Efficient Tet-Dependent Expression of Human Factor IX *in Vivo* by a New Self-Regulating Lentiviral Vector

Elisa Vigna,[1] Mario Amendola,[2,3] Fabrizio Benedicenti,[2] Andrew D. Simmons,[4] Antonia Follenzi,[1,*] and Luigi Naldini[2,3,†]

[1]*Institute for Cancer Research and Treatment, University of Torino Medical School, Strada Provinciale 142, 10060 Candiolo, Turin, Italy*
[2]*HSR-TIGET, San Raffaele-Telethon Institute for Gene Therapy, and [3]Vita Salute San Raffaele University,*
H. San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy
[4]*Cell Genesys, Inc., 500 Forbes Boulevard, South San Francisco, CA 94080, USA*

*Present address: Departments of Medicine and Pathology, Albert Einstein College of Medicine, Ullmann 625,
1300 Morris Park Avenue, Bronx, NY 10461, USA.

[†]*To whom correspondence and reprint requests should be addressed. Fax: +39 02 2643 4668. E-mail: naldini.luigi@hsr.it.*

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Regulation of gene expression represents a long-sought goal of gene therapy. However, most viral vectors pose constraints on the incorporation of drug-dependent transcriptional regulatory systems. Here, by optimizing the design of self-regulating lentiviral vectors based on the tetracycline system, we have been able to overcome the limitations of previously reported constructs and to reach both robust expression and efficient regulation from a single vector. The improved performance allows us to report for the first time effective long-term *in vivo* regulation of a human clotting Factor IX (hF.IX) transgene upon systemic administration of a single vector to SCID mice. We showed that hF.IX expression in the plasma could be expressed to therapeutically significant concentrations, adjusted to different set levels by varying the tetracycline dose, rapidly turned off and on, and completely recovered after each treatment cycle. The new vector design was versatile, as it successfully incorporated a tissue-specific promoter that selectively targeted regulated expression to hepatocytes. Robust transgene expression in the systemic circulation coupled to the ability to switch off and even adjust the expression level may open the way to safer gene-based delivery of therapeutics.

Key Words: gene therapy, lentiviral vectors, regulation of gene expression, Tet-regulated system, hemophilia B, tissue-specific promoter

INTRODUCTION

To be broadly applicable, gene-based delivery of therapeutics requires safe and efficient gene delivery to target cells *in vivo* as well as regulation of the transduced gene expression [1]. The gene product should be expressed to concentrations comprised within a therapeutic window and adjusted to the specific requirements of the disease condition. Moreover, expression should be reversible, allowing for termination of treatment and, if required, repeated treatment cycles. The ability to switch off transgene expression provides an important safety feature, in case adverse events related to therapeutic gene expression develop. These advanced expression features may be best obtained by regulating transcription of the transgene via the exogenous administration of ligands endowed with low toxicity and excellent tissue penetra-tion [2]. Among the currently available drug-regulated gene expression systems, the tetracycline (Tet)-dependent system has been developed to reach remarkable performance *in vitro* [3] and has been extensively tested *in vivo* [4]. The Tet system combines elements of viral and bacterial origin to generate chimeric transcription factors (Tet transactivators, tTA and rtTA, respectively of the "off" and "on" type) that control the activity of a chimeric promoter (P_{tet}) driving transgene expression. For wide application in gene therapy, a regulated vector should contain within itself all components of the system, i.e., an expression cassette for tTA or rtTA and the transgene of interest under the control of an inducible P_{tet} promoter. Such an arrangement avoids the need to deliver two separate vectors into the same cells, a challenging task when gene therapy is administered directly *in vivo,* in particular by the systemic route.

Among the viral vectors being tested in gene therapy applications, lentiviral vectors (LVs) provide several advantages because of their efficient *in vivo* gene transfer, stable integration into the genome of dividing and nondividing cells, long-term sustained transgene expression [5], and apparently limited perturbation of target cell transcription [6] (Turunen et al., submitted for publication). Others and we have previously developed Tet-regulated LVs and tested their performance *in vivo.* Some studies used two separate LVs to express the trans-activator and the transgene. Regulation of transgene expression was obtained *in vivo,* taking advantage of the efficient localized transduction reached by *in situ* LV injection or by transducing hematopoietic stem cells *ex vivo* followed by transplantation [7–9]. Other studies used a single LV to deliver all system components. Kafri et al. [10,11] introduced the expression cassettes for the transgene and for tTA in tandem in the same orientation in LVs and tested gene delivery into the rat brain. Although these authors obtained some level of regulation of transgene expression, a high level of basal expression was retained during doxycycline (Dox) treatment in the off phase. We previously developed a self-regulating LV (LV-TA1/R2) by replacing the transcriptional enhancer sequences in the HIV-1 LTR with seven tandem repeats of Tet operators to drive transgene expression in a Tet-dependent manner ($P_{tet-LTR1}$) and expressing tTA from a constitutive internal phosphoglycerate kinase (PGK) promoter [7]. By this design we reached transgene expression regulation over 2 orders of magnitude and an undetectable expression in the off condition. However, $P_{tet-LTR1}$ expression was significantly lower in the context of a self-regulating vector than when two separate vectors were used to express the transgene and tTA, suggesting occlusion of $P_{tet-LTR1}$ by the neighboring PGK promoter. This effect limited the level of transgene expression reached by the self-regulating LV in the on condition and prevented its efficacious application *in vivo.* More recently, Vogel et al. [12] described a new Tet-dependent LV in which the transgene and rtTA were expressed by two separate internal expression cassettes placed in opposite orientation and separated by an insulator sequence. This vector was used to regulate effectively gene expression (luciferase and tyrosine hydroxylase) in the rat striatum, with low background expression (1–2% of induced level), although high Dox

concentrations were needed to induce expression. Until now, however, no work has reported *in vivo* regulated transgene expression upon systemic delivery of LVs, a challenging task that requires combining high gene transfer efficiency and robust expression within a single regulated vector.

Hemophilia B is a congenital X-linked coagulation disorder due to defective blood clotting Factor IX (F.IX) production. Although it is generally considered that exogenous regulation of a F.IX transgene may not be strictly required, the disease provides a well-established model for testing gene therapy applications [13]. Previous studies have shown that a single intravenous administration of LVs carrying the human F.IX (hF.IX) cDNA allowed the attainment of stable and therapeutic-range levels of hF.IX in mouse plasma [14–17]. The liver and spleen represented the major target sites of systemically administered LV [18–22]. Using an hF.IX transgene, the robustness of gene expression, the regulation extent, and the response time to drug administration may be accurately assessed by monitoring the plasma of treated mice for long periods of time and performing subsequent cycles of induction/repression.
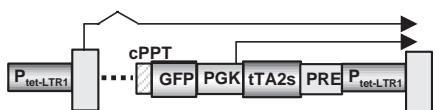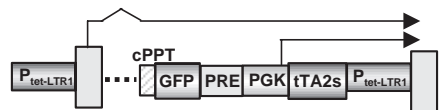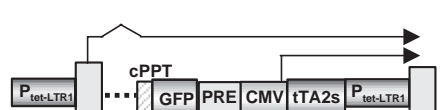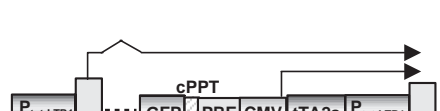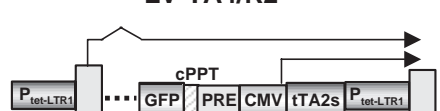
Here we optimized the design of self-regulating Tet-dependent LVs and assessed their performance upon systemic delivery of an hF.IX transgene into immuno-compromised mice. We report long-term transgene expression and robust regulation *in vivo,* reaching therapeutic-range plasma levels of hF.IX in the on state and undetectable levels in the off state. These data indicate that the newly developed self-regulating LVs represent powerful tools for experimental gene transfer and may help to address in the future a major safety and efficacy requirement of gene therapy.

