Glucose-Responsive Gene Delivery in Pancreatic Islet Cells via Recombinant Adeno-Associated Viral Vectors

Ya-Wun Yang[1,3] and Robert M. Kotin[2,4]

Received March 30, 2000; accepted June 6, 2000

Purpose. Recent progress in genetic engineering presents the possibility of providing physiologically regulated glucose metabolism in individuals with diabetes. The objective of this study is to explore the feasibility of obtaining glucose dependent gene expression in the pancreatic β-cell lines via recombinant adeno-associated virus type 2 (rAAV) mediated gene transfer.

Methods. Two transcription cassettes containing the luciferase gene under the control of the rat insulin I gene promoter and the enhanced green fluorescent protein (EGFP) open reading frame under the control of the immediate early gene promoter of human cytomegalovirus (CMV) were placed in series between the inverted terminal repeats (ITRs) of AAV. The rAAV vectors produced were used to transduce pancreatic β-cell line grown in the absence or presence of various concentrations of glucose. Luciferase activity assays were performed at 72 hr post-transduction.

Results. Glucose-responsive reporter gene expression was obtained in both calcium phosphate transfected HIT-T15 and βHC-9 cells, demonstrating regulated luciferase gene expression under control of the insulin gene promoter. At MOI of 100, rAAV-transduced βHC-9 cells exhibited glucose-dependent luciferase activities, which were approximately 4.3 fold higher than those transfected by the calcium phosphate coprecipitation method at 20 mM glucose.

Conclusions. Delivery of the insulin gene promoter via rAAV was shown in this study to result in glucose-dependent control of the reporter gene expression. The results suggest that rAAV is an efficient viral vector for gene transfer into the pancreatic islet cells.

KEY WORDS: recombinant adeno-associated virus; insulin gene promoter; pancreatic islet cells; glucose-responsive gene delivery.

INTRODUCTION

Diabetes mellitus (DM), including insulin dependent and non-insulin dependent DM (IDDM and NIDDM, respectively), is caused by impaired β-cell functions in the islets of Langerhans in the pancreas. Whereas IDDM (type I) diabetes results from the autoimmune -mediated depletion of the β-cells, the molecular mechanisms of NIDDM remain obscure. The disease is characterized by decreased insulin secretion from the pancreatic islet cells in response to glucose. Diabetes patients, unable to regulate blood glucose, often require administration of exogenous insulin for survival. Although insulin replacement restores the normal regulation of glucose metabolism, multiple injection is usually required to achieve controlled blood concentration. Even with multiple daily administrations of insulin, the fluctuations in blood glucose concentrations that occur are often greater than in normal individuals. Therefore, glucose-sensitive release of insulin has been attempted using biomaterials or devices. Unfortunately, the current therapies do not satisfactorily control blood glucose levels as accurately as physiologically secreted insulin from the pancreatic islet cells.

Recent advance in gene transfer technology affords a possible means of improving blood glucose regulation by genetically modifying insulin-producing cells. Genetic regulation of insulin secretion could be achieved by incorporating transcriptionally active genes containing elements necessary for glucose responsiveness in the cells, such that glucose-stimulated insulin secretion would occur over a range of glucose concentrations similar to that required for stimulation of normal islet β-cells (1). It is established that glucose metabolism is required to stimulate insulin secretion (2). In principle, once the key functions of glucose-stimulated insulin secretion are identified, it would be possible to replace destroyed β-cell by engineering cells such that insulin gene regulation is controlled by glucose-responsive regulatory elements (3,4). Engineering glucose-stimulated insulin secretion in non-islet cells has been attempted by transfecting the anterior pituitary cell line AtT-20ins with GLUT-2 cDNA for generation of "artificial β-cells" (5,6). These genetically modified cells were expected to serve as surrogates for islets in diabetes.

However, glucose responsive secretion of insulin from GLUT2 transfected AtT-20ins cell lines is outside the physiological range. Other attempts of using non-islet cells as surrogates for β-cells were unsuccessful. Attention thus has been turned to engineering of insulin-secreting cell lines for insulin replacement in diabetes (3,6). Successful gene transfer into β-cells would have important implication for diabetes gene therapy.

