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Evaluation of Gene Promoters For Liver Expression By Hydrodynamic Gene Transfer

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Abstract

Gene therapy represents a promising treatment for hepatic disease. Most approaches today utilize viral methods to target tissues. While non-viral gene therapy is less prominent, hydrodynamic gene delivery represents a promising approach to direct gene expression to the liver. The purpose of the present study was to evaluate promoters for efficient gene expression in hepatocytes *in vivo* by hydrodynamic delivery and to test the findings in a model of hemophilia A.

Materials and Methods—Human cytomegalovirus (hCMV), chicken beta-actin/CMV enhancer (CAG), elongation factor-1alpha (EF1 α), and phosphoglycerokinase (PGK) promoters were subcloned into plasmids with a luciferase reporter gene. *In vitro* calcium phosphate-mediated transfection of 2×10^5 HEK 293 cells was followed by *in vivo* whole animal bioluminescence and luminometry after hydrodynamic tail vein injection of plasmid DNA. Six-month-old FVB factor VIII-deficient mice were similarly injected with CBA- or EF1 α -promoted constructs containing the factor VIII heavy and light chains and expression was examined.

Results—*In vitro* transfection demonstrated a hierarchy of expression: hCMV-intron > CAG > EF1 α > hCMV > PGK. *In vivo* luminometry demonstrated that the CAG construct produced 2.6x, 3.0x, 3.4x, and >1000x the expression of the hCMV-intron, EF1 α , hCMV, and PGK constructs respectively. Factor VIII (FVIII) plasmid injected hemophilic mice demonstrated higher levels of factor VIII expression with CAG versus EF1 α , confirming the reporter gene studies. All FVIII-deficient mice injected with EF1 α -FVIII or CAG-FVIII plasmids survived after tail clipping.

Conclusions—The CAG promoter/enhancer combination is an excellent alternative to the human CMV promoter for hydrodynamic gene delivery to the liver.

Introduction

Significant advances in gene therapy have been made due to a better understanding of the mechanisms of disease, the improvement of gene delivery systems, both viral and non-viral, and the discovery and improvement of tissue-specific gene regulatory sequences. However, at present, viral-based gene therapy is limited by the development of adaptive immune responses to viral capsid proteins, abrogating the effect of therapy. In addition, certain viral vectors used in gene therapy are not without risk. Some (e.g. retrovirus) integrate semirandomly with a

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preference for actively transcribing genes [1] making them potentially mutagenic. In addition, integration of retroviral vectors in proximity to cellular proto-oncogenes may lead to transactivation through the action of the enhancer/promoter elements in the long terminal repeat [2]. This has occurred in the clinical setting in two of three patients who developed leukemia-like proliferations after retroviral gene therapy for X-linked severe combined immune deficiency [3,4].

Non-viral gene therapies are attractive as these methods lack viral proteins; in general, this greatly reduces the antigenicity of gene delivery and expression. Such methods can be divided into two categories: physical (carrier-free gene delivery) and chemical approaches [5]. Physical methods involve the forcing of plasmid DNA into cells through one of multiple methods: needle injection, electroporation, gene gun, ultrasound, or hydrodynamic delivery. Chemical methods utilize lipid or polymer carriers that complex with DNA to deliver the transgene into cells. [5].

Hydrodynamic gene administration is an attractive option for non-viral gene delivery to the liver. This method has been demonstrated to produce gene expression primarily in liver due to its unique architecture [6]. The methodology of this approach is currently being investigated in large animal models [7]. Experiments in gene therapy have demonstrated that certain regulatory elements lead to higher levels of gene expression. The human CMV promoter is generally considered one of the best promoters for high-level gene expression in tissues of interest. However, several studies have demonstrated that the CMV promoter may be silenced or turned off in hepatocytes [8,9]. As a first step toward producing a better expression cassette for hydrodynamic gene delivery and expression in the liver, we compared several commonly used constitutive mammalian promoters with the CMV promoter to find an alternative that has not been reported to be silenced. We included three housekeeping gene promoters, one as a promoter/enhancer combination. In addition, we examined whether the inclusion of an intron 5' to the cDNA of our luciferase reporter potentiates hepatic expression, as seen in hepatocytes cultured *in vitro* [10]. Finally, we tested our findings in a clinically relevant model, the murine model of hemophilia A, a disease affecting factor VIII production.