RESULTS

Optimizing Tet-Dependent Expression by Self-Regulating Vectors

To overcome the limitations of the previously developed self-regulating LV [7] (LV-TA1/R2, Fig. 1), we modified the type and arrangement of transcription control elements in the vector and tested the performance of the modified constructs by monitoring vector yield, infectivity, and the level and regulation of transgene expression *in vitro.* To compare the different constructs, we measured the

FIG. 1. Tet-dependent self-regulating lentiviral vectors. Left: Scheme of the proviral forms of the HIV-derived vectors studied. Arrows indicate the expected transcripts. $P_{tet-LTR1}$, Tet-dependent promoter made by replacing the U3 sequence from position −418 to −36 relative to R in the long terminal repeat with seven tandem repeats of Tet operators [7]; cPPT, central polypurine tract; GFP, enhanced green fluorescent protein; PRE, posttranscriptional regulatory element of the woodchuck hepadnavirus; PGK, promoter of the human phosphoglycerate kinase gene; EF-1-α, elongation factor 1 promoter; CMV, immediate/early enhancer promoter of the human cytomegalovirus; tTA2s, synthetic gene for Tet transactivator-2. For each type of vector, the 5′ hybrid LTR driving expression of the vector genome in producer cells (Rous sarcoma virus enhancer/promoter, RRL, or CMV enhancer/promoter, CCL, fused to HIV-1 R-U5 [26]), the expression titer in transducing units on HeLa cells (TU)/ml, the particle concentration in HIV-1 Gag p24 equivalent, and the infectivity, calculated from the ratio between TU/ml and ng p24/ml of a representative batch, are shown. Right: GFP expression in HeLa cells transduced by single copy (according to the Poisson distribution) of each vector and treated or not with 10 ng/ml Dox. The percentage and mean fluorescence intensity (MFI) of GFP-positive cells, gated in R3, are indicated.

	5' Hybrid LTR	Titer TUx10^6/ml	p24 ng/ml	Infectivity TU x 10^4/ng p24
LV-TA1/R2	RRL	1.9	120	1.5
LV-TA1.1/R2	RRL	0.07	42	0.17
LV-TA2/R2	RRL	0.5	279	0.11
LV-TA3/R2	RRL	0.73	244	0.3
LV-TA4/R2	RRL	0.82	170	0.48
LV^{CCL}-TA4/R2	CCL	2.5	85	2.94
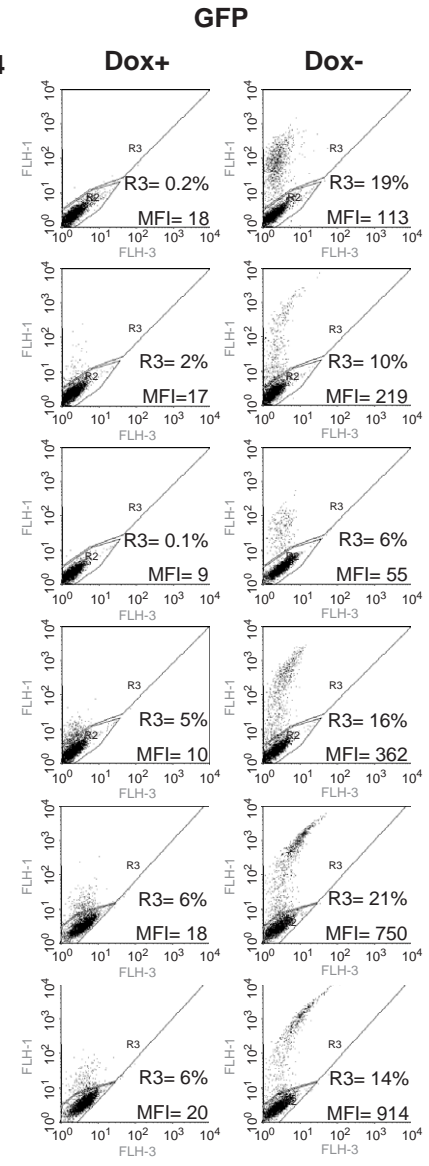
mean fluorescence intensity (MFI) of GFP expression in HeLa cells transduced by single vector copies and grown in the presence or absence of Dox.

First, to enhance selectively expression of the mRNA originated from $P_{tet-LTR1}$, we moved the woodchuck hepadnavirus posttranscriptional regulatory element from the 3' end of the vector to a more central location, between the transgene and the internal expression cassette for the transactivator (LV-TA1.1/R2, Fig. 1). This position was previously described to allow selective accumulation of LTR-driven transcripts with respect to internal promoter-driven transcripts in LVs carrying a transcriptionally targeted LTR [23]. Although this modification doubled the average transgene expression level per vector copy, it also resulted in a decrease in vector titer and infectivity. Second, to reduce $P_{tet-LTR1}$ occlusion, we tested different types of internal promoters to drive tTA expression. We substituted the PGK promoter in LV-TA1.1/R2 with that of the elongation factor 1α (EF1α) [24] (LV-TA2/R2, Fig. 1) or with the immediate early enhancer/promoter of the human cytomegalovirus (CMV, LV-TA3/R2, Fig. 1). In the case of the EF1α promoter, GFP expression levels decreased compared to those obtained with the PGK promoter. In contrast, in the case of the CMV promoter, GFP expression level in the on condition increased between 1.5 and 2 times, maintaining a very low level in the off condition. Third, to enhance $P_{tet-LTR1}$-dependent expression further, we moved the central polypurine tract (cPPT) from upstream to downstream of the transgene, to prevent translation interference (LV-TA4/R2, Fig. 1). In fact, in previous studies analyzing vectors carrying the cPPT element in different positions, we found that the enhancement of vector infectivity was position-independent but that cPPT placement immediately upstream of the transgene decreased its expression [25] (A. Lombardo, A. Follenzi, and L. Naldini, unpublished results). The latter modification allowed a further significant increase in transgene expression, with the LV-TA4/R2 vector reaching an overall sevenfold higher level of gene expression than that obtained by the original LV-TA1/R2 self-regulating vector.

In Vivo Validation of Expression-Optimized Self-Regulating Vector

We then assessed the expression-optimized LV-TA4/R2 vector for the systemic delivery and *in vivo* regulation of a hF.IX transgene. We first analyzed hF.IX expression *in vitro,* transducing HeLa cells with increasing doses of self-regulating vector and comparing the amount of hF.IX secreted into the medium to that obtained after transduction with a standard LV expressing hF.IX from the CMV promoter. When cells transduced by LV-TA4/R2.hF.IX were grown with Dox in the medium, no hF.IX expression was detected. On the other hand, when the same cells were grown without Dox, hF.IX was easily detectable in the medium, although to an average 10-fold

lower level than that obtained from cells transduced with matched amounts of LV-CMV-hF.IX particles (Fig. 2A). The lower yield of hF.IX from the self-regulating vector was not due to lower transgene expression compared to that obtained from the CMV promoter, but to lower vector infectivity, as shown by the lower number of integrated vector copies measured in target cells by real-time PCR (Fig. 2B). This analysis indicated that vector integration per input particle was 18-fold lower for LV-TA4/R2-hF.IX than for LV-CMV-hF.IX ($0.2 \pm 0.04 \times 10^4$ and $3.6 \pm 0.7 \times 10^4$ integrated vector copy/ng p24, respectively, $n = 3$, $P < 0.01$). In fact, when vector expression was normalized to integration level, the average hF.IX secretion per vector copy was found to be 2-fold higher for the regulated vector in the on condition than for the constitutive vector (21.6 ± 1.1 pg hF.IX/ml per vector copy per 24 h for LV-TA4/R2-hF.IX, $n = 4$, and 11.7 ± 2.3 pg hF.IX/ml per vector copy per 24 h for LV-CMV-hF.IX, $n = 3$, $P < 0.01$). In summary, the *in vitro* analysis indicated proficient expression and effective Dox-dependent regulation of the LV-TA4/R2 self-regulating vector. However, because the low vector infectivity limited transduction, administration of high vector doses would be required to reach significant transgene expression levels *in vivo*.