Several approaches for gene transfer to the somatic cells have been explored, including cationic liposomes (7), retroviral vector-mediated gene transduction (8), adenoviral-mediated gene transfer (9), and adeno-associated viral vector system in retina, CNS, skeletal muscle, and liver (10–13). A common desired feature of all these methods is the efficient and stable delivery of genes into the cells. The use of recombinant viruses for gene delivery is a compelling idea that has not been realized due to technical and biological constraints. However, recent advances in vector development may provide the means to repair β-cell dysfunction *in vivo* or *ex vivo*. Limitations of virus vectors require that the appropriate virus system be applied for that indication. Retrovirus vectors require that the cell enters S-phase. Transduction with adenovirus vectors results in short-term expression of the transgene often accompanied by a potent immune response to the vector destroying the transgene expressing cells and thereby preventing future vector re-administration and risking an autoimmune response (14).

[1] National Taiwan University, Taiwan.
[2] Laboratory of Biochemical Genetics, NHLBI, NIH, Bldg. 10; Rm. 7D18, 10 Center Drive, Bethesda, Maryland 20892.
[3] Present address: School of Pharmacy, College of Medicine, National Taiwan University. 1, Jen-Ai Road, Section 1, Taipei 100, Taiwan.
[4] To whom correspondence should be addressed. (e-mail: kotinr@nhlbi.nih.gov)

ABBREVIATIONS: rAAV, recombinant adeno-associated virus; IDDM, insulin-dependent diabetes mellitus; NIDDM, non-insulin dependent diabetes mellitus; EGFP, enhanced green fluorescent protein; CMV, cytomegalovirus; IE, immediate early; ITR, inverted terminal repeat; MOI, multiplicity of infection or multiplicity of transduction

0724-8741/00/0900-1056$18.00/0

Adeno-associated virus type 2 (AAV) is a nonenveloped, non-pathogenic human virus of the parvoviridae family with a single-stranded DNA genome, which infects a wide range of cell types *in vitro*. Recombinant AAV (rAAV) can be generated in a system in which the virus genes are replaced by the desired gene construct. The only virus elements necessary *in cis* are the inverted terminal repeats (ITRs) (15). Studies to date seem to indicate that the vector *per se* is not immunogenic (16). In contrast to wild-type AAV, which among eukaryotic viruses is unique in its ability to integrate into a defined locus on human chromosome 19 (17), rAAV appears to remain episomal. The use of rAAV has not been examined in the pancreatic islet cells. In this study, an AAV vector containing the insulin gene promoter and the luciferase open reading frame (ORF) functioning as a reporter for insulin promoter activity, was used to transduce the pancreatic islet β-cells. Hamster and murine derived insulin producing lines, HIT-T15 and βHC-9 cells were used in this study (18,19). Here we report on the capability of these genetically engineered cells to express luciferase in response to the environmental glucose stimulation.

MATERIALS AND METHODS

Cell Culture

HIT-T15 cell line, derived from hamster islet cells transformed with SV40 T-antigen (18), was obtained from ATCC, while the hyperplastic pancreatic islet cell line βHC-9 was kindly provided by Dr. D. Hanahan (University of California, San Francisco) (19). Both cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Experiments were performed with βHC-9 cells between passages 23 to 29. Cell cultures were maintained in a humidified environment at 37 °C in a 5% CO_2 atmosphere.

Construction of Recombinant Adeno-Associated Viral Vector

The plasmid pSYNT, containing 410 bp of the rat insulin I (rIns-I) gene 5′ flanking region and the coding sequence of chloramphenicol acetyltransferase (CAT), was obtained from Dr. M. S. German (University of California, San Francisco) (2). The 410 bp rIns-I enhancer/promoter region of pSYNT was excised with XbaI/HindIII and inserted into NheI/HindIII sites of pGL3-basic vector (Promega, Madison, WI), upstream of the luciferase gene, to generate pIns.Luc. Plasmid pΔEGFP was produced by deleting the polylinker of pEGFP-N1 (Clontech Inc., Palo Alto, CA) by digestion with BglII and BamHI and religation. This construct contains the open reading frame (ORF) of enhanced green fluorescence protein (EGFP) under the control of the immediate early (IE) gene promoter of human cytomegalovirus (CMV). The 5.2 kb SalI restricted fragment from pIns.Luc containing the insulin gene promoter and the luciferase gene was blunt-ended and ligated by T4 DNA ligase to the 1.8 kb AseI/DraIII digested, blunt-ended fragment containing the EGFP ORF from pΔEGFP. The resulting plasmid pIns.Luc.ΔEGFP was digested with AflII, blunt-ended, and further digested with KpnI. The plasmid pAAV-MCS2.7 (20), containing the inverted terminal repeats (ITR) of AAV2, was digested with HindIII. The linearized DNA was treated with T4 DNA polymerase plus 4 dNTPs to create blunt ends. The blunt ended DNA was subsequently digested with KpnI. The resulting 4.6 kb fragment was ligated to the gel-purified 4.0 kb fragment obtained from pIns.Luc.ΔEGFP. The size of the plasmid is 8.53 kb and the size of the vector genome is 4,485 nt. The schematic representation of the final construct is shown in Fig. 1.

