or was snap-frozen in liquid nitrogen and stored at –80°C for later applications. For *in vivo* application, the retroviral supernatant was concentrated 10-fold at 4°C by ultrafiltration using the Pericor pump and UFP-100-C-MB01 filters (Schleicher and Schuell) as described by the manufacturer.

For retroviral transduction of hepatoma cells, 1.5 ml of retroviral supernatant, 1.5 ml of fresh growth medium and Polybrene (Sigma) at a final concentration of 4 µg/ml were added to 2×10^6 cells in 60 mm dishes. After 6 h culture, the procedure was repeated. At 6 h after the second transduction cycle, the transduction medium was replaced by fresh growth medium.

Viral titre was calculated by transduction of a known number of HepG2 cells with different volumes of retroviral supernatant. We plotted increasing volumes of supernatant from 5 to 1000 µl against percentages of EGFP fluorescent target cells. The titre was calculated from the volumes corresponding to the linear slope of the curve according to the reported formula: viral titre = HepG2 cell number × % of

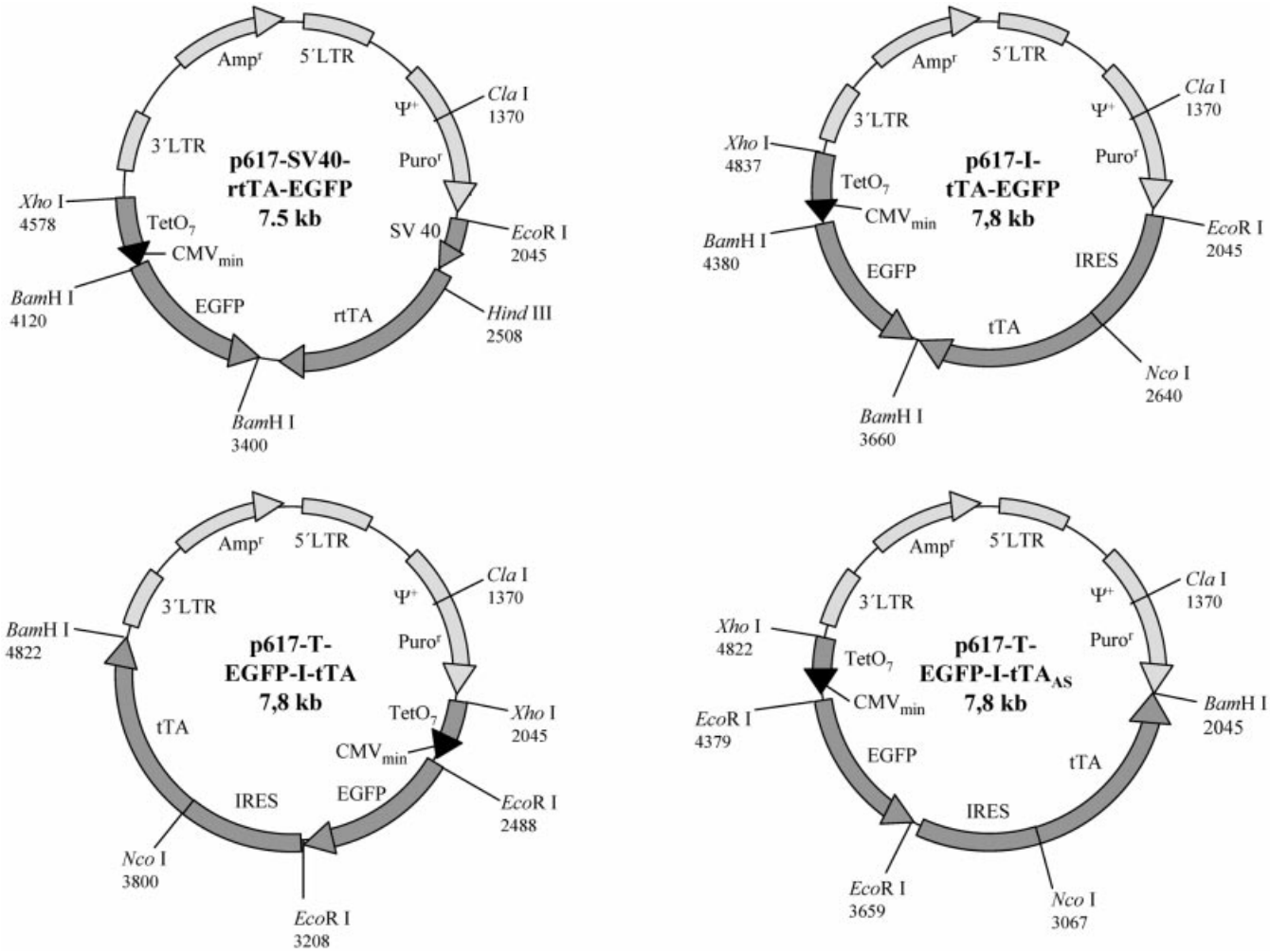

Figure 1. Schematic representation of the constructed retroviral vectors. The positions of unique restriction sites are indicated with numbers.

EGFP-positive cells/volume of supernatant (12). Stable HepG2 cell lines were generated by antibiotic selection with 2 μg/ml puromycin or 500 μg/ml neomycin after retroviral transduction.

To evaluate doxycycline-regulated EGFP expression in HepG2 cells, growth medium was removed and cells were thoroughly washed with phosphate-buffered saline (PBS). Subsequently, cells were incubated with growth medium supplemented or not with 1 μg/ml doxycycline. Three days later, EGFP fluorescence was monitored by fluorescence microscopy.

Adenovirus preparation

The adenoviral vector Ad5-CMVLacZ was kindly provided by Dr D. Brenner, Chapel Hill, NC. To generate high titre viral stocks, 2×10^8 293 packaging cells at 90% confluency were infected at a multiplicity of infection (m.o.i.) of 5–10 p.f.u. per cell. The infected cells were grown for 3–5 days until a strong cytopathic effect could be observed and ~50% cells were detached. The cells were then collected by centrifugation and viral particles were released by four cycles of freezing in liquid nitrogen and rapid thawing at 37°C. For further purification, the virus preparation was subjected to a 2-fold CsCl banding. CsCl banding and determination of infectivity by viral plaquing were performed according to standard protocols.

Virus preparations were stored at –20°C in 25% glycerol, 10 mM Tris–HCl, pH 7.4, and 1 mM $MgCl_2$.

In vivo transduction of hepatocytes and monitoring of doxycycline-regulated gene expression in the liver

Pathogen-free male NMRI-nu/nu mice (aged 6–8 weeks) were obtained from the Animal Research Institute of the Medizinische Hochschule Hannover. All experiments were performed in agreement with German legal requirements.

It has been shown that adenovirus-mediated urokinase gene transfer induces liver regeneration and allows efficient retroviral transduction of >30% of the hepatocytes *in vivo* (13,14). Consequently, we used liver injury by high titre adenovirus to trigger liver regeneration and to facilitate efficient retroviral transduction of hepatocytes *in vivo*. Ad5-CMVLacZ were prepared, purified and titred as described above. Prior to infection, the virus was dialysed twice against a solution containing 10 mM Tris–HCl, pH 8.0, 1 mM $MgCl_2$, 140 mM NaCl at 4°C. Infection of the mice was carried out by administration of 0.25 ml of virus solution into the tail vein at

a total virus load of 1×10^8 p.f.u./g body weight, calculated for ~80% transduction of liver hepatocytes. At 48 h after adenoviral liver injury, 2 ml of concentrated ecotopic retroviral vectors with 4 µg/ml Polybrene (Sigma) were administered by hydrodynamic tail vein injection within 4–10 s.

At 24 h after retroviral transduction, the mice in the Dox+ group received doxycycline by intraperitoneal injection at a dose of 0.5 mg/kg body weight and in the drinking water at a final concentration of 200 µg/ml in 5% (w/v) sucrose (pH 6.0). Three days after retroviral transduction, EGFP expression in Dox– and Dox+ mice was visualized by fluorescence microscopy of the native liver specimen.

