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Research paper

# Evaluation of promoter strength in mouse and rat primary hepatocytes using adenovirus vectors

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**Abstract**

Primary cultured hepatocytes are widely used in the studies of basic and clinical hepatology. Finding an efficient method for gene transfer into primary hepatocytes will be an important advance for these studies. In the present study, we evaluated the activity of an adenovirus vector including promoters for the Rous sarcoma virus (RSV), elongation factor 1α, and cytomegalovirus (CMV) as well as the β-actin promoter/CMV enhancer (CA) using β-galactosidase as a reporter gene. Although RSV and elongation factor 1α promoters had low transcriptional activity in hepatocytes, the CA and CMV promoters had high activity. The CA promoter was the most active, mediating 50.3- and 204.4-fold more activity than the RSV promoter in mouse and rat hepatocytes, respectively. Dose-response studies revealed that transgene activity can be controlled by as much as 1000-fold, by selection of the promoter and the number of infectious particles per cell. These findings should help in the construction of adenovirus vectors for expressing genes of interest in rodent primary cultured hepatocytes.


*Keywords:* Hepatocyte; Adenovirus vector; Promoter; Transgene activity

## 1. Introduction

The liver is a multifunctional and vital organ that consists of parenchymal and nonparenchymal cells such as sinusoidal endothelial, Kupffer, and stellate cells. Hepatocytes have been well studied, and they are known to play a central role in the metabolism of drugs and detoxification of xenobiotics. Because there are few hepatocyte cell lines that have similar characteristics and behavior as intact hepatocytes, primary cultures of hepatocytes have been used for the study of viral hepatitis and the regeneration, physiology, pathobiology, and pharmacology of the liver [1–5]; however, primary hepatocytes progressively lose the liver-specific properties when isolated and cultivated [1,6]. The ectopic expression of genes of interest is very useful for estimation and investigation of the roles and functions of the genes, and an extremely efficient transfection system is needed for research using primary hepatocytes.

Gene transduction techniques include both nonviral and viral vectors. Adenovirus (Ad) vectors are widely used for basic and clinical research and are 100- to 1000-fold more efficient at mediating gene transduction than cationic lip-

*Abbreviations:* RSV, rous sarcoma virus; CMV, cytomegalovirus; CA, β-actin promoter/CMV enhancer; Ad, adenovirus; RT-PCR, reverse transcription polymerase chain reaction; CAR, coxsackievirus and adenovirus receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EF, elongation factor 1α; Ad, adenovirus; LacZ, β-galactosidase; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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ids, an effective nonviral vector [7]. Furthermore, quantitative comparison of transcriptional and post-transcriptional efficiency revealed that Ad vectors are 7000-fold more effective than cationic lipids for the transduction of nuclear DNA [8,9].

Transcription in mammalian cells is controlled by regulatory DNA sequences, including promoters, introns, and polyadenylation signals. An expression cassette, which is optimal, can be specific to each cell line or tissue [10–15]. It is therefore important to select an optimal expression cassette for each experimental model. Although Ad vectors are important in the basic studies of hepatocytes, the characteristics of the expression cassette of Ad vectors have not been fully determined in rodent primary cultured hepatocytes. In the present study, we investigated the ability of several promoters to mediate transgene activity by Ad vectors in mouse and rat primary hepatocytes.

## 2. Materials and methods

### 2.1. Isolation of hepatocytes from mouse and rat liver

Hepatocytes were isolated from male Sprague–Dawley rats and C57BL/6J mice (6 weeks old; Nippon SLC Co. Ltd., Kyoto, Japan) by the collagenase-perfusion method described by Seglen [16]. The cells were suspended in Williams' E medium containing 10% fetal calf serum, 1 nM insulin, and 1 nM dexamethasone. Next, cell viability was assessed by Trypan blue dye exclusion. Cells that were at least 90% viable were used in this study. For transfection, the suspended cells were seeded onto a 12-well plate at $2.5 \times 10^5$ cells/well. The cells were cultured in a humidified 5% $CO_2$ incubator at 37 °C.

