nonviral approaches are effective in expressing transgenes at therapeutic levels and most of them, including plasmid DNA complexes with polymers or lipids/liposomes, generally suffer from unacceptably low levels of transgene expression [2]. By marked contrast, hydrodynamic injection of naked plasmid DNA results in the expression of transgenes at a level high enough to exhibit effects in various experimental models [3–5]. The level of this hydrodynamic injection method is as high as that achieved with adenoviral vectors [6], which are one of the most effective viral vectors developed thus far.

In addition to the level of expression, its duration is another important parameter determining the therapeutic effects of gene transfer, and sustained transgene expression at therapeutic level is necessary for effective gene therapy. Long-term expression will reduce the frequency of dosing, which leads to an improvement in the quality of life of patients receiving gene transfer. Unfortunately, the hydrodynamic injection method, a most efficient method for *in vivo* gene transfer, generally produces shorter transgene expression than the use of viral vectors or other nonviral methods, such as intramuscular injection of naked plasmid DNA [1].

Bacterial DNA and bacterially-derived plasmid DNA have an expected frequency (one out of 16) of CpG dinucleotides, whereas mammalian DNA has a much lower frequency (one out of 50–100) of CpG dinucleotides and its cytosine is highly methylated [7]. Unmethylated CpG dinucleotides, or CpG motifs, which are uncommon in mammalian DNA, stimulate mammalian immune cells through Toll-like receptor 9 (TLR9). This recognition results in the production of pro-inflammatory cytokines, especially when DNA is administered as a DNA/cationic liposome complex [8,9]. Pro-inflammatory cytokines have been reported to reduce the transgene expression in later periods of gene transfer [10,11]. In addition, the cytosine residue of the CpG motif can be a target for methylation by DNA methyltransferases. It has been reported that approximately 30% and 70% of CpG sites in the cytomegalovirus (CMV) promoter/enhancer were methylated at days 1 and 7, respectively, after intramuscular injection of adenoviral vectors [12]. It was concluded that methylation is a major mechanism responsible for the reduced expression from adenoviral vectors. Similarly, CpG methylation could reduce transgene expression from plasmid vectors. This hypothesis has been, at least partially, proven in previous studies using pGZB plasmid vector in which CpG motifs were present only in the Ori and cDNA regions [13]. Recently, it was shown that the deletion of CpG motifs in plasmid improves the duration of *in vivo* transgene expression when administered as a DNA/polymer complex [14]. These lines of experimental evidence suggest that a reduction in CpG content in plasmid DNA comprises a universal method of increasing the duration of transgene expression.

We demonstrated that pGZB plasmid encoding murine interferon (IFN)-$\beta$ or IFN-$\gamma$ (pGZB-Mu$\beta$ and pGZB-Mu$\gamma$, respectively) exhibits a prolonged expression of corresponding IFNs and better inhibitory effects against pulmonary metastasis of tumor cells than conventional, CpG-replete IFN-expressing plasmids [15,16]. A further increase in the duration of transgene expression could be achieved by reducing the remaining CpGs in the plasmids. In the present study, we constructed several types of plasmids with different CpG contents encoding firefly luciferase, and examined the duration of transgene expression after intravenous injection into the tail vein of mice by the hydrodynamic injection method. The effects of CpG methylation and pro-inflammatory cytokines on the expression were also evaluated. Based on the results obtained using luciferase-expressing plasmids, a plasmid vector that achieves persistent transgene expression of IFN-$\gamma$ was successfully developed.

## Materials and methods

### Cell cultures and mice

A mouse colon carcinoma cell line CT-26 was cultured in RPMI1640 supplemented with 10% fetal calf serum. Male 7-week-old BALB/c mice and male 4-week-old ICR mice, approximately 20 g in weight, were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan), maintained on a standard food and water diet under conventional housing conditions, and were used within 1 week of delivery. BALB/c mice were used only in tumor metastasis experiments. All animal experiments were approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences, Kyoto University.

### Plasmid DNA

pcDNA3.1 was purchased from Invitrogen (Carlsbad, CA, USA). pCMV-Luc and pGZB-Luc were as described previously [16,17]. pCMV-Luc(CMV-syn) was constructed by inserting the *Bgl*II/*Hind*III CpG-free CMV-syn promoter fragment of the pGZB-Luc into the *Bgl*II/*Hind*III site of pCMV-Luc. pCpG-Luc was constructed by inserting the *Bgl*II/*Nhe*I firefly luciferase cDNA fragment amplified by the polymerase chain reaction (PCR) from pCMV-Luc into the *Bgl*II/*Nhe*I site of pCpG-mcs (InvivoGene, San Diego, CA, USA). pCpG-ΔLuc was constructed by inserting the *Bgl*II/*Nhe*I firefly luciferase gene and the Sh ble gene fragment from pORF-LucSh-ΔCpG (InvivoGene), which contains no CpG dinucleotides, into the *Bgl*II/*Nhe*I site of pCpG-mcs. pCpG-Luc(CMV-syn) was constructed by inserting the *Sda*I/*Hind*III CpG-free CMV-syn promoter fragment amplified by PCR from the pGZB-Luc into the *Sda*I/*Hind*III site of pCpG-Luc. IFN-$\gamma$-expressing plasmid vectors, pCMV-Mu$\gamma$ and pGZB-Mu$\gamma$, have been described previously [16,17]. pCpG-Mu$\gamma$ was constructed by inserting the *Bgl*II/*Nhe*I IFN-$\gamma$ cDNA fragment amplified by PCR from pCMV-Mu$\gamma$ into the *Bgl*II/*Nhe*I site of pCpG-mcs. Table 1 summarizes the size, number of CpG motifs,

Table 1. Properties of plasmid DNA used in this study

| Plasmid | Size (kbp) | Number of CpG[a] | Promoter | cDNA |
|---|---|---|---|---|
| pCMV-Luc | 7.1 | 846 | CMV | Luciferase |
| pCMV-Luc(CMV-syn) | 6.8 | 772 | CMV-syn | Luciferase |
| pGZB-Luc | 4.5 | 282 | CMV-syn | Luciferase |
| pCpG-Luc | 4.7 | 194 | EF1 | Luciferase |
| pCpG-Luc(CMV-syn) | 4.6 | 194 | CMV-syn | Luciferase |
| pCpG-Δluc | 3.6 | 0 | EF1 | Modified luciferase gene fused to Sh ble gene |
| pCMV-Muγ | 6.4 | 686 | CMV | Mouse IFNγ |
| pGZB-Muγ | 3.3 | 116 | CMV-syn | Mouse IFNγ |
| pCpG-Muγ | 3.5 | 20 | EF1 | Mouse IFNγ |

[a]Numbers of CpG dinucleotides in plasmid DNA are indicated. syn, synthetic.

promoter and cDNA of plasmid DNA used in the present study.

## Methylation of cytosine residues in plasmid DNA

Plasmids (pCMV-Luc, pCMV-Luc(CMV-syn), pGZB-Luc, pCpG-Luc, pCpG-ΔLuc, pCMV-Muγ, pGZB-Muγ and pCpG-Muγ) were treated with *SssI* CpG methylase (New England Biolabs, Ipswich, MA, USA), which methylates cytosine residues of CpG dinucleotides. Mock-methylated plasmids, which were treated in the same manner as methylated ones without the addition of methylase, were used as controls. Methylation of cytosine residues was confirmed by restriction digestion with *HpaII*, a methylation-sensitive restriction enzyme.

## Preparation of DNA/cationic liposome complexes

Cationic liposomes consisting of *N*-[1-(2,3-dioleyloxy) propyl]-*n*,*n*,*n*-timethylammonium chloride and cholesterol in a 1 : 1 molar ratio were prepared as described previously [10]. Cationic liposomes and pcDNA3.1 were mixed in 5% dextrose at a charge ratio (−/+) of 1 : 2.24 and left at 37 °C for 30 min to form a DNA/cationic liposome complex, or lipoplex.

## Hydrodynamic injection of plasmid DNA into mice

Mice received an injection via the tail vein of plasmid DNA dissolved in 1.6 ml of saline over 5 s [3]. In most experiments, the dose of plasmid DNA was set to 0.2–0.3 pmol/mouse, which corresponded to 0.43–1.4 μg of DNA/mouse. To examine the effect of pro-inflammatory cytokines, lipoplex consisting of 25 μg of pcDNA3.1 was injected in a normal manner into the tail vein, 1 h after the hydrodynamic injection of naked plasmid DNA.

## Luciferase assay

At indicated periods after injection, the liver was harvested and homogenized in 5 ml/g of a lysis buffer (0.1M Tris, 0.05% TritonX-100, 2 mM ethylenediaminetetracetic acid, pH7.8). The homogenates were centrifuged at 10 000 *g* for 10 min at 4 °C. Then, 5 μl of the supernatant was mixed with 50 μl of luciferase assay buffer (Picagene; Tokyo-Ink, Tokyo, Japan) and the chemiluminescence was measured with a luminometer (Lumat LB 9507; EG&G Bethhold, Bad Wildbad, Germany).

## Measurement of pro-inflammatory cytokines in the liver

At 2 h after injection of 1 μg of naked plasmid DNA (pCMV-Luc or pCpG-ΔLuc) or lipoplex consisting of 25 μg of pcDNA3.1, the liver was isolated and homogenized in 2 ml of phosphate-buffed saline containing a cocktail of protease inhibitors. Then, the homogenates were centrifuged at 15 000 *g* for 10 min at 4 °C. The concentrations of tumor necrosis factor (TNF)-α and interleukin (IL)-6 in supernatants were measured using enzyme-linked immunosorbent assay (ELISA) kits (TNF-α Mouse ELISA Kit, R&D systems, Minneapolis, MN, USA; BD OptEIA™ Mouse IL-6 ELISA set, BD Bioscience, San Jose, CA, USA).

## Measurement of serum concentrations of IFN-γ

At indicated periods after injection, 50–150 μl of blood was collected from the tail vein. The blood samples were incubated at 4 °C for 2 h to allow clotting and then centrifuged at 8000 *g* to obtain serum. The concentration of IFN-γ in the serum was analysed by ELISA using a commercial kit (Ready-SET-Go! Mouse IFN-© ELISA; eBioscience, San Diego, CA, USA).

## Experimental pulmonary metastasis

CT-26 cells were trypsinized and suspended in Hanks' balanced salt solution (HBSS). Cell suspensions containing $1 \times 10^5$ CT-26 cells in 200 μl of HBSS were injected into the tail vein of syngeneic BALB/c mice to establish experimental pulmonary metastasis. At 4 days after tumor inoculation, plasmid DNA was injected in the tail vein by the hydrodynamic injection method at a dose of

0.2 pmol/mouse. At 21 days after inoculation of tumor cells, mice were sacrificed and the number of metastatic colonies on the lung surface was counted.

## Statistical analysis

Differences were statistically evaluated by Student's *t*-test. $p < 0.05$ was considered statistically significant.

# Results

## Time-course of luciferase activity in the liver after injection of luciferase-expressing plasmid DNA with different CpG contents

To examine the effect of CpG motifs in the promoter region, we compared the expression profiles of two luciferase-expressing plasmid vectors, pCMV-Luc and pCMV-Luc(CMV-syn), both of which have almost identical characteristics (Table 1) except for the CpG motifs in the CMV promoter. Figure 1 shows the time-courses of luciferase activity in the liver after hydrodynamic injection of naked pCMV-Luc or pCMV-Luc(CMV-syn) at a dose of 0.3 pmol/mouse. The luciferase activity in the liver was very high at 6 and 24 h after injection, then declined over time. There were no significant differences in the luciferase activity in the liver, although pCMV-Luc(CMV-syn), the plasmid containing no CpG motifs in the promoter, exhibited a relatively higher activity than pCMV-Luc 3 and 7 days after injection.

Next, expression profiles from other plasmids with different CpG contents were compared. Although the luciferase activity at 1 day after injection of naked plasmid DNA was not so different among the plasmids used, the activity at 3 and 7 days after injection of pCpG-Luc, pCpG-ΔLuc or pCpG-Luc(CMV-syn) was significantly ($p < 0.05$) higher than that of the other kinds of plasmids, including pGZB-Luc. These results showed a trend in which transgene expression from plasmids is prolonged when those with low CpG contents are used for gene transfer.

