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ARTICLE

In Vitro and *in Vivo* Comparative Study of Chimeric Liver-Specific Promoters

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Targeting therapeutic genes to the liver is essential to improve gene therapy protocols of hepatic diseases and of some hereditary disorders. Transcriptional targeting can be achieved using liver-specific promoters. In this study we have made chimeric constructs combining promoter and enhancer regions of the albumin, α1-antitrypsin, hepatitis B virus core protein, and hemopexin genes. Tissue specificity, activity, and length of gene expression driven from these chimeric regulatory sequences have been analyzed in cultured cells from hepatic and nonhepatic origin as well as in mice livers and other organs. We have identified a collection of liver-specific promoters whose activities range from twofold to less than 1% of the CMV promoter in human hepatoma cells. We found that the best liver specificity was attained when both enhancer and promoter sequences of hepatic genes were combined. *In vivo* studies were performed to analyze promoter function during a period of 50 days after gene transfer to the mouse liver. We found that among the various chimeric constructs tested in this work, the α1-antitrypsin promoter alone or linked to the albumin or hepatitis B enhancers is the most potent in directing stable gene expression in liver cells.

Key Words: transcriptional targeting, gene therapy, liver-specific promoters, hydrodynamics-based transfection, polyethylenimine, long-term expression

INTRODUCTION

The liver fulfils a great variety of essential functions in the body, including the synthesis of proteins involved in metabolism, hemostasis, and protection against infection. Many genetic diseases, such as hemophilia A or B, ornithine transcarbamylase deficiency, familial hypercholesterolemia, or α1-antitrypsin deficiency, are associated with altered gene expression in the liver. Although some of these defects can be treated by infusion of the deficient protein, this is expensive, carries a risk of toxicity, and works only temporarily. A simple alternative could be the use of gene therapy to express a functional gene in the liver to replace a needed protein or to block the expression of an altered gene product. Transduction of hepatic cells with appropriate genes, such as immunostimulatory cytokines, can also be useful to induce immune responses against viral hepatitis or liver neoplasms [1,2].

Gene transfer into mammalian hepatocytes has been performed using both *ex vivo* and *in vivo* procedures. The *ex vivo* approach requires harvesting of the liver cells, *in vitro* transduction with long-term expression vectors, and reintroduction of the transduced hepatocytes into the portal circulation [3,4]. *In vivo* targeting has been done by injecting DNA or viral vectors into the liver parenchyma, hepatic artery, or portal vein. Adenoviral vectors, even when administered systemically, target mainly the liver in mice [5] but can also infect lung and skeletal muscle [6,7]. Moreover, the liver specificity of adenovirus has not yet been demonstrated in humans.

Another method to localize gene expression is by transcriptional targeting. The use of proper liver-specific transcriptional elements should restrict the expression of a therapeutic gene to hepatocytes. Some promoters that are active mainly in the liver have been already used for cell-specific gene delivery [8,9]. The major disadvantages for their use in gene therapy are (i) the big size, since many vectors have a restricted cloning space, and/or (ii) the low activity compared to cytomegalovirus (CMV) or long terminal repeat (LTR) promoter sequences, widely used in gene therapy protocols. In an attempt to construct promoters of high liver specificity and activity, we have selected hepatospecific transcriptional sequences using

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the following criteria: (i) short size, (ii) known regulatory factors, (iii) expression independent of tight metabolic or hormonal control, and (iv) non-species-specificity.

The liver promoters used in this study were from mouse albumin, human α1-antitrypsin, and hemopexin genes (Fig. 1A). The albumin is an abundant protein in the adult serum, it is mostly expressed in the liver and it is regulated at the transcriptional level [10]. The albumin promoter shows high homology between different species, including rat, chicken, frog, mouse, and human [11,12]. The human α1-antitrypsin (AAT) gene has been extensively characterized [13–15]. AAT is one of the major proteinase inhibitors in serum and it is synthesized mainly in hepatocytes, although it is also expressed by macrophages during inflammation [16]. Hepatocytes and macrophages transcribe the same coding region of the AAT gene from promoters that are 2 kb apart [17]. The hemopexin is a heme-binding plasma glycoprotein secreted only by the liver whose synthesis is increased during acute infections [18].

The enhancer regions employed in this work were the distal mouse albumin regulatory region and the enhancer II (EII) of the human hepatitis B virus (HBV) (Figs. 2A and 3). The far-upstream enhancer (−10.5 to −8.5 kb) of the albumin gene is required for full hepatocyte-specific expression in transgenic mice [19] and is able to stimulate promoter activity in cultured hepatocytes and in hepatoma cells [20,21]. The EII is responsible for the liver-specific transcription of pregenomic 3.5-kb-long RNA that codes for the core protein and serves as template for viral DNA synthesis [22,23]. This enhancer has been previously used to stimulate activity of the CMV minimal promoter (CMVm) in the context of adenoviral vectors [7]. Even if the hybrid promoter was as strong as the CMV whole regulatory sequence in mouse liver and EII-mediated enhancement was liver specific, expression in other tissues was still detected [7,24].

Our goal was to construct a collection of highly specific chimeric promoters that have different activities in hepatic cells, but low or no function in extrahepatic tissues. Adequate liver expression of a transgene can then be tuned by choosing the right enhancer–promoter combination. In this study we have compared activity and specificity of albumin, AAT, and hemopexin promoters, together with the nonspecific widely used SV40 and CMV promoters. We have analyzed the function and specificity of hepatic enhancers combined with specific and nonspecific promoter sequences in culture cells and in Balb/C and C57BL/6 mice. The broader range of activity and best specificity were achieved when both liver promoter and enhancers were combined. Some of these constructs were also able to conduct stable gene expression in a long-term study, making them good candidates to be used in gene therapy protocols.