RESULTS

Orientation of the tet elements in an autoregulatory loop facilitates tight control of gene expression in a single retroviral vector

We subcloned four different moloney murine leukaemia viral vectors, in which the regulatory elements of the tet system were arranged in different patterns (Fig. 1). The gene of interest in all vectors was EGFP, which could be monitored easily by fluorescence microscopy. In the first retrovirus (p617-SV40-rtTA-EGFP), the expression of the reverse transactivator (rtTA) was controlled by the SV40 promoter, whereas in the second retrovirus (p617-I-tTA-EGFP) the chimeric transactivator (tTA) was translated from a bicistronic RNA, which was transcribed under the control of the 5′-long terminal repeat (LTR). For the third and fourth retroviral vector, we subcloned an autoregulatory cassette, in which tTA was translated from a bicistronic RNA, which was transcribed from the TO promoter and the 5′ LTR (p617-T-EGFP-I-tTA) or from the 3′ LTR (p617-T-EGFP-I-tTA_{AS}). Amphotropic virus supernatants from transient producer Phoenix (293T) cells were used to transduce the highly differentiated hepatoma cell line HepG2, and viral titres were assessed. The viral titres varied from 2×10^7 to 5×10^7 for p617-SV40-rtTA-EGFP, from 3×10^6 to 6×10^6 for p617-I-tTA-EGFP, from 2×10^4 to 4×10^4 for p617-T-EGFP-I-tTA_{AS}, and from 9×10^5 to 2×10^6 for p617-T-EGFP-I-tTA.

The level of EGFP expression was monitored in the presence or absence of doxycycline (Fig. 2).

The p617-SV40-rtTA-EGFP vector showed strong EGFP expression which cannot be regulated by doxycycline. Translation of tTA from a bicistronic RNA appears to improve tet-mediated gene regulation, as demonstrated by the more prominent expression of EGFP in the absence of doxycycline in p617-I-tTA-EGFP-transduced cells, but the background level of EGFP expression in p617-I-tTA-EGFP-transduced cells remains very high. In contrast to p617-SV40-rtTA-EGFP and p617-I-tTA-EGFP, tight control of gene expression was observed for the retroviruses p617-T-EGFP-I-tTA and p617-T-EGFP-I-tTA_{AS}, in which the elements of the tet system were arranged in an autoregulatory loop. Tet-controlled gene expression was stable for at least 20 weeks after retroviral transduction (data not shown). Compared with p617-T-EGFP-I-tTA_{AS}, higher retroviral titres and tighter control of gene expression were obtained with p617-T-EGFP-I-tTA (Fig. 2).

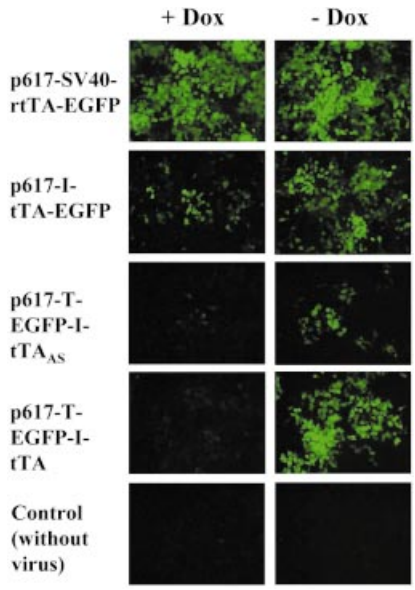

Figure 2. Doxycycline-dependent gene expression by the different retroviruses in highly differentiated HepG2 cells. Retrovirus-transduced cells were grown in the absence or presence of 1 mg/ml doxycycline, and EGFP expression was visualized by fluorescence microscopy.

Modified transactivation domains of the original tet transactivator are necessary to obtain retrovirally transduced clones with controllable gene expression

p617-T-EGFP-I-tTA represents the most appropriate orientation of the tet elements for controlled liver gene therapy. Consequently, we used p617-T-EGFP-I-tTA to investigate the effect of modulated VP16 transactivation domains on tet-controlled retroviral gene expression in HepG2 cells. The original VP16 transactivation domain was exchanged with three different chimeric tet transactivation domains (tTA2, tTA3 and tTA4) that possess lower toxicity compared with VP16. Replacement of VP16 with the transactivation domains tTA2, tTA3 or tTA4 in the autoregulatory cassette of p617-T-EGFP-I-tTA does not influence the tight control of gene regulation by doxycycline after retroviral transduction (data not shown), but only with the modulated VP16 transactivation domains was establishment of stable HepG2 cell clones possible after neomycin selection (Fig. 3B and Table 1). Interestingly, no cell clones with background EGFP expression were observed in the uninduced state. The highest frequency of doxycycline-controllable cell clones was obtained with the retroviral vectors p617neo-T-EGFP-I-tTA3 and p617neo-T-EGFP-I-tTA4. The low toxicity of tTA3 and tTA4 allows selection of HepG2 cell clones with functionally active autoregulatory cassettes, thereby providing strong doxycycline-mediated gene regulation in ~50% of the clones.