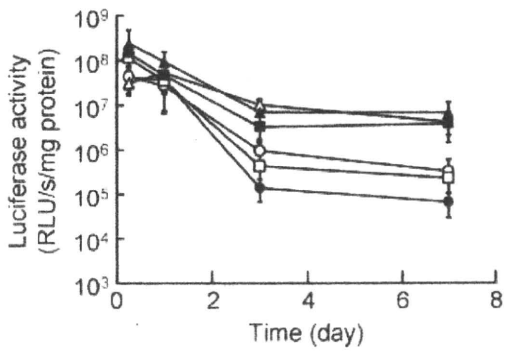


**Figure 1. Time-course of the luciferase activity in the liver after hydrodynamic injection of pCMV-Luc, pCMV-Luc(CMV-syn), pGZB-Luc, pCpG-Luc, pCpG-Luc(CMV-syn) or pCpG-ΔLuc at a dose of 0.3 pmol/mouse. The results are expressed as the mean ± SD of four mice. (●) pCMV-Luc (□) pCMV-Luc(CMV-syn) (○) pGZB-Luc (▲) pCpG-Luc (■) pCpG-Luc(CMV-syn) (△) pCpG-ΔLuc. RLU, relative luminescence unit**

## Effect of cytosine methylation on luciferase expression

Cytosine residues of CpG dinucleotides in pCMV-Luc, pCMV-Luc(CMV-syn), pGZB-Luc, pCpG-Luc and pCpG-ΔLuc were methylated or mock-methylated. All methylated plasmids were resistant to digestion by *Hpa*II, a methylation-sensitive restriction enzyme that recognizes the sequence CCGG, whereas mock-methylated plasmids, except for that of pCpG-ΔLuc, were digested by *Hpa*II (Figure 2A). Figure 2B shows the luciferase activity in the liver at 6 and 48 h after injection of methylated or mock-methylated plasmids. The luciferase activity at 6 h after

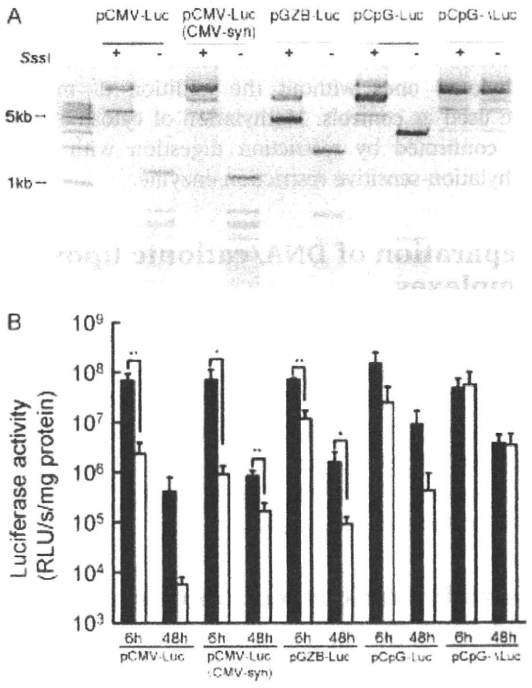


**Figure 2. Effect of cytosine methylation on luciferase activity in the liver after hydrodynamic injection of luciferase-expressing plasmid DNA. (A) pCMV-Luc, pCMV-Luc(CMV-Syn), pGZB-Luc, pCpG-Luc or pCpG-ΔLuc was methylated with *Sss*I methylase or mock-methylated and, then treated with *Hpa*II. Each sample (0.1 μg) was run on a 1% agarose gel and DNA was detected using ethidium bromide. (B) Methylated or mock-methylated pCMV-Luc, pCMV-Luc(CMV-syn), pGZB-Luc, pCpG-Luc or pCpG-ΔLuc was injected into mice at a dose of 0.3 pmol/mouse. Luciferase activity in the liver was measured at 6 or 48 h after gene transfer. The results are expressed as the mean ± SD of four mice. Open columns, methylated plasmid; solid columns, mock-methylated plasmid. *$p < 0.05$, **$p < 0.01$ compared to the mock-methylated group. RLU, relative luminescence unit**

injection (when pCMV-Luc produces a peak luciferase activity) of methylated pCMV-Luc was approximately 1.5 log less than that obtained with mock-methylated pCMV-Luc ($p < 0.01$). At 48 h after injection, an approximately 2 log difference in luciferase activity was apparent between methylated and mock-methylated pCMV-Luc. In addition, the luciferase activity from methylated pGZB-Luc and pCMV-Luc(CMV-syn) was significantly lower than that from mock-methylated pGZB-Luc and pCMV-Luc(CMV-syn), respectively. A similar trend was also observed for methylated and mock-methylated pCpG-Luc, although the differences were not statistically significant. pCpG-ΔLuc, which contains no CpG motifs, exhibited almost identical luciferase activity, irrespective of the treatment with *Sss*I CpG methylase. Therefore, it can be concluded that the differences in transgene expression between methylated and mock-methylated plasmids are due to the results of methylation of the plasmid CpG motifs.

## Effect of pro-inflammatory cytokines on luciferase expression

CpG motifs in plasmid DNA trigger the release of pro-inflammatory cytokines, including TNF-α, even when administered as a naked form [11,15], which was considered to reduce the level of transgene expression through several mechanisms [18,19]. DNA lipoplex induced a large amount of TNF-α secretion in serum after intravenous injection, which was much higher than that induced by the injection of any CpG-replete plasmid in naked form by the hydrodynamic injection method [11]. Therefore, DNA lipoplex was used to induce the production of pro-inflammatory cytokines to examine whether transgene expression is reduced by pro-inflammatory cytokines. Mice received a hydrodynamic injection of naked luciferase-expressing plasmid DNA, followed by an intravenous injection of DNA lipoplex consisting of pcDNA3.1, an empty plasmid DNA, after an interval of 1 h. Figures 3A and 3B show the levels of TNF-α and IL-6, respectively, in the liver at 2 h after injection of DNA lipoplex or naked plasmid DNA. High levels of these cytokines were detected in the liver of mice receiving the DNA lipoplex, and the level of TNF-α was significantly greater than that of mice receiving hydrodynamic injection of a naked plasmid DNA, pCMV-Luc or pCpG-ΔLuc. High levels of these cytokines were also observed in the serum of mice only when the DNA lipoplex was injected (data not shown). Figure 3C shows the luciferase activity in the liver at 6 and 48 h after injection of pCMV-Luc or pCpG-ΔLuc. The luciferase activities expressed from both pCMV-Luc and pCpG-ΔLuc were hardly affected by the administration of DNA lipoplex. These results strongly support the hypothesis that CpG DNA-induced pro-inflammatory cytokines are not a major factor determining the duration of transgene expression.

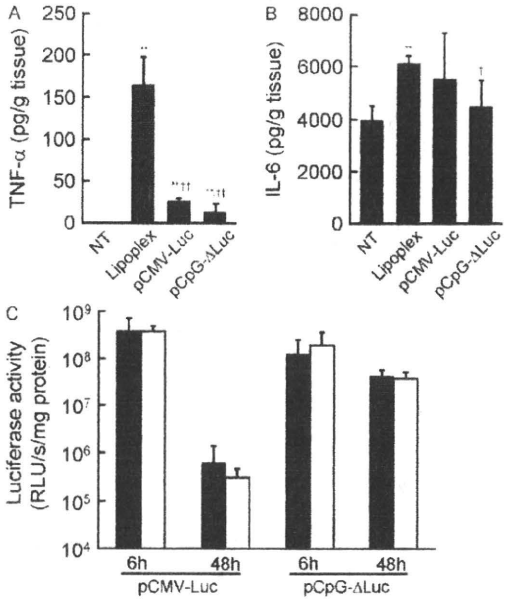


**Figure 3. Effect of DNA-induced pro-inflammatory cytokines on luciferase activity in the liver after hydrodynamic injection of luciferase-expressing plasmid DNA. (A, B) Mice received an intravenous injection of pcDNA3.1 lipoplex at a dose of 25 μg of DNA/mouse or a hydrodynamic injection of pCMV-Luc or pCpG-ΔLuc at a dose of 1 μg of DNA/mouse. At 2 h after injection, the levels of TNF-α (A) and IL-6 (B) in the liver were measured. The results are expressed as the mean ± SD of three mice. NT, no treatment group. The TNF-α level in the NT group was almost negligible. **$p < 0.01$ compared to the NT group; †$< 0.05$, ††$< 0.01$ compared to the lipoplex-treated group. (C) pCMV-Luc or pCpG-ΔLuc was injected into mice at a dose 1 μg/mouse (0.22 and 0.43 pmol/mouse for pCMV-Luc or pCpG-ΔLuc, respectively). Mice were injected with (open columns) or without (solid columns) pcDNA3.1 lipoplex at a dose of 25 μg of DNA/mouse, 1 h after hydrodynamic injection of plasmid DNA. Luciferase activity in the liver was evaluated at 6 or 48 h after gene transfer. The results are expressed as the mean ± SD of three mice. RLU, relative luminescence unit**

## Serum IFN-γ concentration after injection of IFN-γ-expressing plasmid DNA

Figure 4A shows the time-courses of the IFN-γ concentration in mouse serum after hydrodynamic injection of pCMV-Muγ, pGZB-Muγ or pCpG-Muγ at a dose of 0.2 pmol/mouse. Sustained IFN-γ concentrations were observed in mice receiving either CpG-reduced plasmid DNA: pGZB-Muγ or pCpG-Muγ. More than 1000 pg/ml of IFN-γ was detected in the serum from 6–31 days after injection of pCpG-Muγ, or from 6 h to 7 days after injection of pGZB-Muγ, whereas the concentrations were below the detection limit (15 pg/ml) at 36 h or later after injection of pCMV-Muγ. The initial IFN-γ concentration after injection of pGZB-Muγ was much greater than that after injection of pCpG-Muγ ($p < 0.05$). On the other hand, the IFN-γ concentration after injection of pCpG-Muγ was higher than that after injection of pGZB-Muγ ($p < 0.05$) on the third day and thereafter. These profiles of IFN-γ expression from these two plasmid vectors

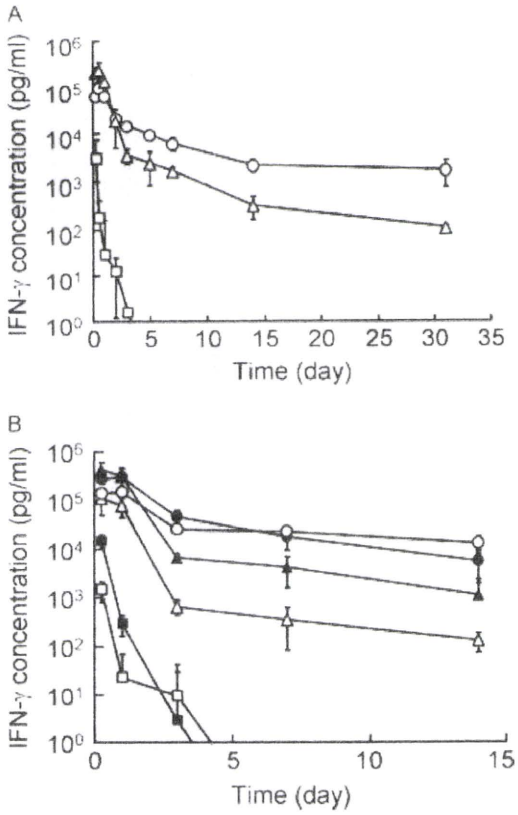


Figure 4. Time-course of the concentration of IFN-$\gamma$ in serum after hydrodynamic injection of IFN-$\gamma$-expressing plasmid DNA. (A) pCMV-Mu$\gamma$, pGZB-Mu$\gamma$ or pCpG-Mu$\gamma$ was injected into mice at a dose of 0.2 pmol/mouse. The results are expressed as the mean ± SD of three mice. □, pCMV-Mu$\gamma$; △, pGZB-Mu$\gamma$; ○, pCpG-Mu$\gamma$. (B) Methylated or mock-methylated pCMV-Mu$\gamma$, pGZB-Mu$\gamma$ or pCpG-Mu$\gamma$ was injected into mice at a dose of 0.2 pmol/mouse. The results are expressed as the mean ± SD of four mice. Open symbols, methylated plasmid; solid symbols, mock-methylated plasmid. □■, pCMV-Mu$\gamma$; △▲, pGZB-Mu$\gamma$; ○●, pCpG-Mu$\gamma$

resulted in a large difference in the peak-to-trough ratio. The ratio calculated for the first 31 days of pCpG-Mu$\gamma$ was 57, which was much smaller than that of pGZB-Mu$\gamma$ (1700), indicating that pCpG-Mu$\gamma$ produces a constant IFN-$\gamma$ concentration over a long period of time.

## Effect of cytosine methylation on IFN-$\gamma$ expression

To examine the effect of methylation on transgene expression from IFN-$\gamma$-expressing plasmids, pCMV-Mu$\gamma$, pGZB-Mu$\gamma$ and pCpG-Mu$\gamma$ were treated with *Sss*I methylase. Figure 4B shows the time-courses of the IFN-$\gamma$ concentration in mouse serum after hydrodynamic injection of methylated or mock-methylated plasmids encoding mouse IFN-$\gamma$ at a dose of 0.2 pmol/mouse. The IFN-$\gamma$ concentration in serum after injection of methylated pCMV-Mu$\gamma$ or pGZB-Mu$\gamma$ was significantly lower than that obtained with mock-methylated pCMV-Mu$\gamma$ or pGZB-Mu$\gamma$. Although the initial levels of the IFN-$\gamma$ concentration after injection of methylated pCpG-Mu$\gamma$ were approximately 55% of those obtained with mock-methylated pCpG-Mu$\gamma$, the level was almost identical to that of mock-methylated pCpG-Mu$\gamma$ at 7 days after injection and thereafter.

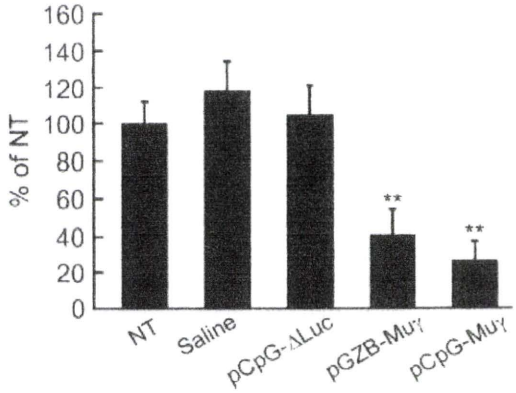


Figure 5. Effect of IFN-$\gamma$ gene transfer on the experimental pulmonary metastasis of CT-26 cells in mice. Tumor-bearing mice were injected intravenously with saline, pCpG-ΔLuc, pGZB-Mu$\gamma$ or pCpG-Mu$\gamma$ at a dose of 0.2 pmol/mouse by the hydrodynamic injection method, 4 days after tumor inoculation. Then, 21 days after tumor inoculation, mice were sacrificed and the number of metastatic colonies on the lung surface was counted. The results are normalized to the value of the no treatment (NT) group and expressed as the mean ± SD of at least four mice. $^{**}p < 0.01$ compared to the NT group

## Effects of IFN-$\gamma$ gene transfer on pulmonary metastasis of tumor cells

At 4 days after inoculation of CT-26 cells into the tail vein, pCpG-ΔLuc, pGZB-Mu$\gamma$ or pCpG-Mu$\gamma$ was injected into mice at a dose of 0.2 pmol/mouse. Figure 5 shows the number of metastatic colonies on the lung surface measured at 21 days after inoculation. pGZB-Mu$\gamma$ and pCpG-Mu$\gamma$ significantly reduced the number of colonies to 41.0 ± 13.5% ($p < 0.01$) and 26.9 ± 10.5% ($p < 0.01$), respectively, of that found in the no treatment group. No significant reduction was obtained by the injection of saline (118.2 ± 16.1%) or pCpG-ΔLuc (104.8 ± 16.7%).