RESULTS

Construction of Reporter Plasmids Containing the Liver Promoters: *In Vitro* Comparative Study

The 5′ flanking region of the albumin gene that confers promoter activity and liver-specific expression is limited to about 170 bp [10]. Deletions, mutations, and footprinting analysis with nuclear extracts or purified proteins have allowed the identification of transcription activators involved in promoter function (Fig. 1A) [11,25–27]. We cloned the region encompassing nucleotides −180 to +16 (relative to the cap site) of the mouse albumin gene (Palb) to drive luciferase expression in the reporter plasmid pGL3 basic. A tissue-specific element (TSE) required for liver function of the AAT promoter was identified between nucleotides −137 and −37. TSE is involved in positive and negative regulation in hepatic and nonhepatic cells, respectively [13,15,28]. The sequence from −261 to +44 contains a distal region (DRI) required for whole promoter activity. Both TSE and DRI were cloned in the pGL3 basic reporter vector (Pa1AT) (Fig. 1A). DNA binding sequences for nuclear proteins involved in hemopexin liver specificity and IL-6 induction have been identified in position −120 to −104 of the gene [29]. Sequence from −175 to +22 of the human hemopexin promoter, necessary to direct cell-specific transcription [18], was isolated and cloned in pGL3 basic vector (Phpx) (Fig. 1A). These pGL3 derivatives were used to transfect well-differentiated hepatoma and hepatocarcinoma cells from different origins: human HepG2 and Hep3B, mouse Hepa1-6, and rat H35. All of them express considerable levels of albumin, AAT, and hemopexin, as a guarantee of hepatocyte differentiation [13,30]. As negative controls we used human nonliver HeLa and HEK293 cells, which do not express these genes [13,31]. To control transfection efficiency and cell recovery, which may vary between experiments and cell lines, we cotransfected the pGL3 derivatives with a plasmid that expresses *Renilla* luciferase under the control of the SV40 promoter and all the results have been corrected for *Renilla* luciferase expression (see Materials and Methods). We have also compared the activities of the liver promoters with those from the strong CMV promoter (Fig. 1B). To determine the effects of the enhancers on the universal CMV and early SV40 promoters, we studied the expression from both, with (CMV and SV40) and without (CMVm and SV40m) their enhancer regions.

CMV was the strongest promoter in all cell types, showing an expression 4- to 40-fold higher than SV40. As expected, promoter strength decreased when the minimal CMV and SV40 promoters were used, but expression levels were well above background. This reduction was dependent both on the promoter and on the cells, from 3 to 12 times in the case of SV40 or 14- to 80-fold for CMV. Unlike CMV and SV40 promoters, which were active in cells from hepatic and nonhepatic origin, Palb, Phpx, and

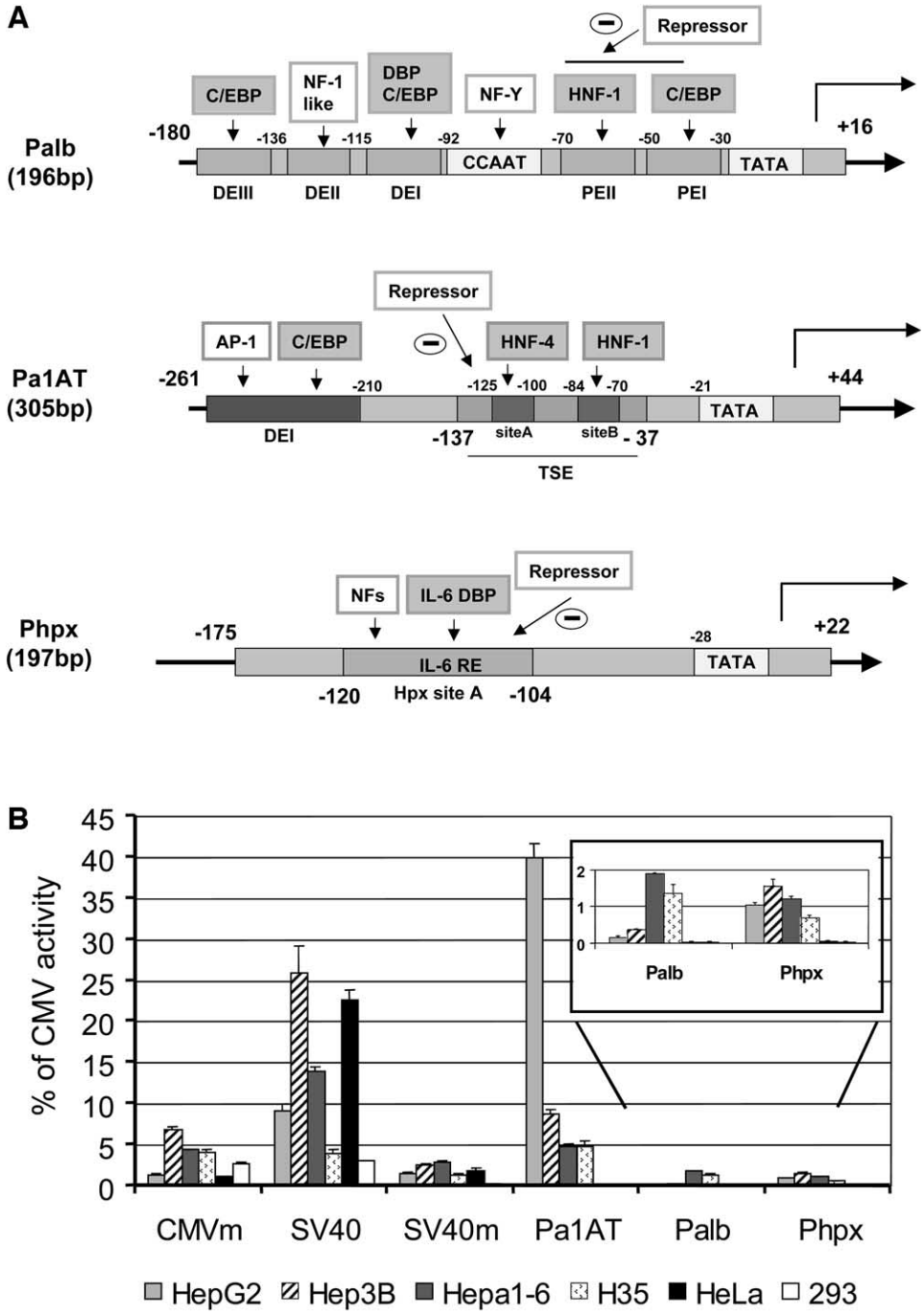

FIG. 1. Activity of universal and liver-specific promoters (A) Schematic structure of the liver promoters. Specific (DBP, D-site binding protein; C/EBP, CCAAT enhancer binding protein α or β; HNF, hepatocyte nuclear factor; IL-6DBP, liver-specific IL-6-dependent DNA binding protein) and nonspecific (NF-1, nuclear factor 1; NF-Y, CAAT binding protein; AP-1) activating transcription factors that bind to each regulatory sequence are indicated for the albumin, Palb [26]; α1-antitrypsin, Pa1AT [14,15]; and hemopexin, Phpx [18,29] promoters. Binding regions of putative repressor factors present in nonhepatic cells are depicted [11,18,28]. Coordinates with respect to the cap site and size (base pairs) of each sequence are indicated. Regulatory elements are shown: DE, distal element; PE, proximal element; TSE, tissue-specific element; IL-6RE, interleukin-6-response element; TATA box. (B) Promoter activity in liver and nonliver cells. Hepatic (HepG2, Hep3B, Hepa1-6, H35) and nonhepatic (HeLa, HEK293) cell lines were transiently transfected with plasmids containing the indicated promoters. Luciferase expression from the liver-specific promoters was compared to the expression driven by the immediate-early cytomegalovirus promoter (CMV), early SV40 promoter, and CMVm and SV40m, which lack enhancer regions. The mean (two independent experiments) of luciferase expression relative to the maximum (100%) CMV activity is represented. The amount of luciferase for CMV promoter was 1965.22 ± 42.74, 1033.60 ± 139.89, 585.90 ± 8.72, 18.24 ± 0.56, 2112.12 ± 78.17, and 9427.15 ± 505.91 for HepG2, Hep3B, Hepa1-6, H35, HeLa, and 293 cells, respectively. Values are expressed as means ± SD. An amplified view of the activity of Palb and Phpx is shown in the small box.