Induction of EGFP gene expression driven by p617neo-T-EGFP-I-tTA4 in the liver of mice

We used the non-toxic p617neo-T-EGFP-I-tTA4 retroviral vector for the evaluation of doxycycline-regulated gene expression in the liver *in vivo*. Recently it has been shown that delivery of plasmid DNA and small interfering RNA (siRNA) into hepatocytes *in vivo* can be significantly

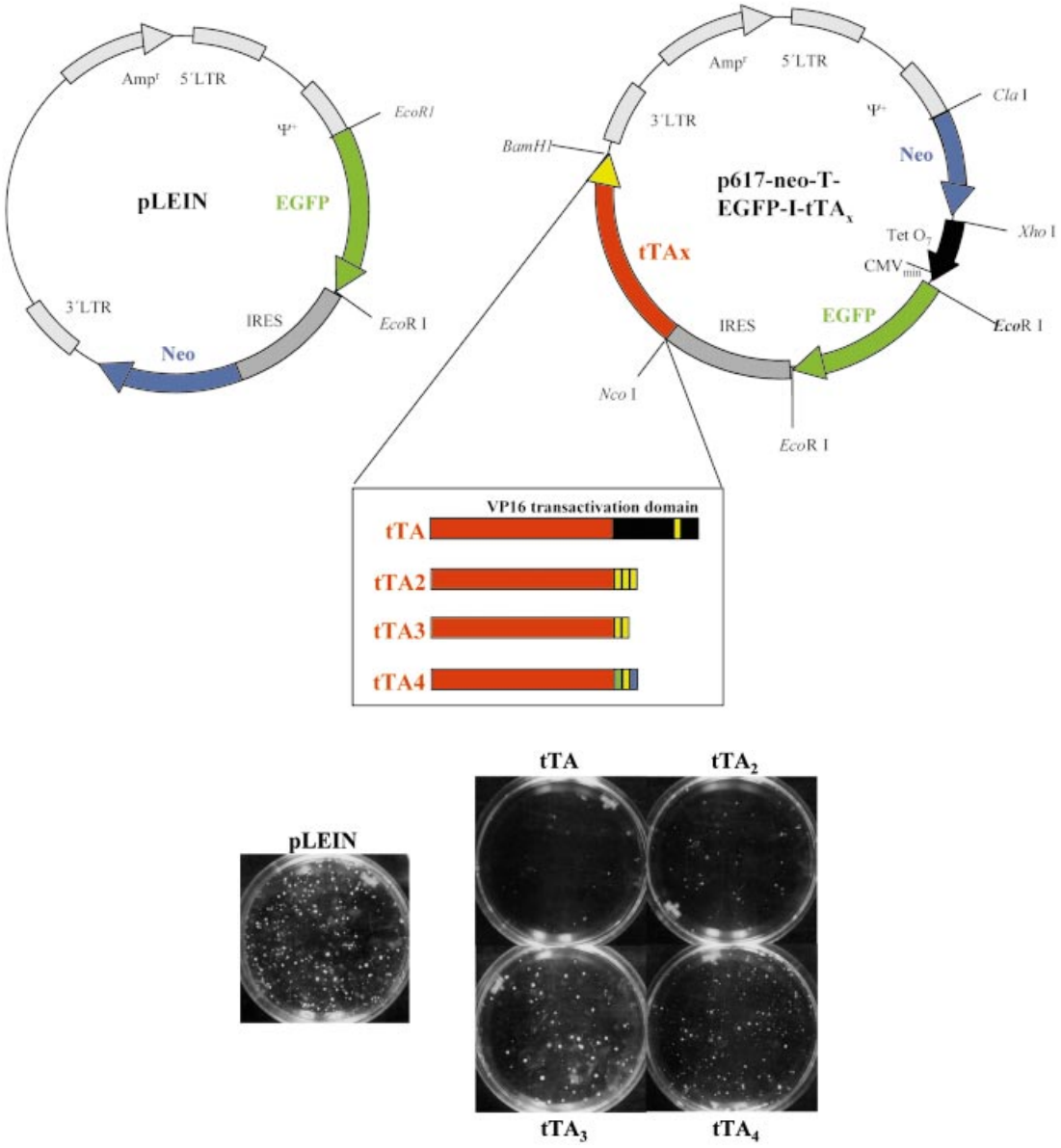

Figure 3. (**A**) Schematic representation of the control vector pLEIN and the doxycycline-controllable retroviral vectors with different VP16-derived transactivation domains. To compare the yield of retrovirally transduced stable cells clones of the controllable vectors with the uncontrollable vector pLEIN, the puromycin resistance gene in p617-T-EGFP-I-tTA was replaced by neomycin cDNA. (**B**) Stable retrovirally transduced HepG2 cell clones after neomycin selection. Replacement of the toxic VP16 transactivation domain by tTA3 and tTA4 facilitates the isolation of retrovirally transduced cell clones in a very high yield. The figure shows a representative example of three independent experiments.

enhanced by hydrodynamic injection. Delivery of a large volume presumably results in a short-term right heart failure and in a return flow of a large volume into the liver, whereby the therapeutic liquid may be squeezed through the endothelial layer of the sinusoids (15). Therefore, we used hydrodynamic injection for *in vivo* transduction of hepatocytes by retroviral vectors. A 2 ml aliquot of concentrated viral suspension of the ecotropic retroviral supernatant was injected within 4–10 s in the tail vein of mice during liver regeneration, 48 h after adenoviral liver injury. After retroviral transduction, three animals were treated with doxycycline and three animals were left untreated. Three days after retroviral transduction, the livers of the animals were analysed by fluorescence