# Discussion

Cytokine-supported tumor immunotherapy is one of the most promising strategies for cancer gene therapy. IFN gene transfer is considered to be useful for gene therapy because IFNs have a variety of biological functions, including antiproliferative and immunomodulatory activities, which are capable of contributing to the host's defense against tumors [20–24]. In fact, IFN genes are being used in clinical as well as preclinical trials of cancer gene therapy. We have reported that hydrodynamic injection of plasmid vectors expressing IFN-$\beta$ or IFN-$\gamma$ is effective in inhibiting the growth of hepatic metastasis of

mouse colon carcinoma CT-26 cells [15]. However, it was also found that the IFN gene transfer by this method was only marginally effective against pulmonary metastasis of the tumor cells. This result may be associated with the transient concentration of the IFNs in the lung tissue as well as in plasma after the injection of the conventional plasmid DNA encoding IFN-$\beta$ or IFN-$\gamma$.

In a previous study, we succeeded in increasing the potency of IFN gene therapy against pulmonary metastasis of tumor cells by using CpG-reduced IFN-expressing plasmid DNA, pGZB-Mu$\gamma$ and pGZB-Mu$\beta$ [16]. These results of the previous study provided experimental evidence that sustained transgene expression of IFN is important for obtaining good therapeutic results with respect to the inhibition of the metastatic growth of tumor cells in mice. However, substantial numbers of CpG motifs remained in the plasmids constructed based on the pGZB vector (Table 1), which would restrict a prolonged expression from the plasmid vectors. Therefore, in the present study, we initially examined the effect of the number and location of CpG motifs on the duration of transgene expression from naked plasmid DNA administered to mice by the hydrodynamic injection method, and then attempted to improve the efficacy of IFN cancer gene therapy by developing IFN-expressing plasmid DNA with the ability to achieve more sustained transgene expression.

Reduction in the number of CpG motifs is widely considered to be an effective means of increasing the duration of transgene expression. However, the impact of the position of the CpG motif in plasmids remains unclear. The results obtained using luciferase-expressing plasmid vectors suggest the following possibilities. When the expression from firefly luciferase-expressing plasmids was compared, the duration of transgene expression correlated well with the total number of CpG motifs in plasmid vectors (Figure 1 and Table 1). Early studies suggested the special importance of CpG motifs in the promoter/enhancer regions [12,25], but the results obtained in the present study suggest that the removal or replacement of CpG motifs in these regions is not sufficient to increase the duration of transgene expression.

The CpG motif-dependent duration of transgene expression suggests that the presence of the CpG motif in plasmid DNA interferes with the transgene expression from the vectors. Several possibilities have been proposed regarding the adverse effects of the CpG motif on transgene expression. A major possibility is methylation of the cytosine residue of CpG motifs by DNA methyltransferases [26,27]. It is reported that methylated CpG motifs recruit methyl-CpG binding proteins, and that transgene expression is transcriptionally repressed [28,29]. Irvine *et al.* [30] reported that methylation in the promoter and coding regions has a combined effect on transcriptional suppression. *In vitro* methylation of plasmid vectors showed a trend for transgene expression being dependent on methylation of the cytosine residue of CpG motifs (Figures 2B and 4B). In addition, the methylation-induced reduction in expression was prominent with plasmids containing many CpG motifs, but was not seen with the non-CpG plasmid DNA, pCpG-$\Delta$Luc. However, detailed examination of the data also indicated that methylation itself is not the whole mechanism behind the suppression of transgene expression. The expression from methylated pCMV-Luc, pGZB-Luc, pCMV-Mu$\gamma$ and pGZB-Mu$\gamma$ decreased with time (Figures 2B and 4B), indicating that additional events, such as the recruitment of methyl-CpG binding proteins, are also involved in methylation-mediated suppression of expression. No significant differences were observed in the profile of transgene expression between methylated and mock-methylated pCpG-$\Delta$Luc (Figure 2B), indicating the importance of reducing the number of CpG motifs in plasmid vectors for sustained transgene expression. The cytosine methylation of pCpG-Luc by *Sss*I methylase resulted in some reduction in the luciferase activity at 48 h after injection, although the activity was not significantly different from that by the mock-methylated counterpart. On the other hand, the time-courses of luciferase activity in mouse liver were almost superimposed between the pCpG-Luc- and the pCpG-$\Delta$Luc-injected groups, at least for the first 7 days after injection (Figure 1). These results could be explained by assuming that the rate and extent of CpG methylation in mouse liver is lower than that induced by *Sss*I methylase, and/or that CpG methylation in the cDNA region has less effects on transgene expression than that in other regions, such as promoter and ori.

Unmethylated CpG motifs in plasmid vectors have been found to stimulate the innate immune response by interacting with the host TLR9, and to trigger the release of pro-inflammatory cytokines [31–33]. These cytokines have been shown to have a variety of effects on transgene expression from vectors [34–36]. Reyes-Sandoval *et al.* [34] reported that CpG motif-mediated immune responses promote the activation of transgene product-specific B and T cells. In addition, it has been reported that vector-induced pro-inflammatory cytokines inhibit transgene transcription directly through shutdown of the promoter [37]. Furthermore, apoptosis of transfected cells may be another reason for the loss of transgene expression induced by the immune response [38]. Although naked plasmid DNA is a weak inducer of pro-inflammatory cytokines following administration to mice compared with DNA lipoplex, it induced the production of TNF-$\alpha$ after hydrodynamic injection [11]. In addition, the level of the cytokine was proportional to the number of CpG motifs in the plasmid DNA and cytosine methylation abolished cytokine production [11]. Therefore, the production of pro-inflammatory cytokines induced by the presence of CpG motifs in plasmid DNA could be another reason for the suppression of transgene expression from plasmids with many CpG motifs. To examine this possibility, DNA lipoplex was used to induce the production of pro-inflammatory cytokines because it is a far better inducer than naked plasmid DNA (Figures 3B and 3C). To avoid the fact that the delivery, or expression, from naked plasmid DNA given by the hydrodynamic injection

method is affected by the administration of DNA lipoplex, as well as following production of pro-inflammatory cytokines, DNA lipoplex was administered 1 h after the hydrodynamic delivery of luciferase-expressing plasmid DNA. Even although a large amount of TNF-$\alpha$ and IL-6 was produced by the administration of DNA lipoplex to mice that had received a hydrodynamic injection of naked plasmid DNA, the luciferase activity in the liver was hardly affected by the treatment (Figure 3). Therefore, it is reasonable to conclude that pro-inflammatory cytokines induced by the CpG motifs of plasmid vectors are not significantly involved in the suppression of transgene expression from naked plasmid DNA.

Reduction of CpG motifs from plasmid vectors was found to be effective in increasing the duration of transgene expression of IFN-$\gamma$, and the plasmid pCpG-Mu$\gamma$ that contains only 20 CpG motifs, all of which are in the cDNA of mouse IFN-$\gamma$, exhibited a very prolonged transgene expression (Figure 4A). The results using methylated plasmids (Figures 2B and 4B) suggest that CpG motifs in the IFN-$\gamma$ cDNA have less effects on transgene expression than those in the firefly luciferase cDNA. This difference in the effect of the CpG motif in the cDNA region between pCpG-Luc and pCpG-Mu$\gamma$ could be explained by the difference in the number of CpG motifs: only 20 motifs in the IFN-$\gamma$ cDNA and 194 in the luciferase cDNA (Table 1). Taken together, these results suggest that methylation of CpG motifs in cDNA region, even if it occurs, does not affect the expression of transgene from plasmid vectors when the CpG motif is only in the cDNA region and its numbers are limited.

The difference in the duration of transgene expression observed in the present study cannot be solely due to the difference in CpG content of the plasmid vector. Another possible major factor is the type of promoter, and there are several concerns relating to the use of CMV promoters [39–41]. Compared with CMV promoters, the human elongation factor 1 (EF1) promoter of pCpG-Luc, pCpG-$\Delta$Luc and pCpG-Mu$\gamma$ appears to achieve a prolonged transgene expression because it is a cellular promoter. It has been shown that the transgene expression from a plasmid containing an EF1 promoter is lower than that from a plasmid containing a CMV promoter, but that it declines much more slowly [42]. In addition, IFN-$\gamma$ is a potent anti-viral agent that has been shown to inhibit the viral transcription of CMV [43,44] and transgene expression from the CMV promoter [45]. However, little difference was observed between the EF1 promoter and the CpG-free CMV promoter with respect to the duration of transgene expression of luciferase (Figure 1). These findings strongly suggest that CpG content is a more important factor than the type of promoter with respect to the duration of transgene expression.

In conclusion, we have demonstrated that the total number of CpG motifs, especially those in the regions outside cDNA, is an important factor determining the sustainability of transgene expression after hydrodynamic injection of naked plasmid DNA into mice. Prolonged expression of IFN-$\gamma$ was successfully achieved, which was effective in inhibiting the experimental pulmonary metastasis of tumor cells. Together with the fact that the plasmid DNA-induced cytokine production is almost completely explained by the presence of the CpG motif in DNA *in vivo* (Yoshida *et al.*, unpublished data), the present study provides experimental evidence that the CpG reduction in plasmid DNA is indispensable for a safe and effective *in vivo* gene therapy for diseases that require sustained transgene expression.

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## References

1. Nishikawa M, Hashida M. Nonviral approaches satisfying various requirements for effective in vivo gene therapy. *Biol Pharm Bull* 2002; **25**: 275–283.
2. Nishikawa M, Takakura Y, Hashida M. Pharmacokinetic considerations regarding non-viral cancer gene therapy. *Cancer Sci* 2008; **99**: 856–862.
3. Kobayashi N, Nishikawa M, Takakura Y. The hydrodynamics-based procedure for controlling the pharmacokinetics of gene medicines at whole body, organ and cellular levels. *Adv Drug Deliv Rev* 2005; **57**: 713–731.
4. Liu F, Song Y, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther* 1999; **6**: 1258–1266.
5. Zhang G, Budker V, Wolff JA. High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum Gene Ther* 1999; **10**: 1735–1737.
6. Jacobs F, Snoeys J, Feng Y, *et al.* Direct comparison of hepatocyte-specific expression cassettes following adenoviral and nonviral hydrodynamic gene transfer. *Gene Ther* 2008; **15**: 594–603.
7. Bird AP. CpG islands as gene markers in the vertebrate nucleus. *Trends Genet* 1987; **3**: 342–347.
8. Yoshida H, Nishikawa M, Yasuda S, Mizuno Y, Toyota H, Kiyota T, Takahashi R, Takakura Y. Cellular activation by plasmid DNA in various macrophages in primary culture. *J Pharm Sci* 2008; **97**: 4575–4585.
9. Yasuda K, Ogawa Y, Yamane I, Nishikawa M, Takakura Y. Macrophage activation by a DNA/cationic liposome complex requires enosomal acidification and TLR9-dependent and independnt pathways. *J Leukoc Biol* 2005; **77**: 71–79.
10. Tan Y, Li S, Pitt BR, Huang L. The inhibitory role of CpG immunostimulatory motifs in cationic lipid vector-mediated transgene expression in vivo. *Hum Gene Ther* 1999; **10**: 2153–2161.
11. Kako K, Nishikawa M, Yoshida H, Takakura Y. Effects of inflammatory response on in vivo transgene expression by plasmid DNA in mice. *J Pharm Sci* 2007; **97**: 3074–3083.
12. Brooks AR, Harkins RN, Wang P, Qian HS, Liu P, Rubanyi GM. Transcriptional silencing is associated with extensive methylation of the CMV promoter following adenoviral gene delivery to muscle. *J Gene Med* 2004; **6**: 395–404.
13. Yew NS, Zhao H, Przybylska M, *et al.* CpG-depleted plasmid DNA vectors with enhanced safety and long-term gene expression in vivo. *Mol Ther* 2002; **5**: 31–38.
14. de Wolf HK, Johansson N, Thong AT, *et al.* Plasmid CpG depletion improves degree and duration of tumor gene expression after intravenous administration of polyplexes. *Pharm Res* 2008; **25**: 1654–1662.
15. Kobayashi N, Kuramoto T, Chen S, Watanabe Y, Takakura Y. Therapeutic effect of intravenous interferon gene delivery with