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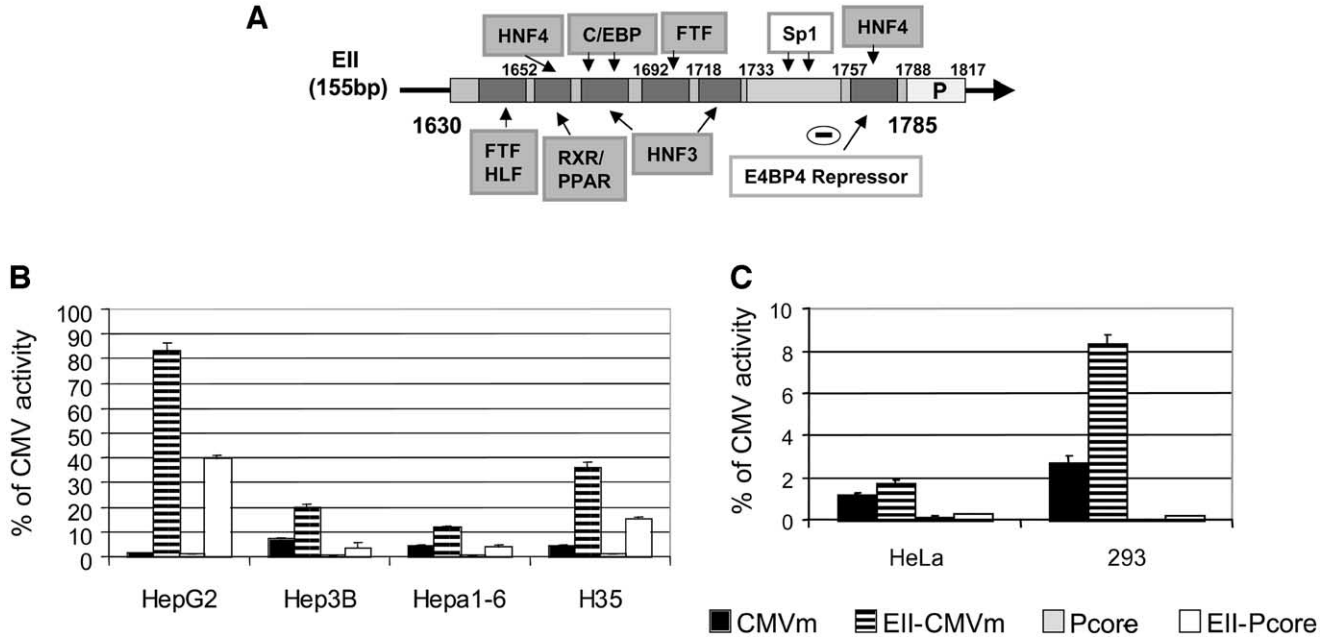


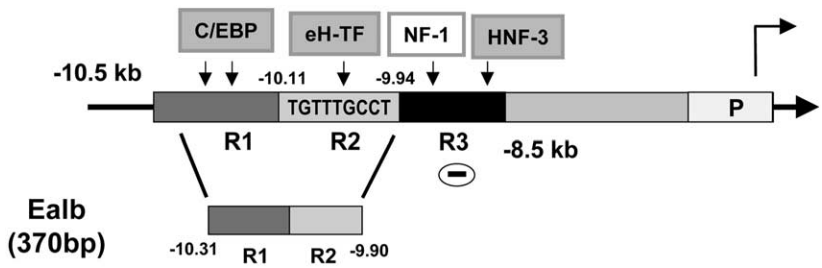
FIG. 2. Influence of hepatitis B enhancer II on promoter activity. (A) The EII region is shown. Binding regions for hepatic (C/EBP; HNFs; HLF, hepatic leukemia factor; FTF, fetoprotein transcription factor; RXRα–PPARα, retinoid X receptor α–peroxisome proliferator-activated receptor α heterodimer) and ubiquitous (Sp1) transcription factors are indicated. These activating factors are required for core protein transcription and pregenomic RNA synthesis [32,33]. The transcriptional suppressor E4BP4 binding site is indicated. This protein is present mainly in nonhepatic cells [49]. P, core promoter. Coordinates are given according to Cheng *et al.* [34]. (B) The activity of Pcore and CMVm promoters with (EIICMVm, EIIPcore) or without enhancer II sequence was analyzed in liver cells. (C) Luciferase expression from the same promoters was analyzed in nonliver cells. The percentage with respect to CMV activity is represented and the absolute value of luciferase expression driven by the CMV promoter is given in the legend to Fig. 1.

Pa1AT were functional only in liver cells. Of the three liver-specific promoters tested, Pa1AT was the strongest, yielding almost 40% of CMV activity in HepG2 cells. Of note, as shown in the small box of Fig. 1B, Palb and Phpx exhibit a marked liver specificity although the expression in hepatic cells is low, even lower than the minimal nonspecific promoters (CMVm and SV40m).

Effect of the EII Liver-Specific Sequence in the Enhancement of Hepatic and Universal Promoters

The HBV EII enhancer has been well characterized by site-directed mutagenesis and was limited to a 155-bp fragment [22,23,32,33] (Fig. 2A). Löser and co-workers [24] used a construct composed of the EII plus the promoter of viral core protein of HBV (two elements that are adjacent in the viral genome [34,35]) and showed that these regulatory sequences were able to augment the activity of the universal CMVm promoter in liver cells. This construct showed, however, some nonspecific activity in cells from nonliver origin [7,24]. To determine whether this nonspecificity was due to the EII element or to the CMVm sequence, we have made several promoter constructs to compare their activity in different cells. Two reporter plasmids were prepared, one containing only the 29-bp core promoter (Pcore) and the other containing EII fused to Pcore (EIIPcore). We also generated a vector link-

FIG. 3. The mouse albumin enhancer (Ealb). This sequence contains three functional regions (R1–R3). Liver-specific transcription factors (eH-TF, C/EBP, and HNF-3) and NF-1 binding places are indicated [20,50]. R3 acts as a negative element [20], hence it was removed from our construct (short fragment). Coordinates with respect to the transcriptional start point (arrow) are shown. P, albumin promoter.



ing EII directly to CMVm (EIICMVm). All these constructs, together with CMV and CMVm, were analyzed for luciferase expression. Results are shown in Figs. 2B and 2C. We observed that EII was able to markedly enhance CMVm activity in liver cells (up to 58 times in HepG2 cells) and also to a lesser extent in nonhepatic cells (up to fourfold). Activity of Pcore alone was liver specific but very low. EII was also able to stimulate the function of Pcore in hepatic human cells from 5.4 (Hep3B) to 106 (HepG2) times (Fig. 2B), but did not affect expression in nonliver cells (Fig. 2C). From these results we conclude that promoter enhancement using EII can be achieved without loss of specificity. Moreover, both the promoter and the enhancer sequences need to be liver specific to reduce maximally extrahepatic gene expression.

