Glucose responsiveness of a reporter gene transduced into hepatocytic cells using a retroviral vector

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Abstract An MMLV-based retroviral vector containing the chloramphenicol acetyl transferase reporter gene under the control of a glucose-dependent internal promoter derived from the L-type pyruvate kinase gene was constructed. After transfection into psi-CRIP packaging cells, clones producing recombinant retrovirus were selected. These retroviruses were used to infect cultured established hepatocytic cells whose endogenous L-type pyruvate kinase gene is transcriptionally regulated by glucose. In the infected cells, the reporter gene was as responsive to glucose as the endogenous L-type pyruvate kinase gene, and the glucose gene activation was time- and concentration-dependent. The possibility to confer a glucose responsiveness on a transgene carried by a retroviral vector provides a powerful tool in the prospect of gene therapy for diabetes mellitus.

Key words: Retroviral vector; Gene therapy; Glucose responsiveness; Hepatocytic cell

1. Introduction

Glucose is a modulator of gene expression in practically all forms of life. In vertebrates, it especially regulates insulin production in pancreas β cells (at both transcription and secretion levels) and activity of various metabolism enzymes in hepatocytes and adipocytes [1]. Some of these effects are mediated by insulin while others require the presence of glucose itself. For instance, genes encoding proteins of the glycolytic and lipogenic pathways respond to glucose through a glucose response element first characterized in the promoter of the L-type pyruvate kinase (L-PK) gene [2–4], and further described in the regulatory regions of several other genes [5–7].

The L-PK gene glucose response element (GlRE-position bp −168 to −144) is a perfect palindrome composed of two non-consensus binding sites for proteins of the MLTF/USF family, separated by 5 bp. The L-PK promoter is constituted of this GlRE which cooperates with a contiguous hepatocyte nuclear factor 4 (HNF4) binding site [2,8], and, in a more proximal position (bp −96 to −68), a hepatocyte nuclear factor 1 (HNF1) binding site which is essential for a tissue-specific activity of the L-PK promoter [9,10]. In hepatocytes, the glucose-dependent activation of the L-PK gene requires the presence of insulin, needed to induce the liver-specific glucose-phosphorylating enzyme, glucokinase [11]. In the cultured mhAT3F cell line, derived from a transgenic mice expressing the SV40 large T antigen under the control of the antithrombin III gene promoter [12,13], the glucose responsiveness of the L-PK gene does not require insulin, probably because, in these cells, glucokinase is replaced by other types of insulin-independent hexokinases [11,14].

Our purpose was to construct a vector able to transfer into cells glucose-responsive genes. We first tested the efficiency of the L-PK promoter used as an internal promoter in an enhancerless retroviral vector in conferring a glucose responsiveness on the chloramphenicol acetyl transferase (CAT) reporter gene at physiological concentrations. Besides, we observed that in mhAT3F cells infected with such a recombinant retrovirus, activations of both the endogenous L-PK gene and the CAT gene by glucose were similar.

2. Materials and methods

2.1. Retroviral vector construction

All plasmids were constructed by standard DNA cloning procedures. Junctions were checked by DNA sequencing. The construct pHSG-(L4L3)4-PKCAT, presented in detail in Fig.1, contains the MMLV constructive frame of pHSGneo and CAT gene coding sequence directed by the rat L-type pyruvate kinase gene promoter (bp −119 to +11) upstream of which four repeats of the L4–L3 fragment (i.e. GlRE and contiguous HNF4 binding site) were ligated. L4–L3 fragment, covering bp −172 to −123 upstream of the L-PK transcription start site, was obtained by PCR [2]. The polyA signal sequence used was that present in the 3'-LTR of MMLV. pHSGneo [15], a MMLV-based retroviral plasmid, and φCRIP, an amphotropic packaging cell line which supplies the structural proteins of MMLV in *trans*, were provided by Dr. A. Weber (Institut Cochin de Génétique Moléculaire, Paris); pRSVneo, in which aminoglycoside-phosphotransferase gene was driven by the Rous Sarcoma Virus LTR promoter, was used for cotransfection.