### 2.2. Reverse transcription polymerase chain reaction (RT-PCR) analysis

The cells were harvested with a cell scraper. Total RNA was extracted from the cells with High Pure RNA Isolation Kit (Roche, Mannheim, Germany). RT-PCR was carried out using a TaKaRa RNA PCR kit (AMV) version 3.0 using an Oligo dT primer according to the manufacturer's instructions (TaKaRa, Shiga, Japan). Primers used here and the sizes of the putative PCR products are listed in Table 1. The PCR conditions were as follows: for mouse coxsackievirus and adenovirus receptor (CAR), 30 cycles of 45 s at 94 °C, 60 s at 57 °C, and 90 s at 72 °C; for mouse αv-integrin, 30 cycles of 45 s at 94 °C, 60 s at 56 °C, and 90 s at 72 °C; for mouse β3-integrin, 35 cycles of 45 s at 94 °C, 60 s at 53 °C, and 90 s at 72 °C; for mouse β5-integrin, 35 cycles for 45 s at 94 °C, 60 s at 57 °C, and 90 s at 72 °C; for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 30 cycles of 45 s at 94 °C, 60 s at 57 °C, and 90 s at 72 °C; for rat CAR, 30 cycles of 45 s at 94 °C, 60 s at 53 °C, and 90 s at 72 °C; for rat αv-integrin, 30 cycles of 45 s at 94 °C, 60 s at 53 °C, and 90 s at 72 °C; for rat β3-integrin, 30 cycles of 45 s at 94 °C, 60 s at 53 °C, and 90 s at 72 °C; for rat β5-integrin, 30 cycles of 45 s at 94 °C, 60 s at 53 °C, and 90 s at 72 °C; for rat GAPDH, 30 cycles of 45 s at 94 °C, 60 s at 53 °C, 90 s at 72 °C.

### 2.3. Preparation of Ad vectors

Ad vectors were prepared by an in vitro ligation method as described previously [17,18]. First, shuttle vectors were constructed by the replacement of the cytomegalovirus (CMV) promoter in pHMCMV5 [19] with the Rous sarcoma virus (RSV) promoter, elongation factor 1α (EF) promoter (derived from pEF/myc/nuc; Invitrogen, Carlsbad, CA,

Table 1
Primers used in this study

| Gene | | Sequence (5′–3′) | Product size (bp) |
|---|---|---|---|
| Mouse CAR | Forward | TGATCATTTTGTATTCTGGA | 211 |
| | Reverse | TTAACAAGAACGGTCAGCAG | |
| Mouse αv-integrin | Forward | CCAGCCTGGGATTGTAGAAG | 105 |
| | Reverse | ACTCCAGTGGGTCATCTTTG | |
| Mouse β3-integrin | Forward | TCTGGCTGTGAGTCCTGTGT | 115 |
| | Reverse | GCCTCACTGACTGGGAACTC | |
| Mouse β5-integrin | Forward | TCGTGTGAAGAATGCCTGTT | 126 |
| | Reverse | GCTGGACTCTCAATCTCACC | |
| Mouse GAPDH | Forward | ACCACAGTCCATGCCATCAC | 452 |
| | Reverse | TCCACCACCCTGTTGCTGTA | |
| Rat CAR | Forward | ATGGATCCTACACCCGAACAGAGGATCG | 280 |
| | Reverse | GCGATTTCGCGTCGCCAGACTTGACAT | |
| Rat αv-integrin | Forward | GGGCTACATCCTAGGCCTTC | 273 |
| | Reverse | GAAGATTCCAGGCAACAGGA | |
| Rat β3-integrin | Forward | CATCACGTCGCAGAACATCT | 279 |
| | Reverse | CTTGGTGGCCGTGTACTTCT | |
| Rat β5-integrin | Forward | TGTGTCTCTGCGGTGTTTGC | 312 |
| | Reverse | CCACGAGAACACCACAACAA | |
| Rat GAPDH | Forward | GGTCGGTGTGAACGGATTTG | 298 |
| | Reverse | GTGAGCCCCAGCCTTCTCCAT | |