naked plasmid DNA in murine metastasis models. *Mol Ther* 2002; **6**: 737–744.
16. Kawano H, Nishikawa M, Mitsui M, *et al.* Improved anti-cancer effect of interferon gene transfer by sustained expression using CpG-reduced plasmid DNA. *Int J Cancer* 2007; **121**: 401–406.
17. Nomura T, Yasuda K, Yamada T, *et al.* Gene expression and antitumor effects following direct interferon (IFN)-gamma gene transfer with naked plasmid DNA and DC-chol liposome complexes in mice. *Gene Ther* 1999; **6**: 121–129.
18. Qin L, Ding Y, Pahud DR, Chang E, Imperiale MJ, Bromberg JS. Promoter attenuation in gene therapy: interferon-gamma and tumor necrosis factor-alpha inhibit transgene expression. *Hum Gene Ther* 1997; **8**: 2019–2029.
19. Ghazizadeh S, Carroll JM, Taichman LB. Repression of retrovirus-mediated transgene expression by interferons: implications for gene therapy. *J Virol* 1997; **71**: 9163–9169.
20. Gansbacher B, Bannerji R, Daniels B, Zier K, Cronin K, Gilboa E. Retroviral vector-mediated gamma-interferon gene transfer into tumor cells generates potent and long lasting antitumor immunity. *Cancer Res* 1990; **50**: 7820–7825.
21. Yanagihara K, Seyama T, Watanabe Y. Antitumor potential of interferon-gamma: retroviral expression of mouse interferon-gamma cDNA in two kinds of highly metastatic mouse tumor lines reduces their tumorigenicity. *Nat Immun* 1994; **13**: 102–112.
22. Singh RK, Gutman M, Bucana CD, Sanchez R, Llansa N, Fidler IJ. Interferons $\alpha$ and $\beta$ down-regulate the expression of basic fibroblast growth factor in human carcinomas. *Proc Natl Acad Sci USA* 1995; **92**: 4562–4566.
23. Albini A, Marchisone C, Del Grosso F, *et al.* Inhibition of angiogenesis and vascular tumor growth by interferon-producing cells: a gene therapy approach. *Am J Pathol* 2000; **156**: 1381–1393.
24. Ikeda H, Old LJ, Schreiber RD. The roles of IFN gamma in protection against tumor development. *Cytokine Growth Factor Rev* 2002; **13**: 95–109.
25. Mutskov VJ, Farrell CM, Wade PA, Wolffe AP, Felsenfeld G. The barrier function of an insulator couples high histone acetylation levels with specific protection of promoter DNA from methylation. *Genes Dev* 2002; **16**: 1540–1554.
26. Hsieh CL. In vivo activity of murine de novo methyltransferases, Dnmt3a and Dnmt3b. *Mol Cell Biol* 1999; **19**: 8211–8218.
27. Bestor TH. The DNA methyltransferases of mammals. *Hum Mol Genet* 2000; **9**: 2395–2402.
28. Hendrich B, Bird A. Identification and characterization of a family of mammalian methyl-CpG binding proteins. *Mol Cell Biol* 1998; **18**: 6538–6547.
29. Fujita N, Shimotake N, Ohki I, *et al.* Mechanism of transcriptional regulation by methyl-CpG binding protein MBD1. *Mol Cell Biol* 2000; **20**: 5107–5118.
30. Irine RA, Lin IG, Hsieh C. DNA methylation has a local effect on transcription and histone acetylation. *Mol Cell Biol* 2002; **22**: 6689–6696.
31. Hemmi H, Takeuchi O, Kawai T, *et al.* A Toll-like receptor recognizes bacterial DNA. *Nature* 2000; **408**: 740–745.
32. Ahmad-Nejad P, Hacker H, Rutz M, Bauer S, Vabulas RM, Wagner H. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. *Eur J Immunol* 2002; **32**: 1958–1968.
33. Chuang TH, Lee J, Kline L, Mathison JC, Ulevitch RJ. Toll-like receptor 9 mediates CpG-DNA signaling. *J Leukoc Biol* 2002; **71**: 538–544.
34. Reyes-Sandoval A, Ertl HC. CpG methylation of a plasmid vector results in extended transgene product expression by circumventing induction of immune responses. *Mol Ther* 2004; **9**: 249–261.
35. Yew NS, Zhao H, Wu IH, *et al.* Reduced inflammatory response to plasmid DNA vectors by elimination and inhibition of immunostimulatory CpG motifs. *Mol Ther* 2000; **1**: 255–262.
36. Kuramoto T, Nishikawa M, Thanaketpaisarn O, Okabe T, Yamashita F, Hashida M. Use of lipoplex-induced nuclear factor-$\kappa$B activation to enhance transgene expression by lipoplex in mouse lung. *J Gene Med* 2006; **8**: 53–62.
37. Chen D, Murphy B, Sung R, Bromberg JS. Adaptive and innate immune responses to gene transfer vectors: role of cytokines and chemokines in vector function. *Gene Ther* 2003; **10**: 991–998.
38. Scheule RK. The role of CpG motifs in immunostimulation and gene therapy. *Adv Drug Deliv Rev* 2000; **44**: 119–134.
39. Herweijer H, Zhang G, Subbotin VM, Budker V, Williams P, Wolff JA. Time course of gene expression after plasmid DNA gene transfer to the liver. *J Gene Med* 2001; **3**: 280–291.
40. Kay MA, Baley P, Rothenberg S, *et al.* Expression of human alpha 1-antitrypsin in dogs after autologous transplantation of retroviral transduced hepatocytes. *Proc Natl Acad Sci USA* 1992; **89**: 89–93.
41. Kay MA, Li Q, Liu TJ, *et al.* Hepatic gene therapy: persistent expression of human alpha 1-antitrypsin in mice after direct gene delivery in vivo. *Hum Gene Ther* 1992; **3**: 641–647.
42. Gill DR, Smyth SE, Goddard CA, *et al.* Increased persistence of lung gene expression using plasmids containing the ubiquitin C or elongation factor 1alpha promoter. *Gene Ther* 2001; **8**: 1539–1546.
43. Kerr IM, Stark GR. The antiviral effects of the interferons and their inhibition. *J Interferon Res* 1992; **12**: 237–240.
44. Gribaudo G, Ravaglia S, Caliendo A, *et al.* Interferons inhibit onset of murine cytomegalovirus immediate-early gene transcription. *Virology* 1993; **197**: 303–311.
45. Jerome SH, Gary AS. Interferon-$\gamma$ inhibits transgene expression driven by SV40 or CMV promoters but augments expression driven by the mammalian MHC I promoter. *Hum Gene Ther* 1995; **6**: 1291–1297.

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# Reactivation of Silenced Transgene Expression in Mouse Liver by Rapid, Large-Volume Injection of Isotonic Solution

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**Abstract**

Rapid, large-volume injection, or so-called hydrodynamic injection, of naked plasmid DNA gives high transgene expression in mouse liver, and this method has been applied to liver-directed gene transfer in humans with slight modifications. To prove that injection-induced biological changes are involved in hydrodynamic injection-induced, high-level transgene expression in mouse liver, isotonic solutions were injected into mice that had received a hydrodynamic injection of plasmid DNA. Transgene expression in the liver was increased by such injections irrespective of the cDNA, promoter, and type of solution. This reactivation was repeatable and detectable even 3 months after gene transfer. Parameters required for reactivation were similar to those required for the hydrodynamic injection of plasmid DNA. Plasmid DNA–polyethyleneimine complex-based transgene expression in mouse liver was also reactivated by the same treatment. DNA microarray and quantitative RT-PCR analyses revealed that the expression of immediate-early response genes c-*fos* and c-*jun* was increased 70- and 100-fold, respectively. Activator protein (AP)-1- or nuclear factor (NF)-κB-dependent transgene expression was increased by an injection of isotonic solutions. These findings indicate for the first time that a rapid, large-volume injection of isotonic solution activates the transcription factors AP-1 and NF-κB in the liver, which in turn increases the transcription of genes delivered by hydrodynamic injection or other methods.

## Introduction

HYDRODYNAMIC DELIVERY of naked plasmid DNA has become a "gold standard" for achieving transgene expression in mouse liver *in vivo* (Kobayashi *et al.*, 2005). This has been applied to study the function of genes of interest in live animals (Ehrhardt *et al.*, 2005). Because this method does not require any lipids or polymers for *in vivo* gene transfer, it has been considered safe for use in clinics to deliver therapeutic genes and short hairpin RNA. Naked plasmid DNA was delivered to human liver by hydrodynamic delivery using balloon-catheters (Khorsandi *et al.*, 2008).

Budker and coworkers reported intravascular delivery of naked plasmid DNA under high pressure to the liver (Budker *et al.*, 1996) or to skeletal muscle (Budker *et al.*, 1998), the mechanism of which could be similar to that of the hydrodynamic injection. Since Liu and coworkers (1999) and Zhang and coworkers (1999) reported hydrodynamic injection-mediated high transgene expression, the mechanism of this gene transfer has been explored in detail (Kobayashi *et al.*, 2001, 2004; Zhang *et al.*, 2004; Budker *et al.*, 2006; Sebestyén *et al.*, 2006). We reported that hydrodynamic injection transiently increases the permeability of the cell membrane (Kobayashi *et al.*, 2004). Zhang and coworkers (2004) reported that liver fenestrae are enlarged and pores are created on cell membranes by injection. Through the pores, plasmid DNA and other membrane-impermeable macromolecular compounds enter cells (Kobayashi *et al.*, 2001). On the other hand, transaminases and green fluorescent protein (GFP) expressed in liver cells were released from the cells (Kobayashi *et al.*, 2004). Suda and coworkers (2007) found that some hepatocytes of mice receiving a hydrodynamic injection have diluted cytoplasm, suggesting that injected solutions have direct access to the cytoplasm.

The level of transgene expression achieved by the hydrodynamic injection of naked plasmid DNA is high compared with other nonviral approaches. It is often evaluated with firefly luciferase, and the amount of this protein, expressed as relative light units (RLU), is used as an indicator of the level of transgene expression. We have developed several approaches to liver-specific gene transfer methods, including sugar-conjugated polymers or liposomes (Nishikawa *et al.*, 1998, 2000; Kawakami *et al.*, 2000; Morimoto *et al.*, 2003) and electroporation-mediated gene transfer (Sakai *et al.*, 2005; Thanaketpaisarn *et al.*, 2005). In these cases, a 1- to 5-mg/kg dose (i.e., 20–100 μg/20-g mouse) of plasmid DNA

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was injected and expression levels of $10^2$–$10^6$ RLU/sec/mg protein were obtained in mouse liver with a plasmid expressing firefly luciferase under the control of the cytomegalovirus (CMV) promoter, pCMV-Luc (Nishikawa *et al.*, 2000). The same plasmid gave $10^9$ RLU/sec/mg protein or higher expression when injected by the hydrodynamics-based procedure at doses of 5 μg/mouse or greater (Kobayashi *et al.*, 2004). The plasmid DNA injected at a dose as low as 10 ng/mouse gave luciferase activity in the liver of $10^6$ RLU/sec/mg protein. Thus, expression is extremely efficient when plasmid DNA is hydrodynamically injected into mice. In other words, no other nonviral gene delivery method is as efficient as those using hydrodynamic injections or viral vectors.

The great discrepancy between hydrodynamic injection and other nonviral gene transfer methods in terms of the level of transgene expression could be attributed to biological changes in the liver induced by the injection procedure. Because the volume and speed of the injections used are quite different from physiological blood flows, physiological and biological conditions can be altered by the injection. Ochiai and coworkers (2007) reported that mice receiving a second hydrodynamic injection showed a greater level of transgene expression compared with those receiving no additional injections. On the basis of these findings, they suggested an involvement of the activation of transcription factors in this observation, but no experimental evidence was provided. Loser and coworkers (1998) demonstrated that silenced transgene expression from a plasmid vector was increased by administration of endotoxin when the transgene was driven by a CMV promoter, suggesting the presence of a mechanism of reactivation of silenced transgene expression.

To prove that any biological changes in mouse liver are involved in the high transgene expression levels obtained by hydrodynamic injection of naked plasmid DNA, mice that had received a hydrodynamic injection of naked plasmid DNA were injected again with an isotonic solution containing no plasmid DNA. Various parameters were evaluated, including the cDNA and promoter of plasmids; the type of isotonic solutions; and the volume, speed, timing, and repeats of additional injections. Here we show that the injection of isotonic solutions reactivates silenced transgene expression in mouse liver. This reactivation was independent of plasmid or solution used, but was dependent on the volume and speed of injection. A hydrodynamic injection of isotonic solutions greatly increased mRNA expression of c-*fos* and c-*jun*, and activated transcription factors activator protein (AP)-1 and nuclear factor (NF)-κB. These findings explain why the hydrodynamic injection of naked plasmid DNA is highly efficient compared with other nonviral gene transfer methods.

## Materials and Methods

### *Chemicals*

Isotonic 0.9% sodium chloride solution was purchased from Otsuka Pharmaceutical Factory (Tokushima, Japan). Ringer's solution was obtained from Fuso Pharmaceutical Industries (Osaka, Japan). TransIT-QR delivery solution was purchased from Mirus Bio (Madison, WI). *In vivo* jetPEI was purchased from Polyplus-transfection (Illkirch, France).

### *Animals*

Male ICR mice (6 weeks old, about 28 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Animals were maintained under conventional housing conditions and received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996). The protocols for animal experiments were approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences of Kyoto University (Kyoto, Japan).

### *Plasmid DNA*

pCMV-Luc (Nishikawa *et al.*, 2000) and pCMV-Muγ (Nomura *et al.*, 1999) were constructed as reported previously. pGL3-control vector and pRL-SV40, which encode firefly luciferase and *Renilla* luciferase under the control of the simian virus 40 (SV40) promoter, respectively, were purchased from Promega (Madison, WI). Mercury pathway profiling luciferase system 1 (pTA-Luc, pAP-1-Luc, pNFκB-Luc, pGRE-Luc, pCRE-Luc, and pSRE-Luc) and pEGFP-N1 encoding enhanced green fluorescent protein (GFP) were purchased from Clontech (Mountain View, CA). pCpG-Luc and pCpG-ΔLuc, which encode firefly luciferase or a firefly luciferase fusion protein, respectively, under the control of the human elongation factor-1 (EF-1) promoter, were constructed with pCpG-mcs (InvivoGen, San Diego, CA) and pORF-LucSh-ΔCpG (InvivoGen) as reported elsewhere (Mitsui *et al.*, unpublished data). pAAT-Luc, a plasmid expressing firefly luciferase under the control of the human $\alpha_1$-antitrypsin (AAT) promoter, was kindly provided by X. Xiao (University of North Carolina, Chapel Hill, NC). Table 1 shows the promoters and the number of binding sites for AP-1 and NF-κB in the plasmids used in the present study.

### *Hydrodynamic injection*

Naked plasmid DNA dissolved in isotonic solutions was injected into the tail vein of mice. Unless otherwise indicated, 10 μg of plasmid DNA dissolved in 2.2 ml (8% of body weight) of 0.9% sodium chloride solution was injected over 5 sec (Liu *et al.*, 1999).

### *Hepatic gene transfer*

Under light anesthesia with diethyl ether, mice received an injection of 10 or 100 μg of naked pCMV-Luc in 20–30 μl of 0.9% sodium chloride solution into the right lobe of the liver. At 4.5 days after gene transfer, mice received an injection of 0.9% sodium chloride solution. In a separate set of experiments, 50 μg of pCMV-Luc was mixed with *in vivo* jetPEI at an *N/P* ratio of 10, and the complex formed was injected into the portal vein (the *N/P* ratio refers to the number of nitrogen residues of jetPEI per DNA phosphate). At 6.5 days after gene transfer, mice received an injection of 0.9% sodium chloride solution. In both cases, luciferase activity in mouse liver was determined 12 hr after the rapid, large-volume injection.

### *Luciferase imaging*

Imaging was performed in a two-step process using WinLight32 software, as reported elsewhere (Hyoudou *et al.*, 2007).

TABLE 1. PROMOTER AND NUMBER OF BINDING SITES FOR AP-1 AND NF-κB IN PLASMID VECTORS[a]

| *Plasmid DNA* | *Promoter* | *cDNA* | *Number of binding sites* | |
|---|---|---|---|---|
| | | | *AP-1* | *NF-κB* |
| pCMV-Luc | Human CMV | Firefly luciferase | 2 | 4 |
| pGL3-control | Human SV40 | Firefly luciferase | 2 | 0 |
| pCpG-Luc | Human EF1 | Firefly luciferase | 7 | 9 |
| pCpG-ΔLuc | Human EF1 | Firefly luciferase fusion protein | 7 | 9 |
| pAAT-Luc | Human AAT | Firefly luciferase | 6 | 0 |
| pRL-SV40 | Human SV40 | *Renilla* luciferase | 3 | 2 |
| pEGFP-N1 | Human CMV | EGFP | 2 | 4 |
| pCMV-Muγ | Human CMV | Murine IFN-γ | 2 | 4 |
| pTA-Luc | TATA box | Firefly luciferase | 0 | 0 |
| pAP-1-Luc | TATA box + AP-1 | Firefly luciferase | 4 | 0 |
| pNFκB-Luc | TATA box + NF-κB | Firefly luciferase | 0 | 4 |
| pGRE-Luc | TATA box + GRE | Firefly luciferase | 0 | 0 |
| pSRE-Luc | TATA box + SRE | Firefly luciferase | 0 | 0 |
| pCRE-Luc | TATA box + CRE | Firefly luciferase | 0 | 0 |

[a]The number of binding sites for AP-1 and NF-κB was counted using databases for transcriptional regulation (Heinemeyer *et al.*, 1998).

