Glucose-Modulated Transgene Expression via Recombinant Adeno-Associated Virus

Ya-Wun Yang,[1,2] Yuan-Chiao Hsieh,[1] and Chih-Kai Chao[1]

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Purpose. The objective of this study was to examine glucose-modulated reporter gene expression via recombinant adeno-associated viral vectors both *in vitro* and *in vivo*.

Methods. Huh7 human hepatoma cells were transduced by recombinant adeno-associated virus (rAAV) vectors containing the luciferase gene under control of the rat insulin I gene promoter and a cytomegalovirus immediate-early promoter driving-enhanced green fluorescence protein gene. The reporter gene expression was evaluated by glucose stimulation either in the absence or presence of insulin secretagogues, including phorbol-12-myristate-13-acetate, dibutyryl cyclic AMP, and forskolin. *In vivo* studies were performed by injecting rAAV into the livers of streptozotocin-induced diabetic C57BL/6J mice followed by measurements of blood glucose concentration and luciferase activity assays 2 weeks after rAAV injection.

Results. At a multiplicity of infection of 500, approximately 66–69% of cells expressed enhanced green fluorescence protein at 48 h post-transduction. Luciferase activities, driven by the insulin gene promoter, in the rAAV-transduced hepatoma cells responded to millimolars of glucose. The addition of phorbol-12-myristate-13-acetate, dibutyryl cyclic AMP, and forskolin increased luciferase expression in the presence of either 1 mM or 25 mM glucose. The stimulation of luciferase activities by these substances was inhibited by the presence of 100 nM staurosporine. Exposure to increments of exogenous insulin up to 10^{-7} M inhibited luciferase gene expression in rAAV-transduced Huh7 cells. The *in vivo* experiments demonstrated good correlation between luciferase activities and blood glucose levels in streptozotocin-induced diabetic animals.

Conclusion. rAAV is a promising vector for hepatic gene therapy for diabetes. Glucose and insulin secretagogues modulated transgene expression in rAAV-transduced hepatoma cells, suggesting that conditions affecting insulin gene promoter function in pancreatic islet beta cells also affect transgene expression in human hepatoma cells conferred with insulin gene promoter. Results obtained from *in vivo* experiments demonstrated that glucose modulated transgene expression can be obtained in rAAV-treated diabetic C57BL/6J mice.

KEY WORDS: diabetes mellitus; gene delivery; glucose-responsiveness; recombinant adeno-associated virus.

INTRODUCTION

Insulin delivery presents a challenge because physiologically responsive insulin therapy for Type I diabetes requires insulin levels to be maintained within a therapeutic range and to respond to environmental hormonal and nutritional changes (1–3). It is known that insulin secretion from pancreatic beta cells primarily is controlled by circulating glucose levels: The beta cells sense glucose concentration through the 5′-flanking sequence of the insulin gene by detecting the levels of the products of glucose catabolism (4,5). Glucose responsiveness in the millimolar concentration range is an important criterion in engineering surrogate beta cells for insulin replacement, and attempts have been directed toward generating clonal islet beta-cell lines (6–8). Over time, however, these cells in culture exhibit either reduced insulin production or a loss of responsiveness to physiological glucose stimulation (9). Efforts using engineered non-beta cells, such as muscle cells or hepatocytes, have therefore been made to produce insulin for the treatment of diabetes mellitus (10–15). Hepatocytes especially have been widely used for studying gene therapy for diabetes because of their ability to synthesize and secrete a wide variety of enzymes that are involved in glucose metabolism. To regulate the secretion of insulin, these non-beta cells often need to be engineered to express the insulin gene under the control of a regulatable promoter (14–17). In an attempt to couple insulin levels to glucose concentrations, efforts have also been made to bioengineer insulin-producing cells through, for example, transfecting insulin-producing hepatoma cells with the glucose transporter GLUT2 (12), overexpressing RINm5F cells with glucokinase (18), or using glucose- and insulin-sensitive promoters (19). Most gene transfer experiments performed in these studies used non-viral techniques, which are often less efficient than virus-mediated methods.

Adeno-associated virus (AAV) is a 4.7-kb single-stranded, non-pathogenic human parvovirus. Wild-type AAV infects a wide variety of mammalian cells, including both dividing and non-dividing cells, without any recognized pathogenicity (20–23). Unlike adenovirus vectors, AAV does not induce serious immune response. Recombinant AAVs (rAAVs), derived from AAV in which the viral genes are replaced by the desired genes of interest and flanked by the inverted terminal repeats, are being developed as a promising gene delivery system (22,23). In a previous study, an rAAV vector was constructed containing the luciferase gene under control of the 410-bp rat insulin I gene promoter and an enhanced green fluorescence protein (EGFP) gene driven by the cytomegalovirus immediate-early promoter, and glucose-responsive luciferase gene expression was demonstrated in rAAV-transduced βHC9 pancreatic islet cells (24).

Type I, or insulin-dependent diabetes mellitus, results from autoimmune destruction of insulin-producing pancreatic islet cells. Gene therapy for insulin-dependent diabetes mellitus, therefore, would be most desirable if insulin production could be obtained from extra-pancreatic sources. At the same time, glucose-induced insulin secretion from the pancreatic islet beta cells is a highly regulated process controlled not only by the glucose concentration but also by the signal transduction crosstalk in the endocrine system (25). For example, cyclic adenosine monophosphate (cAMP), a second messenger produced in the cells in response to hormones and nutrients, also regulates insulin gene transcription.

In the present study, we examined the feasibility of obtaining glucose-modulated transgene expression in Huh7 human hepatoma cells via the rAAV-mediated route. To explore the effects of cAMP-dependent signaling pathway on the insulin promoter-driving luciferase gene in rAAV-

[1] School of Pharmacy, College of Medicine, National Taiwan University, 1, Room 1214, Jen-Ai Road, Section 1, Taipei 100, Taiwan.
[2] To whom correspondence should be addressed. (e-mail: ywyang@ha.mc.ntu.edu.tw)

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transduced Huh7 cells, the effect on regulated transgene expression by a number of substances that are secretagogues for normal islet beta-cells, including phorbol 12-myristate 13-acetate (PMA), dibutyryl cyclic adenosine monophosphate (dbcAMP), and forskolin were also examined. We found that glucose-modulated transgene expression can be obtained in rAAV-transduced human hepatoma cells conferred with the insulin gene promoter. To further examine glucose modulated transgene expression *in vivo*, the rAAV vector, rAAV.Ins.Luc.ΔEGFP, was administered into the livers of streptozotocin (STZ)-induced diabetic C57BL/6J mice. At 2 weeks after viral injection, livers of the animals were harvested and extracted. The luciferase activities determined were shown to correlate well with the animal blood glucose levels. The results presented in this study suggested the potential of treating diabetes mellitus by gene therapy via recombinant adeno-associated viral vectors.

