Regulated expression of mature human insulin in the liver of transgenic mice

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Abstract Transgenic mice expressing either human proinsulin cDNA or mutated proinsulin cDNA in the liver were created. The human proinsulin cDNA was mutated to generate a protein cleavable by the ubiquitous prohormone convertase furin, thus leading to mature insulin peptide. All transgenic lines expressed human C-peptide in the blood, whose level varied according to nutritional conditions. High performance liquid chromatography fractionation of mouse serum revealed that mutant proinsulin was effectively processed into mature insulin in vivo. This transgenic mouse model provides a useful tool for further prospects of gene therapy of insulin-dependent diabetes mellitus.

Key words: Insulin; Furin; L-type pyruvate kinase gene; Transgenic mice; Insulin-dependent diabetes mellitus

1. Introduction

Insulin-dependent diabetes mellitus (IDDM) is caused by destruction of the pancreas islet β cells, leading to insulin deficiency. Different therapeutic strategies based on the substitution of β cells by an 'artificial' insulin producing system in vivo are currently under investigation. In this context, we have created transgenic mice that produce human insulin in the liver in a physiologically regulated manner. The hepatocyte, like the pancreatic β cell, is a secretory cell and possesses both the glucose transporter Glut2 and the glucokinase enzyme, which confers cellular sensitivity to physiological glucose concentrations [1,2]. To achieve physiological metabolic control of insulin production, we used the liver pyruvate kinase (L-PK) promoter to direct the insulin transgene. The gene encoding the glycolytic enzyme L-PK has previously been shown to be transcriptionally regulated, positively by glucose and insulin, and inhibited by glucagon or fasting [3,4]. In contrast to β cells, however, hepatocytes lack the specific proconvertases required to completely process proinsulin into mature insulin, the biologically active form of the peptide [5]. To bypass this inconvenience, we mutated human proinsulin cDNA by generating in the recombinant product cleavable sites for furin, a ubiquitous endoprotease [6]. This strategy has been reported to elicit mature insulin production by a human kidney cell line in culture [7]. Moreover, a point His-to-Asp mutation was introduced at position 10 of peptide B to enhance insulin biological activity [8].

Using this mutated form of proinsulin under the control of the L-PK gene regulatory sequences, we show here that the liver of transgenic mice expresses a mature and biologically active human insulin. Moreover, its production is physiologically regulated, negatively by fasting and positively by a carbohydrate-rich diet.

2. Materials and methods

2.1. Construction of the mutated human proinsulin cDNA

The human wild-type proinsulin cDNA, kindly provided by Dr. Kevin Docherty (Department of Molecular and Cell Biology, Aberdeen, UK), was mutated by PCR as described in Fig. 1A. The oligonucleotides used were: INS5, 5′-CT**C TCG AG**G CCA TGG CCC TGT GG-3′; INS3, 5′-CTG CG**G TCG AC**G TCT AGT TGC AG-3′; ASP1, 5′-CAC CAG GTC **GGA TCC** GCA CAG GTG-3′; ASP2, 5′-CTG TGC **GGA TCC** GAC CTG GTG GAA G-3′; BC1, 5′-CTC CCG CTT GGT CCT GGG TGT GTA G-3′; BC2, 5′-GCA GAG GAC CTG CAG G-3′; CA1, 5′-CTT CTG CCG **GGA TCC** CTC CAG GGC-3′ and CA2, 5′-CTG GAG **GGA TCC** CGG CAG AAG CTG G-3′. Bold letters indicate restriction sites shown in Fig. 1A. The resulting plasmid containing the mutated human proinsulin cDNA was designated pIPS6.

