



# Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA

F Liu, YK Song and D Liu

Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15261, USA

Development of methods that allow an efficient expression of exogenous genes in animals would provide tools for gene function studies, treatment of diseases and for obtaining gene products. Therefore, we have developed a hydrodynamics-based procedure for expressing transgenes in mice by systemic administration of plasmid DNA. Using cDNA of luciferase and  $\beta$ -galactosidase as a reporter gene, we demonstrated that an efficient gene transfer and expression can be achieved by a rapid injection of a large volume of DNA solution into animals via the tail vein. Among the organs expressing the transgene, the liver showed the highest level of gene expression. As high as 45  $\mu$ g of luciferase protein per gram of liver can be achieved

by a single tail vein injection of 5  $\mu$ g of plasmid DNA into a mouse. Histochemical analysis using  $\beta$ -galactosidase gene as a reporter reveals that approximately 40% of hepatocytes express the transgene. The time–response curve shows that the level of transgene expression in the liver reaches the peak level in approximately 8 h after injection and decreases thereafter. The peak level of gene expression can be regained by repeated injection of plasmid DNA. These results suggest that a simple, convenient and efficient method has been developed and which can be used as an effective means for studying gene function, gene regulation and molecular pathophysiology through gene transfer, as well as for expressing proteins in animals.

**Keywords:** transfection; gene therapy; plasmid DNA; gene expression; hydrodynamics-based transfection

## Introduction

The ability to introduce genes into tissues of an animal and to generate sufficient amounts of gene product is critical for the advancement of several areas of study in molecular biology, gene function and regulation, protein structure and function, gene therapy as well as biotechnology in general. Such ability becomes even more important considering the fast pace at which the human genome and other gene cloning projects discover new genes. Transgene expression *in vivo* for gene function studies or for therapeutic purposes will require methods that allow for efficient gene transfer into cells.

The most intensely studied method for the introduction of exogenous genes into animal cells utilizes virus as a carrier.<sup>1–11</sup> The most commonly used viral carriers include retrovirus,<sup>2,3</sup> adenovirus,<sup>4,5</sup> adeno-associated virus,<sup>6,7</sup> herpes virus<sup>8</sup> and others.<sup>9–11</sup> While fairly efficient in transferring genes into the target cells, viral carriers usually require laborious procedures for preparation and purification. In addition, there are concerns about the possibility for recombination with endogenous virus to produce a deleteriously infectious form.<sup>12–14</sup> Some viral carriers induce an immune response and cause side-effects that render the repeated administration problematic.<sup>15,16</sup> Therefore, efforts have been made recently to develop alternative approaches for gene transfer.

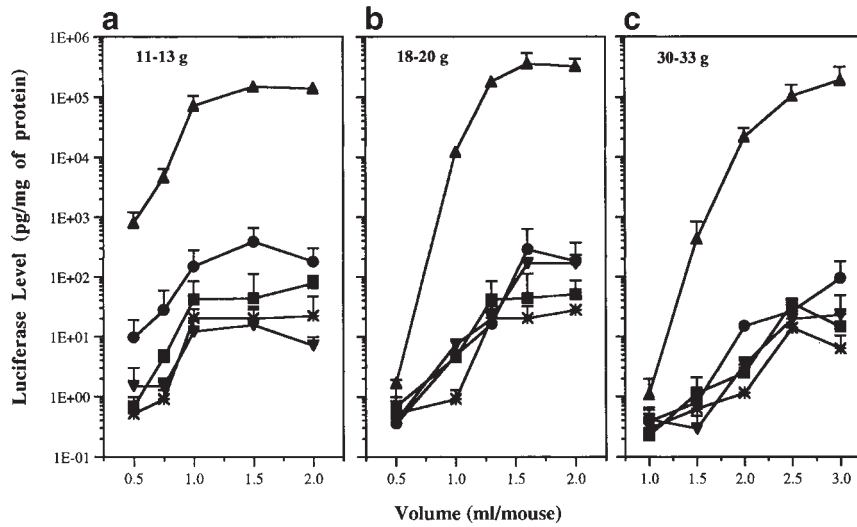
One of the alternatives currently under development is the direct use of naked DNA.<sup>17–23</sup> Compared with virus-

mediated gene transfer systems, introduction of an exogenous gene into cells in the form of naked DNA has many obvious advantages. Preparation of DNA has become routine in many laboratories, and it is easy to obtain significant amounts of DNA without involving laborious procedures. Since DNA is chemically and biologically stable, no sophisticated storage conditions are required. Since the initial report in 1990 of the successful expression of a reporter gene in muscle by the method of intramuscular injection of plasmid DNA,<sup>17</sup> there have been many *in vivo* studies demonstrating successful transgene expression in liver,<sup>18–20</sup> lung,<sup>21</sup> heart,<sup>22</sup> and skin<sup>23</sup> using a similar method. However, the major limitation of this method is that it requires a local regional administration or surgical procedures. In addition, the level of transgene expression resulting from such local regional administration is relatively low and restricted to the injection site. To overcome these problems, we have explored the possibility of expressing exogenous genes in animals using systemic administration of plasmid DNA. We report here the results from a series of experiments designed to identify the important factors that affect the level of transgene expression in mice. These results suggest that systemic administration of plasmid DNA can be a very efficient means for the introduction and expression of exogenous genes in animals.