Based on the above calculations and to compare matching amounts of transducing particles, we injected LV-TA4/R2-hF.IX and LV-CMV.hF.IX into the tail vein of severe combined immunodeficient (SCID) mice in an approximate ratio of 20 to 1 p24 equivalents. We treated the mice or not with Dox in the drinking water and, starting 3 weeks after vector injection, performed weekly and, later, biweekly bleedings to measure the amount of hF.IX in the plasma by immunocapture (Fig. 2C). The mice injected with self-regulating vector ($n = 4$) stably expressed hF.IX to an average level of 30 ng/ml when kept in the on condition, while they showed undetectable hF.IX expression when kept in the off condition. Serial cycles of Dox treatments performed on the same animals induced the expected changes in hF.IX expression with subsequent cycles showing the same basal and induced expression levels, indicating stable maintenance of the vector and robust performance over time *in vivo*. Although the off response to Dox addition was fast, leading to complete shut-off of transgene expression before the subsequent weekly bleeding, full recovery of hF.IX expression after Dox withdrawal was slower, taking at least 3 weeks. This delayed response was probably due to the slow clearance of the drug that accumulated in the tissues during the treatment. The average on level of hF.IX in the mice injected with self-regulating vector was similar to that obtained in mice injected with 20-fold less amount of constitutive vector (29.2 ± 13.7 ng/ml versus 22.9 ± 1.9 ng/ml, respectively, $n = 4$ and 2, $P > 0.1$), while the content of vector DNA in the liver of mice analyzed by real-time PCR 200 days after injection was 0.09 ± 0.01 and
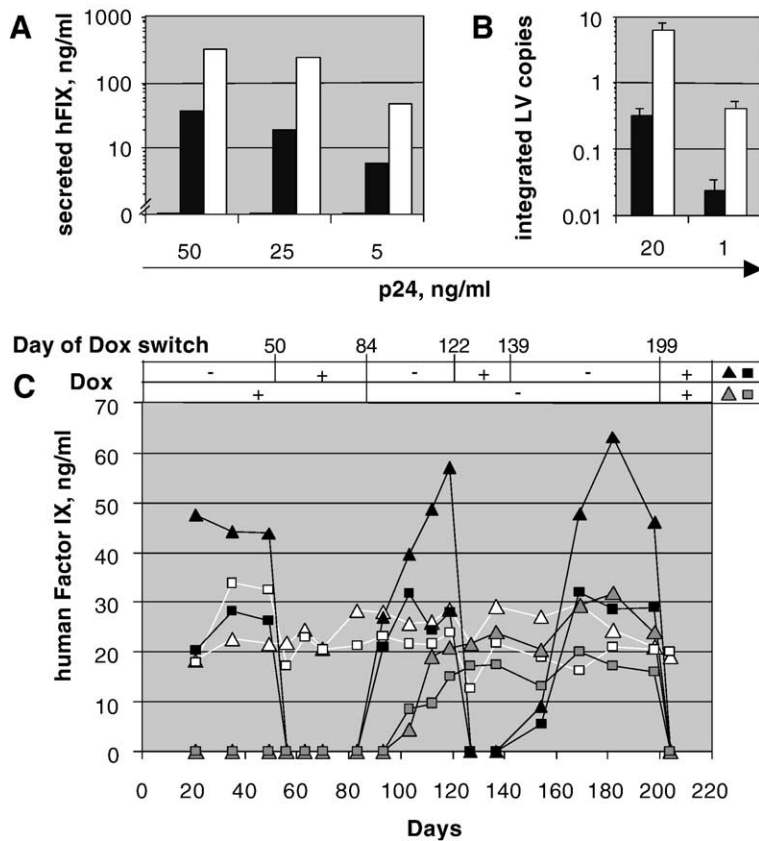
FIG. 2. Human F.IX expression by expression-optimized self-regulating vector. (A) Secretion of hF.IX by HeLa cells transduced by the indicated p24 equivalent of LV-TA4/R2 hF.IX and grown with (gray bars) or without (black bars) 10 ng/ml Dox or by LV-CMV-hF.IX vector (white bars). Data shown are the averages of duplicate determinations from one of two similar experiments. (B) Number of integrated vector copies in the genome of HeLa cells transduced by the indicated p24 equivalent of LV-TA4/R2 hF.IX (black bars) or LV-CMV-hF.IX (white bars) vector, measured by real-time PCR. Data shown are the means ± SD of triplicate determinations. (C) Plasma levels of hF.IX in SCID mice after injection of 25 μg (mice 1 and 2, black and gray triangles) or 20 μg (mice 3 and 4, black and gray squares) of p24 equivalent of LV-TA4/R2.hF.IX and of 0.7 μg (mouse 5, white triangles) or 1.1 μg (mouse 6, white squares) of p24 equivalent of LV-CMV-hF.IX. Mice were treated as indicated above with or without 50 μg/ml Dox in the drinking water. Two control mice were injected with PBS and bled as the others; no hF.IX was detected in their plasma (not shown).

0.25 ± 0.01 ($P < 0.01$) vector copy/diploid genome for the self-regulating and the constitutive vector, respectively. These data were in agreement with the *in vitro* analysis of vector performance, reflecting the lower infectivity but robust expression of regulated vector.

In summary, while the modified self-regulating vector allowed efficient Tet-dependent transgene expression *in vivo,* its application remained limited by the low vector infectivity that required administration of high vector doses to reach the therapeutic-range of hF.IX expression.