2.2. Construction of the plasmids

All plasmids were constructed following a standard DNA cloning procedure [9]. A fragment (from −3200 to +580 bp relative to the start site of transcription) containing the regulatory sequences of the L-PK gene, the first exon, the first intron and the beginning of the second exon was mutated by PCR, transforming the ATG site into TTG (M. Vasseur-Cognet, unpublished results). The resulting *Eco*RI-*Eco*RI fragment was cloned in the bluescript KS$^+$ vector at the *Eco*RI site. This plasmid was designated KS-PK. The L-PK/proINS plasmid was obtained by inserting a 490 bp Klenow-blunted *Spe*I-*Not*I fragment of the proinsulin cDNA into the *Eco*RV-*Not*I cut KS-PK. A Klenow-blunted 330 bp *Xho*I-*Sal*I fragment of the mutated human proinsulin from the pIPS6 plasmid was inserted at the *Eco*RV site of the bluescript KS$^+$ vector. A Klenow-blunted 240 bp *Bam*HI-*Bcl*I fragment of the SV40 polyA was cloned at the blunted *Hin*dIII site of this plasmid. A Klenow-blunted *Xho*I-*Xho*I 570 bp fragment containing the mutated proinsulin and the SV40 polyA was obtained from this plasmid and inserted at the blunted *Spe*I site of the KS-PK. The resulting plasmid was designated L-PK/INSm.

A 330 bp *Xho*I-*Sal*I fragment from the pIPS6 plasmid containing the mutated human proinsulin cDNA was introduced at the *Sal*I site in the polylinker of the empty plasmid containing the promoter of cytomegalovirus (pCMV). This plasmid was designated pCMV-INSm.

The 183PK/CAT construct has been previously described [5]. The KSV2 CAT plasmid, used as a transfection control, contains the chloramphenicol acetyltransferase (CAT) reporter gene directed by the early promoter and enhancer of simian virus 40 (SV40) [10].

2.3. Generation of transgenic mice

The restriction fragments containing the chimeric gene were microinjected into fertilized mouse eggs as previously described [11]. Founders were identified after Southern blot analysis of tail DNAs from 2 week old mice. All subsequent studies were performed on F_1 or F_2 mice. Littermates were used as control. All mice were maintained in accordance with the 'Ministère de l'Agriculture, de la Pêche et de

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l'Alimentation' guide for the care and use of laboratory animals (authorization number 04093, official Dr. Dominique Daegelen-Proux).

2.4. Nutritional treatment

For metabolic studies, animals were on standard diet or fasted for 24 h and refed with a high carbohydrate diet for 18 h. Mice were killed between 9.00 and 11.00 h.

2.5. High performance liquid chromatography (HPLC) separation of serum C-peptide and proinsulin

A 4.6×250 mm C8 Nucleosil column (Touzart and Matignon) was used with a LKB system. Absorbance was monitored at 226 nm. Solvent A was Milli-Q water–0.1% trifluoroacetic acid and solvent B was 80% acetonitrile–0.1% trifluoroacetic acid. Using a flow rate of 1 ml/min, elution was obtained as follow: isocratic at 30% solvent B for 10 min, then developing a linear gradient from 30% to 45% solvent B up to 50 min. Fractions were collected every minute. For RIA determination, fractions lyophilized were reconstituted in a phosphate buffer. The HPLC system was standardized using authentic human C-peptide, insulin and proinsulin.

2.6. Insulin, C-peptide and glucose assays

Blood was collected from the retro-orbital sinus of the mice between 9.00 and 11.00 h and centrifuged at 800×*g* for 10 min. Sera were kept at −20°C until further analysis. Urine from mice on the carbohydrate-rich diet was collected between 9.00 and 11.00 h, kept at −20°C and centrifuged at 800×*g* for 10 min before use. Serum and urine insulin and C-peptide were measured by RIA with respectively an anti-insulin serum, recognizing both mouse and human insulin, and an anti-C-peptide serum recognizing human C-peptide and proinsulin (Behring Diagnostic, France). Serum glucose level was measured using a glucose oxidase colorimetric reaction (Glucose Trinder, Sigma Diagnostic, USA).

2.7. Cell culture conditions

Hepatocytes from 3 days fasted male Sprague-Dawley rats (180–200 g), isolated by the collagenase perfusion method [4,12,13], were plated for 4 h onto 10 cm dishes in a final volume of 10 ml medium 199 (Life Technologies, Inc.) supplemented with penicillin, streptomycin, and 10% (v/v) dialyzed fetal calf serum. The medium was then replaced by a serum-free, glucose-free medium containing 10 mM lactate.

2.8. Cell transfections

Transfection of the isolated hepatocytes with the different plasmids was performed by lipofection using *N*-(1-(2,3-dioleoyloxy) propyl)-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP, Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions.

2.9. CAT assays

Cells were harvested 36 h after transfection. CAT activity assays were performed as described [10,12]. The CAT activity was normalized with respect to the CAT activity of the KSV2 used as a transfection standard. The experiments were performed in duplicate independently three times.

