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A Mechanism of differential expression of GLUT2 in hepatocyte and pancreatic β-cell line

Jae-woo Kim,[1] Yu-kyong Kim[1] and Yong-ho Ahn[1,2]

1 The Institute of Genetic Science and Department of Biochemistry and Molecular Biology, Yonsei University College of Medicine, Seoul 120-752, Korea

2 Corresponding author

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Abbreviations: GLUT2, pancreatic β-cell/liver type glucose transporter; EMSA, electrophoretic mobility shift assay; UPE, upstream promoter element

Abstract

DNase I footprinting assay using liver nuclear extracts revealed six protected regions between nucleotide -600 and +110 and hence named Box I~VI. Upstream promoter element (UPE), a DNA element playing crucial role in transcriptional control of the tissue specific expression of pancreatic β-cell, has been detected within the proximal region of rat GLUT2 promoter. This region is included in Box VI. The protein-DNA interaction in this region (Box VI) was confirmed by mobility shift assay using liver nuclear extracts. Deletion of the region between -585 bp and -146 bp resulted in dramatic changes in promoter activity when they were expressed in liver and β-cell derived cell line. When -585/-I46 construct was expressed in liver, the activity was decreased to 46%, whereas the activity in β-cell line, HIT-T15 cell, was increased by 84% when compared to -146/+190 construct. These opposing phenomena can be explained by the fact that β-cell specifically expresses the UPE binding protein. Assuming that there may be Box VI-binding protein playing negative roles both in hepatocyte and β-cell, and that the protein acts as a negative regulator of GLUT2 gene, the UPE binding protein in the β-cell may overcome the inhibition by binding to the protein.

Keywords: GLUT2 gene expression, hepatocyte, β-cell, upstream promoter element

Introduction

Glucose uptake into mammalian cells is mediated by a family of glucose transporters (Takeda *et al*., 1993). In hepatocytes, pancreatic β-cells, and absorptive epithelial cells of the intestinal mucosa and kidney, the GLUT2 glucose transporter isoform is mainly responsible for glucose entry (Fukumoto *et al*., 1988). It has been shown that the GLUT2 gene expression is regulated differently in hepatocytes and β-cell, especially in diabetic states; the β-cell showed a marked reduction (up to 90%) in GLUT2 expression whereas GLUT2 expression in liver or kidney was unaltered (Thorens *et al*., l990). The regulation of GLUT2 gene expression has been studied *in vitro* by cell culture and in a number of animal models, showing an imbalanced glucose homeostasis (Asano *et al*., 1992; Postic *et al*., 1993). Generally, in diabetic status, it is accepted that there is a strong reduction in GLUT2 expression which is restricted to the pancreatic β-cells while its expression in liver, pancreas, or kidney is unaltered or slightly increased (Johnson *et al*., 1990; Orci *et al*., 1990; Thorens *et al*., 1990; Ohneda *et al*., 1993). The basic changes responsible for GLUT2 down-regulation is not known. A study in which hyperglycemia was controlled to normoglycemic state in Zucker diabetic rats showed the gradual decrease in GLUT2 expression (Orci *et al*., 1990). It is suggested that *in vitro* glucose may directly stimulate GLUT2 gene expression while hyper-glycemia *in vivo* causes a decreased GLUT2 expression in β-cell. To better understand the differential gene expression of GLUT2 between liver and β-cell, we have cloned the promoter region of GLUT2 along with complete intron-exon relationship (Ahn *et al*., 1996).

The aim of this study was to analyze rat GLUT2 promoter elements contributing to transcriptional control in hepatocytes and HIT-TI5 cells, a pancreatic β-cell derived cell line. In this study, we (i) identified DNA-protein interaction sites between nucleotide -600 and +110 by DNase I footprinting, (ii) demonstrated the presence of UPE and AP-1 binding site by electrophoretic mobility shift assay, and (iii) showed the differential role of UPE binding region in liver and β-cell since the transient expression of truncated UPE region showed different effect in hepa-tocyte and β-cell. The presence of UPE in liver resulted in the decrease in GLUT2 promoter activity, whereas it acted as a positive element of GLUT2 expres-sion in β-cell line.