Optimizing Infectivity of Self-Regulating Vector

During vector production, the level of expression of genomic RNA competent for packaging is a critical limiting factor for vector infectivity [26]. To investigate the mechanism responsible for the low infectivity of the modified self-regulating vector, we analyzed RNA expression from the transfer vector construct in producer cells. We compared 293T cells transfected with LV-TA4/R2, the original LV-TA1/R2, and two control vector constructs carrying only one expression cassette, either internal (LV-R1) or driven from the LTR (LV-R2), all of them expressing GFP [7]. Fig. 3A shows that all constructs produced the expected RNA forms in similar ratios. However, self-regulating constructs produced significantly lower levels of all RNA species than the single-cassette vectors, with the newly modified construct further reduced. Because genomic vector expression in producer cells is driven by a chimeric 5' LTR [26] that competes for transcription factors with the vector-internal promoter, we replaced the RSV-derived enhancer region in the RRL-based construct with the stronger CMV enhancer region, generating the corresponding CCL-based TA4/R2 construct (LV^{CCL}-TA4/R2; Fig. 1). The analysis of RNA species in transfected packaging cells indicated higher expression of packaging-competent RNA from the CMV-driven LV^{CCL}-TA4/R2 vector than observed from the previous RRL-driven construct (Fig. 3B). In fact, using this construct we were able to increase the infectivity and production titer of self-regulating vectors, approaching the values obtained by standard lentiviral vectors. LV^{CCL}-TA4/R2 vector titers measured by transgene expression and by integration were comparable, indicating that virtually every integrated self-regulating vector genome efficiently expressed the transgene (not shown). When we used the new construct to express hF.IX in HeLa cells in comparison with LV-CMV-hF.IX, both transgene expression levels (Fig. 3E) and infectivity (Fig. 3F) per unit particle were similar for the self-regulating vector in the on condition and for the constitutive vector (infectivity $4.7 \pm 1.8 \times 10^4$ and $3.6 \pm 0.7 \times 10^4$ integrated vector copies/ng p24 for LV^{CCL}-TA4/
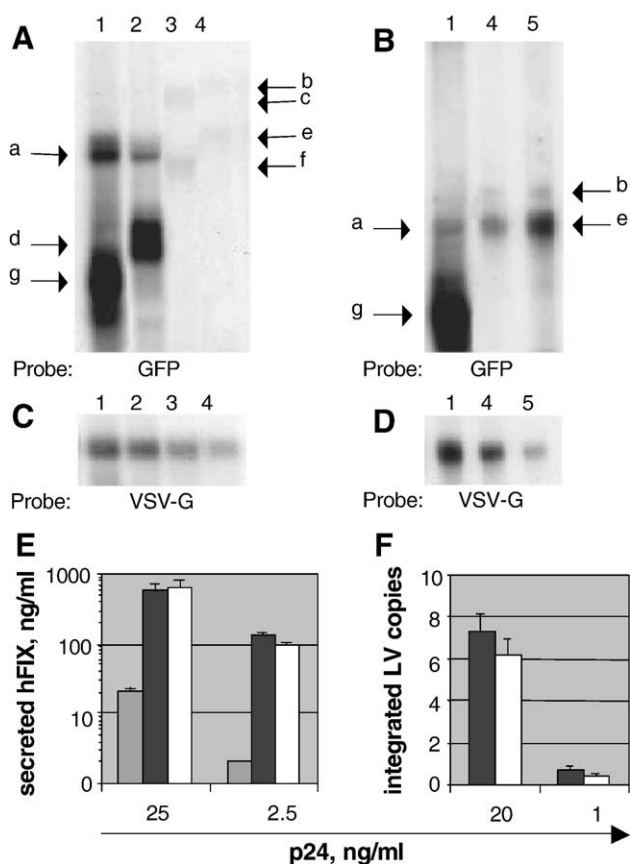
FIG. 3. Production and *in vitro* analysis of optimized self-regulating vectors. (A–D) Northern analyses of poly(A)$^+$ mRNA extracted from 293T cells transfected with the indicated lentiviral transfer vectors carrying GFP as transgene: lanes 1, LV-R1, transfer vector with Tet-dependent internal promoter (P$_{tet-1}$) driving GFP expression; 2, LV-R2, transfer vector with Tet-dependent promoter (P$_{tet-LTR1}$) in the LTR driving GFP expression; 3, LV-TA1/R2, self-regulating vector with P$_{tet-LTR1}$ driving GFP and an internal expression cassette for tTA2s; 4, LV-TA4/R2, expression-optimized self-regulating vector; 5, LV^{CCL}-TA4/R2 production-optimized self-regulating vector (see Fig. 1 and [7] for vector schemes). (A, B) Filters probed for the GFP sequence. Arrows indicate the expected sizes of GFP transcripts originating from the LTR, unspliced (a, b, c) and spliced (d, e, f), and from the internal P$_{tet-1}$ promoter (g). The higher relative intensity of band "e" in B compared to A was due to the absence versus presence, respectively, of Dox during vector production. (C, D) Replicate filters probed for VSV-G sequence. VSV-G RNA was expressed from a separate plasmid cotransfected with the transfer vector plasmids and used to control the efficiency of transfection. (E) Human F.IX secretion by HeLa cells transduced by the indicated p24 equivalent of LV^{CCL}-TA4/R2 hF.IX and grown with (gray bars) or without (black bars) 10 ng/ml Dox or of LV-CMV-hF.IX vector (white bars). (F) Number of integrated vector copies in the genome of HeLa cells transduced by the indicated p24 equivalent of LV^{CCL}-TA4/R2 hF.IX (black bars) or LV-CMV-hF.IX vector (white bars), measured by real-time PCR. Data shown in E and F are the means ± SD of triplicate determinations.

R2-hF.IX and LV-CMV-hF.IX, respectively; $n = 3$, $P > 0.1$). In the presence of Dox, cells transduced by the self-regulating vector reduced hF.IX expression to barely detectable levels, showing a regulation index of up to 70-fold (Fig. 3E). The regulation index was lower (30-fold)

when calculated for the cells transduced with high vector input. This lower ratio of regulation was due to an increase in the amount of hF.IX secreted in the on condition not proportional to the increase in vector input. Nonlinear dependence of secreted hF.IX from vector input at high m.o.i. was observed with different types of vectors (A.F., E.V., unpublished observation) and is probably due to saturation of one or more of the enzymatic steps in the secretory pathway when hF.IX is highly overexpressed. This explanation was confirmed by the maintenance of the linear dependence in the basal level of expression observed in the off condition at all vector m.o.i. tested (see Fig. 3E).

Robust Tet-Dependent *in Vivo* hF.IX Expression upon Systemic Delivery of Self-Regulating Vector
Having optimized both the design and the production of the self-regulating vector, we injected SCID mice with LV^{CCL}-TA4/R2-hF.IX ($n = 8$), LV-CMV-hF.IX ($n = 7$), or PBS only ($n = 4$) and monitored plasma hF.IX expression as described above (Fig. 4 and Table 1). Mice were injected with decreasing vector amounts from 40 to 1.25 μg of p24 equivalent (Figs. 4A to 4D). All vector-injected mice expressed hF.IX in the plasma, when not treated with Dox, to well-detectable levels ($P < 0.01$ for each vector type versus mock-injected mice) and for the long term (latest time point of analysis: 240 days after injection, when they were euthanized for tissue analysis). Plasma hF.IX expression was vector dose-dependent, as shown in Fig. 5A, with the highest dosed mice reaching an average of 160 ng/ml. When treated with Dox in the drinking water (50 μg/ml), the mice injected with 20, 5, or 1.25 μg p24 of self-regulating vector did not express detectable hF.IX, and the mice injected with 40 μg p24 expressed threshold-level, barely detectable hF.IX, showing a regulation index of at least 30-fold. As previously seen in the experiment of Fig. 2C, all treated mice recovered hF.IX expression to before-treatment levels within 3 weeks or more after Dox withdrawal. Recovery to before-treatment levels was similarly observed after each subsequent treatment cycle.