Transfection of HIT-T15 and βHC-9 Cell Cultures

Transfection of HIT-T15 and βHC-9 cells with rAAV vector by the calcium phosphate-mediated method was used to analyze the insulin gene promoter activity by the luciferase reporter system. One day before transfection, approximately 2×10^5 cells were seeded in each well in the 12-well plates and incubated at 37 °C and 5% CO_2. Transfection was carried out with 1 μg of pAAV.Ins.Luc.ΔEGFP plasmid DNA per well using the calcium phosphate transfection system (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instruction. The transfection mixture was added to the cells, and the cells were maintained at 37 °C in 5% CO_2 atmosphere. Twenty-four hours later, the medium was replaced with glucose-free DMEM containing 10% dialyzed fetal calf serum and the desired glucose concentrations. Luciferase activity assay was performed 24 to 48 hours later.

Generation of Recombinant AAV Viruses

Production, purification, and characterization of rAAV were carried out as described previously (21). Briefly, about 2×10^8 exponentially growing COS-7 cells were electroporated with a mixture of 400 μg pAAV.Ins.Luc and 400 μg helper plasmids. The helper plasmid for rAAV packaging contains

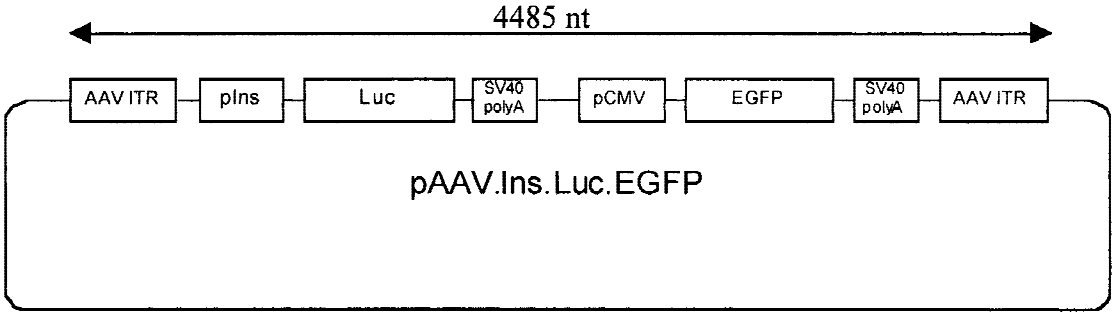

Fig. 1. Schematic diagram of construct of the plasmid, pAAV.Ins.Luc.ΔEGFP, for generation of rAAV. The plasmid is 8.53 kb and the vector genome is 4485 nt in length.

the simian virus 40 (SV40) origin and the AAV *rep* and *cap* genes but lacks the flanking AAV ITR sequence. Electroporation was performed in a 4-mm gap cuvette containing 0.5 ml of the cell-DNA mixture (BTX Instrument, San Diego, CA). Forty-eight hours later, cells were infected with helper wild-type adenovirus 5 at a ratio of approximately 10 infectious adenovirus particles per cell. At 72 hr post-transduction, cells were harvested and centrifuged to remove debris. Purification of rAAV was performed by isopycnic ultracentrifugation in cesium chloride (density = 1.4 g/ml) at 32,000 rpm in a Beckman SW41Ti rotor for 60 hours. Fractions corresponding to refractive index between 1.370 to 1.375 were pooled and re-centrifuged in a Beckman VTi65 rotor at 54,000 rpm for 16 hours. Fractions from the second gradient were collected and dialyzed against 0.9% (w/v) NaCl before use.