2.10. Statistics

Data are given as mean ± S.D. Statistical significance of differences between groups was determined by Student's *t*-test. The level of significance was $P < 0.05$.

3. Results

3.1. Creation of transgenic mice expressing the wild-type or the mutated human proinsulin cDNAs in the liver

The mutations described in Fig. 1B were included in the human proinsulin cDNA. The 5′ flanking sequence (−3200 bp/+580 bp) of the rat L-PK gene was used to drive the expression of the wild-type human proinsulin cDNA (L-PK/proINS) and the mutated proinsulin cDNA (L-PK/INSm) in the liver of transgenic mice. For each transgene, three different lines were studied (Table 1). All transgenic animals are healthy and have a normal reproductive life. Their weight and glycemia are as in controls.

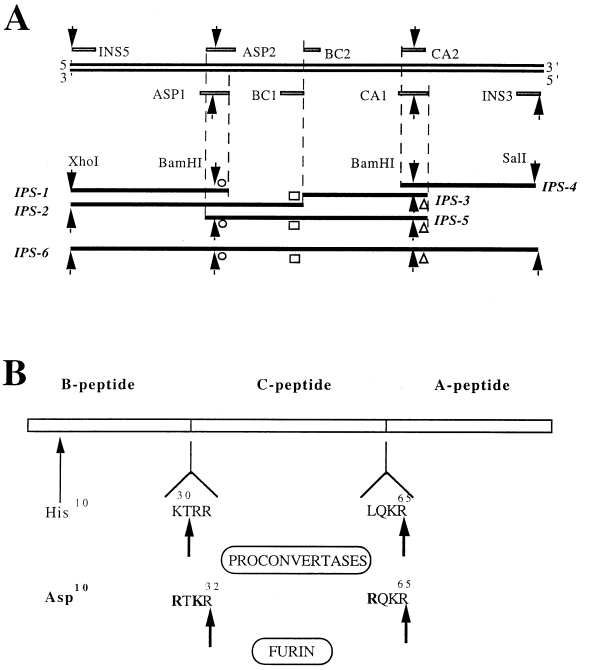

Fig. 1. Schematic representation of the mutated proinsulin cDNA. A: Representation of the strategy used to mutate the human proinsulin cDNA. The pairs of oligonucleotides (INS5, ASP1) and (CA2, INS3) were used to obtain the fragments IPS-1 and IPS-4, respectively (see Section 2 for the oligonucleotide sequences). The pairs of oligonucleotides (INS5, BC1) and (BC2, CA1) were used to obtain the fragments IPS-2 and IPS-3, respectively. The fragment IPS-5 was obtained after a *Bam*HI cut of IPS-2+3 and was then inserted between IPS-1 and IPS-4. The resulting vector containing the entire mutated cDNA was pIPS-6. Arrows indicate new restriction sites. ○ indicates mutation at the His-10 site; □ indicates mutations at the junction of the B and C peptides; △ indicates mutation at the junction of the C and A peptides. B: Representation of the mutation incorporated into the human proinsulin cDNA. Four mutations were incorporated into the human proinsulin cDNA (in bold): His at position 10 of the B chain was replaced by Asp; two mutations were incorporated at the processing site between B and C peptides, converting KTRR into RTKR; LQKR at the processing site between C and A peptides was converted into RQKR.

3.2. The expression of the proinsulin cDNA in L-PK/proINS and L-PK/INSm mice is dependent on the nutritional conditions

All transgenic lines exhibited human C-peptide immunoreactivity in the serum, reflecting the production of C-peptide and/or proinsulin by the liver. As expected, in both L-PK/proINS mice and L-PK/INSm mice, a high carbohydrate diet resulted in a significant increase of serum C-peptide immunoreactivity. By contrast, 24 h fasting resulted in the decrease of C-peptide (Fig. 2). In the L-PK/INSm mice, the stimulatory effect of the high carbohydrate diet was far more important (Fig. 2B). Despite the different serum C-peptide levels, glycemia values for the different metabolic states were similar in controls and transgenic mice (data not shown).