MATERIALS AND METHODS

Materials

PMA, dibutyryl cyclic adenosine monophosphate (dbcAMP), forskolin, staurosporine, and insulin were purchased from Sigma Chemical Co. (St. Louis, Missouri). All cell culture materials were obtained from Life Technologies (Gaithersburg, Maryland).

Cell Culture

Huh7 human hepatoma cells were grown in Dulbecco's modified Eagle medium (DMEM) (GIBCO/BRL, Gaithersburg, Maryland), supplemented with non-essential amino acids, 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg amphotericin B/mL. Cells were maintained in an atmosphere of 95% humidity and 5% CO_2 at 37°C.

Preparation and Titration of rAAV

The rAAV.Ins.Luc.ΔEGFP vector was derived from AAV-type 2 (24). This construct contains the luciferase gene under control of rat insulin I gene promoter and the EGFP gene driven by the cytomegalovirus immediate-early promoter, which were placed in series between the AAV inverted terminal repeats. The production of rAAV stocks was performed as previously described by calcium phosphate-mediated co-transfection of HeLa cells with the packaging plasmid (24,26), followed by infection with adenovirus 5 at an multiplicity of infection (MOI) of 10. The crude virus stocks were double purified by isopycnic ultracentrifugation on CsCl gradient.

The genomic titers in each fraction of the rAAV stocks were serially diluted in 0.5 M NaOH, incubated at 65°C, and dot-blotted onto a nylon membrane. Blots were probed with [α-^{32}P]-dCTP-labeled DNA probes prepared using the random primer DNA labeling method (Life Technologies). The signal intensities of the viral fraction on the autoradiograph were compared with that of the standard plasmid and used to calculate the rAAV vector titers.

Transduction of Human Hepatoma Cells with the rAAV Vectors

To examine the capability of rAAV-transduced hepatoma cells to respond to the stimulation of glucose and the insulin secretagogues, Huh7 cells were plated at 1×10^5 cells/well in 12-well plates and infected with rAAV vectors in a final volume of 100 μL. After 2 h of incubation, 1 mL of complete medium was added. Twenty-four hours later, the medium was removed and replaced with glucose-free medium containing the desired concentrations of glucose and various test substances such as PMA, dbcAMP, forskolin, or insulin. To examine the inhibition effect, staurosporine was added 30 min before addition of the test agents. Mock-infected cultures served as controls. Cells were routinely examined under a Zeiss Axiovert 100TV fluorescence microscope/MC80DX. Unless otherwise indicated, cells were harvested at 48 h post-transduction for analysis of the reporter gene expression.

Kinetics of Glucose-Stimulated Luciferase Expression

To determine the kinetics of glucose-modulated luciferase expression driven by the insulin gene promoter, approximately 1×10^5 Huh7 human hepatoma cells were plated per well in 12-well plates and infected with rAAV at an MOI of 500. Cells were then incubated in glucose-free medium. Twenty-four hours later, fresh medium containing 25 mM glucose was added to stimulate luciferase expression. Cells from triplicate wells were harvested at different time points for up to 48 h and lysed in the cell lysis buffer, followed by luciferase activity assays.

FACS Analysis of EGFP Gene Expression

For detection of EGFP expression in the rAAV-transduced Huh7 cells, cells in 12-well plates were trypsinized, washed with phosphate-buffered saline (PBS), resuspended in PBS, and analyzed by a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry System, San Jose, California), using 488 nm excitation source and 530/30-nm band-pass filter set. A total of 20,000 cells were counted. Data acquisition and analysis were performed by the CellQuest program.

In Vivo Studies

Animal studies were performed on male C57BL/6J mice at 6–8 weeks of age. To obtain various blood glucose levels, animals were injected intraperitoneally with varying doses (0–200 mg) of STZ (5 mg/mL dissolved in 50 mM citrate buffer, pH 4.5), or mock treated with 0.9% NaCl. Blood glucose concentrations of the animals were determined after 6 h of fasting with a One-Touch II portable blood glucose monitor (LifeScan Inc., Milpitas, California). Forty-eight hours after STZ treatment, animals were anesthetized by intraperitoneal injection with chloral hydrate at 400 mg/kg body weight followed by abdominal incision. Recombinant AAV.Ins.Luc.ΔEGFP was delivered to the liver of mice by a single injection of 2×10^{10} rAAV particles in a volume of 200 μL using a 1-mL syringe and 30-gauge needle, and the wound was sutured for recovery. Animals were routinely monitored for changes in weight and blood glucose levels. For the time-course studies, animals with blood glucose levels within 200–

300 mg/dL were selected at random and sacrificed at the designated time points, and the livers were harvested and homogenized for luciferase activity assays. To examine glucose-modulated transgene expression *in vivo*, experiments were performed on the 14th day post-viral injection by grouping the animals into three blood glucose ranges: <100, 100–200, and 200–300 mg/dL glucose. Blood glucose levels were determined after 6 h of fasting. Animals were sacrificed followed by luciferase activity assays of the liver extracts.

Luciferase Activity Assays

Luciferase activities were determined using the Luciferase Reporter Assay System (Promega, Madison, Wisconsin). Briefly, the rAAV-transduced cells in each well were washed with PBS and lysed with 100 μL of lysis buffer. For *in vivo* studies, livers were homogenized with approximately two volumes of chilled lysis buffer and cleared by centrifugation. The extracts were stored at −80°C before assay. For luciferase activity assays, 20 μL of the cell or tissue extracts were combined with 100 μL of luciferase assay buffer. Emitted light was measured for 6 s in a Packard Microplate Scintillation & Luminescence Counter (Meriden, Connecticut). The protein concentration in the cell lysate was determined using Bio-Rad DC Protein Assay Reagents (Bio-Rad Laboratories). Luciferase activities were expressed as counts per second and were normalized by total protein concentrations.

Statistical Analysis

Results are presented as the means ± standard deviation (SD); data were analyzed with either one-way analysis of variance (ANOVA) or Student's *t* test. In conjunction with ANOVA, all pairwise multiple comparison procedure by the Student–Newman–Keuls method was used with a significance level of 0.05.

RESULTS

Glucose competence, a metabolic state that enables the glucose-signaling system to respond to glucose (25), is an important criteria for the design of insulin delivery systems. In the present study, we attempted to evaluate the feasibility of obtaining glucose competence in the hepatoma cells via rAAV-mediated gene transfer and to study the modulation of glucose responsiveness by the insulin secretagogues. To investigate the glucose responsiveness *in vivo*, experiments were performed using STZ-induced diabetic mice.