Determination of Genomic Titers of rAAV Preparations

The titers of the rAAV particles were determined by DNA dot-blot hybridization. The signal intensities of the vector DNA were compared with that of the standard plasmid pAAV.Ins.Luc.ΔEGFP and used to calculate the rAAV vector yield. DNA probes were prepared by the random primer labeling method.

Transduction of Cell Cultures with rAAV

COS-7 and βHC-9 cells were routinely cultured in DMEM containing 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were plated one day before the experiments and transduced with rAAV at an MOI of 100 (MOI – multiplicity of infection or transduction, e.g., an MOI of 1 = 1 particle per cell) for 4 hours, followed by incubation in complete DMEM overnight. Twenty-four hours later, the medium was removed and the cells were incubated in glucose-free DMEM containing 10% dialyzed calf serum with various concentrations of glucose. Cells were routinely examined by epifluorescence microscopy (Zeiss Axiovert 135) and harvested at 72 hr post-transduction, followed by luciferase activity assay. Fluorescence micrographs of the rAAV transduced βHC9 cells were taken with a cooled CCD camera system (Carl Zeiss).

Luciferase Assay

Luciferase activity in the cell lysate was determined using the luciferase assay system (Promega Inc., Madison, WI) according to the manufacturer's instructions and measured in a luminometer (Analytical Luminescence Laboratories, San Diego, CA). Total protein concentration of the cell lysate was determined with the Bio-Rad DC Protein Assay Reagents (Bio-Rad Laboratories, Hercules, CA). Luciferase activity was expressed as relative light units (RLU) normalized to total protein concentration.

Flow Cytometry

Quantitative analysis of the rAAV-transduced cells was carried out by flow cytometry. At 48 hr post-transduction, the cultured cells were washed with cold PBS, trypsinized, and centrifuged at 1,000 rpm for 5 minutes, and fixed in PBS containing 1% paraformaldehyde and 10% fetal calf serum. Flow cytometry analysis was performed using the Coulter EPICS XL-MCL flow cytometer (Coulter Electronics Inc., Miami, FL). A minimum of 20,000 cells was analyzed for each sample. The percentage of GFP positive cells was determined after compensating for autofluorescence using untransduced cells as a negative control.

RESULTS

We constructed a plasmid that contains the luciferase ORF under the control insulin gene promoter and the EGFP ORF under the control of the cytomegalovirus IE gene promoter. These two independent transcription units were flanked by the AAV ITRs. This plasmid was initially used to assess the effects of glucose concentration on reporter gene expression in HIT-T15 and βHC-9 cells and subsequently to prepare the rAAV virus stocks. Recombinant AAV was used to transduce βHC-9 cells.

Transfection of HIT-T15 and βHC-9 Cells with Plasmid Construct by the Calcium Phosphate-Mediated Method

At 48-72 hr post-transfection, HIT-T15 cells showed an increase of insulin gene promoter activity with glucose concentration ranging from 0 to 0.3 mM (Fig. 2). A 4.7-fold higher level of luciferase activity was observed in the extracts of cells grown in 0.25 mM glucose than those grown in 0.05 mM glucose. Similar results were obtained with βHC-9 cells which responded to higher glucose concentration range (Fig. 3). Transfected βHC-9 cells grown in 15 mM glucose exhibited approximately 10.6-fold increase of luciferase activity than those grown in 2.5 mM glucose. Further increase in glucose concentration does not significantly increase luciferase activities further. Control experiments were performed with COS-7 cell line, where increasing glucose concentration did not affect luciferase activity (data not shown). These results

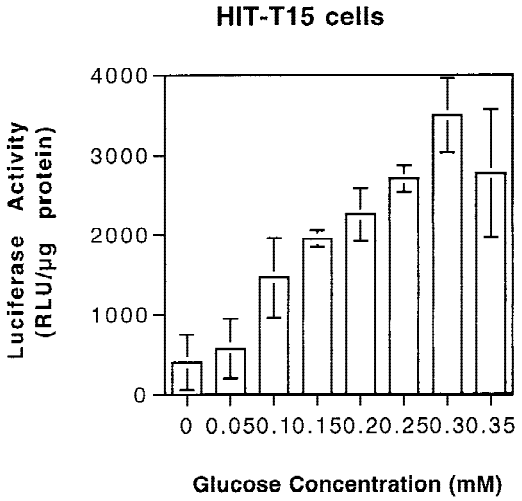

Fig. 2. Effect of glucose concentration on the luciferase activity of transfected HIT-T15 cells. Cultured cells were transfected with 1 μg pAAV.Ins.Luc.ΔEGFP plasmid DNA/well in the 12-well plates by the calcium phosphate coprecipitation method. Luciferase activity was assayed after 48 hr growth in various glucose concentrations. Results are the averages from triplicate wells, with the standard deviation indicated.