USA), or β-actin promoter/CMV enhancer (CA) promoter (kindly provided by Dr. J. Miyazaki, Osaka University, Japan) [20], resulting in pHMRSV5, pHMEF5, and pHMCA5, respectively. The β-galactosidase (LacZ) gene was derived from pCMVβ (Marker Gene, Inc., Eugene, OR, USA) and inserted into pHMCMV5, pHMRSV5, pHMEF5, and pHMCA5, resulting in pHMCMV5-LacZ, pHMRSV5-LacZ, pHMEF5-LacZ, and pHMCA5-LacZ, respectively. Next, I-CeuI/PI-SceI-digested shuttle vectors were ligated with I-CeuI/PI-SceI-digested pAdHM4 [19], resulting in pAd-CMV-LacZ, pAd-RSV-LacZ, pAd-EF-LacZ, and pAd-CA-LacZ, respectively. To generate Ad vector particles, the Ad vector plasmids were digested with PacI, and the resulting digested Ad vector plasmids were transfected into 293 cells with Superfect according to the manufacturer's protocol (Qiagen, Inc., Valencia, CA, USA). Each Ad vector was purified by $CsCl_2$ step gradient ultracentrifugation, followed by $CsCl_2$ linear gradient ultracentrifugation. The concentration of vector particles was determined by a spectrophotometric method [21].

### 2.4. LacZ assay

After 24 h of isolation, the hepatocytes seeded onto a 12-well plate were treated with each Ad vector for 1.5 h. Next, the cells were washed with culture medium and then cultured for an additional 48 h. For 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining assays, the cells were washed with phosphate-buffered saline, fixed with 0.5% glutaraldehyde, and stained with X-gal solution (1.3 mM $MgCl_2$, 15 mM NaCl, 44 mM HEPES, 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, and 0.05% X-gal). The β-galactosidase activity was quantitatively measured with a Luminescent β-Gal Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol, and the activity was normalized by the cell number.

## 3. Results

Ad vectors infect target cells in two steps: (i) attachment of the vector to the cells via the knob domain to CAR on the cell membrane and (ii) internalization of the vector into the cell by interaction with Arg-Gly-Asp motifs of penton base with αvβ3- and αvβ5-integrins on the cell membrane [22–25]. Thus, the expression of CAR and integrins mediates gene transfer by the Ad vector. To examine the use of Ad vectors in hepatocytes, we first confirmed that the cells express these receptors by RT-PCR. As shown in Fig. 1, CAR, αv-integrin, β3-integrin, and β5-integrin were expressed in mouse primary hepatocytes, and CAR, αv-integrin and β5-integrin were expressed in rat primary hepatocytes.

To compare promoter strength, we constructed four Ad vectors containing LacZ as a reporter gene driven by the RSV, EF, CMV, or CA promoter (Ad-RSV-LacZ, Ad-EF-LacZ, Ad-CMV-LacZ, or Ad-CA-LacZ, respectively). We infected primary mouse and rat hepatocytes with each Ad vector at 300 vector particles (VP)/cell, and we checked