Materials and Methods

Animal Procedures

All handling of animals was performed in accordance with the University of California, Los Angeles (UCLA) Chancellor's Committee for Animal Research guidelines. Eight to 12 week old FVB mice were purchased from Charles River Breeding Laboratory (Wilmington, MA) and used for the reporter gene plasmid injections. Mice carrying targeted deletions of exon 16 of the FVIII gene have been described [11] and were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were crossed seven times onto the FVB strain to generate congenic hemophilic animals. These mice were used for the factor VIII plasmid studies at 6 months of age. All of the mice were housed under pathogen-free conditions and food and water were provided *ad libitum*.

Genotyping of Animals

A three-primer PCR was performed to identify hemophilic affected (X_HX_H , X_HY), carrier (X_HX), and wild-type (XX , XY) animals. Genomic DNA was first prepared from tail tips by routine methods and denatured for 5 minutes before amplification. 2.5 μ l of genomic DNA was used for the PCR reactions which were performed by standard methods. A common reverse primer (GAGCAAATTCCTGTACTGA) was used with the forward sequence primer (TGTGTCCCGCCCCCTTCCTTT) to identify the disrupted FVIII gene, as well as with the second forward sequence TGTGTCCCGCCCCCTTCCTTT used to identify the wildtype allele.

All primers were synthesized by Invitrogen (Carlsbad, CA). A 35 cycle PCR amplification (94°C for 30s, 61.2°C for 30s, 72 for 60s) was performed with a two-minute 72°C elongation step upon completion of the cycles.

Construction of Reporter and Factor VIII Plasmids

The factor VIII cDNA was obtained by reverse transcriptase PCR from RNA prepared from human liver. Poly(A) RNA was primed with an oligo (dT) primer, and first-strand synthesis was catalyzed by SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) at 42°C. The cDNA was then amplified with two specific oligonucleotides and Pfu DNA polymerase (Stratagene, La Jolla, CA). Sequencing was performed to confirm integrity by comparing with GENBANK. The DNA sequences encoding the heavy and light chains of human FVIII were constructed as described [12]. The luciferase cDNA was obtained by its excision from pGL3-enhancer plasmid (Promega, Madison WI) at NheI and XbaI.

Diagrams of the plasmid constructs are shown in Figure 1. Most of the plasmids were constructed using the pAAV-hrGFP plasmid (Stratagene, La Jolla, CA). All restriction enzymes were purchased from New England Biolabs (Ipswich, MA). The hCMV-intron-luciferase plasmid was constructed by digesting the pAAV-hrGFP at EcoRI and XhoI to excise the hrGFP cDNA. The luciferase cDNA was inserted through blunt-blunt cloning methods.

The phosphoglycerokinase (PGK) promoter was a gift of Lily Wu (UCLA). It was released from pRRL-U6siLuc-PGK-eGFP at BamHI and XhoI and subcloned into pBluescript (Stratagene, La Jolla, CA) at XhoI and ClaI. PGL3-enhancer plasmid (Promega, Madison, WI) was digested at MluI, subsequently blunted and then digested at BamHI to release luciferase and the SV40 poly A tail. This fragment was then subcloned into pBluescript PGK at EcoRV and BamHI, sites 3' to the PGK promoter.

The hCMV-luciferase plasmid was constructed by digesting pSSV9 (provided by R.J. Samulski, University of North Carolina) at XbaI. pCEP4 (Invitrogen) was digested at SalI releasing a fragment containing the hCMV promoter-multicloning site-SV40 polyadenylation signal. This sequence was subsequently inserted into pSSV9 by blunt end cloning methods followed by insertion of the luciferase cDNA in the multicloning site.