### *Luciferase assay*

Liver homogenates were centrifuged at 13,000 × *g*, and an aliquot of the supernatant was mixed with firefly luciferase assay buffer (Picagene; Toyo Ink, Tokyo, Japan) to detect firefly luciferase activity. The light produced was immediately measured with a luminometer (Lumat LB 9507; Berthold Technologies, Bad Wildbad, Germany). *Renilla* luciferase activity was measured in a similar manner with a Picagene dual kit (Toyo Ink).

### *Detection of GFP-positive cells*

Mice were killed and the livers were then embedded in Tissue-Tek O.C.T. embedding compound (Sakura Finetek, Tokyo, Japan), frozen in liquid nitrogen, and stored in 2-methylbutanol at −80°C. Frozen liver sections (thickness, 10 μm) were made with a cryostat (Jung Frigocut 2800E; Leica Microsystems, Wetzlar, Germany) by a routine procedure. The sections were directly observed by fluorescence microscopy (Biozero; Keyence, Osaka, Japan) without any fixation.

### *Enzyme-linked immunosorbent assay for interferon-γ*

About 100 μl of blood was collected from the tail vein at the indicated times after gene transfer. The blood samples were kept for 2–3 hr to allow clotting and then centrifuged to obtain serum. The concentration of interferon (IFN)-γ in the serum was determined by enzyme-linked immunosorbent assay (ELISA), using a commercial kit (Ready-SET-Go! mouse IFN-γ ELISA; eBioscience, San Diego, CA).

### *Real-time polymerase chain reaction*

Mice were killed and total RNA was extracted from approximately 50-mg liver samples, using Sepasol RNA I super (Nacalai Tesque, Kyoto, Japan). After RNase-free DNase I treatment (Takara, Tokyo, Japan), reverse transcription was performed with SuperScript II (Invitrogen, Carlsbad, CA) and oligo(dT) primer according to the manufacturer's protocol. For a quantitative analysis of mRNA expression, real-time polymerase chain reaction (RT-PCR) was carried out with total cDNA, using a LightCycler instrument (Roche Diagnostics, Basel, Switzerland). The oligodeoxynucleotide primers used for amplification were as follows: c-*fos*, forward (5′-gctgacagatacactccaa-3′) and reverse (5′-gacctccagtcaaatcca-3′); c-*jun*, forward (5′-gtgccaactcatgctaacg-3′) and reverse (5′-gcaaccagtcaagttctcaag-3′); c-*myc*, forward (5′-accaccagcacgactct-3′) and reverse (5′-agacgtggcacctcttga-3′); and *mkp-1*, forward (5′-tcaacgtctcagccaattgtcct-3′) and reverse (5′-cgtccagctttacccggttagtc-3′). Amplification products were detected online via intercalation of the fluorescent dye SYBR green (LightCycler-FastStart DNA master SYBR green I kit; Roche Diagnostics). The cycling conditions were as follows: initial enzyme activation at 95°C for 10 min, followed by 55 cycles at 95°C for 10 sec, 58°C for 5 sec, and 72°C for 12 sec. All cycling reactions were performed in the presence of 3.5 m*M* $MgCl_2$. Gene-specific fluorescence was measured at 72°C.

### *Serum transaminase activities*

Mice received a hydrodynamic injection of 10 μg of naked pCMV-Luc in 0.9% sodium chloride solution and a rapid, large-volume injection of 0.9% sodium chloride solution with a 4-day interval. Blood was serially collected by cutting the tip of the tail. Serum aspartate transaminase (AST) and alanine transaminase (ALT) activities were determined with test reagents (Transaminase CII test Wako; Wako Pure Chemical, Osaka, Japan). Control values were determined with blood obtained from age-matched, untreated mice.

### *Statistical analysis*

Differences were statistically evaluated by Student *t* test or by one-way analysis of variance followed by the Fisher PLSD multiple comparison test. The level of statistical significance was set at $p < 0.05$.

## Results

### *Rapid, large-volume injection reactivates silenced transgene expression in mouse liver*

The expression of firefly luciferase from a plasmid vector pCMV-Luc was transient in mouse liver (Nishikawa *et al.*, 2000), and such transient expression from plasmid vectors was also reported by others (Herweijer *et al.*, 2001). Therefore, this plasmid was injected in naked form into the tail vein of mice by the hydrodynamics-based procedure, and successive images were acquired from the same mice. Figure 1A–J shows luminescent images of mice receiving a single injection of naked pCMV-Luc in 0.9% sodium chloride solution at a dose of 10 $\mu$g/mouse. Two groups of three mice each were used to trace the time course of luminescence intensity in individual mice, and the orders of mice were kept unchanged from Fig. 1A to E (group 1), and from Fig. 1F to J (group 2). Thus, Fig. 1A and F indicates the results of two different groups of mice under identical experimental conditions, which is also the case for Fig. 1B and G. Luciferase activity in the liver was high at 6 hr (Fig. 1A and F) but fell to a low level 3 days after gene transfer (Fig. 1B and G), indicating that the expression is transient. These mice then received a second injection of 0.9% sodium chloride solution containing no plasmids, in the same manner as the hydrodynamic injection. *In vivo* imaging of mice clearly demonstrated that only mice receiving the injection 12 hr before the imaging showed increased luminescence irrespective of the interval between the injections (Fig. 1C–E). Figure 1K shows luciferase activity in the liver of mice 4 days after the hydrodynamic injection of naked pCMV-Luc. The mice were injected with 0.9% sodium chloride solution at the indicated times before the measurement of luciferase activity. Luciferase activity was increased by the second injection irrespective of the time interval between the injection and the assay, and a broad peak was observed from 6 to 24 hr after the second injection. These results are in a good agreement with those reported by Ochiai and coworkers (2007). The level achieved by the second injection was about 3% of the peak level ($1.14 \times 10^8$ RLU/sec/mg protein) of the initial transgene expression measured 6 hr after gene transfer. In addition, the mouse receiving the second injection 3.5 days after gene transfer showed detectable luminescent signals on day 4, but no detectable signals on day 8 or 12 (Fig. 1C–E, the

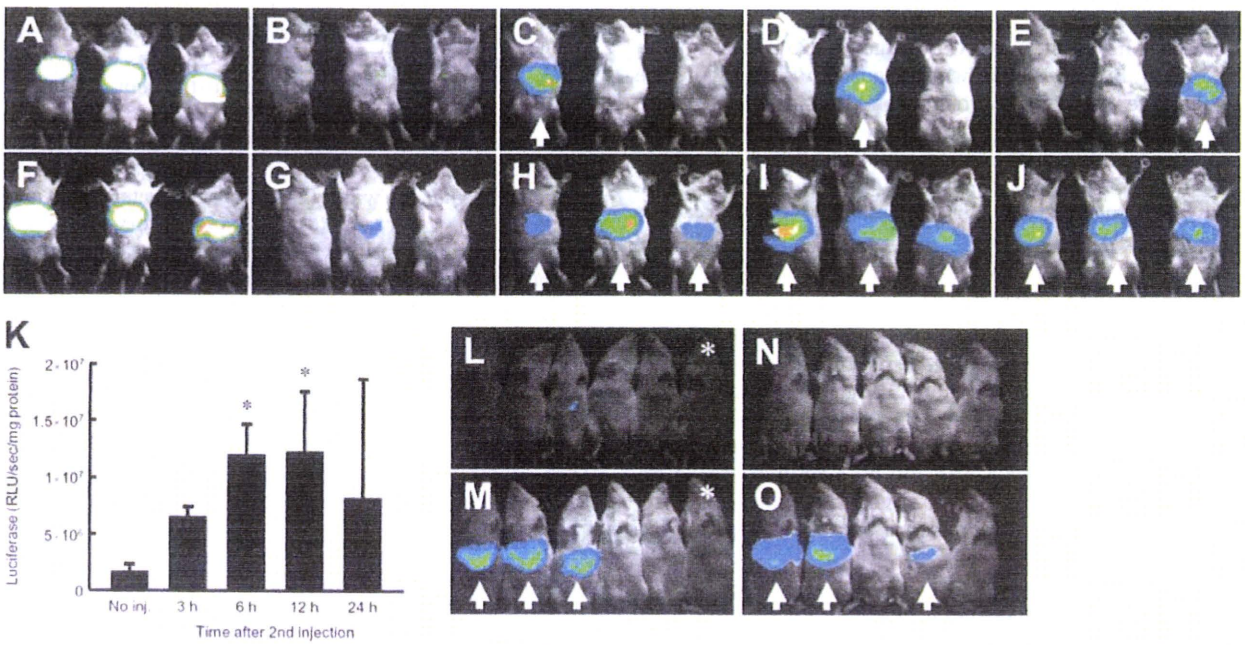


FIG. 1. Time course of transgene expression in mice receiving a hydrodynamic injection of 0.9% sodium chloride solution containing 10 $\mu$g of naked pCMV-Luc. (**A–J**) Luminescent images were taken (**A** and **F**) 6 hr, (**B** and **G**) 3 days, (**C** and **H**) 4 days, (**D** and **I**) 8 days, and (**E** and **J**) 12 days after gene transfer. Arrows indicate that the mice received additional injections of 0.9% sodium chloride solution 12 hr before imaging. In (**A–E**) and (**F–J**), three mice each were used to trace the time course of luminescence intensity in individual mice, and the orders of mice were kept unchanged. (**K**) Mouse liver was excised 4 days after gene transfer, and luciferase activity in the supernatant of liver homogenates was determined. Mice received a rapid, large-volume injection of 0.9% sodium chloride solution containing no plasmid DNA at the indicated times before the assay. Results are expressed as means ± SD of three or four mice. *$p < 0.05$ compared with the no-injection group. (**L–O**) Luminescent images were taken (**L** and **M**) 1 month and (**N** and **O**) 3 months after gene transfer, and the mice indicated with arrows received a rapid, large-volume injection of 0.9% sodium chloride solution containing no plasmid DNA. Before (**L** and **N**) and 12 hr after (**M** and **O**) the injection, luminescent images were obtained. *This mouse was found dead just before the imaging at 3 months after gene transfer because of deep anesthesia, so it was not shown in (**N** and **O**). The orders of mice were kept unchanged in (**L–O**) except for the dead mouse. Because of the reduction in the luminescent intensity with time, different parameters from those in (**A–J**) were used for the detection of luminescence at 1 and 3 months after gene transfer.

mouse on the left). These results suggest that reactivated expression is also transient as observed after the hydrodynamic injection of naked pCMV-Luc.

In a different set of mice, multiple injections of 0.9% sodium chloride solution were performed. Each injection greatly increased the luminescence (Fig. 1F–J), although all mice showed no significant luminescence 12 hr before the additional injections (Fig. 1G; and data not shown). Luminescent images were then obtained 1 and 3 months after the hydrodynamic injection of naked pCMV-Luc. The luminescence in the liver was much higher in mice receiving an injection of 0.9% sodium chloride solution 12 hr before the imaging compared with untreated animals (Fig. 1L–O). Again, reactivation was found to be dependent on the injection of 0.9% sodium chloride solution (indicated by arrows), transient, and repeatable.

*Reactivation depends on plasmid dose, volume, and speed of injection, but not on type of isotonic solutions*

Figure 2A–H shows luminescent images of mice receiving a hydrodynamic injection of naked pCMV-Luc at a dose of 1 $\mu$g/mouse (Fig. 2A–D) and 50 $\mu$g/mouse (Fig. 2E–H). The luminescence intensity detected 6 hr (Fig. 2A and E) or 1 day (Fig. 2B and F) after gene transfer corresponded to the plasmid dose, and much greater intensity was detected in mice receiving the high dose. Thereafter, expression decreased and no significant luminescence was detected 3 days after injection when a fixed set of parameters was used for detection (Fig. 2C and G). A second injection of 0.9% sodium chloride solution greatly increased intensity in the liver, which was again proportional to the plasmid dose with which mice had been injected (Fig. 2D and H). These lumi-