2.2. Cell culture and transfection

The hepatocyte-like cell line mhAT3F was derived from the tumoral liver of transgenic mice expressing the SV40 early gene driven by the liver-specific antithrombin III promoter (ATIII-SV40) [12,13]. These cells were cultured in DMEM/NUT.MIX. F12 (1:1 vol/vol) with glutamax-1 or DMEM/HamF12 (1:1 vol/vol)(Gibco-BRL) with or without D-glucose (Sigma), supplemented with 1 μM dexamethasone, 1 μM triiodothyronine, 20 nM human insulin, 5% (vol/vol) fetal calf serum (FCS). NIH3T3 and φCRIP cells were cultured in DMEM (5.5 mM glucose, 1.0 mM sodium pyruvate) supplemented with 10% (vol/vol) newborn calf serum. Ampicilline, streptomycine and glutamine were added in all media and cultures were incubated at 37°C with 5% (vol/vol) CO_2. φCRIP cells were cotransfected with pHSG(L4L3)4-PKCAT and pRSVneo (molar ratio of the two plasmids is 10:1) by the cationic liposome method (DOTAP, Boehringer Mannheim) and selected by G418 (800 μg/ml). G418 resistant clones were separated for the retrovirus production test.

2.3. Viral RNA preparation and one-step RT-PCR

G418-resistant clone cells were seeded in 90 mm dish and cultured to 100% confluence. The supernatant of culture cells was filtered through 0.22 μ pore membrane, incubated with DNase I (10 μg/ml) at 37°C for 30 min and then centrifuged at 65,000 rpm for 20 min at 4°C. The retroviral RNA was extracted and purified from the pellet by proteinase K and phenol/chloroform, co-precipitated with tRNA. The sample was dissolved in diethylpyrocarbonate (DEPC)-treated water

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containing 1 mM dithiothreitol (DTT), 40 U RNasin/100 μl, and then used for one step coupled RT-PCR [16–18]. The two primers used for amplification were: sense Gag, 5′-ACTCAAATGCCCAATGAAGT-C-3′, corresponding to the Gag region of MMLV (bp 1524 to 1544); antisense CAT, the 5′-TCAACGGTGGTATATCCAGAT-3′, corresponding to the coding region of the CAT gene (bp 35 to 15 with respect to the translation start site). Reverse transcription from the antisense PCR primer was catalyzed by 8 U AMV reverse transcriptase (Promega) for 1 h at 42°C and the subsequent PCR with 2.5 U Taq DNA polymerase (Gibco) was performed for 35–40 cycles. The amplified fragment was expected as a 440 bp-long containing part of the gag gene, 4 repeats of the L4–L3 element, the −119L-PK promoter and the first 69 bp of the CAT gene. When necessary, the RT-PCR fragments were cloned and sequenced.

2.4. Infection by recombinant retrovirus, estimation of the retrovirus titres and CAT assays

RT-PCR positive clones were used to infect the NIH3T3 cells to characterize the infectious capabilities of produced retroviruses: 1 ml supernatant of 100% confluent culture cells was used to infect the logarithmic growth NIH3T3 with 8 μg/ml polybrene. After 3 h incubation the polybrene was diluted to 2 μg/ml with medium, and culture was continued for another 48 h. The genomic DNA of infected mhAT3F cells was extracted and used for PCR amplication using the same primers as those used for RT-PCR. Detection of the specific amplified fragment proved the integration of the retroviral sequences into the genome of infected cells. The retrovirus titre was appreciated by comparing intensities of the amplified DNA bands from genomic DNA of infected NIH3T3 cells and from different dilutions of the retroviral vector (10^2–10^6 molecules). Clones which produced infectious retroviruses were co-cultured with mhAT3F cells: a 60 mm dish, whose bottom was broken away and the brim smeared with silicone oil, was placed upside down to divide the 90 mm dish into two parts; the mhAT3F cells and retrovirus-producing cells were added in the center and the periphery respectively, being careful not to mix the two types of cells. When the cells are well attached onto the dish after 6 h incubation, the small dish was taken out. The medium was changed for another 16 h incubation, then polybrene was added to 8 μg/ml, the cells were cultured for 48 h. CAT activity was measured by thin-layer chromatography [19] and quantitated in cpm by liquid scintillation.