microscopy in order to detect EGFP fluorescence. Livers of animals treated with doxycycline showed almost no EGFP expression, whereas efficient EGFP gene expression was observed in the whole liver, predominantly near the portal fields, in the animals without doxycycline treatment (Fig. 4). This result confirms the tight control of p617neo-T-EGFP-I-tTA4-mediated transgene expression in the liver *in vivo*. As retroviral insertion may trigger development of cancer, we treated six mice with p617neo-T-EGFP-I-tTA4. The animals were then kept under surveillance for 12 months. We did not find any alteration of liver histology, in particular no liver tumours, in these mice (data not shown), indicating a low level of influence of retroviral insertion on hepatocarcinogenesis.

Table 1. Yield of doxycycline-controllable HepG2 cell clones after retroviral transduction and puromycin (p617-SV40-rtTA-EGFP, p617-I-tTA-EGFP, p617-T-EGFP-I-tTA, p617-T-EGFP-I-tTA) or neomycin (p617-neo-T-EGFP-I-tTA, p617-neo-T-EGFP-I-tTA2, p617-neo-T-EGFP-I-tTA3, p617-neo-T-EGFP-I-tTA4) selection

Vector	Number of stable clones	Expression Dox+	Expression Dox–	Dox regulatable clones (%)
p617-SV40-rtTA-EGFP	72	53	53	0
p617-I-tTA-EFFP	23	9	9	0
p617-T-EGFP-I-tTA$_{AS}$	0	0	0	0
p617-T-EGFP-I-tTA	3	0	0	0
pLEIN	84	0	0	0
p617-neo-T-EGFP-I-tTA	7	0	0	0
p617-neo-T-EGFP-I-tTA$_2$	39	0	9	9/39 (23)
p617-neo-T-EGFP-I-tTA$_3$	68	0	33	33/68 (49)
p617-neo-T-EGFP-I-tTA4	65	0	29	29/65 (45)

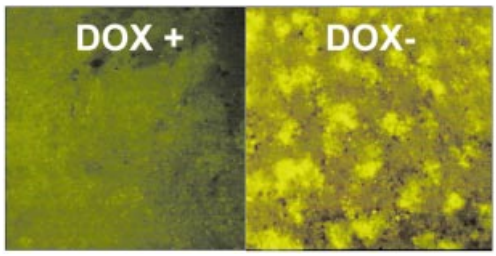

Figure 4. Tight doxycycline-dependent regulation of EGFP expression in the liver *in vivo* by p617-neo-T-EGFP-I-tTA4. Three days after retroviral transduction of hepatocytes with p617-neo-T-EGFP-I-tTA4, EGFP expression was monitored by fluorescence microscopy of the liver surface of mice treated (DOX+) or not treated (DOX–) with doxycycline. The figure shows a representative example of three independent experiments.