Time Course of Transgene Expression and the Effect of rAAV Doses on Transduction Efficiency

To examine the transduction efficiency and the time course of rAAV-mediated transgene expression, cells were transduced with rAAV at an MOI of 500 and monitored for EGFP expression by fluorescence microscopy and flow cytometry. Figure 1A illustrates the time course of EGFP expression in the rAAV-transduced Huh7 cells by FACS analysis. The expression of EGFP gene in the rAAV-transduced Huh7 cells could be detected as early as 12 h post-infection (p.i.) and reached a maximum at 36 to 60 h p.i.. The transduction efficiency of Huh7 cells by rAAV at an MOI of 500, as determined from flow cytometric analysis, was approximately 71% at 36 h post-transduction, demonstrating that rAAV is an efficient vector for delivering the foreign genes into Huh7 cells. The expression levels were stable up to 60–72 h after virus transduction.

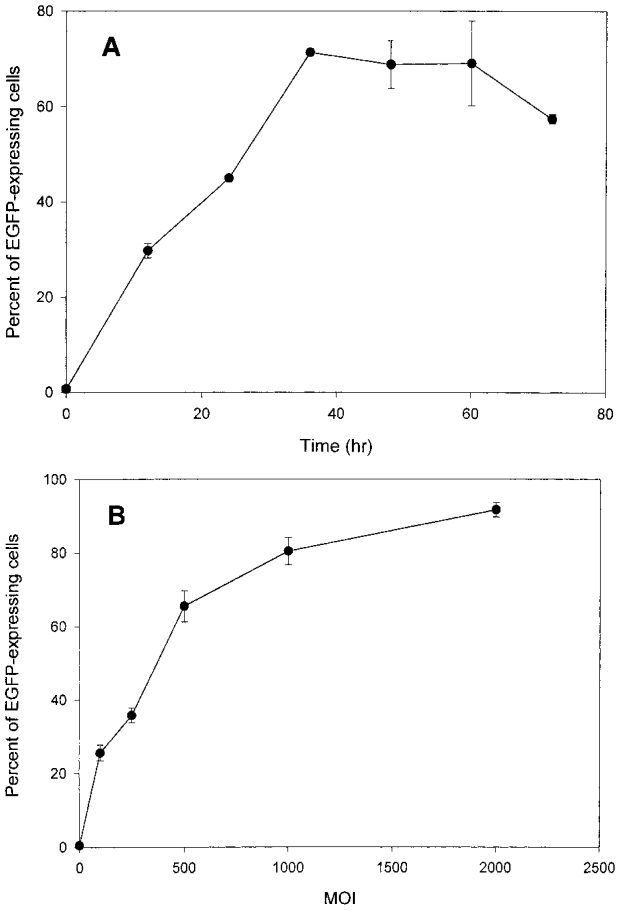

Fig. 1. (A) Flow cytometric analysis of the percentage of EGFP-expressing cells by FACS analysis illustrating the time course of EGFP expression in rAAV-transduced Huh7 cells. (B) Flow cytometric analysis of EGFP expression in Huh7 cells at 48 h post-transduction showing the dependence of the transduction rate in Huh7 hepatoma cells on the dose of transducing rAAV particles (MOI). The percentage of EGFP-expressing cells was calculated by dividing the number of fluorescent cells measured by flow cytometry by the total number of cells counted. Each value shown is the mean ± SD for triplicate wells.

To examine the effect of virus doses on transduction efficiency, Huh7 cells were infected with varying amounts of rAAV and harvested at 48 h post-transduction followed by flow cytometric analysis of EGFP expression. Figure 1B shows the effect of the rAAV doses on the transduction rates in the Huh7 cells by FACS analysis, illustrating an increase in EGFP-expressing cells with increasing MOI. At an MOI of 500, approximately 66% of the cells expressed EGFP. These results demonstrated the high transduction efficiency of rAAV in Huh7 human hepatoma cells.

Response of Luciferase Expression to Glucose Stimulation

To determine whether glucose responsiveness can be obtained in rAAV-transduced Huh7 human hepatoma cells, HeLa cells, serving as the control, and Huh7 hepatoma cells

were transduced with rAAV at an MOI of 500. As shown in Fig. 2, luciferase activity was insensitive to glucose concentrations between 0 and 15 mM in the rAAV-transduced HeLa cells (Fig. 2A), but responded well to the stimulation of glucose in the rAAV-transduced Huh7 cells within the same concentration range (Fig. 2B). This glucose dependence was statistically significant ($P < 0.001$ by ANOVA). Elevated glucose also promoted a rise in luciferase activities. Maximum induction of luciferase activity at 8 mM glucose was approximately 2.2-fold higher than basal activity in the absence of glucose.

Kinetics of Glucose Stimulation in rAAV-Transduced Huh7 Cells

Kinetics experiments were performed to determine the time course of glucose-stimulated luciferase expression in rAAV-transduced Huh7 cells. Figure 3 shows that an elevation in luciferase activities occurred as early as 2 h after glucose stimulation, indicating that this is the time required for transcription and translation of the luciferase gene. Maximal luciferase activity was obtained at 24 h after the addition of glucose, and was sustained for up to 36 h after glucose stimulation.

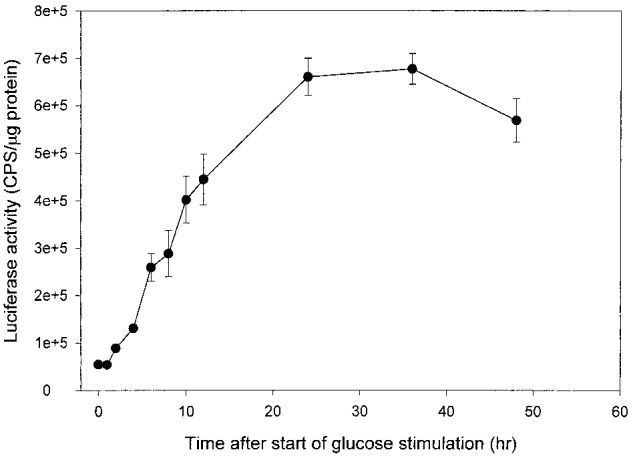

Fig. 3. Kinetics of glucose stimulated luciferase gene expression in rAAV-transduced Huh7 cells. Approximately 1×10^5 cells/well in 12-well plates were infected with rAAV at an MOI of 500 and incubated in glucose-free medium. At 24 h post-transduction, fresh medium containing 25 mM glucose was added (zero time point). Cells were lysed at various intervals as indicated, followed by luciferase activity assays. Each value shown is the mean ± SD for triplicate wells.