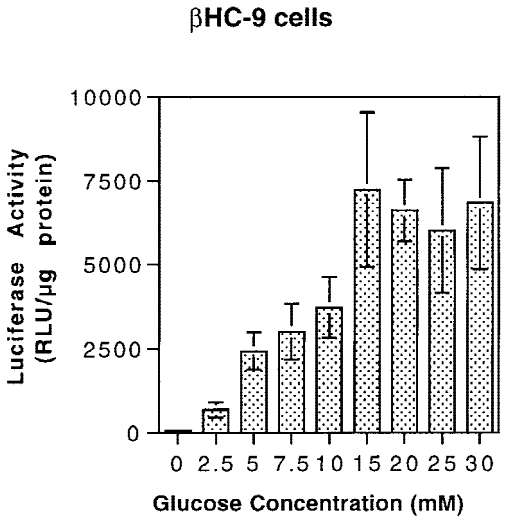

Fig. 3. Effect of glucose concentration on the luciferase activity of transfected βHC-9 cells. Cultured cells were transfected with 1 μg pAAV.Ins.Luc.ΔEGFP plasmid DNA/well in the 12-well plates by the calcium phosphate coprecipitation method. Luciferase activity was assayed after 48 hr growth in various glucose concentrations. Results are the averages from triplicate wells, with the standard deviation indicated.

demonstrated glucose responsive insulin gene promoter activity as indicated by the luciferase expression levels.

rAAV-Mediated Transduction of Insulin Gene Promoter into βHC-9 Pancreatic Cells

The ability of rAAV to transduce a variety of cell types and cell lines *in vitro* has been established (10–13). Here we wish to determine the efficiency of rAAV to transduce pancreatic islet cells *in vitro*. Transduction of βHC-9 pancreatic cells by rAAV.Ins.Luc.ΔEGFP showed green fluorescence produced as early as 24 hours after rAAV transduction. Flow cytometrical analysis of cells transduced with rAAV fractions with refractive indices lying between 1.370–1.377 indicated that transduction efficiency of rAAV in COS-7 cells was much higher than in βHC-9 cells (Table I). The maximum transduction efficiency occurred in the rAAV fraction with refractive index of 1.3735. Transduction of βHC-9 cells with 4 μl of this fraction resulted in approximately 14% of EGFP (+) βHC-9 cells. The number of EGFP-expressing cells reached a maximal level at 48-72 hr post-transduction and remained relatively stable for approximately 5 days. The EGFP fluorescence was found to be located in both cytoplasm and nucleus of the βHC-9 cells (Fig. 4). Three days after rAAV transduction, cells were harvested and luciferase activity assays were performed. Fig. 5 shows the glucose-responsive luciferase activity in the rAAV transduced βHC-9 cells at 72 hr post-transduction. In the presence of 20 mM glucose, the virus transduced cells (MOI = 100) exhibited an approximately 4.3-fold higher luciferase activities than those transfected by the calcium phosphate-mediated method, indicating that gene transfer efficiency is higher via rAAV-mediated method

Table I. Analysis by Flow Cytometry of the Percentage of EGFP(+) COS-7 and βHC-9 Cells 48 Hours After Transduction with rAAV Fractions Obtained from CsCl Gradient Ultracentrifugation

Refractive index	EGFP(+) COS-7 cells (%)	EGFP(+) βHC-9 cells (%)
1.3770	6.3	0.5
1.3760	4.9	0.6
1.3750	11.3	0.8
1.3745	29.1	4.1
1.3735	59.7	14.4
1.3730	52.6	6.6
1.3720	41.8	3.9
1.3710	29.3	3.1
1.370	17.7	3.4

DISCUSSION

Transfer of pancreatic islet cells with the regulatable insulin gene is a conceptually appealing approach for diabetes therapy. Due to the complexity of glucose-sensing and physiological regulation of insulin secretion from the pancreatic beta cells, insulin replacement therapy via gene transfer has been very difficult. One strategy for insulin delivery may be approached by genetically engineering an endocrine cell line that is endowed with regulatable elements for insulin secretion which can be grown *in vitro* in large quantity (22). However, gene transfer to the pancreatic islet cells is inefficient. The quiescent nature of these cells is incompatible with retrovirus vectors which require cell division for efficient gene transfer. Transfection with adenovirus-polylysine (AdpL) or lipid-DNA complexes was reported to be efficient means to