3. Results

3.1. Recombinant retrovirus production

Retroviral vector pHSG(L4L3)4PKCAT and pRSVneo were used to cotransfect the packaging cells φCRIP. Seventy G418-resistant cell clones were analyzed. Eight RT-PCR positive clones showed the capability of producing infectious recombinant retroviruses determined by specific PCR amplification of the expected fragment from NIH3T3 genomic DNA. Out of the eight, we analyzed two clones of retroviruses containing different internal promoter regions: as shown in Fig. 1, clone F6 (5×10^4 infectious retrovirus/ml medium) produced a retrovirus with the complete promoter region (4 repeats of the L4–L3 fragments), clone B8 (5×10^5 infectious retrovirus/ml medium), a retrovirus with a deleted promoter (retaining only one complete L4–L3 fragment and a small part of a second L3 box). Since the same deletion was found in the genomic DNA of retrovirus-producing clones, in the genomic RNA of recombinant retrovirus as well as in the genomic DNA of infected NIH3T3 and mhAT3F cells, the deletion occurred probably early during transfection of the packaging cells and integration of the plasmidic DNA into chromosomal DNA.

Characterization of the retrovirus-producing clones by RT-PCR simplified the cloning procedure and permitted the detection of any deletion between the PCR primers. In addition, measure of retrovirus titres is often difficult when recombinant retroviruses do not contain marker genes for selection or histochemical analyses. Therefore, we estimated the viral titres by a semiquantitative RT-PCR using different dilutions of pHSG (L4L3)4PKCAT as standards.

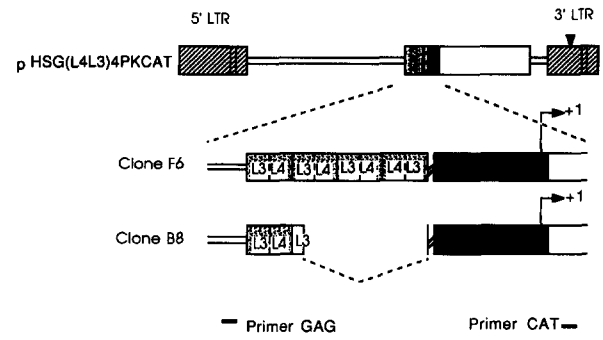

Fig. 1. Retroviral vector and recombinants. The pHSG(L4L3)-4PKCAT is a retroviral plasmid. The Clone F6 and Clone B8 are recombinant retroviral variants containing L-PK gene promoter-derived internal promoters. The hatched rectangle represents the MMLV LTR and the stippled box is the L4(GlRE) and L3(HNF4 binding site) elements of the L-PK gene promoter. The proximal L-PK gene promoter is shown as a shaded rectangle and the CAT gene, an open rectangle. MMLVsequences and polylinker are presented as double horizonal line and double oblique line, respectively. The arrow head above the 3′LTR indicates the enhancer deletion. The start site of transcription is shown by the +1 position. Positions of the primers used for PCR and RT-PCR amplification of the recombinant retrovirus sequence are the primer GAG in 5′ and primer CAT in 3′.

3.2. Dose- and time-dependent glucose induction of the chimeric LPK/CAT gene

Rat hepatocyte-like cell line mhAT3F maintains the capability to respond to glucose by a transcriptional activation of the L-PK gene and is therefore suitable to test any glucose responsiveness of the CAT transgene transferred by the retroviral vector, and therefore the function of the L-PK GlRE in a retroviral context. However, mhAT3F cells were only poorly infectable by recombinant retroviruses (not shown). To enhance the infection efficiency, we co-cultured the mhAT3F cells two times with the retrovirus-producing cells. The L-PK promoter expression is hepatocyte-specific and was not detected in φCRIP packaging cells (not shown). A satisfying infection level was achieved by using clone B8, but not clone F6, whose titre is at least tenfold lower than of clone F8.

As shown in Figs. 2 and 3, the expression of the L-PK/CAT transgene in the infected mhAT3F cells was well induced by glucose, with a half maximal induction obtained for 3–4 mM glucose and a maximal induction for 8 mM glucose. For higher glucose concentrations, CAT activity remained relatively stable. In mhAT3F cells cultured with 17 mM glucose, L-PK gene induction was observed at the 8th hour of incubation, was half-maximal before the 24th hour and maximal at the 48th hour.