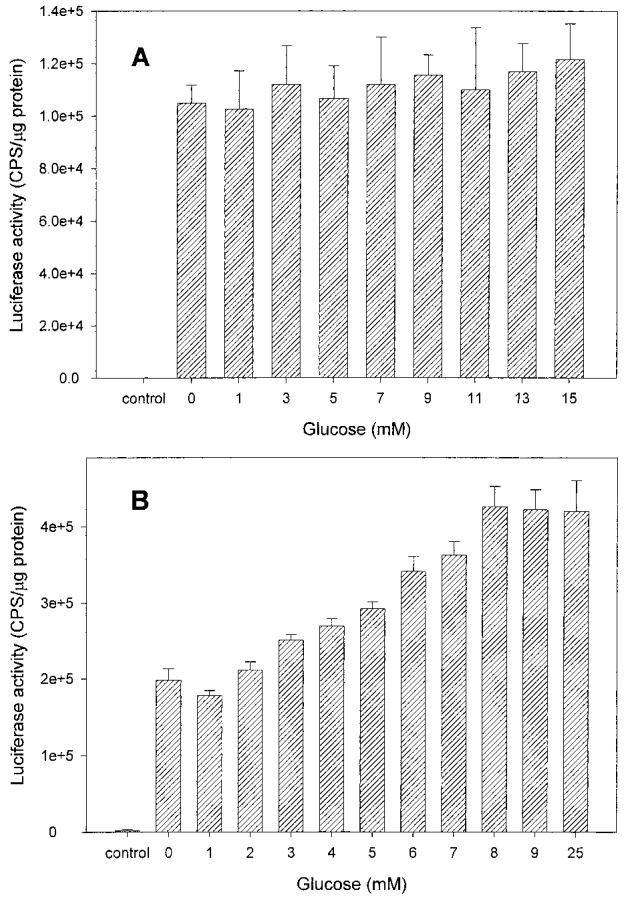

Fig. 2. Effect of glucose concentration on the luciferase expression in rAAV-transduced (A) HeLa and (B) Huh7 cells. Approximately 1×10^5 cells/well in 12-well plates were transduced with rAAV at an MOI of 500. At 24 h post-transduction, cells were subjected to stimulation of varying concentrations of glucose. At 48 h post-transduction, a luciferase activity assay was performed. Untreated cells served as the control. Each value shown is the mean ± SD for triplicate wells.

Effect of PMA, dbcAMP, Forskolin, and Staurosporine on Luciferase Expression, Driven by the Insulin Gene Promoter

To characterize the coupling of glucose responsiveness to cAMP-dependent signal transduction by insulin secretagogues, rAAV-transduced Huh7 cells were stimulated with PMA, dbcAMP and forskolin in the presence of either low (1 mM) or high (25 mM) glucose concentration at 24 h post-transduction, followed by luciferase activity assays 24 h later.

Addition of PMA, a phorbol ester known to stimulate protein kinase C (PKC) activity (27) from 0 to 100 nM elicited a concentration-dependent increase in the luciferase activity in the rAAV-transduced Huh7 cells (Fig. 4A). In the presence of 1 mM glucose, addition of 100 nM PMA caused a 2-fold increase in luciferase activity, which was reduced in the presence of 500 nM PMA. Addition of 25 mM glucose elevated the luciferase activity of all PMA concentrations tested, but the stimulating effect of PMA was somewhat less than that at 1 mM glucose. Figure 4B and C illustrate the stimulating effect of dbcAMP and forskolin on luciferase expression at 1 mM and 25 mM glucose. In the presence of either dbcAMP or forskolin, an increase in glucose concentration from 1 to 25 mM increased the normalized luciferase activities in the rAAV-transduced Huh7 cells. Increases in either dbcAMP or forskolin similarly caused an elevation in luciferase activities. In the presence of 25 mM glucose, the addition of 1 mM dbcAMP increased the luciferase activity approximately twofold. Forskolin exhibited a smaller stimulating effect in 1 mM glucose but induced a greater stimulating effect in the presence of 25 mM glucose. Luciferase activity was enhanced approximately 2.1-fold in the presence of 50 μM forskolin as compared to the control cells grown in the absence of the agent. The stimulating effect of all these substances on luciferase activities were statistically significant (by ANOVA, $P < 0.05$), indicating that the insulinotropic effect of these sub-