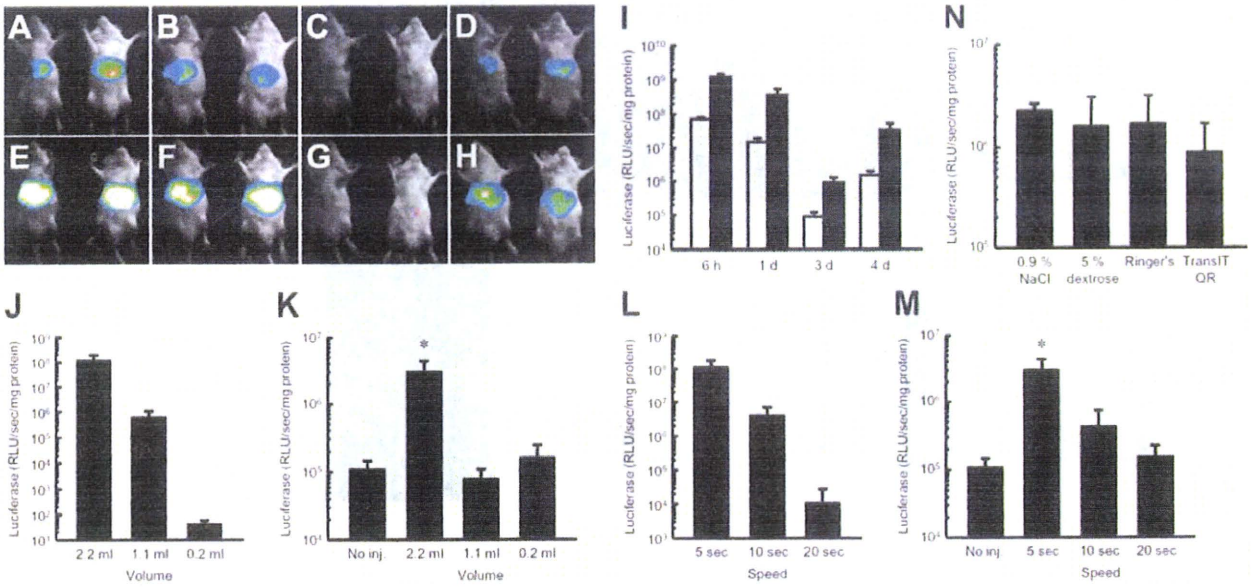


**FIG. 2.** Effect of plasmid dose, volume and speed of injection, and type of isotonic solution on the reactivation of transgene expression in mouse liver. (**A–H**) Luminescent images were taken (**A** and **E**) 6 hr, (**B** and **F**) 1 day, (**C** and **G**) 3 days, and (**D** and **H**) 4 days after a hydrodynamic injection of naked pCMV-Luc at a dose of 1 $\mu$g (**A–D**) or 50 $\mu$g (**E–H**). Each mouse received a rapid, large-volume injection of 0.9% sodium chloride solution containing no plasmid DNA on day 3.5. (**I**) Mice received a hydrodynamic injection of 1 $\mu$g (open columns) or 50 $\mu$g (solid columns) of pCMV-Luc. Then, 3.5 days after gene transfer, mice received a rapid, large-volume injection of 0.9% sodium chloride solution. Luciferase activity in mouse liver was determined at the indicated times after gene transfer. Results are expressed as means ± SD of at least four mice. (**J**) Mice received an injection of 10 $\mu$g of pCMV-Luc dissolved in 2.2, 1.1, or 0.2 ml of 0.9% sodium chloride solution at a speed of 2.2 ml/5 sec. Luciferase activity in mouse liver was determined 6 hr after gene transfer. Results are expressed as means ± SD of at least three mice. (**K**) Mice received a hydrodynamic injection of 10 $\mu$g of pCMV-Luc. Then, 3.5 days after gene transfer, mice received an injection of 2.2, 1.1, or 0.2 ml of 0.9% sodium chloride solution at a speed of 2.2 ml/5 sec. Luciferase activity in mouse liver was determined 12 hr after the second injection. Results are expressed as means ± SD of at least three mice. $^*p < 0.05$ compared with the no-injection group. (**L**) Mice received an injection of 10 $\mu$g of pCMV-Luc dissolved in 2.2 ml of 0.9% sodium chloride solution at a speed of 2.2 ml/5 sec, 2.2 ml/10 sec, or 2.2 ml/20 sec. Luciferase activity in mouse liver was determined 6 hr after gene transfer. Results are expressed as means ± SD of at least three mice. (**M**) Mice received a hydrodynamic injection of 10 $\mu$g of pCMV-Luc. At 3.5 days after gene transfer, mice received an injection of 2.2 ml of 0.9% sodium chloride solution at a speed of 2.2 ml/5 sec, 2.2 ml/10 sec, or 2.2 ml/20sec. Luciferase activity in mouse liver was determined 12 hr after the second injection. Results are expressed as means ± SD of at least three mice. $^*p < 0.05$ compared with the no-injection group. (**N**) Mice received a hydrodynamic injection of 10 $\mu$g of pCMV-Luc. Then, 3 days after gene transfer, mice received an injection of 0.9% sodium chloride solution, 5% dextrose solution, Ringer's solution, or TransIT-QR delivery solution. Luciferase activity in mouse liver was determined 12 hr after the second injection. Results are expressed as means ± SD of four mice.

nescence results were confirmed by the quantitative luciferase assay data (Fig. 2I).

Because the injection volume is a parameter affecting the level of transgene expression by hydrodynamics-based gene transfer (Liu *et al.*, 1999), the effect of the volume of 0.9% sodium chloride solution on luciferase activity in the liver was examined. When the volume of the hydrodynamic injection of naked pCMV-Luc was changed from 2.2 ml (8% of body weight, the hydrodynamic injection) to 1.1 ml (4%) and 0.2 ml (0.7%) at a constant speed of 2.2 ml/5 sec, the expression level decreased in proportion to the volume (Fig. 2J). Less than 1% luciferase activity was obtained when the volume was reduced to half or less, compared with the hydrodynamic injection. The effect of injection volume on reactivation was then examined in mice that had received a hydrodynamic injection of pCMV-Luc 3.5 days before the second injection (Fig. 2K). Injection of 0.9% sodium chloride solution in a reduced volume of 1.1 or 0.2 ml hardly increased luciferase activity in the liver.

We then examined the effects of the speed of injection, another important parameter determining the level of transgene expression. Mice received an injection of 2.2 ml of 0.9% sodium chloride solution containing pCMV-Luc over 5, 10, or 20 sec. Luciferase activity in the liver after injection of pCMV-Luc was proportional to the speed of injection (Fig. 2L). Again, the effect of injection speed on reactivation was examined. Even although the same volume of 0.9% sodium chloride solution was injected into mice that had received the hydrodynamic injection of pCMV-Luc, no significant increase in luciferase activity was observed when 2.2 ml of 0.9% sodium chloride solution was injected over 10 or 20 sec (Fig. 2M).

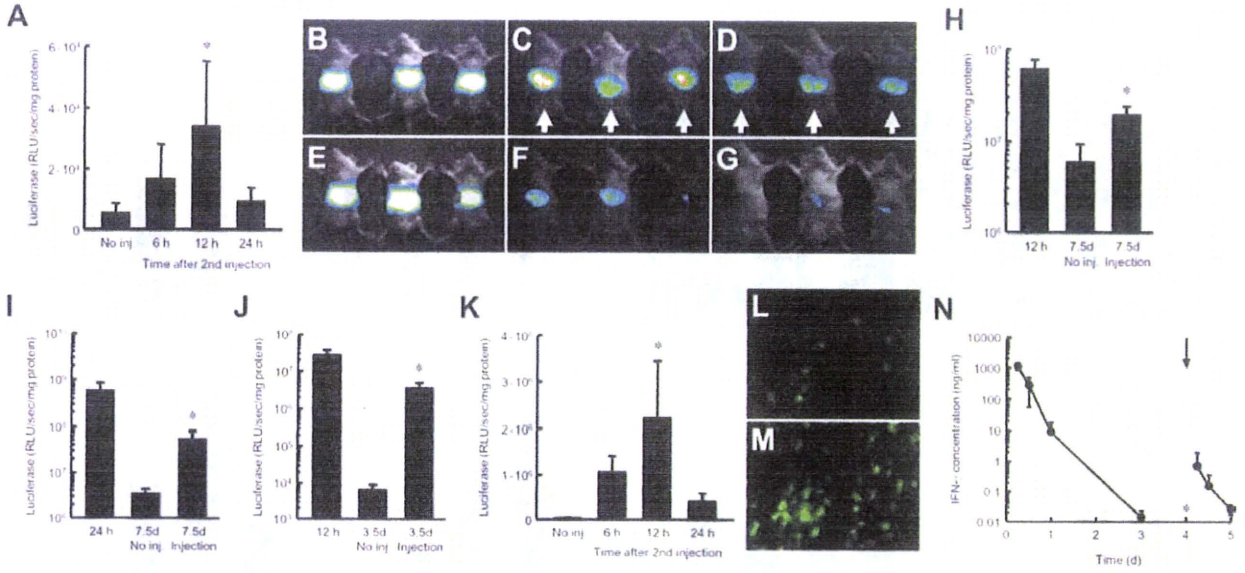


**FIG. 3.** Effect of promoter and cDNA on the reactivation of transgene expression in mouse liver. (**A**) Mice that had received a hydrodynamic injection of 10 $\mu$g of pGL3-control vector were given a rapid, large-volume injection of 0.9% sodium chloride solution at the indicated periods before the luciferase assay. Luciferase activity in mouse liver was determined 4 days after gene transfer (the first injection). Results are expressed as means ± SD of at least three mice. *$p < 0.05$ compared with the no-injection group. (**B–G**) Luminescent images were taken (**B** and **E**) 12 hr, (**C** and **F**) 7.5 days, and (**D** and **G**) 14.5 days after a hydrodynamic injection of naked pCpG-ΔLuc at a dose of 1 $\mu$g. Mice in the upper panels received a rapid, large-volume injection of 0.9% sodium chloride solution containing no plasmid DNA on day 7 and day 14. In (**B–D**) and (**E–G**), three mice each were used to trace the time course of luminescence intensity in individual mice, and the orders of mice were kept unchanged. Arrows indicate that the mice received additional injections of 0.9% sodium chloride solution 12 hr before imaging. (**H–J**) Mice received a hydrodynamic injection of (**H**) 1 $\mu$g of pCpG-ΔLuc, (**I**) 1 $\mu$g of pCpG-Luc, or (**J**) 10 $\mu$g of pAAT-Luc. Seven (**H** and **I**) or 3 days (**J**) after gene transfer, a group of mice received a rapid, large-volume injection of 0.9% sodium chloride solution containing no plasmid DNA. Luciferase activity in mouse liver was determined at the indicated times after gene transfer. Results are expressed as means ± SD of at least four mice. *$p < 0.05$ compared with the no-injection group. (**K**) Mice that had received a hydrodynamic injection of 10 $\mu$g of pRL-SV40 were given a rapid, large-volume injection of 0.9% sodium chloride solution at the indicated times before luciferase assay. Luciferase activity in mouse liver was determined 4 days after gene transfer (the first injection). Results are expressed as means ± SD of at least three mice. *$p < 0.05$ compared with the no-injection group. (**L** and **M**) Mice that had received a hydrodynamic injection of 1 $\mu$g of pEGFP-N1 were (**L**) left untreated or (**M**) injected with a rapid, large-volume injection of 0.9% sodium chloride solution 5 days after gene transfer. On day 6, mice were killed and the frozen liver sections made were observed with a fluorescence microscope. (**N**) Mice that had received a hydrodynamic injection of 20 $\mu$g of pCMV-Mu$\gamma$ were given a rapid, large-volume injection of 0.9% sodium chloride solution 4 days after gene transfer (indicated with an arrow). Blood samples were sequentially obtained from the tail vein, and the concentration of mouse IFN-$\gamma$ in serum was determined by ELISA. Low levels (<0.1 ng/ml) of endogenous IFN-$\gamma$ production were detected in the saline-treated mice (data not shown). Results are expressed as means ± SD of six mice. *The serum IFN-$\gamma$ concentration at 96 hr was below the detection limit of the ELISA.

Because the volume of sodium chloride solution used is almost comparable to the blood volume, the ingredients of the solution used may be involved in this reactivation. Figure 2N shows luciferase activity in the liver of mice receiving a hydrodynamic injection of pCMV-Luc in 0.9% sodium chloride solution. Each type of isotonic solution was injected into mice 3 days after gene transfer and luciferase activity was examined 12 hr after the second injection. In addition to 0.9% sodium chloride solution, 5% dextrose solution, Ringer's solution, and TransIT-QR delivery solution were injected into mice. No significant differences were observed among the groups examined.

### *Silenced expression is reactivated irrespective of promoter or cDNA*

To examine whether reactivation is specific to the plasmid DNA used, reactivation of transgene expression from other plasmid vectors were examined. Figure 3A shows the firefly luciferase activity in mouse liver after a hydrodynamic injection of firefly luciferase-expressing pGL3-control vector under the control of the SV40 promoter. Luciferase activity was significantly increased by the second injection of 0.9% sodium chloride solution. To examine whether reactivation is limited to viral promoters, such as CMV and SV40 promoters, or also applicable to cellular promoters, plasmids expressing firefly luciferase under the control of cellular promoters were used and reactivation was examined as described previously. Figure 3B–G shows the luminescent images of mice receiving a single injection of naked pCpG-ΔLuc, a plasmid expressing a derivative of firefly luciferase under the control of the human EF-1 promoter. The duration of transgene expression from pCpG-ΔLuc was longer than that from pCMV-Luc, but luciferase activity was significantly increased by the second injection (Fig. 3C) and the third injection (Fig. 3D), compared with those receiving no additional injections (Fig. 3F and G). The quantitative measurement of luciferase activity of liver homogenates confirmed these findings (Fig. 3H). Expression of normal firefly luciferase under the control of the human EF-1 promoter was also reactivated by the second injection of 0.9% sodium chloride solution (Fig. 3I). Similar results were obtained with pAAT-Luc (Fig. 3J), a plasmid expressing firefly luciferase under the control of the human AAT promoter. In addition, the expression of *Renilla* luciferase (Fig. 3K), green fluorescent protein (GFP; Fig. 3L and M), and murine interferon-γ (IFN-γ; Fig. 3N) was also reactivated by rapid, large-volume injection of 0.9% sodium chloride solution. These results indicate that reactivation of silenced transgene expression is a universal event irrespective of the cDNA or promoter of plasmid DNA.

### *Reactivation is not dependent on mode of gene transfer*

To examine whether reactivation is unique for plasmid DNA delivered by the hydrodynamic injection, two different methods were used for gene transfer to mouse liver, then 0.9% sodium chloride solution was injected. Low transgene expression was detected 6 hr after gene transfer by direct injection of naked pCMV-Luc into the liver ($6.5 \times 10^3$ RLU/sec/mg protein at 20 μg) or intraportal injection of pCMV-Luc complexed with *in vivo* jetPEI ($8.1 \times 10^5$ RLU/sec/mg protein at 50 μg). Luciferase activity fell over time in both cases, and low levels of activity were detected for the direct injection of naked pCMV-Luc into the liver (Fig. 4A) or for pCMV-Luc/*in vivo* jetPEI complex (Fig. 4B) on day 5 or 7, respectively. Activity was significantly increased by a rapid, large-volume injection of 0.9% sodium chloride solution 12 hr before the assay.