Construction of Chimeric Liver-Specific Promoters

To increase gene expression levels in hepatic tissue we decided to use strong liver-specific promoters bound to specific enhancers. The far-upstream mouse albumin gene enhancer (Ealb) contains both positive and negative regulatory regions and the DNA binding sites for liver-specific factors [19,20]. We used the positive regions of 370-bp-long (Fig. 3) for the design of new promoter constructs. We have fused Ealb or EII with the three liver promoters shown in Fig. 1 (Palb, Pa1AT, and Phpx) in all the possible combinations (Fig. 4A). The activity relative to CMV of each chimera or of promoters alone is represented in Fig. 4B. Western blot analysis to detect the luciferase protein driven by each construct in HepG2 cells has been carried out from the most representative samples to visualize and confirm the luciferase activity observed (Fig. 4C). The results show that both enhancers work in liver cells, but not in HeLa or 293 cells. EII was able to stimulate expression from all three promoters: Palb was enhanced up to 253-fold, Phpx up to 16-fold, and Pa1AT up to 6.2-fold. On the other hand, Pa1AT was the only promoter able to be enhanced by the albumin distal sequence, suggesting a minimum requirement of promoter activity for the function of Ealb. In contrast to Pa1AT, Palb and Phpx were practically unmodified by Ealb both in hepatic and in nonliver cells. Interestingly, Ealb–Pa1AT fusion was less active than Pa1AT alone in 293 and HeLa cells, suggesting a possible role for inhibitors acting on this enhancer in nonhepatic cells (Fig. 4B, small box).

In summary, if we order these promoter–enhancer sequences in a decrescent order of expression, the strongest would be EII-Pa1AT, then Ealb-Pa1AT and EII-Palb followed by EII-Phpx, Pa1AT, and Phpx. In all cases, the level of expression in nonliver cells driven by them was very low, indicating that combining liver transcriptional regulatory sequences is a good strategy for engineering liver-specific promoters. Different expression levels observed in the hepatic cells of various species could be due to varying amounts of required transcription factors, as well as to the presence or absence of other factors (i.e., p53) that could influence gene expression. Additionally, promoter activity could be affected by the degree of differentiation of each cell type [25,36].

***In Vivo* Study of the Activity of Chimeric Promoters in the Liver and Duration of Gene Expression Driven by These Regulatory Sequences**

Administration of naked plasmid DNA by the hydrodynamics-based system has been demonstrated to be an efficient and rapid method for *in vivo* comparative studies [37,38]. With this procedure and using 20 μg of a β-galactosidase reporter plasmid we have confirmed a transfection efficiency between 10 and 20% (data not shown), similar to that previously reported by other authors [37,39,40]. In our study we have used this methodology to analyze the expression driven by each of the most representative promoters: EII-Pa1AT, Ealb-Pa1AT, EII-Palb, EII-Phpx, Pa1AT, Ealb-Phpx, and Phpx, together with the CMV and EIICMVm that served as controls. We injected plasmids into Balb/C mice, prepared liver extracts, and measured luciferase activity. To ensure that DNA uptake was comparable in all cases, we isolated total DNA from livers of treated animals and detected plasmid DNA by Southern blot analysis (Fig. 5A). Similar amounts of DNA were observed in most of the samples obtained from three representative mice. Differences, likely attributable to animal-to-animal variation or manipulation, were comparable in all groups of animals. According to the luciferase expression data obtained 6 h postinjection of the plasmids (Fig. 5B), we could divide the assayed promoters into three statistically significant groups: (i) the highly active, represented by CMV, EII-Pa1AT, EII-CMVm, and Ealb-Pa1AT; (ii) the moderately active, EII-Palb, EII-Phpx, and Pa1AT ($P < 0.05$); and (iii) the poorly active, Ealb-Phpx and Phpx ($P < 0.001$). In all cases, even in the last group, a considerable level of gene expression was observed. These *in vivo* data are in good agreement with the results observed in culture cells (see above).

Duration of promoter activity was then tested in C57BL/6 mice using the AAT gene as reporter. Serum levels of AAT driven by each tested promoter were measured every 2 to 7 days (Fig. 5C). During the steady state (after the initial upsurge of gene expression following hydrodynamic administration of the plasmids) Phpx activity decreased rapidly with time, while Pa1AT alone or combined with EII and Ealb was more stable. These three sequences, which were the most powerful in the long-term study, allowed the obtainment, at day 50, of serum levels of the secretable reporter protein of 70, 30, and 20 μg/ml with Ealb-Pa1AT, EII-Pa1AT, and Pa1AT, respectively. Although initially EII-Pa1AT showed the highest activity, after 3 weeks it tended to decrease, to reach values similar to those of Pa1AT alone, suggesting a possible inactivation of the EII viral enhancer.