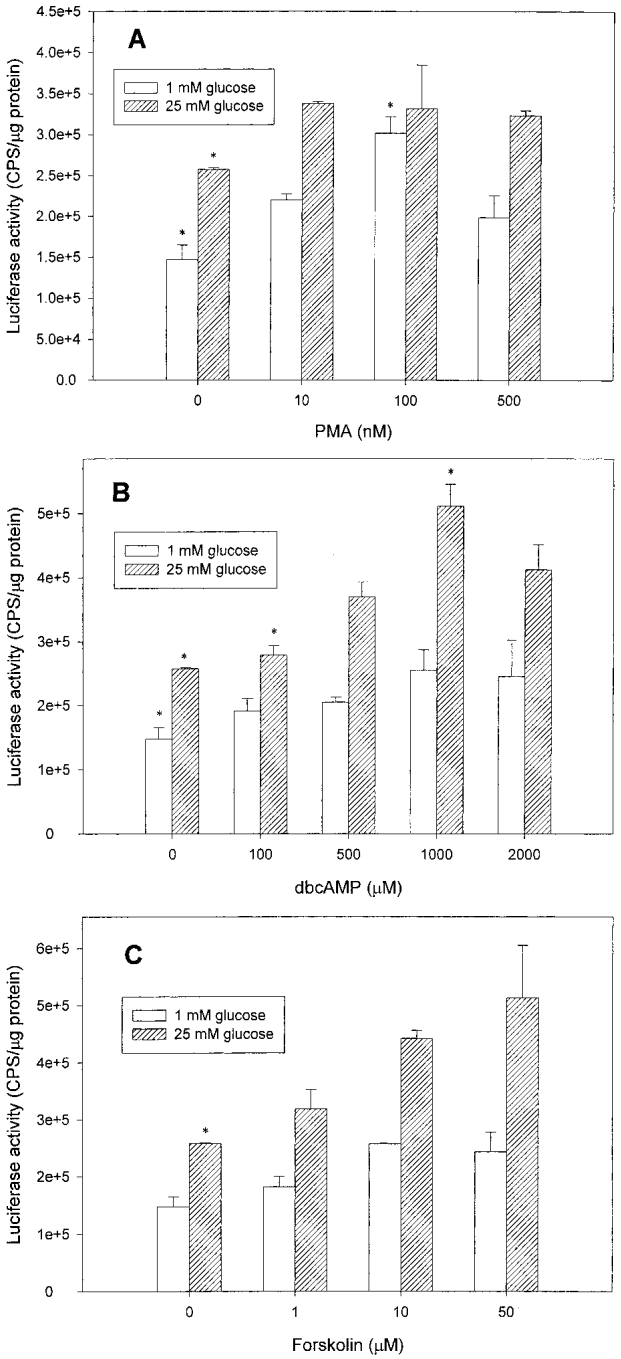

Fig. 4 A–C. Effect of (A) PMA, (B) dbcAMP, and (C) forskolin on insulin gene promoter driving luciferase expression in rAAV-transduced Huh7 hepatoma cells in the presence of 1 mM (blank bars) or 25 mM glucose (hatched bars). Approximately 1×10^5 cells/well in 12-well plates were infected with rAAV at an MOI of 500. Twenty-four hours later, fresh medium containing various concentrations of glucose and the stimulating chemicals was added. At 48 h post-transduction, cells were lysed, followed by luciferase activity and protein assays. Each value shown is the mean ± SD for triplicate wells. *, significantly different from all other means within the same glucose concentration group ($P < 0.05$ by ANOVA).

stances in pancreatic islet cells can be obtained in hepatoma cells (28).

To further investigate the modulation of glucose response via the protein kinase pathway, rAAV-transduced cells were pretreated with staurosporine, a potent protein kinase inhibitor, followed by treatment with insulin secretagogues, including PMA, dbcAMP, and forskolin. Addition of 100 nM staurosporine stimulated luciferase activity in the presence of 25 mM glucose and blocked the stimulatory effect by PMA, dbcAMP and forskolin (Fig. 5).

The Effect of Insulin on Insulin Gene Promoter Driving Luciferase Gene Expression

To determine the effect of insulin on insulin promoter-driving transgene expression, rAAV-transduced Huh7 cells were subjected to stimulation by both glucose and insulin at 24 h post-transduction. Cells were harvested 24 h later, followed by luciferase activity assays. Exposure of rAAV-transduced Huh7 cells to insulin up to 10^{-7} M decreased the luciferase activities at the glucose concentration range tested (Fig. 6).

In Vivo Studies

Time course studies were performed by injecting STZ-treated mice with rAAV. Animals were routinely monitored for weight and blood glucose. Blood glucose levels were determined after 6 h of fasting. Random blood glucose rose sharply 1 to 2 days after STZ administration. At the designated time points, animals treated with 150 mg/kg STZ and exhibiting average blood glucose levels within 200–300 mg/dL glucose were sacrificed. The luciferase activities in the livers were determined, which were shown to increase with time up to the 19th day post-infection (Fig. 7), indicating that 2 weeks may be sufficient for detecting the luciferase activities in rAAV-treated mice.

The glucose responsiveness in rAAV-transduced animals therefore were performed on the 14th day after viral injection. Treatment of animals with 0–150 mg/kg STZ resulted in average blood glucose ranging from 85–300 mg/dL. To increase the range of blood glucose concentration, we have attempted to increase the STZ dose to 200 mg/kg. However, eight animals made diabetic with 200 mg/kg STZ exhibited sustained hyperglycemic and died in less than 1 week, which precluded from further examination. The animals treated with 0–150 mg/kg STZ thus were partitioned into three ranges according to their average blood glucose levels: low (<100 mg/dL), medium (100–200 mg/dL), and high (200–300 mg/dL) glucose. The blood glucose levels of mice in each group were recorded, and the averages are shown in Fig. 8A. Animals from each group were sacrificed and the livers were harvested, extracted, and analyzed for luciferase activities. The STZ-induced diabetic mice without rAAV treatment did not express significant levels of luciferase activities. Figure 8B shows that luciferase activities in rAAV-treated mice were elevated, compared with animals without rAAV treatment, and correlated well with blood glucose concentrations. These results demonstrated that glucose-modulated transgene expression can be obtained in rAAV-treated diabetic mice.

DISCUSSION

Glucose metabolism activates a variety of intracellular signaling pathways and plays a central role in regulating insulin gene transcription. In this study, transduction of Huh7 human hepatoma cells with rAAV carrying the insulin gene