3.3. Mutated human proinsulin is processed by the liver of transgenic mice

In order to analyze the processing of proinsulin in the liver of transgenic mice, HPLC and RIA were performed on the

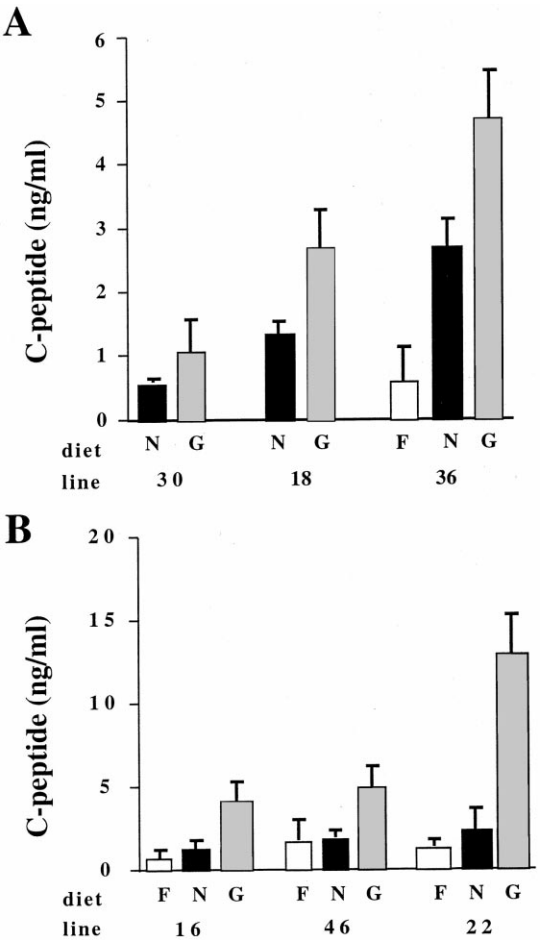

Fig. 2. Detection of human C-peptide in transgenic mice in different nutritional conditions. The human C-peptide immunoreactivity was evaluated in transgenic lines subjected to 24 h of fasting (F), on a standard diet (N) or on a high carbohydrate diet (G). A: L-PK/proINS transgenic lines. B: L-PK/INSm transgenic lines. Bars represent the mean ± S.D. of all measurements ($n \geq 8$).

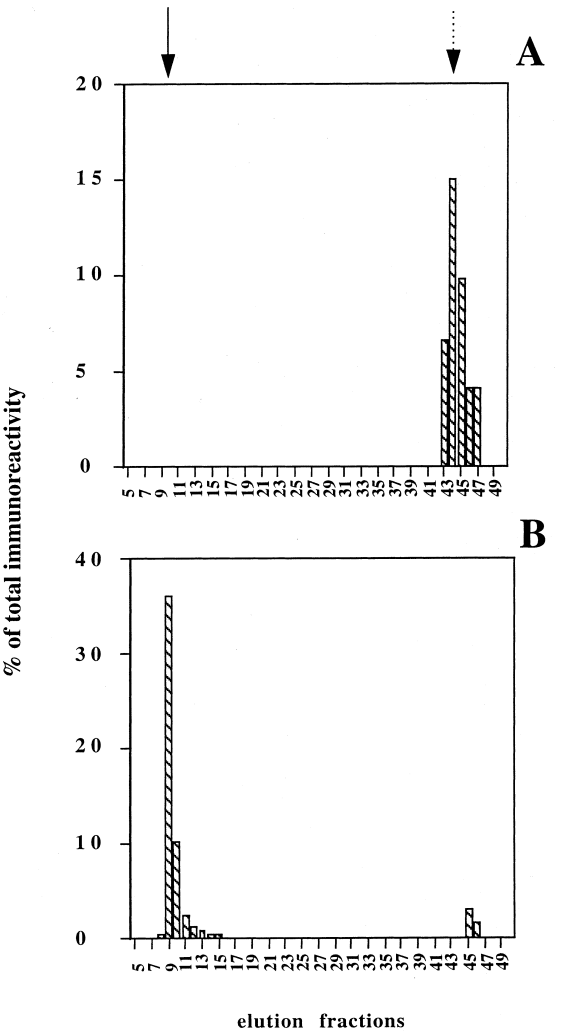

Fig. 3. HPLC analysis of C-peptide immunoreactive products in the serum of transgenic mice. The plain arrow indicates C-peptide peaks and the dotted arrow indicates proinsulin peaks. A: L-PK/proINS transgenic mice (line 36). B: L-PK/INSm transgenic mice (line 22).