Interestingly, EII-Phpx activity decreased about 20 times between days 1 and 16 but remained stable until

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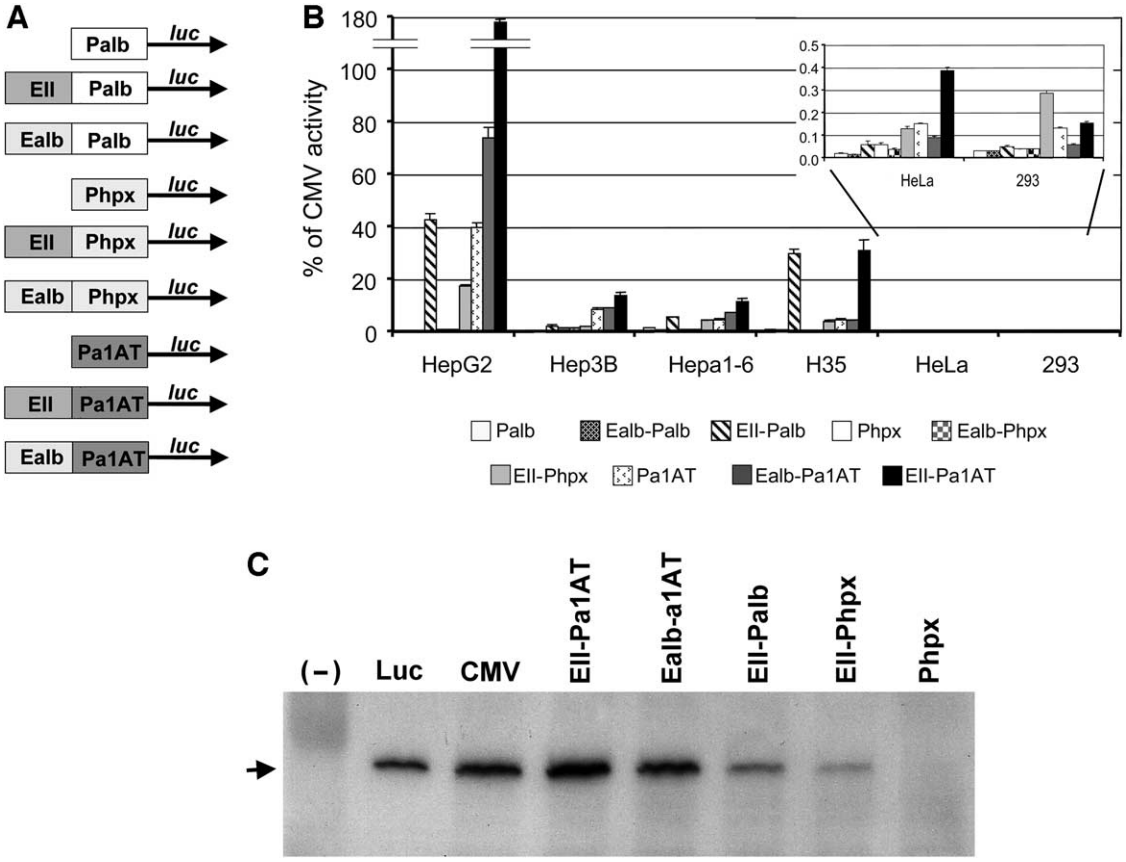

FIG. 4. Activity and specificity of chimeric promoters. (A) Constructed promoter/enhancer fusions. Albumin (Palb), α1-antitrypsin (Pa1AT), and hemopexin (Phpx) promoter sequences have been fused to EII and Ealb regions in all possible combinations to drive luciferase expression. (B) Liver and nonliver cells were transiently transfected with the indicated plasmids. Means ± SD of luciferase expression driven from the chimeric promoters relative to CMV activity (100%) are represented. The values of ng luciferase/mg total cell protein obtained with the CMV promoter in each cell line are given in the legend to Fig. 1. An amplified view of promoter activity in HeLa and HEK293 cells is shown in the small box. (C) Detection of luciferase protein in HepG2 cells. Cells were transfected as above and luciferase detection was performed by Western blot analysis. Luc, pure luciferase (2 ng) and (−) nontransfected cells. Arrow indicates the position of luciferase protein.

day 50, indicating that in this promoter context the EII sequence was active and was able to stabilize hpx promoter function. EII-Palb showed activity and long-term behavior almost identical to those of EII-Phpx (not shown). With respect to the CMV promoter we observed a progressive diminution of its activity with time (Fig. 5C). The values of the secretable reporter protein at day 50 (about 2 μg/ml) were comparable for these three sequences (CMV, EII-Palb, and EII-Phpx).

Analysis of the Activity of Chimeric Promoters in Extrahepatic Tissues

To test promoter specificity, we next studied the activity of the regulatory sequences in organs other than the liver. Transgene expression using the hydrodynamics-based gene transfer method has been described in lung, spleen, kidney, and heart [39] and thus these organs were extracted and analyzed for luciferase activity. Low levels of expression were observed with each liver-specific construct compared to the CMV promoter (Table 1). CMV was more active in kidney than in the three other organs, had similar activity in lung and spleen, and produced little expression in heart. The activity observed with EII-CMVm was 10 times less than with CMV in heart, but only 3 times less in spleen. Combinations of liver-specific enhancers and promoters yielded activities close to background levels, making it difficult to differentiate from the basal expression coming from the pGL3basic vector alone (not shown). In general, we found that the strength of a promoter in the liver is proportional to its extrahepatic functionality.

Since hydrodynamics gene delivery is more effective in transfecting hepatocytes than other tissues [39] we decided to use polyethylenimine (PEI) as vector to deliver