## Results

### *Relationship between the level of gene expression and the volume of administration*

One of the critical parameters for a successful expression of transgene by systemic administration of plasmid DNA



**Figure 1** Volume effect on the level of gene expression in liver (▲), kidney (●), spleen (▼), lung (■) and heart (\*). Three groups of mice with different weight (11–13 g, 18–20 g and 30–32 g) were intravenously injected with 10  $\mu$ g of pCMV-Luc plasmid in various volumes of saline. Luciferase protein in different organs was determined 8 h after DNA injection. Error bar represents the s.e.m. of mean from three animals.

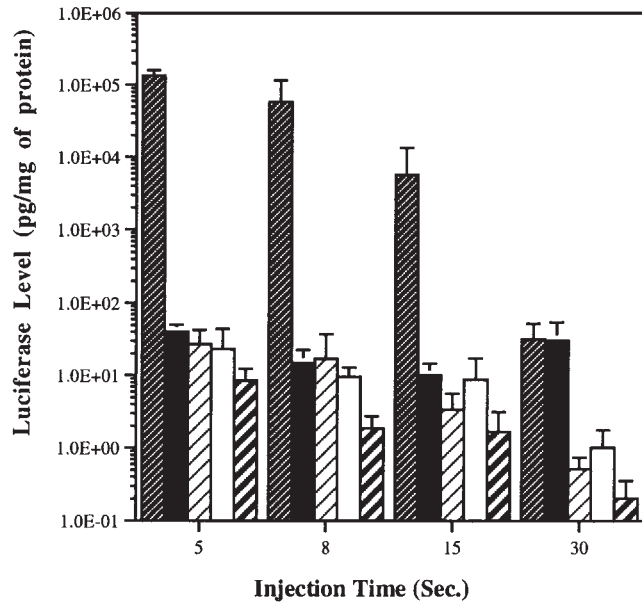
is the volume of DNA solution administered. The volume effect on the level of gene expression in different organs was examined using the luciferase gene as a reporter and the results are shown in Figure 1. These data show clearly that the level of luciferase protein expressed in different organs (lung, spleen, heart, kidneys and liver) increased as the volume of injected DNA solution was increased. The volume needed for a maximal level of gene expression varied depending on the body weight of the animals used. Optimal gene expression was obtained at approximately 1.2, 1.6 and 3.0 ml for animals with body weights of 11–13, 18–20 and 30–32 g, respectively. Among the organs examined, the level of gene expression in the liver is the highest as indicated by the product of approximately 300 ng of luciferase protein per milligram of extracted protein, representing 45  $\mu$ g of luciferase protein per gram of liver. These data suggest that optimal transgene expression requires an injection volume that is approximately 8–12% of the body weight.

**Effect of injection time**

Another important parameter affecting the level of transgene expression is the injection rate. Data presented in Figure 2 show that a rapid injection usually resulted in a higher level of transgene expression. Using an injection volume of 1.6 ml per mouse (18–20 g) containing 10  $\mu$ g of pCMV-Luc plasmid DNA, the level of gene expression was found to decrease with increasing injection time. For example, the level of luciferase in the liver of animals receiving the DNA solution administered in approximately 5 s was 140 ng/mg, compared with approximately 0.03 ng/mg when the same volume and DNA amount were injected into an animal over 30 s. An approximately six-fold increase in the injection time results in a more than 4500-fold decrease in the level of gene expression in the liver. A less, but significant, decrease in the level of transgene expression was also observed in the kidney, spleen, heart and lung.

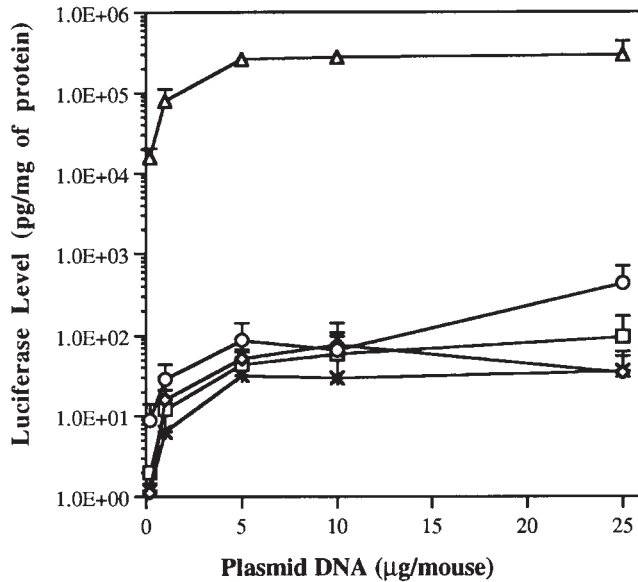
**DNA dose-response**

DNA dose effect on the level of gene expression was also assessed. Data presented in Figure 3 show that a signifi-



**Figure 2** Effect of injection time on the level of gene expression. Mice (18–20 g) were intravenously injected with 10  $\mu$ g of plasmid DNA in 1.6 ml saline. The injection time was varied from 5 to 30 s. Mice were killed 8 h after injection and luciferase protein in the extracts of liver (▨), kidney (■), spleen (▧), lung (□) and heart (▩) was measured. Each column represents the mean  $\pm$  s.e.m. from three animals.

cant level of luciferase protein 8 h after injection was detected in all internal organs including the lung, heart, liver, spleen and kidney when the amount of plasmid DNA injected was as low as 0.2  $\mu$ g per mouse. The luciferase protein in all examined organs increased with increasing the amount of plasmid DNA injected and reached a saturation level at approximately 5  $\mu$ g pCMV-Luc plasmid DNA per mouse. Further increase of the DNA dose up to 25  $\mu$ g, although it increased the level of transgene expression in kidney six-fold, did not result in a significant increase of luciferase protein level in other organs. The amount of luciferase protein expressed at the