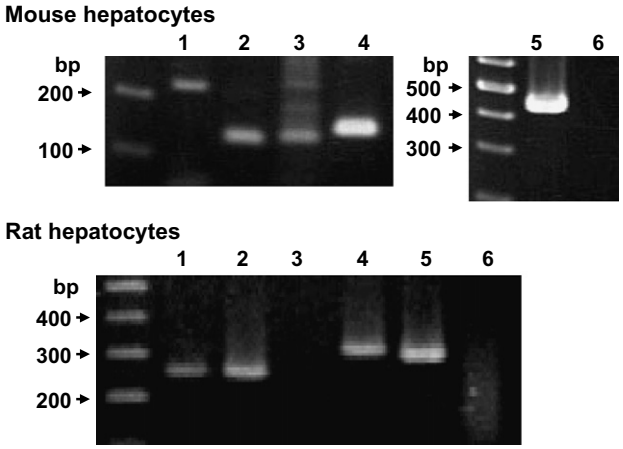


Fig. 1. RT-PCR analysis of receptors for Ad vectors. Total RNA was isolated from hepatocytes, purified with High Pure RNA Isolation Kit, and amplified by RT-PCR. Upper and lower panels are the data from mouse and rat hepatocytes, respectively. The left-most lane shows a 100-bp ladder, and the other lanes are as follows: lane 1, CAR; lane 2, αv-integrin; lane 3, β3-integrin; lane 4, β5-integrin; lane 5, GAPDH; lane 6, sample lacking reverse transcription. GAPDH was used as an endogenous control.

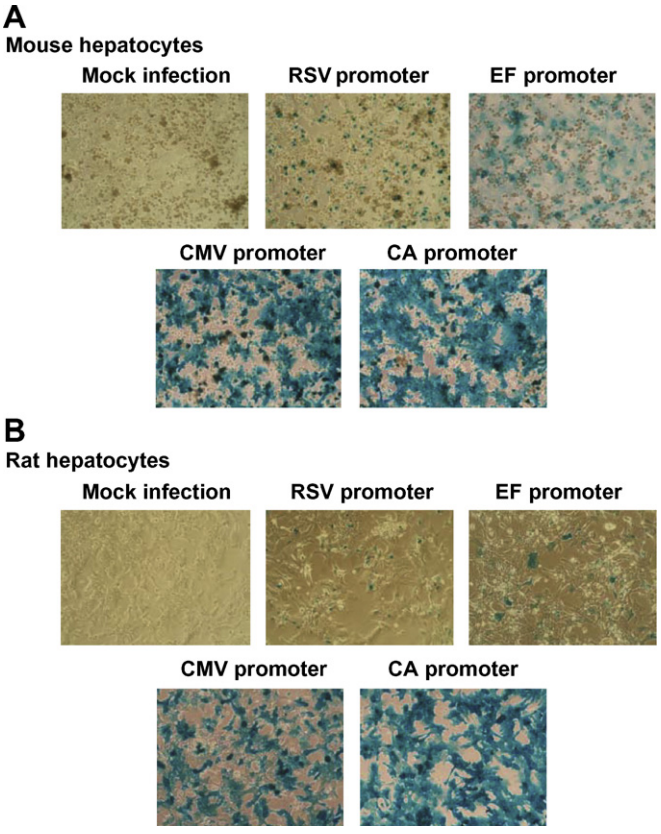


Fig. 2. X-gal staining of Ad vector-transduced rodent primary hepatocytes. Primary cultured hepatocytes isolated from the livers of mouse (A) and rat (B) were seeded onto 12-well plates. The cells were transfected with Ad-RSV-LacZ, Ad-CMV-LacZ, Ad-CA-LacZ, or Ad-EF-LacZ at 300 VP/cell for 1.5 h. The cells were washed with culture medium, cultured for an additional 48 h, fixed, and stained with X-gal.

the transduction of LacZ into the cells by staining them with X-gal after 48 h of transfection. As shown in Fig. 2, the cells infected with Ad-CA-LacZ or Ad-CMV-LacZ