We analyzed the specific expression activity *in vivo* of the different vectors tested (LV-CMV-hF.IX, LV^{RRL}-TA4/R2-hF.IX, and LV^{CCL}-TA4/R2-hF.IX) by comparing the amount of hF.IX expression in plasma per input vector particle. For this analysis, we compared mice injected with vector doses equal to or higher than 5 μg p24 equivalents, because the specific expression activity was relatively independent of the injected vector dose within this range of values (see Table 1). Table 2 shows that LV^{RRL}-TA4/R2-hF.IX had lower specific activity *in vivo* than either LV-CMV-hF.IX ($P < 0.01$) or LV^{CCL}-TA4/R2-hF.IX ($P < 0.05$), while we found no statistically significant differences between LV-CMV-hF.IX and LV^{CCL}-TA4/R2-hF.IX ($P > 0.1$). These data confirmed *in vivo* the gain in specific activity (threefold) obtained using the CMV-driven compared to the RSV-driven LV-TA4/R2-hF.IX construct,
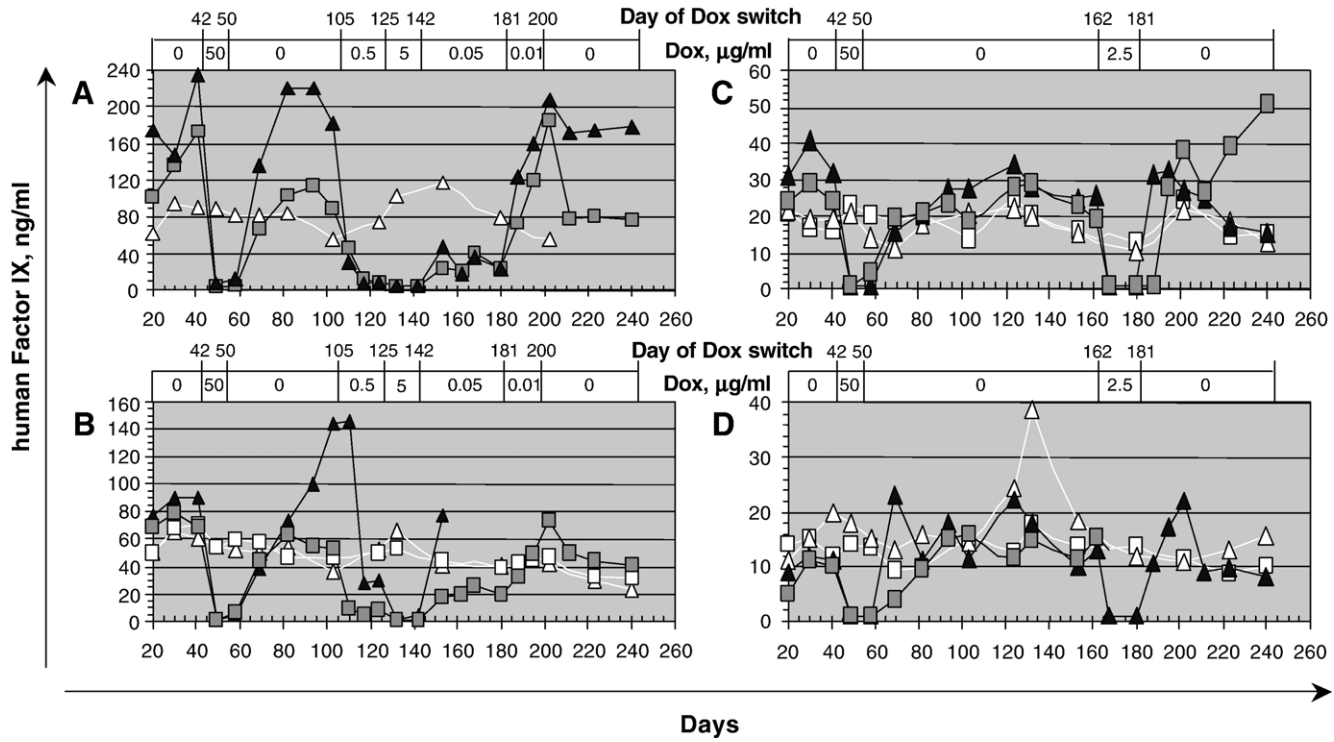
FIG. 4. Tet-dependent hF.IX expression *in vivo* by optimized self-regulating vector. Plasma levels of hF.IX in SCID mice after injection of (A) 40 μg, (B) 20 μg, (C) 5 μg, or (D) 1.25 μg of p24 equivalent of LV[CCL]-TA4/R2 hF.IX (gray squares and black triangles) or LV-CMV-hF.IX (white squares and triangles) vector. Mice were treated as indicated above with Dox in the drinking water. Four control mice were injected with PBS and bled as the others; no hF.IX was detected in their plasma (not shown). Three of the mice died before the end of the experiment (as indicated by the absence of complete follow-up) because of thymoma development, an expected finding in SCID mice of that age.

TABLE 1: Human Factor IX expression and vector content in treated mice

Mouse	Vector	Vector dose (μg p24)	Plasma hF.IX (ng/ml)	Specific activity[a]	Vector copies/genome Liver	Vector copies/genome Spleen	Follow-up (days)
1	LV-CMV	40	82	2.05	0.16 ± 0.01	ND[b]	202
5	LV^{CCL}-TA4/R2	40	128	3.2	0.18 ± 0.01	0.34 ± 0.01	240
13	LV^{CCL}-TA4/R2	40	193	4.8	0.21 ± 0.01	0.26 ± 0.01	240
2	LV-CMV	20	51	2.55	0.01 ± 0.01	0.31 ± 0.04	240
9	LV-CMV	20	53	2.65	0.11 ± 0.01	0.49 ± 0.03	240
6	LV^{CCL}-TA4/R2	20	95	4.75	ND[b]	ND[b]	155
14	LV^{CCL}-TA4/R2	20	65	3.25	0.06 ± 0.01	0.35 ± 0.05	240
3	LV-CMV	5	18.5	3.7	0.33 ± 0.05	0.44 ± 0.01	240
10	LV-CMV	5	19.5	3.9	0.22 ± 0.01	0.31 ± 0.02	240
7	LV^{CCL}-TA4/R2	5	29.5	5.9	ND[b]	ND[b]	240
15	LV^{CCL}-TA4/R2	5	24.5	4.9	0.18 ± 0.01	ND[b]	240
4	LV-CMV	1.25	13.5	10.8	0.16 ± 0.01	0.06 ± 0.002	240
11	LV-CMV	1.25	16.5	13.2	0.18 ± 0.01	0.09 ± 0.001	240
8	LV^{CCL}-TA4/R2	1.25	14	11.2	0.16 ± 0.01	0.09 ± 0.001	240
12	LV^{CCL}-TA4/R2	1.25	15	12	0.19 ± 0.01	ND[b]	187

[a] Average amount of hF.IX in plasma divided by the amount of vector injected.
[b] Sample not available for the analysis for technical reasons.

although the gain was less marked than *in vitro,* most likely because many different factors affect vector performance *in vivo* in addition to vector infectivity. Notably, the specific activity of the CCL-driven vector matched that of the constitutive vector both *in vitro* and *in vivo*.

We then investigated whether it was possible to adjust the level of plasma hF.IX according to the dose of Dox and treated the mice injected with the two highest vector doses (*n* = 3) with increasing concentrations of Dox in the drinking water, starting from 0.01 μg/ml and reaching 5 μg/ml. As shown in Figs. 4A and 4B, plasma hF.IX expression stabilized at a lower level for each increasing dose regimen until complete suppression was reached at 5 μg/ml. The dose-dependent suppression of hF.IX expression is better represented in Fig. 5B as a percentage of the expression reached in each mouse in the absence of Dox treatment. We obtained an expression level corresponding to 70, 25, and 5% of that observed without treatment by administering 0.01, 0.05, and 0.5 μg/ml Dox, respectively. Because 5 μg/ml Dox in the drinking water was enough to switch-off transgene expression completely, the 50 μg/ml dose used in the experiments shown in Figs. 2C and 4 above was unnecessarily high. We then checked whether using a lower drug concentration to suppress expression allowed faster recovery upon withdrawal. We treated the mice injected with the two lowest vector doses (*n* = 3) with 2.5 μg/ml Dox in the drinking water, a high enough dose to extinguish hF.IX expression completely, and then measured the time required to recover full hF.IX expression upon drug withdrawal (Fig. 5C). One of the mice recovered hF.IX expression in only 6 days, while the other two mice were fully expressing after 12 days, a considerably faster time than that observed after treatment with the higher Dox dose.