**Figure 3** DNA dose-dependent luciferase gene expression. Various amounts of plasmid DNA (pCMV-Luc) in 1.6 ml saline were injected into each mouse within 5 s. The level of luciferase gene expression was determined 8 h after injection in liver ( $\Delta$ ), kidney ( $\circ$ ), spleen ( $\diamond$ ), lung ( $\square$ ) and heart ( $\times$ ). Error bars represent s.e.m. from three mice.

dose of 5  $\mu\text{g}$  pCMV-Luc plasmid DNA per mouse is 300 ng per milligram of extracted protein from the liver, 0.1 ng from kidney, 0.06 ng from spleen, 0.04 ng from lung and 0.03 ng from heart, respectively. For liver, this level represents 45  $\mu\text{g}$  per gram of liver in a mouse with a body weight of 18–20 g. More than a thousand-fold higher transgene expression in the liver than those obtained in other organs suggests that liver is the major site for transgene expression.

#### Histochemical analysis of gene expression in the liver

To elucidate the population and location of cells in the liver that express the transgene, pCMV-LacZ plasmid DNA was injected into mice and liver cells expressing the  $\beta$ -galactosidase gene were identified by X-gal staining of liver sections. Compared with control animals which received saline only (Figure 4a), blue cells were easily seen in liver sections from animals injected with pCMV-LacZ plasmid DNA (Figure 4c, e and g). There is a clear co-relationship between the number of blue cells in each section and the amount of plasmid DNA each animal received. There are approximately 40% of cells expressing  $\beta$ -gal at a DNA dose of 25  $\mu\text{g}$  per mouse (Figure 4g). It appeared at higher magnification that these cells were mainly hepatocytes as identifiable by their polygonal shape and round nuclei. However, blue cells with less obvious characteristics of hepatocytes at higher magnification were also seen. At this stage it is not certain whether these cells belong to different types of liver cells or the same type of hepatocytes that show some morphological difference under our experimental conditions. Figure 4b, d, f and h were sections stained with hematoxylin/eosin to identify the potential liver damage. Compared with normal mouse without injection, there are no obvious histological changes seen in liver sections of animals injected with either saline (Figure 4a, b) or

saline containing different amounts of pCMV-LacZ plasmid (Figure 4c–h).