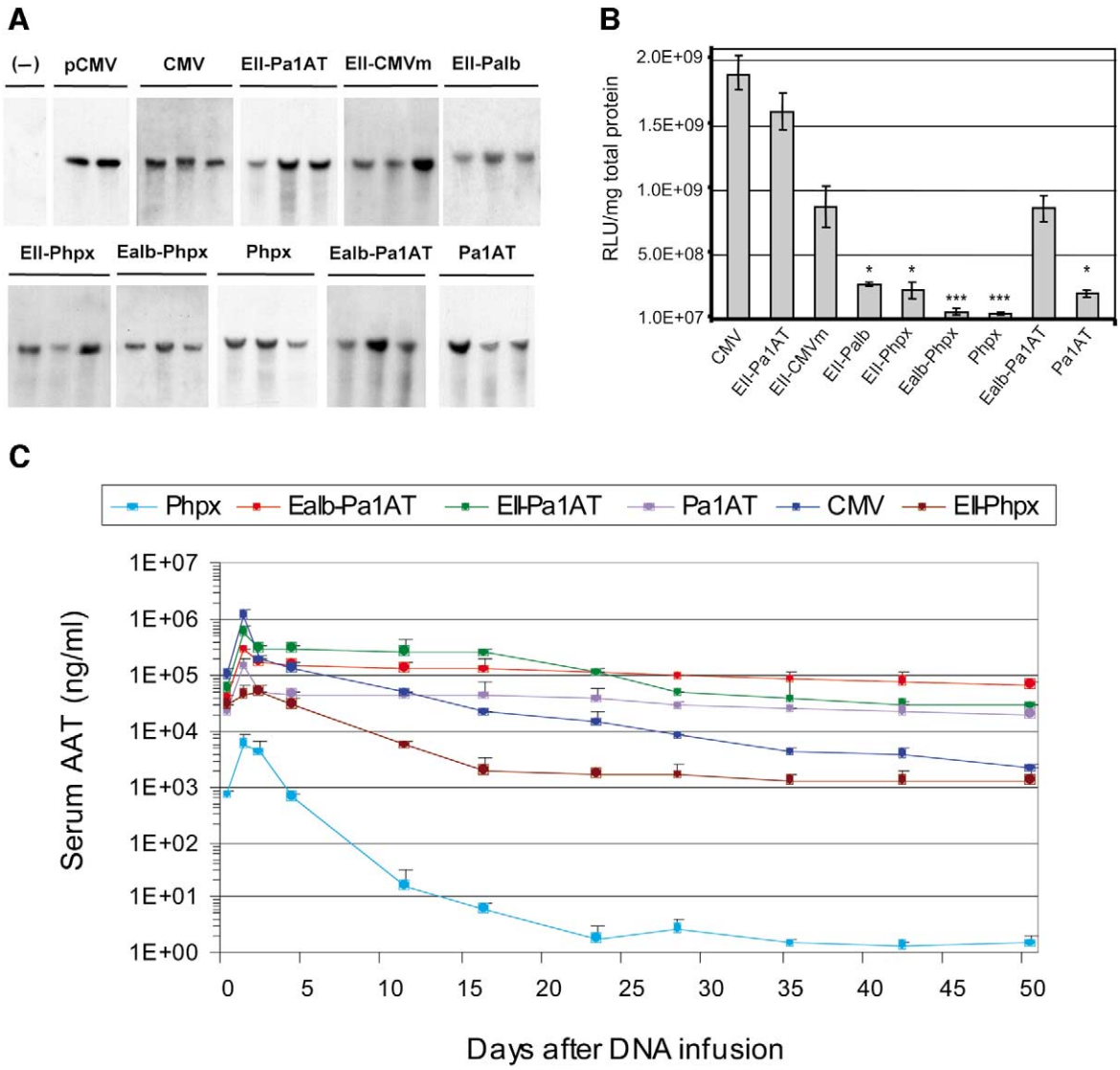

FIG. 5. *In vivo* activity of chimeric promoters. (A) 20 μg of plasmids containing the selected promoters was transferred into Balb/C mice ($n = 4–6$) by hydrodynamics intravenous injection. Total DNA was recovered from livers of untreated (−) and treated mice 6 h postinjection and digested with *Kpn*I. 15 μg of DNA was then loaded on an agarose gel and plasmid bands were detected by Southern blot. 10 ng of CMV-luc plasmid (pCMV) was used as positive control. (B) Luciferase activity (relative light units) was measured in the same liver extracts. Mean ± SD of each experiment is represented. Statistical analysis performed with SPSS version 9.0 software showed significant differences between the groups $*P < 0.05$ and $***P < 0.001$. (C) Long-term expression study. Plasmids containing the AAT gene driven by the indicated promoters were administrated to C57BL/6 mice ($n = 5$). Blood samples were collected at the indicated days and concentration of AAT in serum was determined. Day 0 corresponds to 6 h after DNA injection.

the plasmid constructs into the lung [41]. Southern blot analysis of lung extracts demonstrated similar plasmid uptake efficiency with both CMV and EII-Pa1AT (Fig. 6A). As shown in Fig. 6B, luciferase expression in the lung from all chimeric promoters was three orders of magnitude lower than from CMV. This low activity was similar to that observed in the previous experiment (Table 1). This indicates that when we achieve a high *in vivo* transfection efficiency of extrahepatic tissues, the specificity of liver-specific promoters is manifested more clearly. Incidentally, we observed, in agreement with other reports [24], that EIICMVm, which manifested the highest activity in all nonhepatic organs analyzed, was only 20 times less active than CMV in lung (Fig. 6B).

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TABLE 1: Extrahepatic activity of chimeric promoters

	Kidney		Lung		Heart		Spleen	
	RLU luc/mg prot[a]	%CMV[b]	RLU luc/mg prot[a]	%CMV[b]	RLU luc/mg prot[a]	%CMV[b]	RLU luc/mg prot[a]	%CMV[b]
CMV	$8.98 \times 10^5 \pm 4.73 \times 10^4$	100.00	$7.20 \times 10^4 \pm 8.98 \times 10^3$	100.00	$3.30 \times 10^4 \pm 6.29 \times 10^3$	100.00	$4.43 \times 10^4 \pm 1.08 \times 10^4$	100.00
EII-Pa1AT	$1.11 \times 10^4 \pm 4.50 \times 10^3$	1.24	$4.18 \times 10^3 \pm 1.26 \times 10^3$	5.81	$3.18 \times 10^3 \pm 1.08 \times 10^3$	9.63	$7.61 \times 10^3 \pm 2.52 \times 10^3$	17.19
Ealb-Pa1AT	$1.03 \times 10^4 \pm 2.22 \times 10^3$	1.15	$1.83 \times 10^3 \pm 2.84 \times 10^2$	2.54	$2.68 \times 10^3 \pm 1.57 \times 10^2$	8.11	$5.05 \times 10^3 \pm 4.42 \times 10^2$	11.41
EII-Phpx	$1.10 \times 10^4 \pm 5.45 \times 10^3$	1.23	$2.21 \times 10^3 \pm 5.86 \times 10^2$	3.07	$7.19 \times 10^2 \pm 3.86 \times 10^2$	2.18	$1.51 \times 10^3 \pm 2.97 \times 10^1$	3.42
EII-Palb	$1.08 \times 10^4 \pm 6.40 \times 10^3$	1.20	$1.35 \times 10^3 \pm 6.20 \times 10^2$	1.88	$9.99 \times 10^2 \pm 4.97 \times 10^2$	3.03	$1.11 \times 10^3 \pm 7.52 \times 10^2$	2.50
EII-CMVm	$1.20 \times 10^5 \pm 3.19 \times 10^4$	13.40	$1.85 \times 10^4 \pm 3.09 \times 10^3$	25.75	$3.41 \times 10^3 \pm 1.93 \times 10^3$	10.31	$1.32 \times 10^4 \pm 7.92 \times 10^2$	29.93

The plasmids containing the indicated promoters were delivered to mice by the hydrodynamic procedure. Kidney, lung, heart, and spleen were harvested 6 h after DNA infusion and luciferase activity was determined in the organ extracts.
[a]Relative light units of luciferase per milligram of total cell proteins.
[b]Percentage of chimeric promoter activity relative to CMV (promoter and enhancer).

DISCUSSION

Restriction to the liver of the synthesis of adequate amounts of a therapeutic protein may be advantageous for the effective treatment of inherited or acquired diseases. The fact that the liver produces a great variety of essential proteins that are not synthesized by other organs or tissues indicates the existence of a strict regulation of such genes like albumin, α1-antitrypsin, and hemopexin. In the present work we have selected the transcriptional regulatory sequences of these genes in an attempt to define a set of liver-specific promoters with different activities for application in gene therapy protocols.