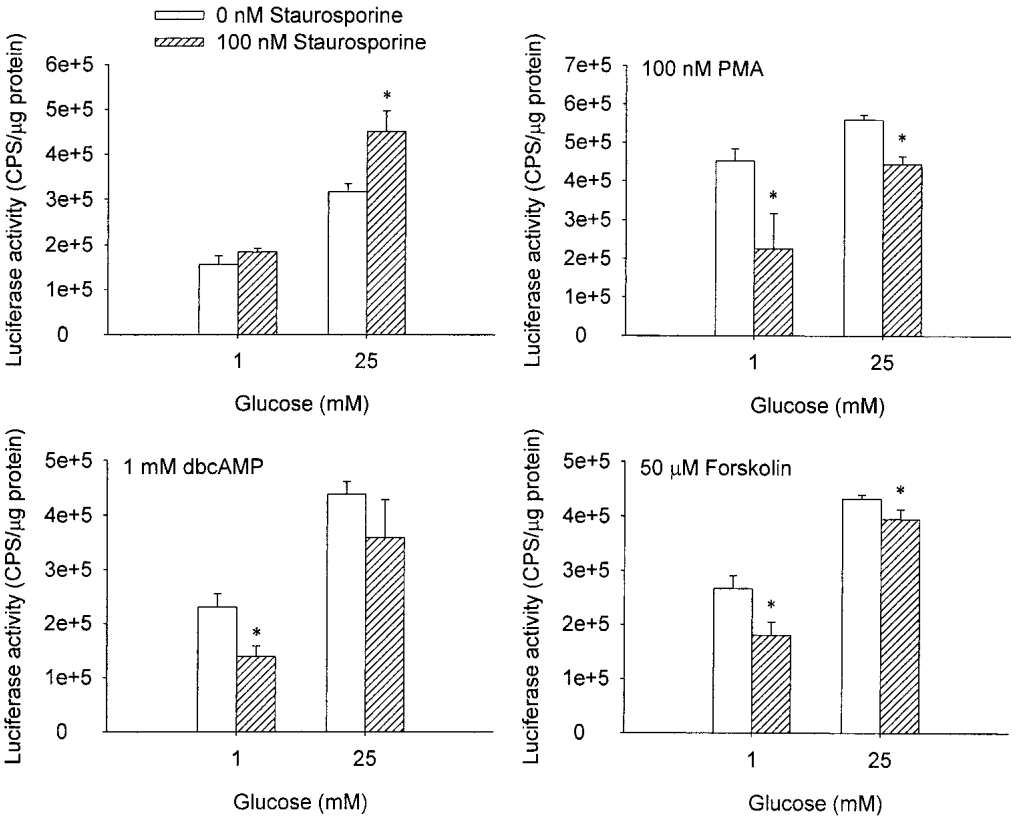

Fig. 5. Effect of staurosporine on luciferase activity in rAAV-transduced Huh7 cells treated with PMA, dbcAMP, or forskolin in the absence (blank bars) or presence of 100 nM staurosporine (hatched bars). Approximately 1×10^5 cells/well in 12-well plates were infected with rAAV at an MOI of 500. Twenty-four hours later, fresh medium containing the desired concentration of glucose and 100 nM staurosporine was added. PMA, dbcAMP, or forskolin was added 30 min later. At 48 h post-transduction, cells were lysed, followed by luciferase activity and protein assays. Each value shown is the mean ± SD for triplicate wells. *, significantly different from other means within the same glucose concentration group ($P < 0.05$ by student *t*-test).

promoter resulted in regulated luciferase expression in response to glucose concentration. The transduction efficiency, as determined from flow cytometric analysis of the EGFP-expressing cells, demonstrated that rAAV is an efficient vector for gene delivery to Huh7 cells. Stable transfection of HepG2ins cells with the glucose transporter GLUT2 was previously shown to result in glucose-stimulated insulin secretion and potentiation of the secretory response to non-glucose secretagogue (12). Here, we showed that glucose-responsive transgene expression can be obtained in hepatoma cells without introduction of glucose transporters, confirming the previous view that glucose transport is not rate limiting for insulin secretion (4).

The maximum luciferase activity obtained in the rAAV-transduced Huh7 cells at 8 mM (equivalent to 150 mg/dL) glucose was approximately 2.2-fold higher than that of the control cells grown in the absence of glucose (Fig. 2B), which was in good agreement with levels reported for transfected HIT-T15 pancreatic islet cells (28,29). The results shown in Fig. 3 demonstrate that exposure of rAAV transduced Huh7 cells to 25 mM glucose for 2 h resulted in induction of luciferase gene expression, which was much faster than the chloramphenicol acetyl transferase gene expression driven by the L-pyruvate kinase gene promoter in retrovirus transduced hepatocytes (shown to be about 8 h after glucose stimulation)

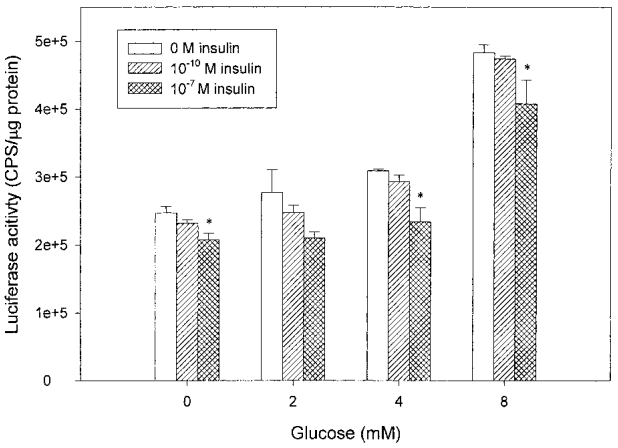

Fig. 6. Effect of exogenous insulin on insulin gene promoter-driving luciferase expression in rAAV-transduced Huh7 cells. Approximately 1×10^5 cells/well in 12-well plates were infected with rAAV at an MOI of 500 and maintained in the glucose-free medium. At 24 post-infection, cells were subjected to stimulation of various concentrations of glucose and insulin. Cells were harvested 24 h later, followed by luciferase activity assays. Each value shown is the mean ± SD for triplicate wells. *, significantly different from other means within the same glucose concentration group ($P < 0.05$ by student *t*-test).

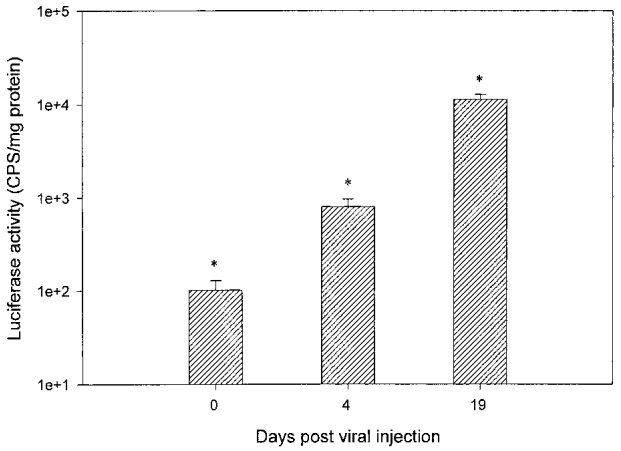

Fig. 7. Time course of luciferase expression in rAAV-treated diabetic mice. The animals were injected with 2×10^{10} rAAV particles in a volume of 200 μL into the livers at 48 h after STZ treatment. At the desired time points, the animals with blood glucose concentration ranging from 200 to 300 mg/dL were sacrificed. The livers were harvested, extracted, and analyzed for luciferase activities. Results shown are means ± SD for each time point (n = 3; animals were made diabetic with 150 mg/kg of STZ) *, luciferase activities were significantly different from other groups in rAAV-treated mice ($P < 0.05$ by one-way ANOVA).