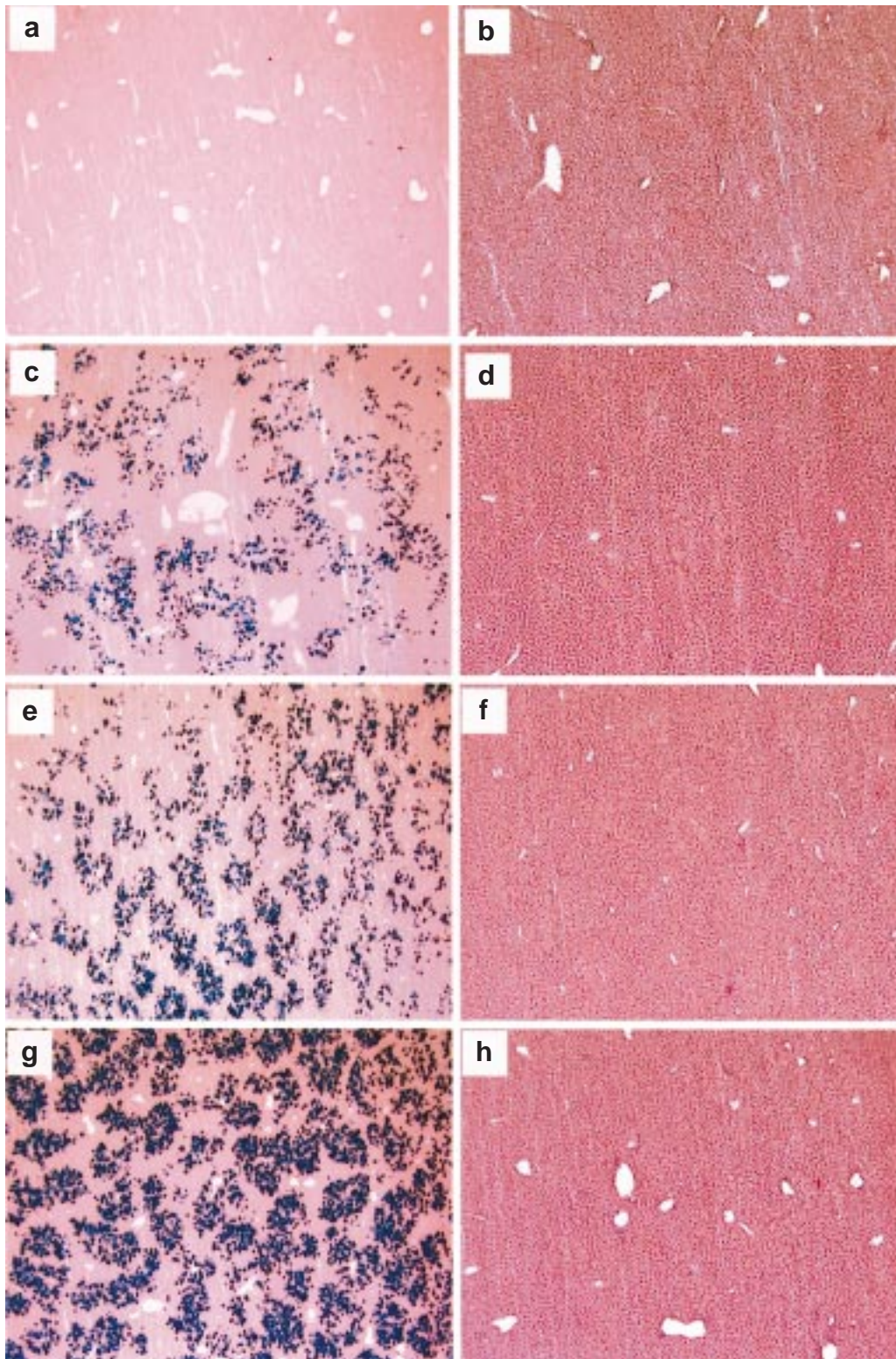
#### Measurement of toxicity

The potential toxic effects of our procedure on animals were assessed by two independent approaches. The first one employs the measurement of animal growth. The rationale of this approach is that growth rate would be affected in these mice, which were still in their fast growth stage, if the injection is toxic. Five animals for each group were injected with saline with or without 10  $\mu\text{g}$  of pCMV-Luc plasmid DNA. The body weight of each animal was measured for 6 consecutive days after injection. Data presented in Figure 5 suggest that growth rates of animals in the control group and the groups injected with either saline or saline containing 10  $\mu\text{g}$  pCMV-Luc plasmid DNA were not statistically different, as analyzed by using one-way analysis of variance (ANOVA) test ( $P > 0.05$ ).

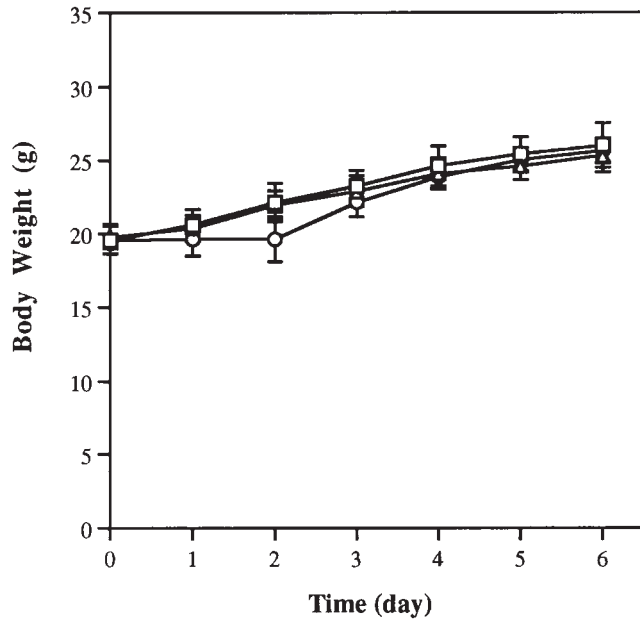
A comprehensive clinical biochemistry test was also performed. These tests include the determination of serum biochemistry including the major ion concentration ( $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Cl}^-$ ); major protein concentration (albumin and the total protein); and the concentration of liver specific enzymes including alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and total bilirubin. Both short- (1 day) and long (7 days)-term effects of DNA administration on these parameters were evaluated. Data presented in Table 1 show that all the biochemical parameters evaluated were in the normal range as compared with those of normal animals with the exception of ALT value on day 1. A transient increase of the ALT value to 177 on day 1 was seen in animals injected with either saline or saline with DNA, compared with approximately 50 in normal animals. It was noted that the ALT value falls into a normal range 3 days after the injection. Animals injected with either saline or saline containing plasmid DNA gave an identical pattern of ALT changes (data not shown), suggesting that the increased ALT value is not caused by plasmid DNA or gene product.

#### Effect of different promoters on time-dependent gene expression

Time-dependent gene expression for the CMV-driven expression system (pCMV-Luc) was compared with a system driven by a liver-specific promoter of human  $\alpha$ 1-antitrypsin. Data presented in Figure 6 show that both gene constructs exhibited very similar gene expression patterns. A significant amount of luciferase was seen as early as 2 h after injection. It reached a maximal level in approximately 8 h and declined soon after. It is apparent that the decline in the rate of luciferase expression varied at different time periods after injection. For example, there was a 2400-fold decrease in the amount of luciferase in the liver from 8 h to 72 h and only four-fold decrease from 72 to 144 h. Six days after the DNA administration, the amount of luciferase in the liver was approximately 0.06 ng/mg of extracted protein, while its level in other organs dropped to the background level. When comparing the two expression systems with different promoters, there was no significant difference between their peak expression level and the persistence of luciferase gene expression.



**Figure 4** Histochemical analysis of  $\beta$ -galactosidase gene expression in liver. Mice were injected with 1.6 ml saline containing various amounts of pCMV-LacZ plasmid DNA. Animals were killed 8 h after injection and liver sections were made using cryostat. Sections (a, c, e and g) were stained with X-gal solution followed by eosin for counterstain. Sections (b, d, f and h) were stained by a standard hematoxylin/eosin staining method. Sections were made from animals each receiving 0 (a, b), 0.5 (c, d), 2.5 (e, f) and 25  $\mu$ g (g, h) of pCMV-LacZ (25 $\times$ ).



**Figure 5** Effect of large volume injection on animal weight. Each mouse was injected with 1.6 ml saline or saline containing 10 µg of pCMV-Luc plasmid DNA. Animal weight was measured before and after the injection. Control group (△) represents mice without receiving injection. Testing animals include those receiving 1.6 ml saline per mouse with (□) or without (○) 10 µg of pCMV-Luc plasmid DNA. Error bars represent s.e.m. from five mice.