After establishing that long-term Dox-dependent expression of hF.IX had been obtained in the treated

mice, we analyzed the liver and spleen, the major sites of vector uptake, of all long-term survivors for vector DNA content by real-time PCR (Table 1). All treated mice showed easily detectable vector DNA in liver and spleen 8 months after injection, consistent with the observed long-term hF.IX expression, whether by constitutive or self-regulating vector. Although mice were injected with decreasing vector doses, and expressed hF.IX in the plasma in a vector dose-dependent manner, the liver and spleen vector DNA contents were similar and relatively high (on average 0.2 copy per diploid genome for liver and 0.3 copy per diploid genome for spleen) in all mice except for the mice that were injected with the lowest vector dose and showed significantly lower vector DNA in the spleen (0.06 copy per genome, $P < 0.01$). These data may indicate that by increasing the dose of systemically administered vector, transduction targets in the liver and spleen become saturated and other organs increasingly contribute to transgene expression. In summary, the optimized self-regulating LV allowed robust, long-term, and regulated transgene expression *in vivo,* with a vector dose dependency similar to that observed with a constitutively expressed vector.

Self-Regulating Lentiviral Vector with Tissue-Specific Expression

To explore further the potential of the newly optimized self-regulating vector design, we investigated the possibility of regulating transgene expression *in vivo* in a tissue-restricted manner. We replaced the internal CMV promoter driving tTA2s expression in the LV^{CCL}-TA4/R2 vector with a synthetic promoter (ET) that was shown to target transgene expression to hepatocytes (LV^{CCL}-TA5/R2; Fig. 6A). The ET promoter was assembled from enhancer sequences selected from randomly ligated hepatocyte-specific transcription factor binding sites and the
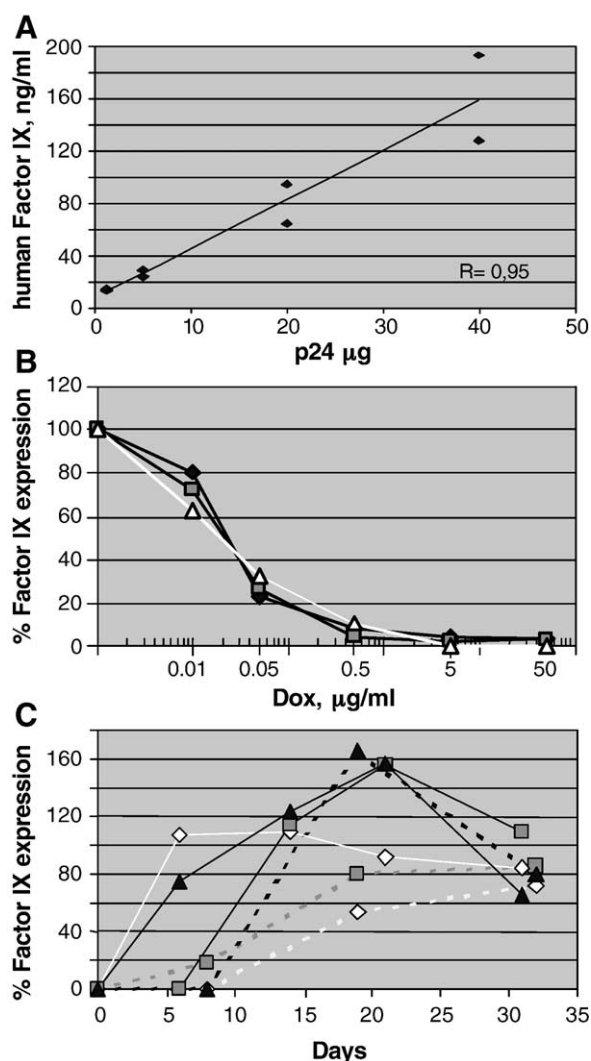
FIG. 5. *In vivo* vector dose dependence, Dox dose dependence, and response time of hF.IX expression. (A) Linear dependence between the vector dose injected into the mice and the plasma hF.IX concentration. The Pearson correlation coefficient for linear regression analysis is shown. Each dot represent one mouse, $n = 8$. (B) Plasma hF.IX levels in mice (diamonds, mouse 5; squares, mouse 13; triangles, mouse 14) treated at different times with the indicated Dox concentrations in the drinking water, expressed as percentage of the level measured in the same mouse in the absence of treatment. (C) Response time to Dox withdrawal in mice (diamonds, mouse 7; triangles, mouse 8; squares, mouse 15) pretreated with 50 μg/ml (dotted lines) or 2.5 μg/ml (continuous lines) Dox in the drinking water. Plasma hF.IX levels are expressed as percentage of the level measured in the same mouse before drug treatment. Day 0 is when Dox was withdrawn. Data in A, B, and C are from mice shown in Fig. 4 and Table 1.

murine transthyretin promoter. The LV^{CCL}-TA5/R2 was produced to very high titer and infectivity (titer 5.5×10^7 TU^{HuH7}/ml and infectivity 1.4×10^5).

We transduced increasing amounts of LV^{CCL}-TA5/R2-GFP vector into a panel of human cell lines derived from hepatocarcinomas, such as HepG2 (Fig. 6B) and HuH7

(Fig. 6C), and from nonhepatic tissues or tumors (Fig. 6D). The LV^{CCL}-TA5/R2 vector drove very high levels of GFP expression in hepatocarcinoma-derived cells. In these cells, GFP expression was suppressed to low levels by the addition of Dox, with a 20- to 50-fold difference in GFP MFI between cells treated or not with Dox, in the cultures transduced with both high and low vector doses. On the other hand, only low levels of GFP expression were detected in nonhepatocarcinoma cell lines, independent of whether the cells were treated with Dox, indicating basal activity of the $P_{tet-LTR1}$ and lack of expression of the tTA2s inducer protein in nontarget cells. A small fraction of cells (between 1 and 5% of the GFP-positive cells), however, showed inducible expression, consistent with our previous finding that LVs carrying tissue-specific promoters show nonspecific transcriptional activity in a small fraction of integrations, most likely due to enhancer/promoter trapping ([27,43]). As expected, when we used very high doses of LV^{CCL}-TA5/R2-GFP to transduce nonhepatic cell lines, the fraction of cells displaying inducible expression increased, consistent with the higher average copy number per cell (not shown).

We then injected the LV^{CCL}-TA5/R2-hF.IX vector into SCID mice ($n = 6$) at increasing doses, as shown in Fig. 6E. In the absence of Dox, we obtained high level of hF.IX expression in the plasma, with the highest dosed mice reaching on average 210 ng/ml. In the presence of Dox (5 μg/ml in the drinking water), the expression was downmodulated to undetectable or barely detectable levels depending on vector input, showing a 35-fold *in vivo* regulation index. Upon Dox withdrawal, all mice recovered hF.IX expression to before-treatment levels (>80% on average) within 1 week.

In summary, these data indicate that the optimized self-regulating lentiviral vector design can incorporate tissue-specific regulation and reach both robust expression and efficient regulation *in vivo*.