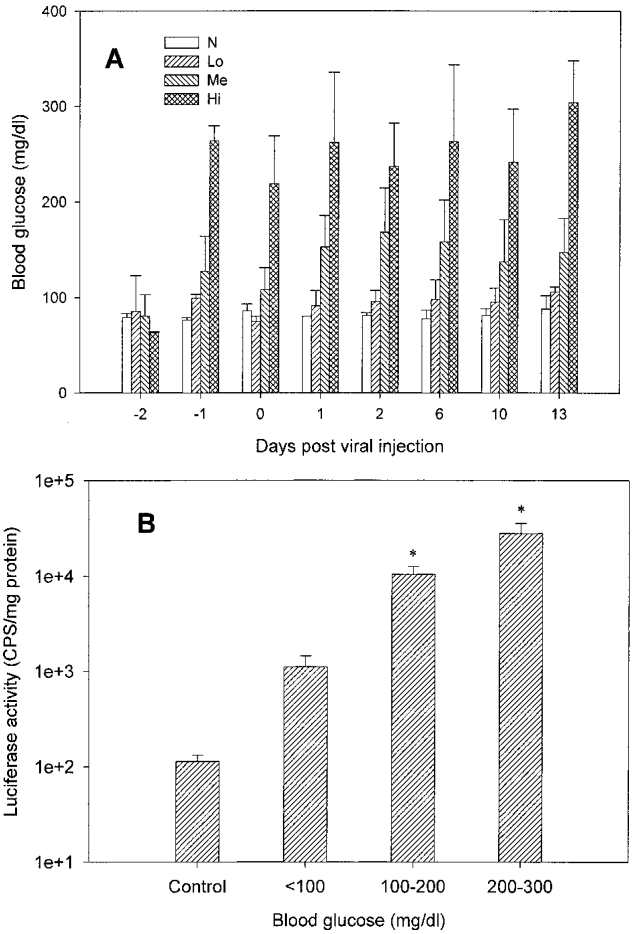

Fig. 8. (A) Mean blood glucose concentrations in mock-treated mice (N), STZ-, and rAAV-treated mice exhibiting low (Lo) blood glucose (<100 mg/dL), medium (Me) blood glucose (100–200 mg/dL), and high (Hi) blood glucose (200–300 mg/dl) on the 14th day after rAAV injection. Each data point represents the contribution of at least three animals. Results shown are means ± SD for each group (n ≥ 3). (B) Luciferase activities in the livers of rAAV-treated mice as a function of blood glucose concentration. Animals were partitioned into three blood glucose ranges on the 14th day after rAAV injection. Livers from each group were harvested, extracted, and luciferase activities were determined. Animals treated with normal saline served as the control. At least three animals were included in each group for analysis of luciferase activities. Results shown are means ± SD for each group. *Luciferase activities were significantly different from other groups of different blood glucose concentration range ($P < 0.05$ by one-way ANOVA).

(10), but was close to the reported times for pancreatic beta cells, in which induction started about 80–100 min after glucose stimulation (29). The data obtained in the present study suggest that relatively short-term regulation of insulin gene transcription by glucose can be obtained in rAAV transduced Huh7 cells.

Hormonal regulation of glucose-induced insulin secretion from pancreatic islet beta-cells results from complex interplay between glucose metabolism and the second messenger-mediated signaling system. In the present study, addition of PMA, dbcAMP, and forskolin increased the insulin promoter-driving luciferase expression, in the presence of either 1 mM or 25 mM glucose (Fig. 4). In most cases, co-incubation of glucose with these agents showed an additive effect. The phorbol ester PMA is known to directly stimulate protein kinase C activity. Dibutyryl cAMP (dbcAMP) is a membrane-permeable cAMP analogue, whereas forskolin is an adenylate cyclase activator that stimulates insulin gene promoter activity mediated by protein kinase A (PKA). The modulation of glucose response by these substances (Fig. 4) demonstrates the effect of the cAMP-signaling system on metabolic regulation of the insulin promoter (30). The transcriptional response to glucose through the insulin promoter is a complex process and is not fully understood. Cyclic AMP inducibility of the cAMP response element of rat insulin I gene promoter was previously shown to be mediated by cAMP response element-binding proteins or closely related proteins (31), which increase insulin release through protein phosphorylation by cAMP-dependent PKA and Ca^{2+}-sensitive diacylglycerol-dependent PKC (32). In the present study, the presence of 100 nM staurosporine, a protein kinase inhibitor, stimulated luciferase activity at a high (25 mM) glucose concentration (Fig. 5), which differed from the observation in the HIT-T15 beta-cells showing opposite effect (28). The reasons for this discrepancy are unclear. On the other hand, stimulation of glucose response by PMA, dbcAMP, and forskolin was inhibited in the presence of 100 nM staurosporine, confirming the role of protein kinases in metabolic regulation of the insulin promoter. Taken together, our results indicate that regulated transgene expression can be obtained in rAAV-transduced hepatoma cells conferred with the insulin gene promoter. The presence of exogenous insulin in the rAAV-transduced Huh7 cells also inhibited luciferase gene expression (Fig. 6), suggesting that insulin promoter-driving transgene expression in the hepatoma cells was modulated not only by the glucose signal transduction system, but also by insulin itself.

Gene therapy for diabetes requires the coordination between the transgenic insulin production with fluctuated blood glucose levels. To investigate the *in vivo* glucose responsiveness via viral vectors used in the present study, animal ex-

periments were performed using the STZ-induced diabetes mice. Luciferase activities in the livers of rAAV-treated diabetic animals, indicative of transgene expression driven by the insulin gene promoter, were shown to correlate well with the blood glucose concentrations (Fig. 8B). Thus, the present model demonstrated efficacious regulation of transgene expression *in vivo*.

In summary, we have demonstrated in this study that recombinant adeno-associated virus is a promising vector for hepatic gene therapy for diabetes. Glucose responsiveness at physiological concentrations can be obtained in rAAV-transduced Huh7 human hepatoma cells. Transcriptional activation of the reporter gene expression was found to be initiated within 2 h after glucose stimulation *in vitro*, suggesting that relatively short term glucose signaling can be obtained, which would allow rapid control of insulin secretion in the genetically modified surrogate islet cells in response to circulating glucose concentration in patients with diabetes. Expression of the insulin promoter-driving luciferase gene was inhibited in the presence of 100 nM exogenous insulin, suggesting feedback inhibition of insulin gene transcription by insulin itself at high concentrations. Insulin secretagogues affected the luciferase expression in the rAAV-transduced cells, indicating that conditions affecting insulin secretion in pancreatic islet cells also affect insulin gene promoter-driving transgene expression in rAAV-transduced hepatoma cells. The *in vivo* experiments demonstrated good correlation between transgene expression and blood glucose levels in rAAV-treated diabetic mice, suggesting the potential of rAAV-mediated gene therapy for diabetes mellitus.