*Effect of repeated injection*

The possibility of maintaining the level of gene expression by repeated administration was assessed. Three groups of 15 mice were injected with 10 µg of pCMV-Luc plasmid per animal on day zero. The first group was killed at 2, 8, 24, 72 and 144 h after the injection, respectively, with three mice per time-point. The second and third group of mice was injected with the same amount of plasmid DNA for a second time on day 6 and a last injection was performed on day 12 for the third group. The luciferase protein level in each organ was analyzed at the indicated time. Data presented in Figure 7 show that almost an identical pattern of time-dependent gene expression resulted from each of the three injection schemes. The level of luciferase protein started to accumulate as early as 2 h after the DNA

administration, reached the peak level in 8 h and declined thereafter.

*Protection effect of hydrodynamics-based procedure against DNA degradation*

DNA degradation by serum and cellular nucleases has been considered as one of the major factors responsible for the lack of successful transfection *in vivo* by conventional method of intravenous injection.<sup>24</sup> To examine whether the high level transgene expression obtained in the liver using rapid injection of large volume of DNA solution is due to a protection mechanism of injected plasmid DNA, we have compared the levels of plasmid DNA in the liver after tail vein injection of 10 µg of pCMV-Luc plasmid DNA into each mouse using either the conventional method (200 µl per mouse) or rapid injection of large volume of DNA solution (1.6 ml per mouse). Data in Figure 8 summarize the results of a Southern analysis designed to assess the amount and forms of injected plasmid DNA in mouse liver as a function of time. Figure 8 shows that degradation of injected plasmid DNA occurs quickly upon injection as indicated by the smear bands in lane 2 and 8 where the liver samples were collected 1 min after the injection. Judging from the density of the observed DNA band in Figure 8, it is clear that degradation of plasmid DNA in the liver is much faster in animals that were injected with pCMV-Luc plasmid DNA by the conventional method (lanes 2–7). Ten minutes after DNA injection, there is no intact plasmid DNA left in the liver, suggesting that the injected plasmid DNA has already been degraded. In contrast to the conventional method of injection, there is a significant level of intact plasmid DNA found in liver samples (lanes 8–13) from animals where the rapid injection of large volume DNA solution was used. More importantly, it is clear that presence of plasmid DNA in these samples persists for at least 2 h without significant decrease in its amount. It is likely that these DNA molecules have already been transferred inside the liver cells and are responsible for the high level of transgene expression seen in the liver.

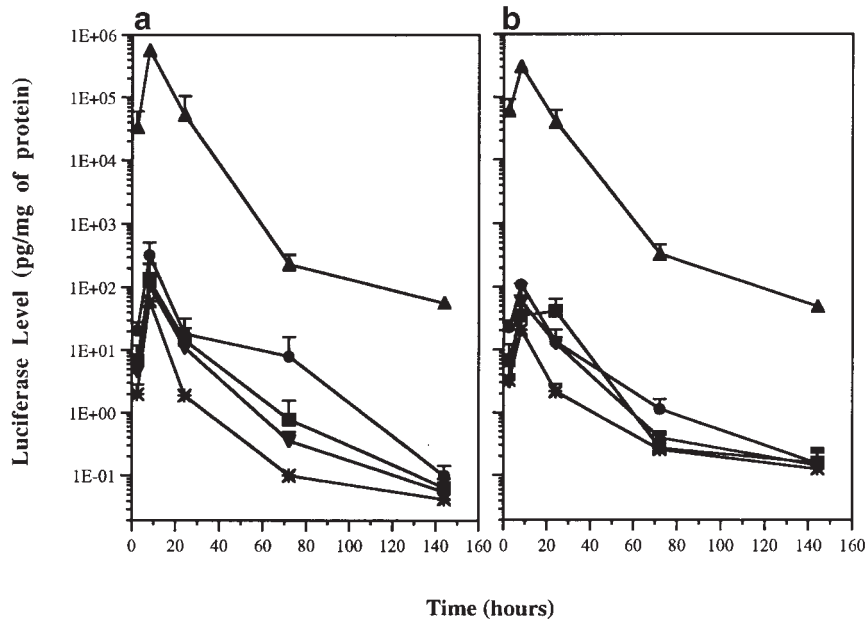
*Discussion*

Expression of exogenous genes *in vivo* by direct use of plasmid DNA was first demonstrated by Wolff and colleagues<sup>17</sup> who showed in their study that a significant

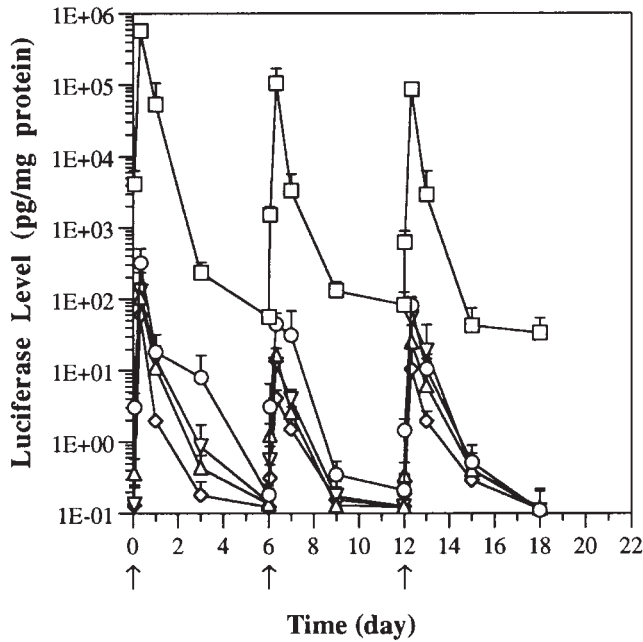
**Table 1** Effect of large volume injection on serum biochemistry