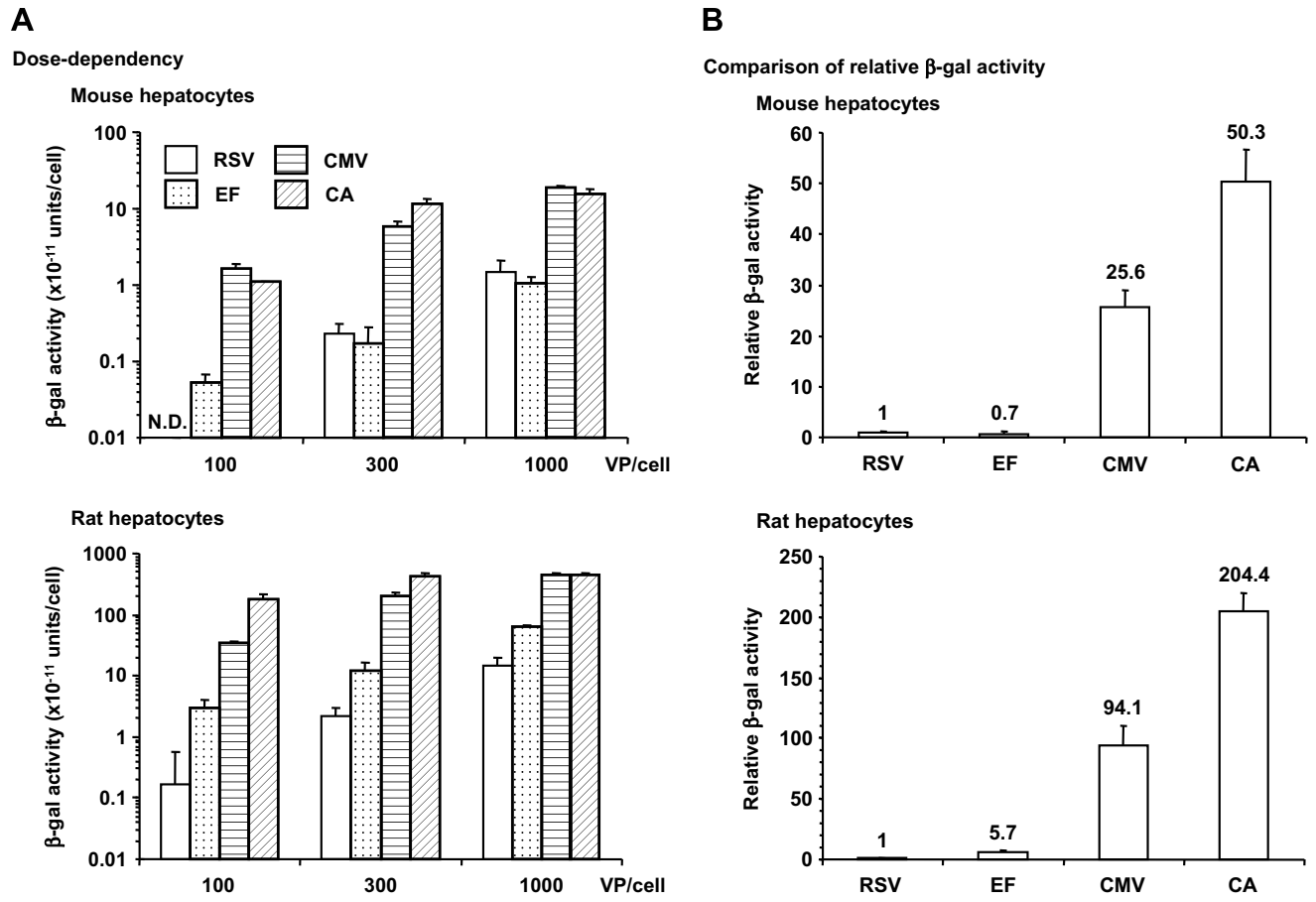


Fig. 3. Dose-dependent transduction activity in Ad vector-transduced rodent primary hepatocytes. (A) Dose-dependence of Ad vectors on transgene activity. Mouse and rat primary hepatocytes were treated with the indicated Ad vectors at the indicated concentration for 1.5 h. After washing with medium, the cells were cultured for 48 h and then harvested for the measurement of β-galactosidase (β-gal) activity using a luminescence assay kit. β-gal activity was normalized by the number of cells. (B) Comparison of relative transgene activity. The β-gal activity of each Ad vector at 300 VP/cell was expressed relative to the activity of Ad-RSV-LacZ. The numbers above each column indicate the relative β-gal activity. Data are means ± SD ($n = 3$). N.D., not detected.

were stained with X-gal, but few of those infected with Ad-RSV-LacZ or Ad-EF-LacZ were stained (Fig. 2A and B). Most of the hepatocytes were stained with X-gal when they were infected with Ad-CA-LacZ or Ad-CMV-LacZ at 1000 VP/cell (data not shown).