DISCUSSION

The liver is an important organ for targeting gene therapies for genetic diseases. An essential feature of any gene delivery system is the ability to regulate the expression of the delivered therapeutic gene. The use of liver-specific promoters is suggested for physiological gene regulation in liver gene therapy approaches. However, the activity of liver-specific promoters is often too low to obtain therapeutic levels of the gene product, and promoter interference may silence gene transcription after retroviral insertion. Consequently, the ability to regulate expression of therapeutic genes via exogenous stimuli will have some substantial advantages in somatic liver gene therapy. The combination of a tightly controlled inducible system, such as the tet system, and retrovirus-mediated gene transfer may be an attractive option for this approach, since retroviral infection guarantees the integration of all regulatory elements between the retroviral LTRs. Tet-repressible transgene expression from single retroviral vectors has been described, but selection of internal promoters and orientation of the tet elements strongly affect proviral genome stability, viral titres and regulation of transgene expression (5–8). Two studies demonstrated that placing the TO promoter in the antisense orientation near the 3′ LTR results in high viral titres and tightly controlled transgene expression in several cell lines (6,8). Therefore, we first investigated doxycycline-dependent expression of EGFP by the retroviral vector p617-SV40-rtTA-EGFP, which has a similar structure to the retroviruses described by Paulus *et al.* (8) and Hwang *et al*. (6), in highly differentiated hepatoma cells. In contrast to previous studies, we observed almost no doxycycline-dependent gene regulation using this retroviral vector, confirming previous observations that the tet system has encountered limitations in hepatocytes (3,16). The use of self-inactivating (SIN) vectors in which the viral 5′ LTR enhancer and promoter control elements are deleted post-infection has allowed us to overcome the leakiness of tet vectors, but viral titres of SIN vectors are significantly lower and are a limitation for successful *in vivo* application (5).

To develop a retroviral tet system which is more applicable in liver gene therapy, we have subcloned different retroviral vectors in which the regulatory elements of the tet system were arranged in different patterns. Our results demonstrate that only the organization of the tet system in a sense-orientated autoregulatory unit results in high retroviral titres and in tight regulation of gene expression in highly differentiated hepatoma cells.

However, it was not possible to establish a tet-controllable stable HepG2 cell line after puromycin or neomycin selection with the VP16 transactivation domain of the original tet system, which appears to be related to the toxicity of the original VP16 transactivator. In the context of an autoregulatory cassette, toxic effects of the original tet transactivator due to transcriptional squelching have been described (9). However, active fragments of VP16 having reduced interactions with cellular transcription factors can be used to control gene expression by the tet or dox system (10,11). Recently, tight control of gene expression has been obtained by novel reverse transactivators with plasmids (17) and in systems composed of two lentiviruses (18,19). In addition, it has been demonstrated that the novel transactivators can be advantageously substituted for the complete VP16 domain in an autoregulatory single AAV vector system (20). To avoid toxicity in hepatoma and liver cells, we subcloned three versions of p617-T-EGFP-I-tTA with different tet transactivators. The use of modified non-toxic tetracycline transactivators within the autoregulatory loop of a single retrovirus enables us to isolate cell clones with doxycyline-dependent gene expression in a very high yield, without any background gene expression. Since we demonstrated tight controllable gene expression not only in highly differentiated

hepatoma cells *in vitro*, but also in differentiated hepatocytes *in vivo*, our new retroviral vector system appears to be suitable for many applications in molecular biology or gene therapy of liver diseases.