sera of L-PK/proINS mice and L-PK/INSm mice on a carbohydrate-rich diet. The anti-human C-peptide antiserum was used to selectively distinguish between the human peptides produced in transgenic mice. One single peak (fractions 43–47) corresponding to human proinsulin was identified in the serum of transgenic mice expressing the wild-type human proinsulin (Fig. 3A). By contrast, in the serum of mice expressing the mutated human proinsulin, in addition to a small peak corresponding to proinsulin (fractions 45–46), one major peak (fractions 8–15) was identified (Fig. 3B). This major peak corresponds to the C-peptide variant derived from the mutated human proinsulin, which appears earlier than the wild-type human C-peptide (expected in fractions 19–20) in the chromatogram. This feature is due to the replacement of the canonical neutral Leu-32 by a basic Arg residue (Fig. 1).

The complete absence of C-peptide in the L-PK/proINS mouse serum was in contrast with the minimal processing of

Table 1
Characteristics of the transgenic lines expressing the human proinsulin cDNA

	Line	Copies	Weight (g)		Glucose (g/l)	Insulin (μU/ml)	
			M	F		N	G
L-PK/proINS	30	6	ND	ND	1.91 ± 0.26*	ND	ND
	18	16	ND	ND	ND	ND	ND
	36	30	25.8 ± 3.6*	20.7 ± 0.6*	1.85 ± 0.29*	1.48 ± 1.7*	29.1 ± 4.3*
L-PK/INSm	16	30	28.4 ± 2.6*	22.2 ± 1.2*	1.91 ± 0.29*	15.8 ± 3.2*	27.8 ± 5.6*
	46	34	28.8 ± 3.6*	25.7 ± 2.9*	1.76 ± 0.26*	ND	ND
	22	130	26.5 ± 3.3*	22.9 ± 1.4*	1.72 ± 0.41*	15.6 ± 2.6*	28.4 ± 8.8*
Control			25.7 ± 1.7	21.9 ± 1.8	2.02 ± 0.23	14.2 ± 2.2	28 ± 2.8

Each value represent the mean ± S.D. of all measurements ($n \geq 8$). M: male; F: female; N: standard diet; G: high carbohydrate diet. *$P > 0.05$ vs. control.

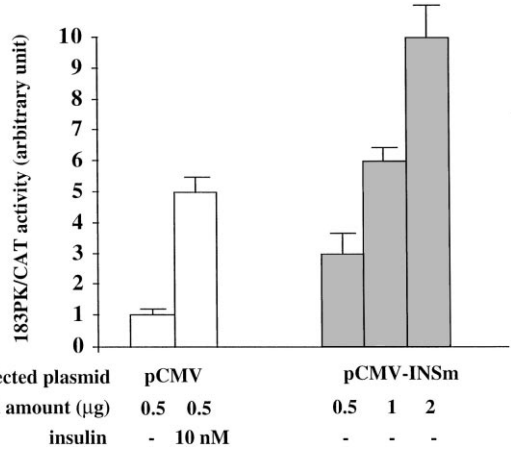

Fig. 4. Stimulatory effect of insulin cDNA expression on L-PK promoter activity. The expression of the 183PK/CAT reporter gene was evaluated in primary cultured hepatocytes, in the presence of pCMV alone or with 10 nM exogenous insulin, and increasing amounts of pCMV-INSm. Similar results were obtained in three independent experiments.

proinsulin previously reported in the liver [5,14]. Since C-peptide is known to accumulate in the urine, transgenic mouse urines were analyzed for C-peptide. Expectedly, C-peptide immunoreactivity was detected in the urines of L-PK/INSm mice (5.44 ± 4.27 ng/ml). It was also detected in the urines of L-PK/proINS mice, although to a lesser extent than in L-PK/INSm mice ($0.69 \pm$ O.6 ng/ml), confirming that a low proinsulin processing can indeed occur in the liver.

Together, these results strongly demonstrate that the mutated proinsulin can be efficiently processed in the liver of LPK/INSm mice.