Time after injection (day)	Serum concentration								
	Na <sup>+</sup> (mM)	K <sup>+</sup> (mM)	Cl <sup>-</sup> (mM)	Alb (mg/l)	T.Prot (mg/l)	ALP (U/l)	AST (U/l)	ALT (U/l)	T.Bili (mg/l)
1	145 (1)	6.5 (0.2)	107 (2)	23 (2)	46 (3)	176 (39)	183 (17)	177 (56)	2 (1)
3	ND	ND	ND	ND	ND	ND	ND	58 (14)	ND
7	147 (2)	7.6 (0.4)	107 (1)	26 (2)	52 (3)	201 (32)	164 (29)	46 (9)	4 (1)
Normal mice	144 (1)	7.7 (0.8)	107 (1)	23 (3)	48 (4)	227 (40)	157 (43)	45 (3)	3 (2)

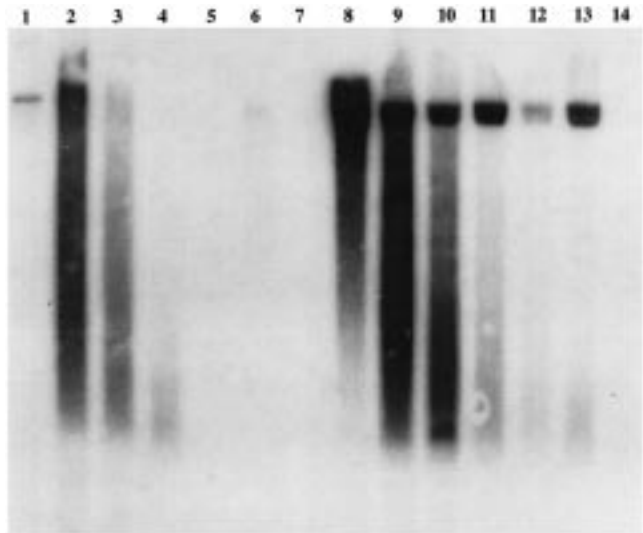
Alb, albumin; T.Prot, total protein; ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; T.Bili, total bilirubin; ND, not determined. Data represent mean (s.d.) from five animals.



**Figure 6** Effect of different promoters on time-dependent gene expression in liver (▲), kidney (●), spleen (▼), Lung (■) and Heart (\*). Two groups of mice (18–20 g) were intravenously injected with 10 µg of either pCMV-Luc (a) or pAAT-Luc (b) plasmid DNA in 1.6 ml saline. Animals were killed at the indicated time after injection. Luciferase protein in the various organs was examined. Each data point presents the mean ± s.e.m. from three animals.



**Figure 7** Effect of repeated injection on the level of luciferase in different organs. Mice were injected with 10 µg of pCMV-Luc plasmid DNA on day zero and then divided into three groups. The first group of animals was killed at the appropriate time without receiving additional injection. The rest of the animals received the second injection (in 1.8 ml saline) of the same amount of plasmid DNA on day 6 and the second group of animals was then killed at different times. The last group of animals received the third injection (in 2.0 ml saline) on day 12 and was killed thereafter at the appropriate time. Luciferase protein level in liver (□), kidney (○), spleen (▼), lung (△) and heart (\*) was measured. Each data point presents the mean ± s.e.m. from three animals. Arrows indicate the time of injection.



**Figure 8** Effect of different methods of DNA administration on the rate of DNA degradation in the liver. Each mouse was injected with 10 µg of pCMV-Luc plasmid using either conventional method or hydrodynamics-based procedure. Lane 1 is the positive control with 200 ng of pCMV-Luc plasmid DNA. Lanes 2–7 represent liver samples collected at 1, 5, 10, 30, 60 and 120 min, respectively, from animals receiving DNA under conventional methods (200 µl per mouse). Lanes 8–13 represent liver samples collected at 1, 5, 10, 30, 60 and 120 min, respectively, from animals injected with DNA using hydrodynamics-based procedure. Lane 14 is negative control with DNA extracted from a normal mouse liver.

level of gene expression can be achieved in muscle cells by intramuscular injection of plasmid DNA. Following this initial discovery, many studies have reported success in expressing exogenous genes using local regional administration.<sup>18–23</sup> In this report, we have demonstrated that an efficient gene transfer and high level of transgene expression can also be achieved by systemic administration of plasmid DNA in animals by a rapid injection of DNA in large volume (Figures 1 and 2). Up to 300 ng of transgene product per milligram of extracted protein was obtained from the liver of a mouse after a single injection of as little as 5 µg of plasmid DNA (Figure 3). Approximately 40% of liver cells were found to be transfected as judged by X-gal staining of liver sections (Figure 4) from animals injected with pCMV-LacZ. This level of transgene expression achieved in whole animals is among the highest ever achieved in the liver via systemic administration of DNA.