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REFERENCES

1. Gossen,M. and Bujard,H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl Acad. Sci. USA*, **89**, 5547–5551.
2. Gossen,M., Freundlieb,S., Bender,G., Muller,G., Hillen,W. and Bujard,H. (1995) Transcriptional activation by tetracyclines in mammalian cells. *Science*, **268**, 1766–1769.
3. Furth,P.A., St Onge,L., Boger,H., Gruss,P., Gossen,M., Kistner,A., Bujard,H. and Hennighausen,L. (1994) Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter. *Proc. Natl Acad. Sci. USA*, **91**, 9302-9306.
4. Kistner,A., Gossen,M., Zimmermann,F., Jerecic,J., Ullmer,C., Lubbert,H. and Bujard,H. (1996) Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. *Proc. Natl Acad. Sci. USA*, **93**, 10933–10938.
5. Hofmann,A., Nolan,G.P. and Blau,H.M. (1996) Rapid retroviral delivery of tetracycline-inducible genes in a single autoregulatory cassette. *Proc. Natl Acad. Sci. USA*, **93**, 185–5190.
6. Hwang,J.J., Scuric,Z. and Anderson,W.F. (1996) Novel retroviral vector transferring a suicide gene and a selectable marker gene with enhanced gene expression by using a tetracycline-responsive expression system. *J. Virol.*, **70**, 8138–8141.
7. Iida,A., Chen,S.T., Friedmann,T. and Yee,J.K. (1996) Inducible gene expression by retrovirus-mediated transfer of a modified tetracycline-regulated system. *J. Virol.*, **70**, 6054–6059.
8. Paulus,W., Baur,I., Boyce,F.M., Breakefield,X.O. and Reeves,S.A. (1996) Self-contained, tetracycline-regulated retroviral vector system for gene delivery to mammalian cells. *J. Virol.*, **70**, 62–67.
9. Gallia,G.L. and Khalili,K. (1998) Evaluation of an autoregulatory tetracycline regulated system. *Oncogene*, **16**, 1879–1884.
10. Baron,U., Gossen,M. and Bujard,H. (1997) Tetracycline-controlled transcription in eukaryotes: novel transactivators with graded transactivation potential. *Nucleic Acids Res.*, **25**, 2723–2729.
11. Urlinger,S., Baron,U., Thellmann,M., Hasan,M.T., Bujard,H. and Hillen,W. (2000) Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. *Proc. Natl Acad. Sci. USA*, **97**, 7963–7968.
12. Limon,A., Briones,J., Puig,T., Carmona,M., Fornas,O., Cancelas,J.A., Nadal,M., Garcia,J., Rueda,F. and Barquinero,J. (1997) High-titer retroviral vectors containing the enhanced green fluorescent protein gene for efficient expression in hematopoietic cells. *Blood*, **90**, 3316–3321.
13. Lieber,A., Vrancken Peeters,M.J., Meuse,L., Fausto,N., Perkins,J. and Kay,M.A. (1995) Adenovirus-mediated urokinase gene transfer induces liver regeneration and allows for efficient retrovirus transduction of hepatocytes *in vivo*. *Proc. Natl Acad. Sci. USA*, **92**, 6210–6214.
14. Patijn,G.A., Lieber,A., Meuse,L., Winther,B. and Kay,M.A. (1998) High-efficiency retrovirus-mediated gene transfer into the livers of mice. *Hum. Gene Ther.*, **9**, 1449–1456.
15. Herweijer,H. and Wolff,J.A. (2003) Progress and prospects: naked DNA gene transfer and therapy. *Gene Ther.*, **10**, 453–458.
16. Shockett,P., Difilippantonio,M., Hellman,N. and Schatz,D.G. (1995) A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice. *Proc. Natl Acad. Sci. USA*, **92**, 6522–6526.
17. Lamartina,S., Silvi,L., Roscilli,G., Casimiro,D., Simon,A.J., Davies,M.E., Shiver,J.W., Rinaudo,C.D., Zampaglione,I., Fattori,E., Colloca,S., Gonzalez Paz,O., Laufer,R., Bujard,H., Cortese,R., Ciliberto,G. and Toniatti,C. (2003) Construction of an rtTA2(s)-m2/tts(kid)-based transcription regulatory switch that displays no basal activity, good inducibility and high responsiveness to doxycycline in mice and non-human primates. *Mol. Ther.,* **7**, 271–280.
18. Koponen,J.K., Kankkonen,H., Kannasto,J., Wirth,T., Hillen,W., Bujard,H. and Yla-Herttuala,S. (2003) Doxycycline-regulated lentiviral vector system with a novel reverse transactivator rtTA2S-M2 shows a tight control of gene expression *in vitro* and *in vivo*. *Gene Ther.*, **10**, 459–466.
19. Vigna,E., Cavalieri,S., Ailles,L., Geuna,M., Loew,R., Bujard,H. and Naldini,L. (2002) Robust and efficient regulation of transgene expression *in vivo* by improved tetracycline-dependent lentiviral vectors. *Mol. Ther.*, **5**, 252–261.
20. Fitzsimons,H.L., Mckenzie,J.M. and During,M.J. (2001) Insulators coupled to a minimal bidirectional tet cassette for tight regulation of rAAV-mediated gene transfer in the mammalian brain. *Gene Ther.*, **8**, 1675–1681.