### *mRNA expression of c-fos and c-jun in mouse liver is upregulated by hydrodynamic injections*

Injection-induced reactivation of transgene expression suggests that the expression efficiency of plasmid DNA is upregulated by injections. Therefore, we examined the changes in the mRNA expression of endogenous genes in the liver. A DNA microarray analysis using mouse genome 430A 2.0 chips (Affymetrix Japan, Tokyo, Japan) revealed that the expression of many genes was changed by a hydrodynamic injection of 0.9% sodium chloride solution containing no plasmids. Table 2 summarizes 20 genes, the expression of which was greatly activated or repressed by the injection. Among those changed, c-*fos* and c-*jun* were the genes whose expression was greatly increased 1 hr after the injection. Thus, the expression of c-*fos*, c-*jun*, and other immediate-early genes (c-*myc* and *mkp-1*) was examined by quantitative RT-PCR. Figure 5A shows the time course of mRNA expression levels of these immediate-early genes in mouse liver after a rapid, large-volume injection of 0.9% sodium chloride solution. The levels of c-*fos* and c-*jun* mRNAs were considerably increased soon after the injection: about 70- and 100-fold increases were observed in the mRNA levels of c-*fos* and c-*jun*, respectively. The expression of c-*myc* and *mkp-1* was not so greatly changed.

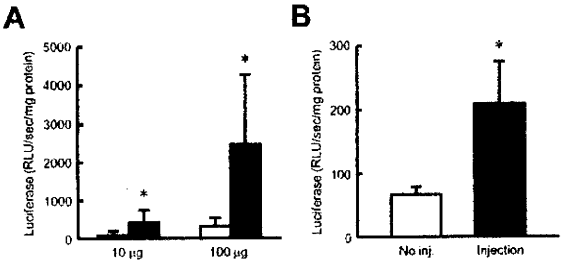


**FIG. 4.** Effect of the mode of gene transfer on the reactivation of transgene expression in mouse liver. (A) Mice that had received a tissue injection of 10 or 100 μg of naked pCMV-Luc into the liver were given a rapid, large-volume injection of 0.9% sodium chloride solution 4.5 days after gene transfer. Luciferase activity in mouse liver was determined 12 hr after the second injection. Results are expressed as means ± SD of at least five mice. $^*p < 0.05$ compared with the no-injection group. Open columns, no additional injection; solid columns, 0.9% sodium chloride solution injection. (B) Mice that had received an injection of 50 μg of pCMV-Luc/*in vivo* jetPEI complex (an *N*/*P* ratio of 10) into the portal vein were given a rapid, large-volume injection of 0.9% sodium chloride solution 6.5 days after gene transfer. Luciferase activity in mouse liver was determined 12 hr after the second injection. Results are expressed as means ± SD of three mice. $^*p < 0.05$ compared with the no-injection group.

TABLE 2. GENES WHOSE EXPRESSION IS GREATLY INFLUENCED BY HYDRODYNAMIC INJECTION OF 0.9% SODIUM CHLORIDE SOLUTION[a]

| Category | Entrez gene ID | Representative public ID | Gene symbol | Description | NT | HD | HD/NT ratio |
|---|---|---|---|---|---|---|---|
| Upregulated | 14281 | AV026617 | *Fos* | FBJ osteosarcoma oncogene | *16.5* | 4496.4 | 272.5 |
| | 12702 | BB241535 | *Socs3* | Suppressor of cytokine signaling 3 | 27.3 | 2101.8 | 77.0 |
| | 13654 | X06746 | *Egr2* | Early growth response 2 | *1.0* | 66.0 | 66.0 |
| | 240913 | BB443585 | *Adamts4* | A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 4 | *0.7* | 44.5 | 63.6 |
| | 11910 | BC019946 | *Att3* | Activating transcription factor 3 | *21.1* | 1323.3 | 62.7 |
| | 75571 | AK006554 | *Spata9* | Spermatogenesis associated 9 | *0.2* | 11.3 | 56.5 |
| | 20296 | AF065933 | *Ccl2* | Chemokine (C-C motif) ligand 2 | *1.5* | 82.5 | 55.0 |
| | 20310 | NM_009140 | *Cxcl2* | Chemokine (C-X-C motif) ligand 2 | *5.3* | 280.4 | 52.9 |
| | 216643 | BC027245 | *Gabrp* | γ-Aminobutyric acid (GABA-A) receptor, pi | *0.6* | 31.5 | 52.5 |
| | 16476 | NM_010591 | *Jun* | Jun oncogene | 79.2 | 3722.9 | 47.0 |
| Downregulated | 66775 | BB521459 | *Ptplad2* | Protein tyrosine phosphatase-like A domain containing 2 | 11.5 | *0.2* | 0.017 |
| | 68022 | AV169581 | *2210407P 21Rik* | RIKEN cDNA 2210407P21 gene | 11.7 | *0.2* | 0.017 |
| | 12763 | AW112184 | *Cmah* | Cytidine monophospho-*N*-acetylneuraminic acid hydroxylase | 18.0 | *0.3* | 0.017 |
| | 77012 | AK018056 | *5730596P 11Rik* | RIKEN cDNA 5730596P11 gene | 12.3 | *0.2* | 0.016 |
| | 319366 | AI452210 | *C920008N 22Rik* | RIKEN cDNA C90008N22 gene | 26.0 | *0.4* | 0.015 |
| | 27083/ 434794/ 619334 | NM_021365 | *Xlr4b/ Xlr4a/ Xlr4e* | X-linked lymphocyte-regulated 4B/ X-linked lymphocyte-regulated 4A/ X-linked lymphocyte-regulated 4E | 98.8 | *1.5* | 0.015 |
| | 236604/ 320951 | AV212090 | *4933439C 20Rik/ Pisd* | RIKEN cDNA 4933439C20 gene/ Phosphatidylserine decarboxylase | 31.1 | *0.4* | 0.013 |
| | 14853 | NM_008179 | *Gspt2* | $G_1$ to S phase transition 2 | 54.5 | *0.7* | 0.013 |
| | 327766 | BB469903 | *Tmem26* | Transmembrane protein 26 | 20.3 | *0.2* | 0.010 |
| | 94094 | BI653857 | *Trim34* | Tripartite motif protein 34 | 85.2 | *0.8* | 0.009 |

[a]Expression profiles of whole mouse liver were obtained with Mouse Genome 430A 2.0 chips (Affymetrix Japan K.K.). An untreated mouse (NT) and a mouse receiving a hydrodynamic injection of 0.9% sodium chloride solution (HD) were used for the analysis. The liver was collected 1 hr after the injection for the HD mouse. Using the expression levels of the NT and HD mice, the ratio of expression was calculated, and 10 genes mostly activated and 10 genes mostly repressed are listed. Italicized values indicate an "absent" call by the Affymetrix software.

### *AP-1- and NF-κB-dependent transgene expression is increased by hydrodynamic injections*

c-Fos and c-Jun are the components of AP-1, and the c-Fos:c-Jun heterodimer binds to the 12-*O*-tetradecanoylphorbol 13-acetate response element, or AP-1-binding site, and then activates transcription synergistically (Bannister *et al.*, 1994; Hess *et al.*, 2004). To examine changes in the activity of transcription factors, plasmid vectors that contained binding sequences for transcription factors, that is, AP-1, NF-κB, the glucocorticoid response element (GRE), the serum response element (SRE), or the cAMP response element (CRE), were injected into mice. Mice injected with pAP-1-Luc or pNFκB-Luc showed significantly greater luciferase activity compared with those injected with a control plasmid, pTA-Luc (Fig. 5B). Mice injected with other plasmids showed less luciferase activity, suggesting a lower contribution of GRE, CRE, and SRE to the event. We then examined whether AP-1 and NF-κB are activated by hydrodynamic injections. Figure 5C shows the time course of luciferase activity in the liver of mice injected with pTA-Luc, pAP-1-Luc, or pNFκB-Luc. Luciferase activity in the liver was significantly increased by the second injection in mice receiving pAP-1-Luc or pNFκB-Luc. The AP-1-dependent expression of firefly luciferase was much greater than that dependent on the NF-κB activity. Together with the fact that all the plasmid vectors except for pTA-Luc have binding elements for either or both AP-1 and NF-κB (Table 1), these results suggest that the activation of these two transcription factors influences the reactivation of silenced transgene expression.

### *Repeated hydrodynamic injections can be performed with limited additional damage*

To estimate possible liver damage induced by repeated hydrodynamic injections, serum AST and ALT activities

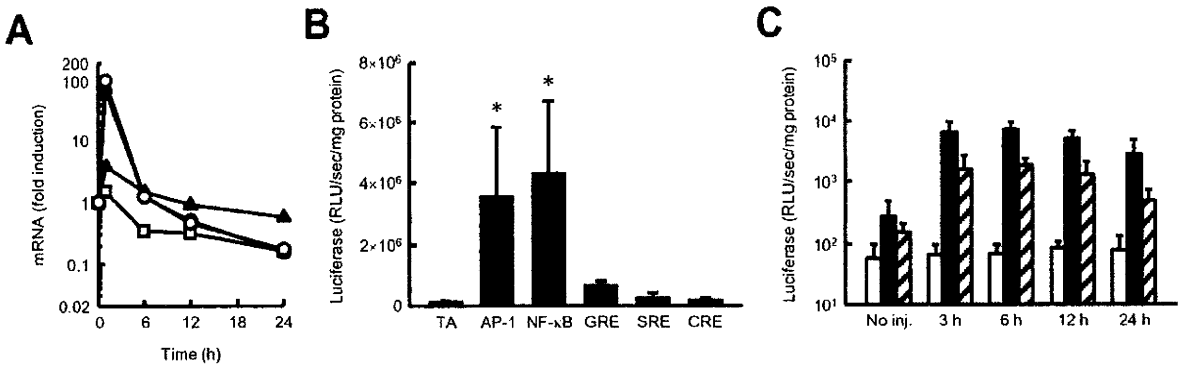


FIG. 5. Effect of hydrodynamic injections on the mRNA expression of immediate-early genes and the activity of transcription factors. (**A**) Total mRNA extracted from the liver of mice receiving a hydrodynamic injection of 0.9% sodium chloride solution was subjected to quantitative RT-PCR analysis. Each mRNA level was normalized on the basis of the expression level of *gapdh* in each sample. The *x*-fold induction was then calculated on the basis of the mRNA levels in untreated mice and those receiving an injection. Results are expressed as the mean of three mice. Solid circles, *c-fos*; open circles, *c-jun*; open squares, *c-myc*; solid triangles, *mkp-1*. (**B**) Mice were injected with a firefly luciferase-expressing plasmid DNA at a dose of 10 $\mu$g/mouse by the hydrodynamics-based procedure. Luciferase activity in mouse liver was determined 6 hr after gene transfer. Results are expressed as means ± SD of three mice. $^*p < 0.05$ compared with the no-injection group. TA, pTA-Luc; AP-1, pAP-1-Luc; NF-κB, pNFκB-Luc; GRE, pGRE-Luc; SRE, pSRE-Luc; CRE, pCRE-Luc. (**C**) Mice that had received a hydrodynamic injection of 10 $\mu$g of firefly luciferase-expressing plasmid DNA were injected rapidly with a large volume of 0.9% sodium chloride solution 3 days after gene transfer. Luciferase activity in mouse liver was determined at the indicated times after the second injection. Results are expressed as means ± SD of four mice. Open columns, pTA-Luc; solid columns, pAP-1-Luc; hatched columns, pNFκB-Luc.

were measured. Mice received two hydrodynamic injections with a 4-day interval, and serum samples were serially obtained from injected and untreated mice. As reported in previous papers (Liu *et al.*, 1999; Kobayashi *et al.*, 2004), the first hydrodynamic injection of naked pCMV-Luc resulted in a transient increase in the both serum AST and ALT levels (Fig. 6). The second injection of 0.9% sodium chloride solution induced less change in AST and ALT, and the serum levels of these enzymes returned to normal within 7 days of the first injection. To confirm whether a second hydrodynamic injection is as effective as the first injection in terms of gene delivery, we compared luciferase activity in mouse liver 6 hr after hydrodynamic injection of pCMV-Luc. Luciferase activity in the liver of mice receiving a hydrodynamic injection of 0.9% sodium chloride solution followed by a hydrodynamic injection of pCMV-Luc was 1.23 (±0.49) × $10^8$ RLU/sec/mg protein, whereas that of mice receiving only a hydrodynamic injection of pCMV-Luc was 3.21 (±0.74) × $10^8$ RLU/sec/mg protein. Therefore, the second injection would have an impact on liver similar to that of the first hydrodynamic injection insofar as the delivery of plasmid DNA is concerned.

## Discussion

Transgene expression at a level high enough to exhibit its therapeutic activity is a major challenge for *in vivo* gene transfer, especially when using plasmid-based nonviral vectors. The development of intravascular delivery methods for naked plasmid DNA (Budker *et al.*, 1996, 1998) as well as the following hydrodynamic injection of naked plasmid DNA, developed in 1999 (Liu *et al.*, 1999; Zhang *et al.*, 1999), has greatly increased the level of transgene expression achieved by plasmid DNA-based approaches. Ochiai and coworkers (2007) reported that the level of transgene expression in mouse liver was increased by a second hydrodynamic injection, and suggested involvement of the activation of transcription factors by such injections. The present study indicates for the first time that this hydrodynamic injection of isotonic solution activates transcription factors AP-1 and NF-κB in mouse liver, which would, at least partially, explain the high transgene expression achieved by this injection method.

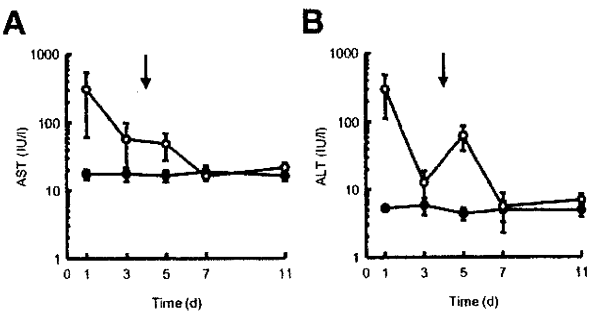


FIG. 6. Serum AST and ALT levels in mice receiving hydrodynamic injections. Mice that had received a hydrodynamic injection of 10 $\mu$g of pCMV-Luc were given a rapid, large-volume injection of 0.9% sodium chloride solution 4 days after gene transfer (indicated with an arrow). Blood was serially collected 1, 3, 5, 7, and 11 days after the first injection, and (**A**) serum AST and (**B**) serum ALT levels were measured. Results are expressed as means ± SD of more than four mice. Solid circles: age-matched, untreated mice; open circles: injected mice.