Transgene expression was observed in all examined internal organs including the lung, spleen, heart, kidney and liver with the highest level seen in the liver. The level of transgene expression is determined mainly by the volume and injection rate (Figures 1 and 2). These results may be explained by the hydrodynamic and anatomical flow of the injected DNA solution. Under ordinary conditions where plasmid DNA is slowly injected into a mouse via the tail vein, the DNA molecules enter the blood stream and are carried into the heart and then distributed to other organs. Due to the presence of the large amount of nuclease in the blood, these DNA molecules are quickly degraded in circulation. This is perhaps one of the important reasons for the lack of a significant level of transgene expression by a routine tail vein injection of plasmid DNA. However, the dynamic flow of the plasmid DNA will be different when a large volume and high injection rate are used. In mice with body weight of 18–20 g, an injection volume of 1.6 ml per mouse is almost equivalent to the total blood volume of the animal (blood volume is estimated to be 7.3% of the body weight for mice<sup>25</sup>) and the injected DNA solution is likely to accumulate in the inferior vena cava when the injection rate exceeds the cardiac output. As a result, a high hydrostatic pressure develops in the inferior vena cava. Such hydrostatic pressure, being proportional to the volume and the speed of injection, will force the flow of DNA solution into tissues such as the liver, kidney and heart that are directly linked to the inferior vena cava. Since the liver is the largest organ in the body with an expandable structure, a large portion of DNA solution will be forced into the liver in a direction that is opposite to that of the regular circulation. This will result in a direct exposure of DNA molecules to liver cells before their being mixed with blood. More importantly, persistent presence of intact plasmid DNA in the liver as shown in Figure 8 suggest that some of plasmid DNA molecules are likely transferred inside the liver cells by the hydrodynamic process during DNA administration. The function of the hydrodynamics-based procedure in transferring gene into the liver cells is likely to be the most critical element involved in the hydrodynamics-based transfection.

Considering that the hepatocytes are the major type of liver cells expressing the transgene (Figure 4), and the sinusoidal structure of the liver, the hydrostatic pressure in the liver induced by the large volume of DNA solution injected may also play a critical role in allowing the DNA

molecules to reach the hepatocytes. By blocking the regular blood flow using surgical procedures, Zhang *et al.*<sup>19</sup> have shown in mice that there is a clear correlation between the level of transgene expression and the hydrostatic pressure in the liver. The authors have interpreted these results to indicate that the pressure may increase the permeability of liver fenestra, which otherwise prohibit the plasmid DNA from reaching the hepatocytes. While such an explanation is scientifically sound, additional studies are needed to examine whether/how such pressure facilitates the entry of the plasmid DNA into the hepatocytes.

The injection volume required to reach a maximal level of gene expression is dependent on animal weight (Figure 1). About 1.2 ml is required to achieve a level of gene expression at 180 ng/mg in animals with a body weight of 10–13 g, compared with approximately 3.0 ml for a similar level of gene expression in animals whose body weight is 30 g or greater. This correlation is likely related to the blood volume and the cardiac capacity of the animal. As younger animals have a smaller cardiac output, a smaller injected volume is sufficient to reach the hydrostatic pressure required for a high level of gene expression. It appears that a ratio of injection volume to animal weight of 8–12 ml per 100 g is required for an optimal level of transgene expression (Figure 1).

Promoter strength is one of the important parameters determining the level of gene expression.<sup>26</sup> Among the many promoters that have been evaluated in mice, we have selected cytomegalovirus immediate-early promoter (CMV) and human  $\alpha$ 1-antitrypsin promoter for our studies. While these two promoters are both effective in expressing genes, human  $\alpha$ 1-antitrypsin promoter has been considered to be liver specific<sup>27</sup> in comparison to the CMV promoter which is less tissue selective. It is evident from the data in Figure 4 that both promoters were not only equally effective in expressing the luciferase gene but also exhibited an almost identical time-response curve. In both cases, the level of gene expression peaked at 8 h after DNA administration and dropped quickly thereafter. A much slower decrease in luciferase level was seen between 3 and 6 days. These results suggest that there are two phases in the decline of the luciferase level. Southern hybridization analysis of total DNA purified from the liver indicated that a quick decline in the amount of luciferase gene in the liver occurs within 3 days after DNA administration (data not shown). The coincidence between the decline of luciferase level and the amount of gene in the liver would suggest that the quick decline of transgene level is most likely caused by the loss of transgene in the transfected cells. Data in Figure 7 show that the peak level of gene expression can be regained by repeated DNA administration.

In spite of these seemingly harsh conditions used for introducing DNA molecules into the liver cells, our toxicity tests showed that all animals used in our experiments appeared to recover well from the procedure. With the exception of transient increase of serum concentration of ALT, all of the clinical biochemistry tests conducted, including the serum concentration of ALP, AST, T.Bili, Na<sup>+</sup>, K<sup>+</sup>, Cl<sup>-</sup>, total protein and albumin, showed no sign of toxicity to the animals both in short (1 day) and long (1 week) term. The transient increase of ALT concentration in serum suggests that some minor liver damage occurred under our experimental conditions. The damage

is apparently caused by the large volume of saline injected and is not related to either the injected plasmid DNA or gene product as the increased serum ALT concentration was seen in animals injected with both saline and saline containing plasmid DNA. Importantly, these animals appear to recover from such minor liver damage quickly. Three days after the injection, the ALT value falls into the normal range (Table 1).

In summary, a simple, convenient and efficient method to introduce and express exogenous genes in whole animals by systemic administration of DNA has been developed. While it may be arguable at this stage of development whether the technique described here is of any use in human gene therapy, the high level of gene expression achieved by a simple tail vein injection of plasmid DNA into mice may prove to be extremely useful for testing the function of different genes, or its mutated forms, within the context of whole animals. One of the greatest advantages of this technique, compared with other vector-mediated gene delivery systems such as viral and nonviral systems, is that this simple transfection technique does not need long and laborious steps necessary for virus production or for preparation of non-viral vectors. We believe that the method reported here provides a convenient tool that allows researchers to analyze, in whole animals, the regulation of gene expression, the function of gene products, and more specifically the molecular mechanisms involved in many genetic and acquired diseases. In addition, this method will be complementary to the well-established bacterial expression system for proteins. The current bacterial expression system, although effective in producing many types of eukaryotic proteins, is limited by the lack of its ability to provide appropriate post-transcriptional modifications to the expressed proteins. The method described in this report may also prove useful considering the need for studying the function of more and more new genes discovered from the human genome project and through other gene discovery programs worldwide.