The chick β -actin/CMV enhancer (CAG)-luciferase plasmid was derived by first excising the hCMV promoter at MluI and SacII from pAAV-hrGFP. The CAG promoter was excised at AccI and ApaI from pturboCre (provided by M. Jiang, UCLA). The CAG promoter was inserted and ligated by blunt-blunt cloning procedures to create the intermediate plasmid CAG-hrGFP. The GFP cDNA was then excised at XhoI and EcoRI; in its place, the luciferase cDNA was inserted by blunt-blunt methods.

To construct the EF1 α -luciferase plasmid vector, pSSV9 was digested at XbaI and a fragment containing the EF1 α promoter, multicloning site, and growth hormone polyA was excised from pEF/myc/nuc/GFP (Invitrogen) at KpnI and SrfI and inserted by blunt end ligation. The GFP-nuclear targeting signal-myc cDNA was excised at PstI and XbaI and luciferase was inserted by blunt end ligation. The factor VIII heavy and light chains were similarly inserted.

The intermediate cloning plasmid CAG-hrGFP was used to create both the CAG-FVIII heavy chain and CAG-FVIII light chain plasmids. The CAG-hrGFP plasmid was digested at restriction sites BstEII and EcoRI to remove the hrGFP cDNA as well as the hGH PA tail. The heavy and light chains of factor VIII and their accompanying hGH polyA tail were amplified by PCR using Platinum Taq Hi-Fidelity DNA Polymerase (Invitrogen, Carlsbad, CA) and sequence specific primers. The PCR products were blunted and cloned into the intermediate cloning plasmid.

Plasmid Injection

Plasmid DNA was recovered by alkaline lysis and subsequently purified by an anion exchange resin per manufacturer's instruction. (Qiagen, Valencia, CA). Plasmid DNA was injected by hydrodynamic technique as previously described [13,14]. In brief, 15 µg of endotoxin-free plasmid DNA was prepared in 2 ml of sterile pharmaceutical grade saline at room temperature. Mice were restrained and the lateral tail vein was accessed with a 21 gauge needle (Tyco Healthcare Group, Mansfield, MA). Administration of the solution was performed in 10 seconds or less without extravasation; each group was represented by three or more animals.

Bioluminescent Imaging

Twenty hours after plasmid injection, mice were placed on a warming table and administered luciferase substrate (D-luciferin, 150 mg/kg IP). Five to 10 minutes later the isoflurane anesthetized mice were imaged using the bioluminescence optical imager (IVIS 200; Xenogen, Alameda, CA). Mice were immediately placed in a light-tight chamber and baseline gray-scale body-surface images were taken. Afterwards, photons emitted from the firefly luciferase reaction within the animal were acquired. Maximal luciferase signals were semiquantified using Living Image 2.5 (Xenogen) and IGOR (Wavemetrics, Lake Oswego, OR) image analysis softwares.

Assays for Luciferase Activity

In vitro transfection was performed utilizing HEK 293 cells (Stratagene, La Jolla, CA) in duplicate. 2×10^5 cells were plated in 6-well plates in DMEM supplemented with 10% bovine growth serum. Twenty-four hours later, cells were transfected in duplicate by calcium phosphate-mediated transfection. One day after transfection, cells were lysed and analyzed for luciferase expression by luminometry. The assay was performed in triplicate and recorded as relative light units (RLU).

Plasmid-injected mice were euthanized by isoflurane overdose followed by cervical dislocation. The lower right portion of the anterior right lobe of the liver was placed in flat bottom 2.0 ml small capped tubes (USA Scientific, Ocala, FL) with 500 µl of luciferase lysis buffer (Promega, Madison, WI) and placed on ice. Tissues were homogenized with a hand-held homogenizer (Omni International, Marietta, GA). Specimens were centrifuged at 13,200 rpm for 5 minutes and then placed at 4°C. A Sirius Luminometer (Berthold Detection Systems, Oak Ridge, TN) was used to measure total light emission according to the manufacturer's instructions. All samples were measured for 10 seconds. Luciferase levels were determined in triplicate by luminometry and recorded as relative light units (RLU).