Despite the volume and speed of injection being beyond the range of physiological conditions, hydrodynamic injection of naked plasmid DNA is tolerable, at least in mice, and can even be applied to humans when some delivery modifications are considered. In actual fact, clinical applications of the concept of this gene delivery method have already been performed in the United Kingdom, using balloon-catheters (Khorsandi *et al.*, 2008). In the present study, we have demonstrated that additional hydrodynamic injections induce less change in serum AST and ALT levels compared with the first injection (Fig. 6). The reason for the difference has not been fully explained yet, but these findings increase the possibility of repeated applications of rapid, large-volume injections not only for repeated gene transfer but also for reactivation of silenced transgene expression.

The type of solutions injected may affect physiological and biological parameters, so that typical isotonic solutions were used for the second injection. Ringer's solution contains sodium chloride, potassium chloride, and calcium chloride at the same concentrations as in body fluids, whereas 5% dextrose contains only dextrose (glucose) and no salts. The ingredients of TransIT-QR delivery solution have not been disclosed. No significant differences were observed in the level of transgene expression when any one of these solutions was injected (Fig. 2N). Therefore, the ingredients seem to have little effect on the reactivation of silenced transgene expression in mouse liver.

Activation of transcription factors and subsequent transgene activation under various experimental and clinical conditions have been reported. Administration of endotoxin increased silenced transgene expression when the transgene was driven by a CMV promoter (Loser *et al.*, 1998). Radiation is another stimulus that greatly activates CMV promoter-driven transgene expression (Vereecque *et al.*, 2003). Ischemia followed by reperfusion of blood flow also activates various transcription factors, such as NF-κB (Kim *et al.*, 2005). Partial hepatectomy is also a well-known treatment to activate transcription factors involving inflammatory processes (Taub, 1996). We found that a hydrodynamic injection is another way of increasing the mRNA expression of immediate-early genes c-*fos* and c-*jun*. Expression of other molecules was also greatly changed by the injections; many upregulated and downregulated genes were identified and 10 genes in each category are listed in Table 2. Changes in the expression of genes other than immediate-early genes would also be involved in the reactivation of silenced transgene expression by hydrodynamic injections, but the details need further study. In the present study we examined the expression of immediate-early genes such as c-*myc* and *mkp-1*, but except for c-*fos* and c-*jun* their expression did not change markedly. These findings led us to examine whether hydrodynamic injection activates transcription factors, including AP-1. Changes in AP-1 activity, due to changes in the expression of AP-1 family members, posttranslational modification, or both, occur in response to a wide variety of events (Wisdom, 1999). As expected from the mRNA expression data, AP-1- and NF-κB-dependent transgene expression was greatly increased by hydrodynamic injections (Fig. 5B), demonstrating the activation of these transcription factors.

Results of the present study have clearly demonstrated that the reactivation of silenced transgene expression is a universal phenomenon relating to the hydrodynamic injections, because reactivation was observed with various types of plasmid vectors examined (Fig. 3). There are several concerns relating to the use of the CMV promoter (Kay *et al.*, 1992a,b; Herweijer *et al.*, 2001), one of the promoters most frequently used in the present study. However, reactivation was not limited to the CMV promoter, but was also applicable to the SV40 promoter, another viral promoter, and cellular promoters, such as the human EF-1 promoter and the human AAT promoter (Fig. 3). Transgene expression from plasmids having the EF-1 promoter was prolonged compared with that from plasmids having the CMV, SV40, or AAT promoter (data not shown), but expression from EF-1 promoter-containing plasmids was also reactivated (Fig. 3B–I). On the other hand, the extent of reactivation was highly dependent on the type of promoters and/or other experimental conditions, such as plasmid dose. Although further studies are needed to elucidate the reasons for the differences, the extent of reactivation may reflect the responsiveness of each promoter to the stimuli induced by hydrodynamic injections. Together with the results after direct injection of naked pCMV-Luc and after intraportal injection of pCMV-Luc complexed with *in vivo* jetPEI (Fig. 4), these results strongly suggest that rapid, large-volume injection of isotonic solution reactivates silenced transgene expression in mouse liver irrespective of the various conditions for gene transfer.

Another important finding of this study was that administered plasmid DNA could remain for a relatively long period of time in a form that could be activated transcriptionally, even when the expression became undetectable by standard detection methods. An early study by Herweijer and coworkers (2001) also reported that plasmid DNA remained even after expression from plasmids became undetectable. The expression of firefly luciferase could be detected even on day 90 after gene transfer (Fig. 1O), when mice received a rapid, large-volume injection of 0.9% sodium chloride solution 12 hr before imaging. These results suggest that part of the DNA delivered to liver cells remains undegraded within cells for a long period of time. Thus, reactivation, not readministration, of plasmid vectors could be an effective approach to achieving sustained, or pulsatile, transgene expression. Stimuli other than large-volume injections might be available if they can induce the activation of transcription factors similar to hydrodynamic injection.

In conclusion, we have demonstrated for the first time that a rapid, large-volume injection of isotonic solution increases the mRNA levels of c-*fos* and c-*jun*; activates transcription factors AP-1 and NF-κB; and reactivates silenced transgene expression in mouse liver, when the genes are driven by the CMV, SV40, EF-1, or AAT promoter. The present study has clearly shown that AP-1 and NF-κB are actually activated in mouse liver and that reactivation is a universal phenomenon relating to hydrodynamic injection. These findings shed new light on the mechanism of regulation of transgene expression after *in vivo* gene transfer and open up novel opportunities to control transgene expression in the liver without administering additional vectors.

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## Author Disclosure Statement

The authors declare no conflict of interest.

## References


Bannister, A.J., Brown, H.J., Sutherland, J.A., and Kouzarides, T. (1994). Phosphorylation of the c-Fos and c-Jun HOB1 motif stimulates its activation capacity. Nucleic Acids Res. 22, 5173–5176.

Budker, V., Zhang, G., Knechtle, S., and Wolff, J.A. (1996). Naked DNA delivered intraportally expresses efficiently in hepatocytes. Gene Ther. 3, 593–598.

Budker, V., Zhang, G., Danko, I., Williams, P., and Wolff, J.A. (1998). The efficient expression of intravascularly delivered DNA in rat muscle. Gene Ther. 5, 272–276.

Budker, V.G., Subbotin, V.M., Budker, T., Sebestyén, M.G., Zhang, G., and Wolff, J.A. (2006). Mechanism of plasmid delivery by hydrodynamic tail vein injection. II. Morphological studies. J. Gene Med. 8, 874–88.

Ehrhardt, A., Xu, H., Huang, Z., Engler, J.A., and Kay, M.A. (2005). A direct comparison of two nonviral gene therapy vectors for somatic integration: *In vivo* evaluation of the bacteriophage integrase $\phi$C31 and the Sleeping Beauty transposase. Mol. Ther. 11, 695–706.

Heinemeyer, T., Wingender, E., Reuter, I., Hermjakob, H., Kel, A.E., Kel, O.V., Ignatieva, E.V., Ananko, E.A., Podkolodnaya, O.A., Kolpakov, F.A., Podkolodny, N.L., and Kolchanov, N.A. (1998). Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL. Nucleic Acids Res. 26, 362–367.

Herweijer, H., Zhang, G., Subbotin, V.M., Budker, V., Williams, P., and Wolff, J.A. (2001). Time course of gene expression after plasmid DNA gene transfer to the liver. J. Gene Med. 3, 280–291.

Hess, J., Angel, P., and Schorpp-Kistner, M. (2004). AP-1 subunits: Quarrel and harmony among siblings. J. Cell Sci. 117, 5965–5973.

Hyoudou, K., Nishikawa, M., Kobayashi, Y., Kuramoto, Y., Yamashita, F., and Hashida, M. (2007). Analysis of *in vivo* nuclear factor-$\kappa$B activation during liver inflammation in mice: Prevention by catalase delivery. Mol. Pharmacol. 71, 446–453.

Institute of Laboratory Animal Resources, National Research Council (1996). Guide for the Care and Use of Laboratory Animals, 7th ed. (National Academies Press, Washington, D.C.).

Kawakami, S., Fumoto, S., Nishikawa, M., Yamashita, F., and Hashida, M. (2000). *In vivo* gene delivery to the liver using novel galactosylated cationic liposomes. Pharm. Res. 17, 306–313.

Kay, M.A., Li, Q., Liu, T.J., Leland, F., Toman, C., Finegold, M., and Woo, S.L. (1992a). Hepatic gene therapy: Persistent expression of human $\alpha_1$-antitrypsin in mice after direct gene delivery *in vivo*. Hum. Gene Ther. 3, 641–647.

Kay, M.A., Baley, P., Rothenberg, S., Leland, F., Fleming, L., Ponder, K.P., Liu, T., Finegold, M., Darlington, G., Pokorny, W., and Woo, S.L. (1992b). Expression of human $\alpha_1$-antitrypsin in dogs after autologous transplantation of retroviral transduced hepatocytes. Proc. Natl. Acad. Sci. U.S.A. 89, 89–93.

Khorsandi, S.E., Bachellier, P., Weber, J.C., Greget, M., Jaeck, D., Zacharoulis, D., Rountas, C., Helmy, S., Helmy, A., Al-Waracky, M., Salama, H., Jiao, L., Nicholls, J., Davies, A.J., Levicar, N., Jensen, S., and Habib, N. (2008). Minimally invasive and selective hydrodynamic gene therapy of liver segments in the pig and human. Cancer Gene Ther. 15, 225–230.

Kim, S.J., Varghese, T.K., Zhang, Z., Zhao, L.C., Thomas, G., Hummel, M., and Abecassis, M. (2005). Renal ischemia/reperfusion injury activates the enhancer domain of the human cytomegalovirus major immediate-early promoter. Am. J. Transplant. 5, 1606–1613.

Kobayashi, N., Kuramoto, T., Yamaoka, K., Hashida, M., and Takakura, Y. (2001). Hepatic uptake and gene expression mechanisms following intravenous administration of plasmid DNA by conventional and hydrodynamics-based procedures. J. Pharmacol. Exp. Ther. 297, 853–860.

Kobayashi, N., Nishikawa, M., Hirata, K., and Takakura, Y. (2004). Hydrodynamics-based procedure involves transient hyperpermeability in the hepatic cellular membrane: Implication of a nonspecific process in efficient intracellular gene delivery. J. Gene Med. 6, 584–592.

Kobayashi, N., Nishikawa, M., and Takakura, Y. (2005). The hydrodynamics-based procedure for controlling the pharmacokinetics of gene medicines at whole body, organ and cellular levels. Adv. Drug Deliv. Rev. 57, 713–731.

Liu, F., Song, Y., and Liu, D. (1999). Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. Gene Ther. 6, 1258–1266.

Loser, P., Jennings, G.S., Strauss, M., and Sandig, V. (1998). Reactivation of the previously silenced cytomegalovirus major immediate-early promoter in the mouse liver: Involvement of NF$\kappa$B. J. Virol. 72, 180–190.

Morimoto, K., Nishikawa, M., Kawakami, S., Nakano, T., Hattori, Y., Fumoto, S., Yamashita, F., and Hashida M. (2003). Molecular weight-dependent gene transfection activity of unmodified and galactosylated polyethyleneimine on hepatoma cells and mouse liver. Mol. Ther. 7, 254–261.

Nishikawa, M., Takemura, S., Takakura, Y., and Hashida, M. (1998). Targeted delivery of plasmid DNA to hepatocytes *in vivo*: Optimization of the pharmacokinetics of plasmid DNA/galactosylated poly(L-lysine) complexes by controlling their physicochemical properties. J. Pharmacol. Exp. Ther. 287, 408–415.

Nishikawa, M., Yamauchi, M., Morimoto, K., Ishida, E., Takakura, Y., and Hashida, M. (2000). Hepatocyte-targeted *in vivo* gene expression by intravenous injection of plasmid DNA complexed with synthetic multi-functional gene delivery system. Gene Ther. 7, 548–555.

Nomura, T., Yasuda, K., Yamada, T., Okamoto, S., Mahato, R.I., Watanabe, Y., Takakura, Y., and Hashida, M. (1999). Gene expression and antitumor effects following direct interferon (IFN)-$\gamma$ gene transfer with naked plasmid DNA and DC–chol liposome complexes in mice. Gene Ther. 6, 121–129.

Ochiai, H., Fujimuro, M., Yokosawa, H., Harashima, H., and Kamiya, H. (2007). Transient activation of transgene expression by hydrodynamics-based injection may cause rapid decrease in plasmid DNA expression. Gene Ther. 14, 1152–1159.

Sakai, M., Nishikawa, M., Thanaketpaisarn, O., Yamashita, F., and Hashida, M. (2005). Hepatocyte-targeted gene transfer by combination of vascularly delivered plasmid DNA and *in vivo* electroporation. Gene Ther. 12, 607–616.

Sebestyén, M.G., Budker, V.G., Budker, T., Subbotin, V.M., Zhang, G., Monahan, S.D., Lewis, D.L., Wong, S.C., Hagstrom, J.E., and Wolff, J.A. (2006). Mechanism of plasmid delivery by hydrodynamic tail vein injection. I. Hepatocyte uptake of various molecules. J. Gene Med. 8, 852–873.

Suda, T., Gao, X., Stolz, D.B., and Liu, D. (2007). Structural impact of hydrodynamic injection on mouse liver. Gene Ther. 14, 129–137.

Taub, R. (1996). Liver regeneration. 4. Transcriptional control of liver regeneration. FASEB J. 10, 413–427.

Thanaketpaisarn, O., Nishikawa, M., Yamashita, F., and Hashida, M. (2005). Tissue-specific characteristics of *in vivo*

electric gene: Transfer by tissue and intravenous injection of plasmid DNA. Pharm. Res. 22, 883–891.
Vereecque, R., Saudemont, A., Wickham, T.J., Gonzalez, R., Hetuin, D., Fenaux, P., and Quesnel, B. (2003). γ-irradiation enhances transgene expression in leukemic cells. Gene Ther. 10, 227–233.
Wisdom, R. (1999). AP-1: One switch for many signals. Exp. Cell Res. 253, 180–185.
Zhang, G., Budker, V., and Wolff, J.A. (1999). High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. Hum. Gene Ther. 10, 1735–1737.
Zhang, G., Gao, X., Song, Y.K., Vollmer, R., Stolz, D.B., Gasiorowski, J.Z., Dean, D.A., and Liu, D. (2004). Hydroporation as the mechanism of hydrodynamic delivery. Gene Ther. 11, 675–682.

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