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REFERENCES

1. D. Mitanchez, B. Doiron, R. Chen, and A. Kahn. Glucose-stimulated genes and prospects of gene therapy for type I diabetes. *Endocrine Rev.* **18**:520–540 (1997).
2. F. Levine and G. Leibowitz. Towards gene therapy of diabetes mellitus. *Mol. Med. Today* **5**:165–171 (1999).
3. C. B. Newgard. Perspectives in diabetes. Cellular engineering and gene therapy strategies for insulin replacement in diabetes. *Diabetes* **43**:341–350 (1994).
4. M. S. German. Glucose sensing in pancreatic islet beta cells: The key role of glucokinase and the glycolytic intermediates. *Proc. Natl. Acad. Sci. USA* **90**:1781–1785 (1993).
5. M. S. German and J. Wang. The insulin gene contains multiple transcriptional elements that respond to glucose. *Mol. Cell Biol.* **14**:4067–4075 (1994).
6. R. F. Santerre, R. A. Cook, R. M. Crisel, J. D. Sharp, R. J. Schmidt, D. C. Williams, and C. P. Wilson. Insulin synthesis in a clonal cell line of simian virus 40-transformed hamster pancreatic beta cells. *Proc. Natl. Acad. Sci. USA* **78**:4339–4343 (1981).
7. H. Efrat, S. Linde, and H. Kofod. Beta-cell lines derived from transgenic mice expressing a hybrid insulin gene-oncogene. *Proc. Natl. Acad. Sci. USA* **85**:9037–9041 (1988).
8. F. Radvanyi, S. Christgau, S. Baekkeskov, C. Jolicoeur, and D. Hanahan. Pancreatic β-cell cultured from individual preneoplastic foci in a tumorigenesis pathway: A potentially general technique for isolating physiologically representative cell lines. *Mol. Cell Biol.* **13**:4223–4232 (1993).
9. S. A. Clark, B. L. Burnham, and W. L. Chick. Modulation of glucose-induced insulin secretion from a rat clonal β-cell line. *Endocrinology* **127**:2779–2788 (1990).
10. R. Chen, B. Doiron, and A. Kahn. Glucose responsiveness of a reporter gene transduced into hepatocytic cells using a retroviral vector. *FEBS Lett.* **365**:223–226 (1995).
11. A. Valera, C. Fillat, C. Costa, J. Sabater, J. Visa, A. Pujol, and F. Bosch. Regulated expression of human insulin in the liver of transgenic mice corrects diabetic alterations. *FASEB J.* **8**:440–447 (1994).
12. A. M. Simpson, G. M. Marshall, B. E. Tuch, L. Maxwell, B. Szymanska, J. Tu, S. Beynon, M. A. Swan, and M. Camacho. Gene therapy of diabetes: glucose-stimulated insulin secretion in a human hepatoma cell line (HEPG2ins/g). *Gene Ther.* **4**:1202–1215 (1997).
13. G. D. Simonson, D. J. Groskreutz, C. M. Gorman, and M. J. MacDonald. Synthesis and processing of genetically modified human proinsulin by rat myoblast in primary cultures. *Hum. Gene Ther.* **7**:71–78 (1996).
14. L. Gros, L. Montoliu, E. Rui, L. Lebrigand, and F. Bosch. Regulated production of mature insulin by non-β cells. *Hum. Gene Ther.* **8**:2249–2259 (1997).
15. L. Gros, E. Rui, L. Montoliu, O. Maria, L. Laurence, and F. Bosch. Insulin production by engineered muscle cells. *Hum. Gene Ther.* **10**:1207–1217 (1999).
16. D. Lu, H. Hoshino, and T. Takeuchi. Regulatable production of mature insulin from a hepatocyte cell line: Insulin production is up-regulated by cAMP and glucocorticoids, and down-regulated by insulin. *FEBS Lett.* **399**:37–42 (1996).
17. D. Lu, H. Tamemoto, H. Shibata, and T. Takeuchi. Regulatable production of insulin from primary-cultured hepatocytes: Insulin production is up-regulated by glucagon and cAMP and down-regulated by insulin. *Gene Ther.* **5**:888–895 (1998).
18. M. Tiedge, M. Elsner, N. H. McClenaghan, H.-J. Hedrich, D. Grube, J. Klempnauer, and S. Lenzen. Engineering of a glucose-responsive surrogate cell for insulin replacement therapy of experimental insulin-dependent diabetes. *Hum. Gene Ther.* **11**:403–414 (2000).
19. P. M. Thulé, J. Liu, and L. S. Phillips. Glucose regulated production of human insulin in rat hepatocytes. *Gene Ther.* **7**:205–214 (2000).
20. G. Podsakoff, Jr. K. K. Wong, and S. Chatterjee. Efficient gene transfer into nondividing cells by adeno-associated virus-based vectors. *J. Virol.* **68**:5656–5666 (1994).
21. D. W. Russell, A. D. Miller, and I. E. Alexander. Adeno-associated virus vectors preferentially transduce cells in S phase. *Proc. Natl. Acad. Sci. USA* **91**:8915–8919 (1994).
22. R. M. Kotin. Prospects for the use of adeno-associated virus as a vector for human gene therapy. *Hum. Gene Ther* **5**:793–801 (1994).
23. T. R. Flotte and B. J. Carter. Adeno-associated virus vectors for gene therapy. *Gene Ther.* **2**:357–362 (1995).
24. Y.-W. Yang and R. M. Kotin. Glucose-responsive gene delivery in pancreatic islet cells via recombinant adeno-associated viral vectors. *Pharm. Res.* **17**:1056–1061 (2000).
25. G. G. Holz and J. F. Habener. Signal transduction crosstalk in the endocrine system: pancreatic β-cells and the glucose competence concept. *Trends Biochem. Sci* **17**:388–393 (1992).
26. Y.-W. Yang and Y.-C. Hsieh. Protamine sulfate enhances the transduction efficiency of recombinant adeno-associated virus-mediated gene delivery. *Pharm. Res.* **18**:922–927 (2001).
27. S. J. H. Ashcroft, P. Hammonds, and D. E. Harrison. Insulin secretory responses of a clonal cell line of simian virus 40-transformed B cells. *Diabetologia* **29**:727–733 (1986).
28. S. Goodison, S. Kenna, and S. J. H. Ashcroft. Control of insulin gene expression by glucose. *Biochem. J.* **285**:563–568 (1992).
29. B. Leibiger, T. Moede, T. Schwarz, and G. R. Brown. Short-term regulation of mature insulin gene transcription by glucose. *Proc. Natl. Acad. Sci. USA* **95**:9307–9312 (1998).
30. P. B. Daniel, W. H. Walker, and J. F. Habener. Cyclic AMP signaling and gene regulation. *Annu. Rev. Nutr* **18**:353–383 (1998).
31. A. Eggers, G. Siemann, R. Blume, and W. Knepel. Gene-specific transcriptional activity of the insulin cAMP-response element is conferred by NF-Y in combination with cAMP response element-binding protein. *J. Biol. Chem* **273**:18499–18508 (1998).
32. W. Yu, T. Niwa, T. Fukasawa, H. Hidaka, T. Senda, Y. Sasaki, and I. Niki. Synergism of protein kinase A, protein kinase C, and myosin light-chain kinase in the secretory cascade of the pancreatic β-cell. *Diabetes* **49**:945–952 (2000).