Protein assay

Samples were centrifuged at 13,200 rpm for 5 minutes and dilutions prepared in 96-well plates. A Lowry-based protein assay was performed (Bio-Rad, Hercules, CA) and specimens were analyzed on a microplate reader (BioTek, Winooski, VT) at 750 nm. Samples were performed in duplicate and compared with protein standards. Mean and standard error of the mean (SEM) were calculated. Luciferase levels by luminometry were normalized after protein concentrations of the liver samples were determined. Luciferase activity was graphed for each plasmid as RLU per microgram of tissue protein to normalize the results for differences in tissue sample mass.

Clotting Assays

Determination of factor VIII levels was performed in duplicate by sandwich ELISA per manufacturer's instructions (Enzyme Research Laboratories, South Bend, IN) (n=3 per group). Functional factor VIII was assessed by sterile clipping of the tip of the mouse tail under

isoflurane anesthesia. Mice were monitored for the development of clot or persistent bleeding (n=4 per group).

Statistics

All data was reported as mean $\pm$ SEM. Statistical evaluation was performed using the two-tailed t-test. Differences were considered significant at $p < 0.05$.

Results

In Vitro HEK293 Studies

Plasmids containing the luciferase reporter gene and a promoter (hCMV, hCMV-intron, CAG, EF1 α , or PGK) were used for *in vitro* transfection of human embryonic kidney 293 cells (figure 2). Each transfection used equal amounts of plasmid. *In vitro* studies demonstrated that each of the promoters produced biologically active luciferase detectable by luminescence in comparison with a control non-luciferase plasmid (GFP). The hCMV-intron ($8.5 \times 10^{10} \pm 5.1 \times 10^9$ RLU) and CAG constructs ($7.8 \times 10^{10} \pm 7.9 \times 10^9$ RLU) produced the highest levels of luminescence and statistically there was no difference between these two groups ($p=0.13$). *In vitro* luciferase expression was greatly reduced with the EF1 α promoter ($5.3 \times 10^{10} \pm 4.5 \times 10^9$ RLU), which showed 63% of the hCMV-intron expression. This was followed by the hCMV vector ($4.3 \times 10^{10} \pm 3.4 \times 10^9$ RLU), whose performance was 50% of the hCMV-intron plasmid. The PGK ($1.5 \times 10^9 \pm 6.5 \times 10^7$ RLU) vector showed only marginal activity over control as its expression was only 2% of the maximally detected expression of the hCMV-intron vector. With the exception of the CAG promoter, all other constructs had statistically less expression than the hCMV-intron plasmid ($p < 0.0001$).

In Vivo Bioluminescent Imaging

Twenty hours after hydrodynamic injection, animals were imaged by whole animal bioluminescent imaging. The results demonstrate that each luciferase plasmid could produce visible amounts of bioluminescence in the liver (figure 3). The CAG plasmid produced the highest levels of bioluminescence with $1.7 \times 10^{10} \pm 1.1 \times 10^{10}$ photons emitted per second (p/sec) through the region of interest (figure 4). This was followed by the EF1 α ($4.3 \times 10^9 \pm 2.2 \times 10^9$ p/sec), hCMV-intron ($2.1 \times 10^9 \pm 1.7 \times 10^9$ p/sec), and hCMV ($1.1 \times 10^9 \pm 6.8 \times 10^8$ p/sec) plasmids, which produced 25%, 12%, and 6% of the maximally detected emission, respectively. While the EF1 α vector produced larger amounts of light expression than hCMV-intron, the difference was not statistically significant. The differences between EF1 α and hCMV-intron and between hCMV-intron and hCMV were also not statistically significant. The PGK promoter produced the smallest expression of the group ($5.0 \times 10^7 \pm 4.9 \times 10^7$ p/sec) with only 0.3% of the light emission when compared with the CAG promoter.