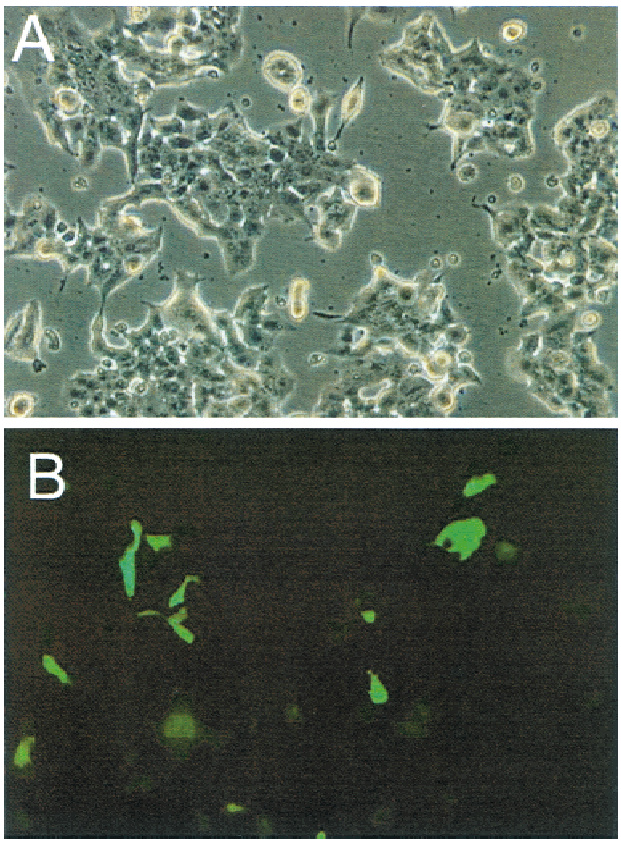

Fig. 4. Green fluorescent protein expression in βHC-9 cells 24 hours after transduction with rAAV at an MOI of 100 under (A), transmitted light and (B), fluorescence light.

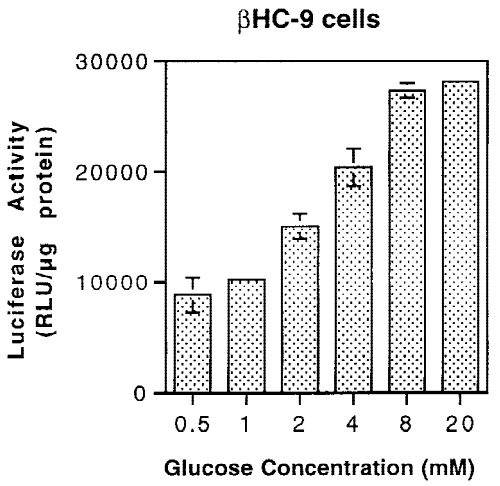

Fig. 5. Effect of glucose concentration on the luciferase activity of βHC-9 cells transduced with rAAV. βHC-9 cells were infected with rAAV at an MOI of 100. At 72 hr post-infection, the cells were washed, harvested, and lysed, followed by luciferase activity assay. Results are the averages from duplicate wells, with the standard deviation indicated.

achieve transient expression of chloramphenicol acetyl transferase (CAT) gene construct in the human pancreatic islet cells *in vitro* (23). Other studies on gene transfer to human pancreatic endocrine cells reported that adenovirus is the most potent vector for *ex vivo* overexpression of foreign gene in adult endocrine pancreatic cells (24). Adenoviral gene delivery, however, is often complicated by the cellular immune response.