DISCUSSION

In this work, by optimizing the choice and arrangement of *cis*-regulatory sequences in the backbone of self-

TABLE 2: Specific vector expressing activity *in vivo*

Vector	n	Specific activity[a] (ng F.IX/p24)	SD	Student *t* test	P
LV-CMV	5	2.97	0.79	LV-CMV vs. LV^{RRL}-TA4/R2	<0.01
LV^{RRL}-TA4R2	4	1.25	0.49	LV-CMV vs. LV^{CCL}-TA4/R2	>0.1
LV^{CCL}-TA4/R2	9	3.49	1.76	LV^{RRL}-TA4/R2 vs. LV^{CCL}-TA4/R2	<0.05

[a] Average amount of hF.IX in plasma (in ng/ml) divided by the amount of vector injected,
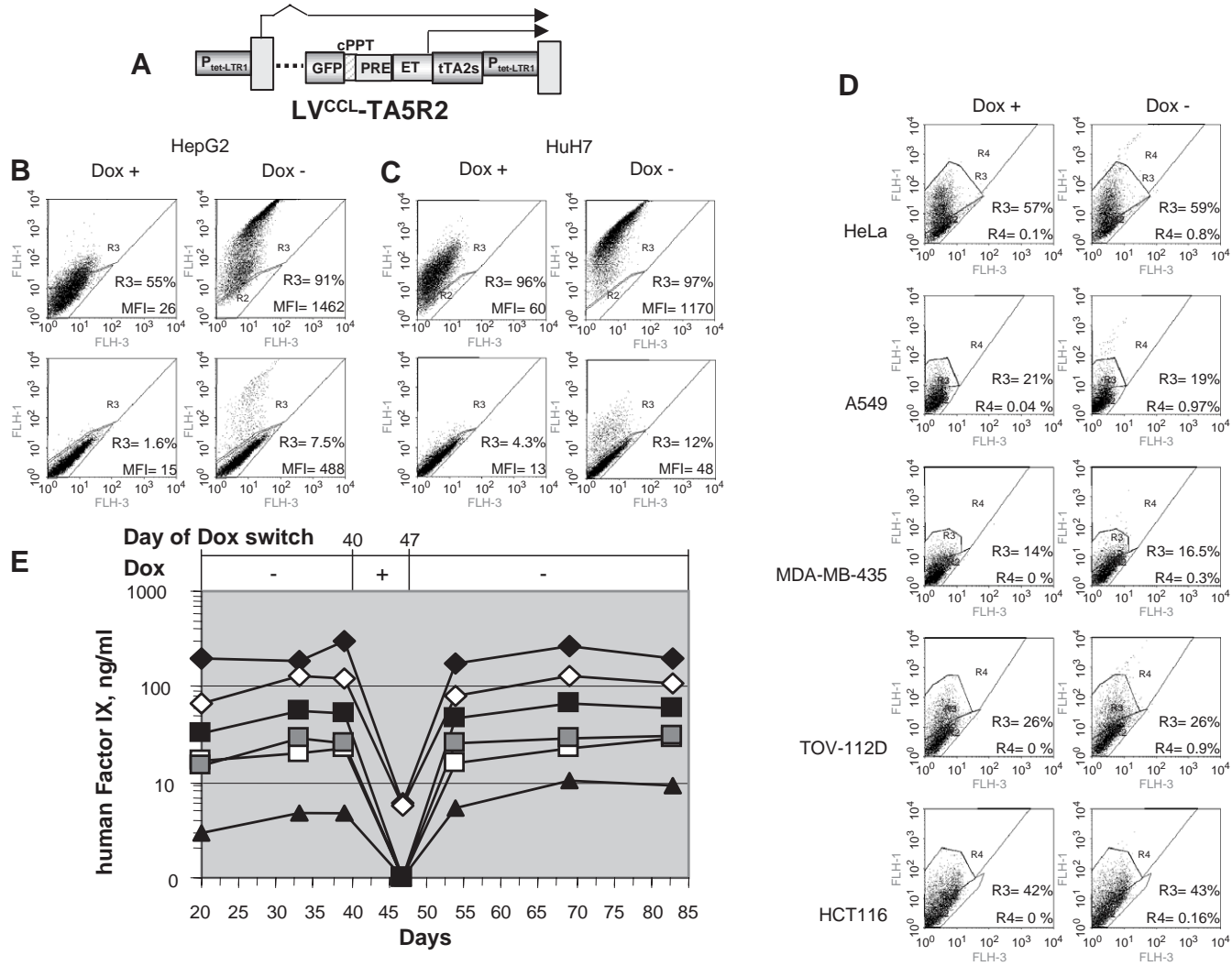
FIG. 6. Hepatocyte-specific self-regulating lentiviral vector. (A) Scheme of the proviral form of the vector; ET, synthetic hepatocyte-specific enhancer fused to the murine transthyretin promoter. Other abbreviations as in Fig. 1. (B) GFP expression in HepG2 cells transduced with 7 (top) or 0.2 ng/ml (bottom) p24 equivalent of LV^{CCL}-TA5/R2 vector and treated with Dox (10 ng/ml) or untreated. The percentage and MFI of GFP-positive cells, gated in R3, are indicated. (C) GFP expression in HuH7 cells transduced with 13 (top) or 0.13 ng/ml (bottom) p24 equivalent of LV^{CCL}-TA5/R2 and treated and analyzed as in B. (D) GFP expression in nonhepatic cell lines transduced by LV^{CCL}-TA5/R2, treated, and analyzed as in B. All cells were transduced with 4 ng/ml p24 equivalent of vector except for HeLa cells, for which 13 ng/ml was used. Representative data from two or three analyses performed with similar results. (E) Plasma levels of hF.IX in SCID mice after injection of 28 (diamonds), 14 (squares), or 7 μg (triangles) of p24 equivalents of LV^{CCL}-TA5/R2. Mice were treated with Dox (5 μg/ml) in the drinking water as indicated. Two control mice were injected with PBS and bled as the others; no hF.IX was detected in their plasma (not shown). Please note that a logarithmic scale was used to better visualize the response of all mice in the same graph.

regulating lentiviral vectors based on the Tet-dependent gene expression system, we have been able to overcome the limitations of previously reported constructs [7,10, 12,28].We have reached both robust expression, as shown by matching the frequency, level, and vector dose dependency of gene expression observed for vectors carrying the constitutive CMV promoter, and efficient regulation, as shown by suppressing expression by at least 30 times upon administration of tetracycline compounds. These substantial improvements were obtained without compromising the titer and infectivity of the vector, thus without limiting its potential applications. As expected from the high level of expression obtained by the new self-regulating vectors, a residual level of expression was detectable in the off condition. The possible consequence of this basal transgene expression should be taken into account if the new self-regulated vectors are to be used to express inducibly proteins that are toxic or active at very low concentrations.

Importantly, the new vector design appeared to be versatile, as the internal constitutive CMV promoter driving transactivator expression could be successfully replaced by a tissue-specific promoter. Using a self-regulating vector expressing tTA from a new hepatocyte-specific promoter, we could effectively limit transgene expression in the majority of nonhepatocyte cells and target high-level regulated expression to hepatocyte-derived cells, among the cell lines tested *in vitro*. Remarkably, this vector reached robust and Dox-regulated expression of hF.IX upon *in vivo* administration. The possibility of selectively targeting transgene expression to specific cell types may be crucial in some gene therapy applications in which widespread transgene expression may cause toxicity or other adverse effects.

Compared to other systems based on the delivery of more than one vector, the use of a single regulated vector provides several advantages. Not only does it allow a wider choice of target tissues and routes of administration, but it also improves the performance of the system. In fact, using a single vector, the presence of all components necessary to regulate transgene expression is ensured in all transduced cells, thus enabling effective induction. In contrast, if separate vectors are used, a fraction of the transduced cells will lack the transactivator. These cells will contribute only to the basal and not to the induced transgene expression level, thus decreasing the overall regulation index of the system. This feature is particularly relevant when delivering therapeutics, such as mitogens and cytokines, that exert biological activity at very low concentrations and may trigger adverse effects when expressed at high level and/or for prolonged times. In addition, single-vector delivery will limit the total number of integrations into the host genome.