## Materials and methods

### Materials

pCMV-Luc plasmid containing firefly luciferase cDNA driven by CMV promoter was constructed in the laboratory of Dr Leaf Huang (Department of Pharmacology, University of Pittsburgh School of Medicine). Plasmid pAAT-Luc (expression of luciferase gene driven by human  $\alpha$ 1-antitrypsin promoter) was provided by Dr Xiao Xiao (Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine). Plasmid DNA was purified by the method of CsCl-ethidium bromide gradient centrifugation<sup>28</sup> and kept in saline at  $-20^{\circ}\text{C}$  until use. The purity of the plasmid preparations was checked by absorbance at 260 and 280 nm and 1% agarose gel electrophoresis. The assay kit for luciferase activity was purchased from Promega (Madison, WI, USA). The reagents used in histochemical analysis of  $\beta$ -galactosidase gene expression were from Sigma (St Louis, MO, USA). CD-1 mice were purchased from Charles River (Wilmington, MA, USA).

### *In vivo* gene expression

Mice were injected with plasmid DNA in saline at the indicated dose, volume of saline, and injection speed via

tail vein. Unless otherwise mentioned, DNA injection was completed in less than 5 s and the level of gene expression was assessed 8 h after injection of plasmid DNA. Different organs including the liver, spleen, lung, heart and kidney were dissected from dead animals using the standard surgical procedures. One milliliter of lysis buffer (0.1 M Tris-HCl, 2 mM EDTA and 0.1% Triton X-100, pH 7.8) was added to the whole organ for kidney, spleen, lung and heart. For liver, the same volume of lysis buffer was added to a piece of liver with wet weight of approximately 200 mg. Each organ was homogenized for 15–20 s with the Tissue Tearor at maximal speed and the tissue homogenates were then centrifuged in a microcentrifuge for 10 min at 13 000 *g* at  $4^{\circ}\text{C}$ . The protein concentration of the supernatant was determined by using Coomassie Blue Plus Protein assay kit. For luciferase assay of the liver extract, the supernatant was further diluted for 60-fold using HEPES buffer. Ten microliters of supernatant of lung, heart, spleen and kidney homogenates and diluted liver extract were mixed with 100  $\mu\text{l}$  of luciferase assay reagent and the luciferase activity was measured in a luminometer (AutoLumant LB 953, EG & G, Salem, MA, USA) for 10 s. Luciferase activity obtained as the relative light units (RLU) was converted to luciferase mass using standard curve established using reagents and procedure from Analytic Luminescence Laboratory (ALL, San Diego, CA, USA). The amount of luciferase protein was calculated using the equation derived from the standard curve in which luciferase protein (pg) =  $7.98 \times 10^{-5}$  RLU + 0.093 ( $R^2 = 0.9999$ ).

Histochemical analysis of  $\beta$ -galactosidase gene expression was performed according to previously published procedure.<sup>19</sup> In brief, 8 h after injection of pCMV-lacZ, animals were killed and the liver was dissected and immediately frozen using dry ice. Ten-micron cryosections were made using Cryostat (CMT 950 Series, Research Manufacturing Company, Tucson, AZ, USA). The sections were stained in X-gal solution (400  $\mu\text{g}/\text{ml}$ ) for 2 h followed by eosin for counterstain. Hematoxylin/eosin were used for regular histological analysis.

### Measurement of clinical biochemistry parameters

Serum was prepared by separation of the coagulated whole blood of animals 1 or 7 days after injection of 1.6 ml of saline or DNA solution. Serum concentration of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (T.Bili),  $\text{Na}^+$ ,  $\text{Cl}^-$ ,  $\text{K}^+$ , albumin (Alb) and total protein (T.Prof) were measured by automated analyzer Vitros 950 (Johnson & Johnson Clinical, Rochester, NY, USA) in the clinical chemistry laboratory at the Presbyterian Hospital, University of Pittsburgh Medical Center.

### Southern analysis

Animals were injected with 10  $\mu\text{g}$  pCMV-Luc plasmid DNA each via tail vein using either conventional method (200  $\mu\text{l}$  per mouse) or hydrodynamics-based procedure (1.6 ml per mouse). The liver from each animal was dissected and immediately frozen on dry ice. Approximately 200 mg of liver sample were used for DNA extraction using the previously published procedure.<sup>29</sup> Twenty-five micrograms of total DNA from each sample were digested using 10 units of *Hind*III for 1 h at  $37^{\circ}\text{C}$  and then loaded on 1% agarose gel. DNA bands were then

transferred overnight on to GeneScreen Plus membrane. After prehybridization with sonicated salmon sperm DNA in prehybridization buffer (50% deionized formamide, 250 mM sodium phosphate, 250 mM NaCl, 1 mM EDTA, 100 µg/ml sonicated salmon sperm DNA), the membrane was incubated at 42°C for 24 h with <sup>32</sup>P-labeled probe which was prepared from full luciferase gene cut out from pCMV-Luc plasmid DNA and using a random primed DNA labeling kit (Dupont NEN, Boston, MA, USA). The hybridized bands were visualized by autoradiography.

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