Although adeno-associated virus vectors are very effective in transducing a variety of cell lines (see above), methods of gene transfer in pancreatic islet cells using rAAV vectors have not been explored. In the present study, we examined whether glucose-responsive gene expression can be achieved via recombinant adeno-associated viral vectors. Results obtained from calcium phosphate transfected HIT-T15 and βHC-9 cells showed a remarkable level of glucose-responsive luciferase gene expression, demonstrating specific expression of the insulin gene promoter in the pancreatic islet cells. The HIT-T15 cells are a clonal cell line of pancreatic islet beta cells derived from SV40-transformed Syrian hamster pancreatic islets (18), whereas βHC-9 cells were derived from the hyperplastic islets of transgenic mice harboring SV40 large tumor antigen gene in the islets (19). Both cell lines secrete insulin in response to glucose in a concentration-dependent manner. The maximal increase in luciferase secretion in response to glucose in the prAAV-transfected HIT-T15 cells occurred at subphysiological glucose concentration (300 μM), while prAAV-transfected βHC-9 cells expressed higher levels of luciferase and responded to a physiological glucose concentration (15 mM). These results were obtained from early passages of the cells. Continuous passage of these pancreatic islet cell lines, however, leads to a significant loss of glucose response, and necessitates the use of freshly thawed cells which were frozen at early passages.

Expression of the luciferase gene under the insulin gene promoter is also achieved by transducing the βHC-9 cells with rAAV vectors. Increasing viral vector doses led to higher gene targeting rates. The transduction efficiency of rAAV at an MOI of 100 was shown to be higher than those achieved by the calcium phosphate-mediated method.

Genetic modification of insulinoma cell lines by "iterative engineering" has been recently developed in diabetes research with the objective of being able to create insulin-secreting cell line which simulates the performance of the normal islet beta cells (25). Studies on transplantation of islet-like cell clusters (ICCs) under the kidney capsule of nude mice have demonstrated the possibility that expanded populations of human pancreatic endocrine cells may also be used for transplantation to treat diabetes (26). The development of efficient gene transfer method to β-cells therefore is of importance if genetic modification of β-cells becomes necessary before transplantation. The results presented in this study demonstrated the feasibility of obtaining glucose-responsive transgene expression in the pancreatic islet cells by the recombinant adeno-associated viral vectors.

ACKNOWLEDGMENTS

We are grateful to Dr. Michael German of the University of California, San Francisco for providing the pSYNT plasmid, and to Dr. Douglas Hanahan of the University of California, San Francisco for providing the βHC-9 cell line. We wish to thank members of the Kotin Lab, Drs. R. Smith and M. Schmidt for helpful discussions. The technical assistance from Dr. Y.-J. Liu on isolation of rAAV is acknowledged. We also wish to thank Ms. Martha Kirby for the excellent flow cytometry analysis. Y.-W. Yang was on leave from National Taiwan University, supported by the National Science Council, Taiwan.

REFERENCES

1. K. Docherty. Gene therapy for diabetes mellitus. *Clin. Sci.* **92**: 321–330 (1997).
2. 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).
3. C. B. Newgard. Cellular engineering and gene therapy strategies for insulin replacement in diabetes. *Diabetes* **43**:341–350 (1994).
4. C. B. Newgard, S. Clark, H. BeltrandelRio, H. E. Hohmeier, C. Quaade, and K. Nomington. Engineered cell lines for insulin replacement in diabetes: Current status and future prospects. *Diabetologia* **40**(Suppl. 2):S42–27 (1997).
5. S. D. Hughes, J. H. Johnson, C. Quaade, and C. B. Newgard. Engineering of glucose-stimulated insulin secretion and biosynthesis in non-islet cells. *Proc. Natl. Acad. Sci. USA* **89**:688–692 (1992).
6. D. Mitanchez, B. Doiron, R. Chen, and A. Kahn. Glucose-stimulated genes and prospects of gene therapy for type I diabetes. *Endocrin. Rev.* **18**:520–540 (1997).
7. P. L. Felgner, T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold, and M. Danielsen. Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA* **84**:7413–7417 (1987).
8. Q.-L. Hao, P. Malik, R. Salazar, H. Tang, E. M. Gordon, and D. B. Kohn. Expression of biologically active human factor IX in human hematopoietic cells after retroviral vector-mediated gene transduction. *Hum. Gene Ther.* **6**:873–880 (1995).
9. M. E. Csete, P. Y. Benhamou, K. E. Drazan, L. Wu, D. F. McIntee, R. Afra, Y. Mullen, R. W. Busuttil, and A. Shaked. Efficient gene transfer to pancreatic islets mediated by adenoviral vectors. *Transplantation* **59**:263–268 (1995).
10. R. R. Ali, M. B. Reichel, A. J. Thrasher, R. J. Levinsky, C. Kinnon, N. Kanuga, D. M. Hunt, and S. S. Bhattacharya. Gene transfer into the mouse retina mediated by an adeno-associated viral vector. *Hum. Mol. Genet.* **5**:591–594 (1996).
11. P. D. Kessler, G. M. Podsakoff, X. Chen, S. A. McQuiston, P. C.