3.4. Mutated proinsulin is biologically active

L-PK promoter is controlled by both glucose and insulin [3,4]. The biological activity of the engineered insulin was then evaluated by its ability to activate in vitro the transcription of the L-PK gene. For that purpose, isolated rat hepatocytes were cotransfected with a CAT reporter gene under the control of the L-PK promoter (183PK/CAT construct) and either empty pCMV or pCMV-INSm. CAT activity measured in transfected cells with the 183PK/CAT gene and the empty pCMV was strongly increased by 10 nM exogenous human insulin (Fig. 4). In pCMV-INSm transfected cells, a high CAT activity was also detected, whose level was dependent on the amount of transfected plasmid (Fig. 4). These results clearly demonstrate that insulin produced by the mutated proinsulin cDNA is a biologically active compound.

3.5. Evaluation of total plasma insulin in transgenic mice

The serum insulin was measured by RIA using an antiserum which recognizes both human and mouse insulin. In the serum of non-transgenic and transgenic mice, insulin levels were similar, on a standard diet or after a carbohydrate-rich diet (Fig. 1). Since insulinemia of transgenic mice corresponds to both human and murine insulin, this result suggests that endogenous insulin production may be inhibited by the presence of the new designed peptide. This point is in full agreement with the absence of hypoglycemia observed in transgenic mice, and the previously reported inhibitory effect of insulin itself on insulin gene transcription [15].

4. Discussion

The liver provides an excellent target organ to produce extrapancreatic insulin. This approach could be of therapeutic value for gene therapy of IDDM. Such a strategy requires (i) insulin gene targeting in the liver, (ii) physiological regulated expression of the hormone, (iii) processing of proinsulin into the biologically active insulin, a condition which cannot be fulfilled naturally by hepatocytes.

We therefore used the regulatory sequences of the L-PK gene, which we have previously reported to target numerous genes in the liver in vivo and to be transcriptionally controlled by glucose, insulin and glucagon [11,16–18]. Moreover, mutations in the human proinsulin cDNA were generated allowing cleavage of proinsulin to insulin by furin, an endoprotease abundantly expressed in the liver. Furin has previously been shown to process proinsulin into insulin, ex vivo, in a non-pancreatic β cell line [8]. Our current results clearly demonstrate that human insulin can also be efficiently produced and processed in vivo by the liver. Moreover, this production appears fairly regulated by physiological glucose concentrations.

Other in vivo studies aimed at expressing the insulin gene in extrapancreatic tissues have already been reported [19,20]. In the first, the constitutive CMV promoter was chosen, leading to a non-regulated synthesis of proinsulin [19]. In the second, the phosphoenolpyruvate carboxykinase (PEPCK) promoter, which is positively regulated by glucagon, was used [20]. This point could be embarrassing in the case of hypoglycemia, since the resulting glucagon secretion could further induce insulin release, leading to subsequent aggravation of hypoglycemia. By contrast, the transgene inhibition observed in our fasted transgenic mice overcomes this problem and provides a safer experimental device. Moreover, these two in vivo studies used proinsulin, which was not converted into mature insulin. These strategies would probably have been improved by a processing system as used in our model. We have demonstrated that the engineered human insulin produced by our transgene is biologically active. It efficiently activates the expression of a reporter gene under the control of the L-PK gene promoter, which stimulation is strictly dependent on insulin [3,4]. This is in agreement with the results of Groskreutz et al., demonstrating that the maturation of proinsulin into insulin by the furin in a kidney cell line leads to a biologically active peptide [8].

In our study, despite the high level of human C-peptide detected in transgenic mice, the insulinemia was similar in transgenic and non-transgenic mice. Note that insulinemia in transgenic mice corresponded to both murine and human insulin. This suggests that transgenic expression of human insulin by the liver could have inhibited the endogenous expression of pancreatic insulin. Similar observations have been reported by Bucchini et al. in transgenic mice expressing human insulin gene in β cells [21]. This point was further supported by in vitro studies demonstrating the feedback inhibition of insulin gene transcription by insulin itself [15].

In contrast to pancreatic β cells, the constitutive pathway is the only pathway used by hepatocytes to release circulating proteins [5]. Thus, the regulated insulin release from the storage vesicles of the β cell that normally occurs after a meal may probably be absent in the liver cells. In this context, at this stage of our strategy, engineering hepatocytes for gene therapy of IDDM would probably not provide a means to strictly

maintain glycemia in the normal range. However, it could allow for the restoration of an endogenous insulin secretion sufficient to avoid acute decompensation.

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