The robust performance of the new self-regulating vectors allowed us to report for the first time to our knowledge effective long-term *in vivo* regulation of hF.IX expression upon systemic administration of a single gene therapy vector. We also showed that the hF.IX expression level could be adjusted to different set levels by varying the orally administered dose of a tetracycline compound. The switch-off kinetic after drug administration was rapid, while the recovery at the end of the drug treatment was slower but complete, and it could be shortened by decreasing the drug concentration used for suppression. Another study recently reported Tet-regulated hF.IX expression, although to progressively decreasing levels, in the first 2 months after injection of two separate adenoviral vectors [29]. Lentivirus- and adenovirus-based vectors are two gene delivery platforms that differ extensively in vector biology, transgene expression features, and host responses, thus representing complementary rather than alternative tools in the gene therapy field.

It is conceivable that the vector design developed in this work can also be used to deliver a Tet-on expression system, which in principle should provide a preferable pharmacokinetic profile for certain *in vivo* applications [2]. However, the rtTA molecules developed until now have shown some limitations in inducibility and Dox responsiveness [30]; thus the performance of a Tet-on self-regulating vector will have to be tested.

Robust transgene expression in the systemic circulation coupled to the possibility of switching expression off and on, and even to adjust the expression level, opens the way to gene-based delivery of therapeutics for which high systemic concentration must be reached in a tightly controlled manner. Further studies are needed to clarify whether such a powerful approach now available for the experimental study of biological therapeutics can also be exploited in the future for human gene therapy. In fact, early work raised concerns about the possible toxicity of the chimeric transactivators used in the Tet system, which contain powerful transactivation domains derived from viral proteins [31]. In agreement with more recent studies [30,32–34], the results reported here indicate stable long-term maintenance and expression of the protein, most likely due to the use of improved transactivator versions [35]. It remains likely, however, that administration of self-regulating lentiviral vectors to immunocompetent hosts will trigger an immune response directed against the foreign transgene products capable of clearing the transduced cells, as has been observed in several studies [17,21,36,37]. As recently reported, restricted transgene expression to hepatocytes may provide a strategy to alleviate such responses [17,38–40]. Further studies in immunocompetent mice using the new hepatocyte-targeted LV-TA5/R2 vector described here and an appropriate reporter gene will address such a crucial issue and help define the full potential impact of the advancement in vector design reported here.

MATERIALS AND METHODS

Plasmids and vectors. All self-regulating transfer vector plasmids were derived from the RRL-based plasmid LV-TA1/R2 [7], using standard molecular cloning techniques. Sequence information and cloning details are available upon request. The 5′ hybrid CCL LTR used to replace RRL in LV-TA4/R2 was previously described [26].

The Enh1mTTR (ET) promoter was generated by fusing a synthetic hepatocyte-specific enhancer to the murine transthyretin promoter [41]. The synthetic enhancer was generated by random ligation of synthetic oligonucleotides coding for binding sites of the following hepatic transcription factors: DPB, C/EBP, HNF1, HNF3, HNF4, and HNF6. The strategy of random assembly of transcription factor binding sites found in promoters active in a given tissue has previously been used to isolate muscle-specific control elements [42]. A 200- to 2000-bp size fraction of the ligation mixture was inserted into a lentiviral vector upstream of a pair of Sp1 binding sites and a minimal human albumin promoter (Accession No. M13075 (984–1083 bp)) driving the expression of GFP. Lentiviral vector from this library was used to transduce HuH7 cells, which were single-cell sorted for GFP expression. The transduction was performed at a low m.o.i., which favored single-copy integrants. After selecting for only the highest expressing clones, we isolated the synthetic elements by PCR and recloned them into the parental enhancerless vector (to ensure that high expression was not due to position effects of the initial lentiviral integration). Lentiviral vector was generated from individual clones, and the synthetic enhancers from vectors that continued to give high GFP expression in HuH7 cells were sequenced. The nucleotide sequence of the synthetic enhancer that gave the highest level of GFP expression was deposited with GenBank under Accession No. AY661265. This fragment was cloned into a lentiviral vector upstream of the murine transthyretin promoter (M19523 (100–109 bp) and M19524 (128–350 bp)) to generate pRRLsin.cPPT.ET.GFP. The ET promoter was then cloned into plasmid LV^{CCL}-TA4/R2 replacing the internal CMV promoter to generate LV^{CCL}-TA5/R2.

VSV-pseudotyped vector stocks were produced by transient transfection of the selected transfer plasmid, the packaging plasmids pMDLg/pRRE and pRSV.REV, and the VSV envelope plasmid pMD2.G (15, 6.5, 2.5, and 3.5 μg, respectively, for 10-cm dishes) in 293T cells as described [14], except that cells were grown in the presence of 10 ng/ml Dox and 1 mM Na butyric acid (both from Sigma). End-point expression titer was determined in HeLa cells by FACS analysis of GFP (FACSCalibur; Becton–Dickinson Immunocytometry Systems). Viral particle concentration was measured by HIV-1 p24 core profile ELISA (NEN Life Science Products).

Cells. Human HeLa, lung carcinoma A549, colon carcinoma HCT 116, ovarian carcinoma TOV-112D, and hepatocellular carcinoma HepG2 and HuH7 cells were from ATCC (Rockville, MD, USA); human breast carcinoma MDA-MB435 cells were from the Georgetown University Tissue Culture Shared Resource (Washington, DC, USA); HeLa and 293T cells were grown in IMDM, A549 and HCT 116 in RPMI, HepG2 and HuH7 in DMEM. TOV-112D cells were cultured using a 1:1 mixture of MCDB 105 medium and medium 199 plus 15% FBS (all from Sigma). All other culture media were supplemented with 10% FCS (Gibco BRL).

Northern blot analysis. RNA was isolated from 293T cells 48 h after transfection by RNAWiz lysis (Ambion), chloroform extraction, and isopropanol precipitation, and poly(A)$^+$ mRNA was purified using a mRNA isolation kit (Roche Molecular Biochemicals). Five micrograms of mRNA were separated in a 1.2% agarose–formaldehyde gel, capillary transferred to a nylon membrane (Hybond-N; Amersham), probed with a ^{32}P-labeled GFP or VSV-G sequence probe, washed in 0.1× SSC/0.1% SDS at 45°C, and exposed to X-ray film.

Animal experiments. SCID mice were purchased from Charles River Laboratories (Calco, Milan, Italy) and maintained under germ-free conditions. At 7 weeks of age, mice were injected with vectors encoding hF.IX or PBS (0.5 ml) in the tail vein. For Dox treatment, the drug was added to the drinking water plus 5% sucrose and changed twice weekly. All animal procedures were done according to protocols approved by

the Institutional Bioethical Committee and the Italian Ministry of Health.

Determination of hF.IX concentration. Human F.IX concentration was measured in mouse plasma from citrate-treated bleedings or in cell culture media collected 24 h after medium change and at least 3 days after transduction, using the Asserachrom IX:Ag ELISA kit (Boehringer Mannheim). The lower level of confidence for hF.IX detection was at a concentration of 3.2 ng/ml, as the lowest reliable concentration measured by the assay was 1.6 ng/ml and the minimal sample dilution in the test was twofold.

Quantification of vector DNA by real-time PCR. Vector copies per genome were quantified by real-time PCR from template DNA extracted from HeLa cells at least 15 days after transduction or from liver and spleen collected from mice immediately after lethal CO_2 inhalation, as previously described [17].

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