We observed that the *in vitro* expression driven by each promoter alone was specific but substantially lower than that of the ubiquitously active CMV promoter and enhancer, which is commonly used to construct gene therapy vectors. To increase the strength of the liver-specific promoters we produced a collection of chimeric sequences by combining these promoters with enhancers from the albumin and hepatitis B virus genes. Our data allowed us to categorize the resulting promoters into three groups: (i) highly active, which are capable of driving expression at levels similar to that of CMV sequence; (ii) moderately active, being about 5-fold lower than CMV; and (iii) weakly active, which can drive expression at levels 25-fold less than CMV. However, when we analyzed *in vivo* the long-term expression of a reporter gene driven by these constructs we found that the hierarchy of the promoter strength changed due to a decrease in the activity of CMV, while most of the liver-specific constructs direct stable gene expression possibly because of their constitutive function. The analysis of extrahepatic activity of these promoters showed that all of them exhibited marked liver specificity.

We found that Pa1AT constitutes a strong liver-specific promoter, a finding that has been previously reported [42]. However, in the context of retroviral plasmid, a similar α1-antitrypsin promoter region has demonstrated only a weak activity [43], an observation that could be due to interference by retroviral LTR *in vivo* and/or to the particular transgene used by the authors. From our data Ealb-Pa1AT, EII-Pa1AT, and Pa1AT are the best regulatory sequences to ensure a constant and high level of gene expression in the liver. These promoters appear to be more suitable for this purpose than EIICMVm, a construct

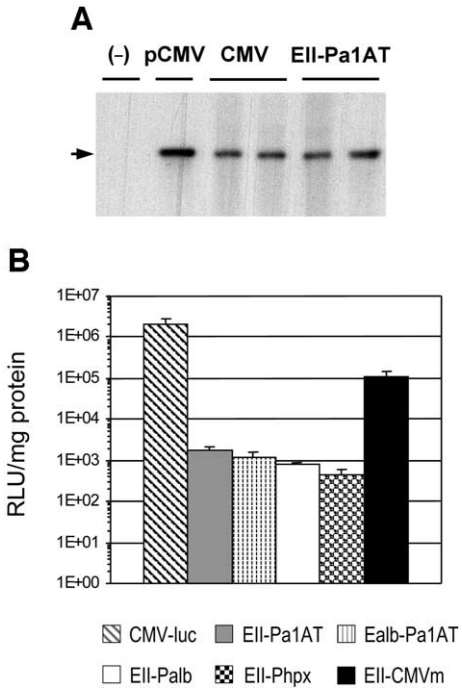

FIG. 6. Extrahepatic activity of chimeric promoters. (A) 25 μg of plasmids containing the indicated promoters was transferred to mice ($n = 4$) in a PEI/glucose solution. EII-Pa1AT and CMV were used as representative samples to monitor transfection of the lung. 15 μg of DNA extracted from lungs was loaded on an agarose gel and plasmid bands were detected by Southern blot. 10 ng of CMV-luc (pCMV) plasmid digested with *Kpn*I was carried as positive control. (−) Untreated animal. Arrow indicates the position of linear plasmid DNA. (B) Luciferase (RLU) activity was measured in the same lung extracts after PEI-mediated transfection with the indicated promoter-containing plasmids.

that, although it has been proposed as a good candidate to direct gene expression to the hepatic tissue, shows less specificity than the above-mentioned sequences due to the presence of the CMVm element (Fig. 2 and [7]).

Our data indicate that the mouse albumin enhancer, which has been differently reported as very potent or poorly active [27,44], was active only when combined with a strong promoter like Pa1AT and not with Palb or Phpx. The lack of enhancement on these two promoters could be due to the fact that in our constructs it was inserted right upstream of the promoter, a position that is different from the distant situation that it has in the original gene [20]. With respect to the viral EII, its activity changes in the context of different promoters since it seems to stabilize Phpx (which has low activity and shuts down after 2 weeks) but does not increase the potency at the steady state of Pa1AT, which is very stable by itself.

It has been hypothesized that viral promoters are not adequate to direct long-term gene expression in the liver because of *in vivo* shutdown as a result of DNA methylation processes or because of the presence of transcription factor binding sites whose cognate proteins are not expressed in the quiescent liver [3,45]. Moreover, some cytokines like interferon α or γ are capable of inhibiting the CMV promoter [46], and consequently CMV transcriptional regulatory sequences would not be appropriate to construct vectors encoding these cytokines or products that activate their synthesis. In our hands, the activity of CMV decays until day 50, an observation that has been previously reported by Zhang *et al.* [37] using the same animal strain, method for transfection of liver cells, and reporter gene. However, in the mentioned work stabilization at a low level of activity was observed after 60 days up to 6 months [37].

In summary, our work has identified a number of liver-specific promoter sequences that may be valuable tools for targeting heterologous genes to the liver when adequate levels of therapeutic proteins need to be produced.