Next, to evaluate the dose-dependence of Ad vector efficiency, we measured β-galactosidase activity in cells infected at 100, 300, and 1000 VP/cell. The β-galactosidase activity increased in a dose-dependent manner for each of the Ad vectors in both mouse and rat hepatocytes (Fig. 3A), but the transcriptional activities differed among the promoters. Fig. 3B shows the β-galactosidase activity for each of the promoters relative to Ad-RSV-LacZ in cells infected at 300 VP/cell. The CA promoter had the highest transcriptional activity (50.3- and 204.4-fold in mouse and rat hepatocytes, respectively).

## 4. Discussion

Ad vectors have been widely used in basic and clinical studies of the liver, and liver function is often investigated using primary hepatocytes isolated from mouse and rat. However, there is little information on Ad vector-mediated gene expression in primary hepatocytes. In the present study, we evaluated the use of Ad vectors with several different promoters for transfecting rodent primary hepatocytes. We found that the CA promoter mediates strong transcriptional activity.

The first step for the expression of genes in mammalian cells is the binding of transcriptional factors to the promoter sequence upstream of the genes. This binding of transcriptional factors differs according to the promoter sequences. For example, Ad vectors with the CMV and EF promoters have very low and high transduction efficiency, respectively, in mouse embryonic stem cells [18]. In contrast, we found here that an Ad vector with the CMV promoter effectively expressed the LacZ gene in rodent hepatocytes, but they did not when they had the EF promoter. These different promoter activities suggest that different transcriptional factors participate in gene expression by mouse embryonic stem cells and mouse primary hepatocytes. Furthermore, the similar profiles of promoter strength in mouse and rat hepatocytes suggest that the two cell types utilize similar transcription factors.

In vivo studies in mice have shown that the CMV promoter has 4- to 10-fold higher transcriptional activity than the CA promoter in the liver [13], but in the current study, the CA promoter was approximately 2-fold more effective than the CMV promoter in rodent primary hepatocytes. This discrepancy might be due to the distribution of Ad vectors in the in vivo assay. Because systemically administrated Ad vectors distribute into parenchymal and nonparenchymal cells, the transgene activity may reflect the transcription of genes in not only hepatocytes but also Kupffer, stellate, and sinusoidal endothelial cells [26–28]. These nonparenchymal cells play important roles in hepatic processes, and the optimization of their transcriptional cassette is of interest for future studies. Another possible explanation for the difference in the in vitro and in vivo results is a difference in transcriptional factors between intact and primary hepatocytes. Indeed, the isolation of hepatocytes induces a prompt decline in certain liver-specific genes [6].

The expression of genes in mammalian cells is controlled by biochemical machinery including the promoter, enhancer, intron, and polyadenylation (PA) signal. To compare promoter activity, we added the PA signal of bovine growth hormone. Further, the optimization of the enhancer, intron, and PA signal is needed to prepare an ideal Ad vector for hepatocytes. The regulation of expression levels is also important for investigations using transgenes, and various systems have been developed for this purpose [29–32]. Xu et al. developed regulated transgene systems based on Ad vectors using elements that can be controlled by tetracycline, ecdysone, and antiprogestin [33]. Although these controllable systems are useful for basic research in rodent primary hepatocytes, chemical reagents must be added to regulate the gene expression. In the present study, we developed Ad vector systems regulated by several promoter sequences, and we found that the expression can differ by as much as 1000-fold according to the promoter sequence and the concentration of the Ad vector used to infect the rodent primary hepatocytes. The different expression profiles for each Ad vector showed to be useful for further basic hepatology research.

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