Colosi, L. A. Matelis, G. J. Kurtzman, and B. J. Byrne. Gene delivery to skeletal muscle results in sustained expression and systemic delivery of a therapeutic protein. *Proc. Natl. Acad. Sci. USA* **93**:14082–14087 (1996).
12. T. J. Sferra, G. Qu, D. McNeely, R. Rennard, K. R. Clark, W. D. Lo, and P. R. Johnson. Recombinant adeno-associated virus-mediated correction of lysosomal storage within the central nervous system of the adult mucopolysaccharidosis type VII mouse. *Hum. Gene Ther*. **11**:507–519 (2000).
13. R. O. Snyder, C. H. Miao, G. A. Patijn, S. K. Spratt, O. Danos, D. Nagy, A. M. Gown, B. Winther, L. Meuse, L. K. Cohen, A. R. Thompson, and M. A. Kay. Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. *Nat. Genet*. **16**:270–276 (1997).
14. T. A. Smith, B. D. White, J. M. Gardner, M. Kaleko, and A. McClelland. Transient immunosuppression permits successful repetitive intravenous administration of an adenovirus vector. *Gene Ther.* **3**:496–502 (1996).
15. R. J. Samulski, L. S. Chang, and T. Shenk. Helper-free stocks of recombinant adeno-associated viruses: Normal integration does not require viral gene expression. *J. Virol*. **63**:3822–3828 (1989).
16. G. Christofori, P. Naik, and D. Hanahan. A second signal supplied by insulin-like growth factor II in oncogne-induced tumorigenesis. *Nature* **369**:414–418 (1994).
17. R. M. Kotin, M. Siniscalco, R. J. Samulski, X. D. Zhu, L. Hunter, C. A. Laughlin, S. McLaughlin, N. Muzyczka, M. Rocchi, and K. I. Berns. Site-specific integration by adeno-associated virus. *Proc. Natl. Acad. Sci. USA* **87**:2211–2215 (1990).
18. 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).
19. 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).
20. V. R. Braddon, J. A. Chiorini, S. Wang, R. M. Kotin, and B. J. Baum. Adeno-associated virus-mediated transfer of a functional water channel into salivary epithelial cells *in vitro* and *in vivo*. *Hum. Gene Ther.* **9**:2777–2785 (1998).
21. J. A. Chiorini, C. M. Wendtner, E. Urcelay, B. Safer, M. Hallek, and R. and R. M. Kotin. High-efficiency transfer of the T-cell co-stimulatory molecule B7-2 lymphoid cells using high-titer recombinant adeno-associated virus vectors. *Hum. Gene Ther.* **6**: 1531–1541 (1995).
22. H. E. Hohmeier, H. BeltrandelRio, S. A. Clark, R. Henkel-Rieger, K. Normington, and C. B. Newgard. Regulation of insulin secretion from novel engineered insulinoma cell lines. *Diabetes* **46**:968–977 (1997).
23. J. Saldeen, D. T. Curiel, D. L. Eizirik, A. Andersson, E. Strandell, K. Buschard, and N. Welsh. Efficient gene transfer to dispersed human pancreatic islet cells in vitro using adenovirus-polylysine/DNA complexes or polycationic liposomes. *Diabetes* **45**:1197–1203 (1996).
24. G. Leibowitz, G. M. Beattie, T. Kafri, V. Cirulli, A. D. Lopez, A. Hayek, and F. Levine. Gene transfer to human pancreatic endocrine cells using viral vectors. *Diabetes* **48**:745–753 (1999).
25. S. A. Clark, C. Quaade, H. Constandy, P. Hansen, P. Halban, S. Ferber, C. B. Newgard, and K. Normington. Novel Insulinoma cell lines produced by iterative engineering of GLUT2, glucokinase, and human insulin expression. *Diabetes* **46**:958–967 (1997).
26. G. M. Beattie, T. Otonkoski, A. D. Lopez, and A. Hayek. Functional β-cell mass after transplantation of human fetal pancreatic cells: Differentiation or proliferation? *Diabetes* **46**:244–248 (1997).