Whole Tissue Luminometry

Initial *in vivo* bioluminescent results were followed by liver-specific luminometry studies where liver tissue was homogenized and analyzed for luciferase expression. Results were normalized for overall liver protein. The basic expression profile was highly similar to and in general correlated with the whole animal bioluminescent studies. The CAG vector produced the highest levels of expression, $4.4 \times 10^5 \pm 1.6 \times 10^5$ RLU/ μ g of liver protein. The hCMV-intron vector demonstrated expression levels that was 39% of the CAG vector ($1.7 \times 10^5 \pm 7.7 \times 10^4$ RLU/ μ g protein). This difference was statistically significant ($p=0.02$). The EF1 α plasmid produced 33% of the maximally detected luminometry levels of CAG ($1.5 \times 10^5 \pm 9.9 \times 10^4$ RLU/ μ g protein) followed by hCMV ($1.3 \times 10^5 \pm 6.6 \times 10^4$ RLU/ μ g protein) at 29% of the maximally detected levels. These were all statistically lower than the CAG plasmid ($p < 0.0001$). The PGK vector produced luciferase levels more than a thousand-times less than CAG

($1.9 \times 10^2 \pm 1.8 \times 10^2$ RLU/ μ g protein). The *in vivo* liver-based luminometry is the most accurate assessment of promoter efficiency in the liver as the expression of luciferase is directly measured in the tissue. These results suggest that the CAG vector is the most effective construct for high-level constitutive expression in the liver.

In Vivo FVIII Production

Plasmids containing the factor VIII (FVIII) heavy or FVIII light chain were constructed with either the CAG promoter or the EF1 α promoter. Affected hemophiliac mice were then administered, by hydrodynamic injection, either the CAG-FVIII heavy/light combination, EF1 α -FVIII heavy/light combination, or saline as a control. Both the CAG and EF1 α -injected mice produced detectable levels of FVIII; the CAG promoted vectors produced levels 27.1% of normal while the EF1 α vectors produced levels at 20.6% of normal (figure 6). Saline controls exhibited no FVIII production (table 1).

To confirm that the factor VIII produced was in fact functional in correcting the abnormality of hemophilia A, factor VIII knock-out mice underwent hydrodynamic injection with either the EF1 α -FVIII combination, the CAG-FVIII combination, or saline (n=4/group). They were challenged by tail-clipping 16 hours after injection. Fifteen hours later, all 4 mice in both the EF1 α and CAG group were active and demonstrated no tail bleeding. In contrast, saline-injected control mice experienced continued blood loss and subsequently expired.

Discussion

Gene therapy is defined as the transfer of genetic material to treat disease or improve the clinical status of patients [15]. The potential for treating disease is boundless. Unfortunately, none of the present gene therapy vector systems is ideal and of these, the viral vector have often been affected by the host innate and adaptive immune systems resulting in abrogation of expression.

Hydrodynamic gene delivery to the liver, a non-viral method, was discovered 10 years ago when large volumes of DNA solutions were rapidly infused into the tail vein of mice resulting in high levels of gene expression in the liver. More recently, this technique has been modified for use in a large animal model [7]. Injection volumes usually used are about 10% of body weight, and to be effective, must be delivered rapidly. Swelling of the liver by the infusion, with short-term disruption of the plasma membrane of the hepatocyte, is likely a very important mechanism for DNA entry into hepatocytes [16]. Efficacy is in part thought to be related to the high permeability of the hepatic sinusoidal endothelium, which is thin and easily disrupted, allowing for DNA to access the parenchyma which is in close proximity [17]. With time, these close and DNA is trapped inside the cells.

CMV is one of the strongest viral promoters *in vitro*. Unfortunately, *in vivo* the CMV promoter has been silenced at the mRNA level [18] within several weeks in multiple organ systems [9, 19, 20]. Other studies have demonstrated that when combined with other regulatory elements, the CMV promoter is not silenced in liver and [21] or other tissues [19]. Using more efficient and non-silenced promoters is desirable. Therefore, we chose to test the CMV promoter, the CMV promoter with globin intron, the CMV chicken β -actin hybrid promoter with the globin intron (CAG), the PGK promoter, and the EF1 α promoter in mouse liver. We compared the activity of the luciferase reporter gene produced by the different promoters with the high-expressing CMV promoter. In addition, we then chose to test two of these vector plasmids that contain promoters that are not silenced (CAG with the highest expression by reporter gene studies, and EF1 α) in a clinical model of a human disease that affects the liver, hemophilia A.