4. Discussion

Up to now, retroviral vectors have especially been used to direct stable unregulated expression of a transgene from retroviral LTR or internal cellular promoters [20]. However, it has been shown that the regulatory regions of the phosphoenolpyruvate carboxykinase (PEPCK) gene transferred through a

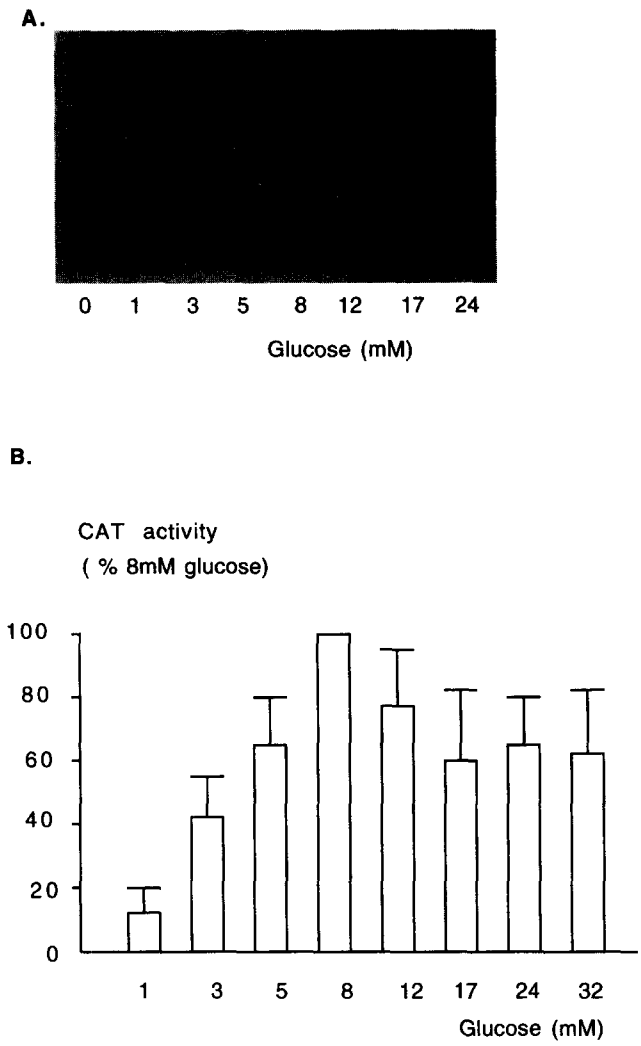

Fig. 2. Dose-dependent glucose induction of LPK/CAT gene expression in mhAT3F cells. The mhAT3F cells infected by co-culture with the clone B8 were seeded at 10^5 cells per 90 mm dish and cultured for 72 h in media containing different concentrations of glucose. (A) Autoradiogram of the CAT activities. (B) Relative CAT activities (mean ± S.D.). Values between 0 and 17 mM glucose are means of 7 independent assays, values at 24 and 32 mM glucose are means of 2 assays only. The CAT activity (cpm) without glucose was set as zero. The CAT activities were expressed as percentages of the CAT activity presented with 8 mM glucose (100%).

retroviral vector ex vivo [21] and in vivo [22] could still confer a transcriptional stimulation by cyclic AMP and glucocorticoids and inhibition by insulin on a reporter gene. We show, in this report, that the L-PK gene GIRE also functions in a retroviral context, being able to confer the glucose responsiveness on a reporter gene.

The dose-dependent glucose effect on the activation of the L-PK/CAT transgene in infected mhAT3F cells shows a half maximal activation at 3–4 mM glucose, which is lower than that in hepatocytes [23]. The reason could be, at least in part, the replacement in mhAT3F cells of the low affinity glucokinase by high affinity hexokinase isozymes [11,14]. Nevertheless, mhAT3F cells still synthesize the Glut-2 glucose transporter (Antoine et al., personal communication) whose affinity for glucose is relatively low (K_m = 10 mM). This could be explain why glucose concentration for a half maximal activation is intermediate in mhAT3F cells between that of hepatocytes and that expected for cells synthesizing the Glut-1 transporter and hexokinases 1 or 2 [24–26]. The time course of glucose-dependent activation of the transgene confirms that the phenomenon is relatively delayed in mhAT3F cells, as already reported for the endogenous L-PK gene and transfected L-PK/CAT plasmids [14].

The major interest of the retroviral vector reported here, capable to transfer into hepatocytic cells a transgene positively regulated by glucose, is in the prospect of gene therapy for insulin-dependent diabetes mellitus (IDDM). Indeed, gene therapy using the insulin gene could transform cells in vivo into insulin-secreting biopumps. Yet, these cells should be capable to express the insulin transgene under the control of physiological glucose concentration, which requires, among other conditions, that cell possess a glucose sensor system [26–28]. Such a