MATERIALS AND METHODS

Plasmid construction. Mouse albumin enhancer and promoter fragments were isolated by PCR amplification of mouse genomic liver DNA according to the method of Arrigo *et al.* [47] and inserted into the pRcCMV*luc* vector (pRcCMV backbone, Invitrogen Life Technologies, + luciferase gene (*Hin*dIII–*Sal*I) from pGEM*luc,* Promega, Madison, WI). Primers used to detect the albumin enhancer were ATCGATAGATCTTCTGCTTCTCTCAGT and CCGCGGTCCCCGTGTACTCAT. Primers used for promoter detection were CCGCGGACAGCTCCAGATGGCAA and AAGCTTAGTGGGGTTGATAGGA. The 754-bp enhancer and the 206-bp promoter fragments were purified from 2% low-melting agarose gel, treated with *Sst*II, ligated, and redigested with *Bgl*II and *Hin*dIII. The CMV promoter was removed from pRcCMV*luc* with the same enzymes and substituted by the albumin 951-bp enhancer/promoter fragment, resulting in the pRcALB*luc* vector. To keep the same backbone in all constructs, promoter and enhancer sequences were isolated by PCR or enzymatic digestion from the original plasmids and reinserted into the multicloning site of a promoterless pGL3 basic vector (Promega) to drive firefly luciferase expression. DNA amplification was performed with 2.5 units of *Pfu* (Stratagene, La Jolla, CA), 1 μg of template, and 1 μl of each 20 μM oligo in a total volume of 100 μl. All inserted sequences were verified by DNA sequencing using RV3 and GL2 primers (Promega). Mouse albumin enhancer (Ealb) and promoter (Palb) were amplified from pRcALB*luc* using oligos TCCCCTCGAGGTTCCTAGATTACAT, CATACCAGATCTACAGCCACATACT and GCGGAGATCTCCAGATGGCAAACA, CGTTACTAGTGGATCCGAGCT, respectively. Human α1-antitrypsin (Pa1AT) and hemopexin (Phpx) promoters were isolated from plasmids pα1AT305 and pHpx526, respectively [15,18]. Oligos for Pa1AT amplification were GAGATCTGTACCCGCCACCCCCT and GCCAAGCTTTTCACTGTCCCAGG. Oligos for Phpx isolation were GGGGCAGATCTCGGGAAAAAGGAGTCT and CTCCCATTTTAAGCTTCCTTAGCT. The 227-bp EIIPcore *Sma*I–*Xho*I fragment and HBV enhancer II region (EII) were isolated from pBSCP plasmid [7]. For EII amplification primers GGACGTCGACCTGAGGTAATTA and CAGCCTCGAGGTACAAAGACCTTTAAC were used. The 29-bp Pcore fragment was constructed by hybridization of two complementary oligos: 5′-GGG**CATAAATTGGTCTGC**GCACCAGCACCAA. Nucleotides sufficient for initiation of both precore and pregenomic RNA synthesis are indicated in boldface [34]. The human CMV promoter–enhancer region spanning position +75 to position −675 was used as control [48]. The 104-bp minimum CMV promoter (position −53 to +51) was isolated from pEIICMVm [7] using CGCGCTCGAGGTAGGCGTGTACGGT and CCGAGATCTTCTATGGAGGTCAAA oligos. Created restriction sites are underlined. pGL3 promoter and pGL3 control plasmids (Promega) contain the early SV40 promoter (SV40m) or the promoter/enhancer region (SV40), respectively. Plasmids containing the human α1-antitrypsin reporter gene were obtained by substitution of the *Hin*dIII–*Bam*HI luciferase poly(A) sequence in the pGL3-derived vectors by the *Hin*dIII–*Xho*I AAT poly(A) fragment of plasmid pRSV.hAAT.bpA [38].

Cell cultures, DNA transfections, and luciferase assays. Cells were obtained from ATCC and ECACC and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, penicillin G (100 U/ml), streptomycin (100 μg/ml), and 2 mM L-glutamine at 37°C in 5% CO_2/air. Reagents were purchased from Gibco BRL (Paisley, Scotland). Transient transfections were performed by the calcium phosphate precipitation method starting from approximately 2×10^5 cells/well in 12-well dishes with 1 μg of each DNA. Plasmids were purified with the Concert Nucleic Acid Purification System (Gibco BRL). In all cases, 20 ng of a plasmid that has the *Renilla* luciferase gene driven by the SV40 promoter (pRL-SV40; Promega) was included in the assay to monitor transfection efficiency. After 32 h, cells were washed with PBS and harvested in 250 μl of Passive Lysis Buffer (PLB; Promega). Firefly and *Renilla* luciferase activity was measured in a Berthold Lumat LB 9507 luminometer from 20 μl of lysate using the Dual-Luciferase Reporter Assay System (Promega). The standard curve equation obtained with purified luciferase (Promega) was generally ng luc $= 4 \times 10^{-8} \times$ RLU $- 0.0005$ ($R^2 = 0.9952$). Total protein concentration was calculated using the Bio-Rad protein assay. The standard curve equation with BSA (Sigma, Steinheim, Germany) was generally μg protein $= 9.5157 \times OD_{570\ nm} - 3.7505$ ($R^2 = 0.9902$).

In vivo gene delivery and determination of luciferase expression in the liver and other organs. Plasmid DNAs were delivered into mice organs by the hydrodynamics method [39,40]. Four to six Balb/C females were used in each group. Twenty micrograms of DNA in 1.6 ml saline was intravenously injected in a time range of 6 to 7 s. Six hours later mice were sacrificed and liver, lung, kidney, spleen, and heart were extracted and mechanically homogenized with an Ultra-Turrax T25 in 1.5 ml (liver) or 0.8 ml (other organs) of PLB (Promega). Samples (0.3 ml) were centrifuged at 14,000 rpm for 10 min at 4°C and luciferase activity was measured in concentrated or diluted supernatants. For the long-term study, plasmids containing the human α1-antitrypsin reporter were purified with the Endotoxin Free Maxi Kit (Qiagen, Hilden, Germany) and administrated to C57BL/6 mice (five females per group) as before. Serum levels of AAT were measured by ELISA [37]. Transfer to the lung of DNA complexed to 22-kDa linear PEI was performed by intravenous injection of 400 μl of a solution containing 5% glucose, 25 μg of DNA, and 7.5 μl of 100 mM PEI (amine/phosphate ratio 10). Four Balb/C mice (7- to 8-week-old females) per group

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were used. Eight hours after injection, animals were sacrificed and luciferase was measured in lungs and other organs.

Southern blot analysis. Homogenized organs (0.5 ml) were mixed with 1 volume of 2× buffer (10 mM Tris HCl, pH 8, 20 mM EDTA, 2% SDS) with proteinase K (0.1 mg/ml) and incubated at 55°C for 5 h. The mixture was then treated three times with phenol:chloroform and DNA was precipitated with 3 M sodium acetate–ethanol and resuspended in 200 μl of deionized water. Fifteen micrograms of total DNA was loaded on a 0.7% agarose gel after treatment with *Kpn*I and RNase A (25 ng/μl). Denatured DNA was transferred to a nylon membrane (Hybond-N+; Amersham Pharmacia Biotech, Ireland) and bands were detected after hybridization of a 1.7-kb-long digoxigenin–dUTP PCR labeled probe (25 ng/ml) performed with the PCR DIG Labeling Mix (Roche, Basel, Switzerland), using a sheep anti-DIG-AP diluted 1:10,000 and the CSPD reagent (Roche). Oligos for probe preparation were AACATAAAGAAAGGCCCGC and CACTGCATTCTAGTTGTGG, which pair with pGL3 plasmid sequences.

Western blot analysis. Transfected HepG2 cells were lysed as described above and 7.5 μg of protein was loaded on a 12% SDS–polyacrylamide gel in Laemmli sample buffer. Proteins were separated by electrophoresis and transferred onto a Hybond-P membrane (Amersham Pharmacia Biotech, England). The membrane was blocked overnight at 4°C in BL buffer (PBS, 0.05% Tween, 5% nonfat milk) and incubated with 1:1500 diluted goat anti-firefly luciferase polyclonal antibody (Promega) followed by 1:2000 secondary anti-goat IgG–HRP (Sigma, St. Louis, MO) reaction. Immunoreactive proteins were visualized on exposed Hyperfilm ECL (Amersham) using the Western Lighting Chemiluminescence Reagent (Perkin–Elmer, Zaventem, Belgium).

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