The data demonstrate that the CAG promoter produces high levels of reporter gene both *in vivo* and *in vitro*. *In vitro* analysis demonstrated that the CAG and hCMV-intron promoters

were superior to EF1 α , hCMV, and PGK promoters. The *in vivo* analysis largely matched this assessment. The bioluminescent imaging data demonstrated that CAG was the highest expressing plasmid, followed by EF1 α , hCMV-intron, hCMV, and PGK. Bioluminescent imaging also confirmed the liver as the primary site of luciferase expression by hydrodynamic gene delivery. In addition, the data obtained from the noninvasive bioluminescent imaging largely paralleled the expression detected by direct hepatic luminometry where the CAG sequence performed significantly better than the other promoters. In the hemophilia A mouse, a murine model of a human disease affecting the liver, the CAG-promoted-FVIII expression produced higher levels of FVIII than the EF1 α -FVIII construct. Phenotypic correction was obtained demonstrating the production of biologically active FVIII.

Strategies for improving gene expression are important as vectors are developed for gene therapy. Strong non-silenced promoters will result in enhanced gene expression necessary for gene therapy to be successful. Our data show that adding a 3' intron to the CMV promoter does increase expression both *in vitro* and *in vivo*. Combining hydrodynamic injection with bioluminescent imaging for luciferase results in a powerful noninvasive tool to evaluate gene therapy promoters and other regulatory elements without having to euthanize animals. Our data demonstrate that this method correlates well with organ expression by whole tissue luminometry.

In conclusion, the CMV chicken β -actin hybrid promoter with the globin intron was more efficient than the human CMV promoter in the liver. This promoter may be beneficial in increasing gene expression in hydrodynamic and other methods of gene transfer for liver diseases such as those of secretory proteins (e.g. hemophilia A, hemophilia B, α 1-antitrypsin deficiency) or of the urea cycle. Each would be well served by achieving fairly high levels of transgene expression in the liver. Further investigation of other regulatory elements to prolong expression (e.g. OriP/EBNA-1) are underway.

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Figure 1.

Diagram of vector constructs, to scale; from top to bottom are displayed the hCMV-intron, hCMV, CAG, EF1 α , and PGK plasmids used for reporter gene studies. Below these are the CAG and EF1 α factor VIII heavy chain/light chain vectors used for *in vivo* factor VIII studies.

Figure 2. Luciferase expression (16 hours after transfection) in HEK 293 cells, shown according to plasmid. The relative light units (RLU) are plotted on the y axis. Experimental groups represent the average of experiments performed in duplicate. The line above each bar indicates the SEM.

Figure 3. Expression of hepatic luciferase production by bioluminescent imaging, 20 hours after hydrodynamic intravenous injection of 15 μ g of plasmid vector. The pseudocolor scale is at the right with red indicating regions of highest expression and purple-blue indicate areas of lowest expression. Units are photons/sec/cm²/steradian.

Figure 4.

Expression of hepatic luciferase production by measurement of light emission 20 hours after hydrodynamic intravenous injection of 15 µg of plasmid DNA. Light emission is detected through a 1 cm diameter region of interest (ROI) centered over the area of highest expression. Photons per second through the ROI is plotted on the y axis. The line above each bar indicates the SEM.

Figure 5. Expression of hepatic luciferase by tissue luminometry 20 hours after hydrodynamic intravenous injection of 15 μg of plasmid DNA. RLU/μg protein is plotted on the y axis. Each group represented three or more animals. The line above each bar indicates the SEM.

Figure 6. Comparison of factor VIII production by CAG and EF1 α promoters 24 hours after hydrodynamic intravenous injection of 15 μ g of factor VIII light chain and 15 μ g of factor VIII heavy chain plasmid vector. Each group represents three animals. Percent of normal human concentration of factor VIII is plotted on the y axis. The line above each bar indicates the SEM.

Table 1

Survival of animals after tail clipping 15 hours after hydrodynamic intravenous injection of 15 µg of plasmid DNA. Each group represents four animals.

	CAG-HVIII Injected	EF1α-HFVIII Injected	Saline-Injected
Number of Mice Injected	4	4	4
Number Alive at 15 hours	4 (100%)	4 (100%)	0 (0%)