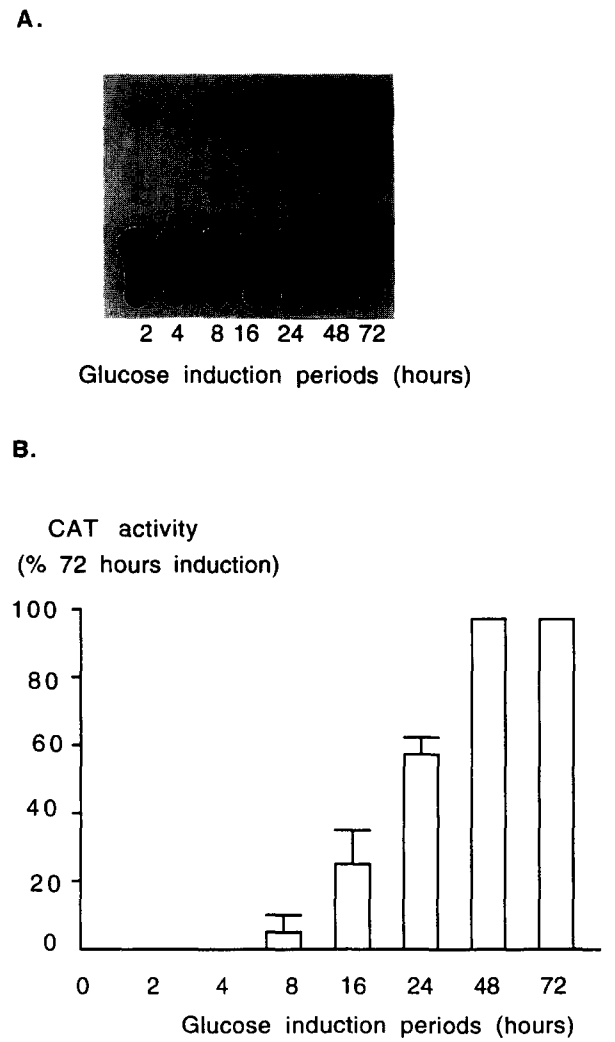

Fig. 3. Time course of LPK/CAT gene activation by glucose in mhAT3F cells. The mhAT3F cells infected by co-culture with clone B8 were seeded at 10^5 cells per 90 mm dish and incubated for 72 h. 17 mM glucose was added in the media 72, 48, 24,16, 8, 4, 2 h, respectively, before harvesting the cells. (A) Autoradiograms of the CAT activity. (B) Relative CAT activities (mean ± S.D.). The CAT activity without glucose was set as zero and glucose induction during different periods were represented as percentages of the value at the 48th hour set as 100%. All values are means of three independent experiments.

system is composed of the Glut-2 glucose transporter and glucokinase. Only two cell types possess both activities, pancreas β cells and hepatocytes [29,30]. Some genes expressed in the liver, in particular the L-type pyruvate kinase gene, are submitted to a physiological control by glucose. Therefore, it should be feasible to program hepatocytes to express the insulin gene under the same type of control. Since the unprocessed insulin gene product, proinsulin, is much less active than processed insulin, this strategy would require either to modify the insulin gene to render it cleavable by the hepatocyte proteolytic machinery [31,32], or to co-transfer into hepatocytes genes for pancreas β cell-specific convertases [33,34]. Infection of hepatocytes by retroviral vectors can be made either ex vivo [21,35–40] or in vivo [22,41,42], resulting in both cases in the presence in the liver of some insulin-secreting cells. We can expect that insulin secretion into the portal venous system, in which insulin is physiologically secreted under normal conditions, will be an advantage for this type of strategy. Besides, the lag period observed ex vivo between glucose signal and transcriptional activation of a transgene controlled by the L-PK regulatory region might not reflect the expression of the same transgene in vivo, which will be continuously stimulated by blood glucose. Reinstoration of an active insulin secretion by such a type of gene therapy, even though a normal glycoregulation may not be reinstored, should surely improve the symptoms of IDDM patient whoseendogenous pancreas insulin production is nil. A last, but not least, advantage of directing in vivo the insulin gene expression by the L-PK GlRE is that this sequence mediates an immediate blockage of gene transcription in hepatocytes by glucagon (through cyclic AMP) [2,4]; therefore, an insulin transgene could be readily blocked at the transcriptional level by hyperglucagonemia secondary to hypoglycemia, which should avoid the risk of sustained hypoglycemia due to the surexpression of the insulin transgene.

In conclusion, this paper demonstrates that it is feasible to use a retroviral vector to transfer into hepatocytic cells transgenes activated by glucose. This result seems to be promising in the prospect of development of gene therapy for insulin-dependent diabetes mellitus.

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