Materials and Methods

General methods

Standard procedures were carried out by the methods

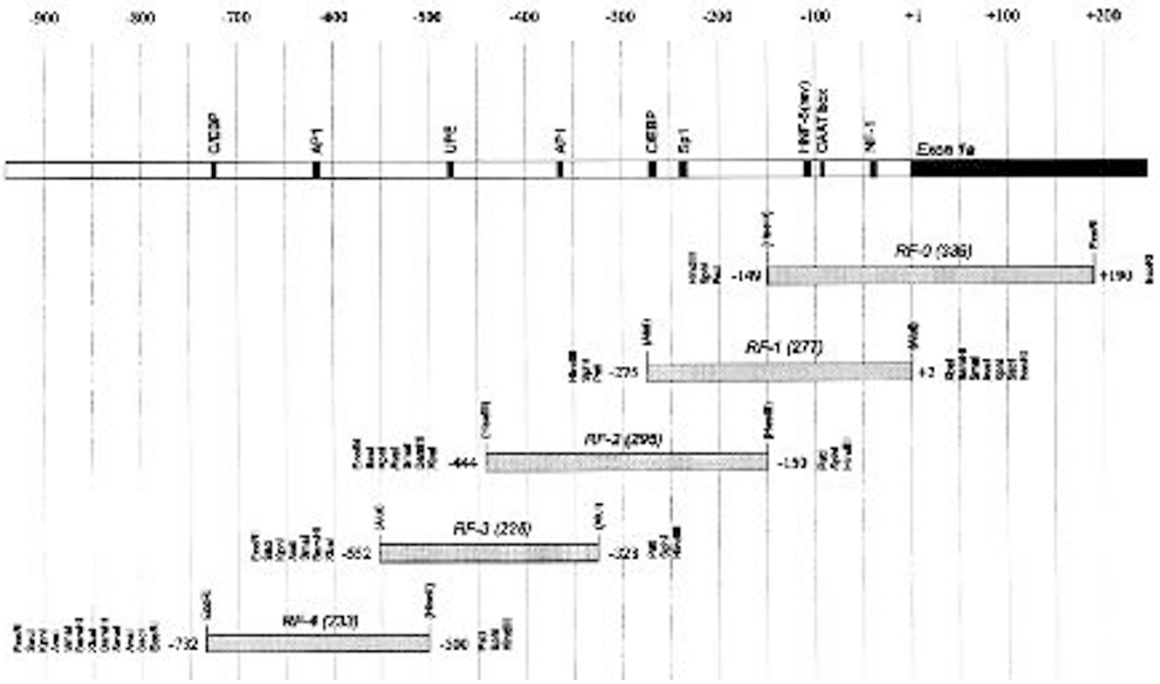

Figure 1. Preparation of promoter fragments for DNase I footprinting. For best binding of proteins to promoter regions, the DNA was digested in a way to generate the size about 300 bp using various restriction enzymes. The resulting fragments were named RF-0, RF-1, RF-2, RF-3,and RF-4. The enzyme sites were obtained by computer analysis of rat GLUT2 promoter region (Ahn *et al*., 1995). The digested fragments were subcloned into pGEM4Z vector.

described by Sambrook *et al.* (1989). The promoter region of rat GLUT2 was obtained by Ahn *et al.* (1995). The DNAs were subcloned into pGEM4Z vector (Promega, WI) for DNA probe preparation.

Hepatocyte culture, transfection and luciferase assay

Hepatocytes were isolated from male Sprague-Dawley rats by collagenase perfusion method (Berry and Friend, l969). Hepatocyte suspensions were plated on 6 cm-dishes in a final volume of 4 ml of modified William's E media (lacking glucose and methyl linoleate supplemented with 6.6 mM glucose, 0.1 unit/ml insulin, 10 nM dexamethasone, 10% fetal calf serum, 26 mM sodium bicarbonate, 100 units/ml penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B). After 6-hour attachment period, transfection was performed using lipofectin. Briefly, 10 μg of luciferase plasmid, 1.5 μg of pRSV β-gal plasmid and 30 μl of lipofectin in 2 ml of the media lacking serum were mixed and added to the hepatocytes in culture plates. After 14-hour incubation period, the remaining liposome-DNA complexes in the culture fluid were removed. The media was replaced with modified William's E media of the same composition but without fetal calf serum. Seventy two hours after transfection, the cells were scraped, resuspended in 100 μl of 0.25 M Tris-Cl, pH 7.8, and disrupted by freezing and thawing. Suspension of disrupted cells was centrifuged at 12,000 *g* for 5 min, and the extract was collected. 30 μl of cell extract was used for β-galactosidase activity and 50 μl of extract was heated at 65˚C for 10 min. Particulates were removed by centrifugation at 12,000 *g* for 5 min and the supernatants (50 μl) were used for luciferase activity (Promega, WI). The luciferase activities were normalized with respect to β-galactosidase activity.

Preparation of DNA probes

A DNA fragment -600/+110 region of rat GLUT2 promoter was digested with various restriction enzymes to produce DNA fragments sizing about 300 bp to use as probes for DNase I footprinting. The fragments were named RF-0, RF-1, RF-2, RF-3, and RF-4 ,and were subcloned into the multiple cloning site of pGEM4Z (Promega, WI, Figure 1). DNA fragments (500 ng) were made 5'-overhang and 3'-overhang at the same time and labeled using 2 μl of 10× Klenow buffer, 2 μl of 4 mM dCTP/dGTP/dTTP, 6 μl of [α-^{32}P]dATP, and 5 units of Klenow enzyme and brought the volume to 20 μl using deionized H_2O. The labeling reaction was carried out at 37˚C for 10 min and chased for another 5 min at 37˚C by adding 2 μl of 4 mM dATP. The labeled DNAs were separated from polyacryl-amide gel electrophoresis. The

labeled fragments were obtained by electroelution and counted radioactivity.

Preparation of rat liver nuclear extracts

Rats were sacrificed and nuclear extracts were prepared by the method described by Gorski *et al*. (1986). The livers were minced and homogenized in 40 ml of homogenation buffer (10 mM HEPES, pH 7.6, 15 mM KC1, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 M sucrose, 10% glycerol, 1.0 mM DTT, 0.5 mM PMSF, and 1% trasylol) using Teflon homogenizer and centrifuged for 30 min at 30,000 rpm using Ti 70 rotor. The pellet was homogenized again in 30 ml of homogenation buffer and centrifuged again. The nucleus was lysed in 5 ml of nuclear lysis buffer (20mM HEPES, pH 7.6, 26% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT), incubated at 4˚C for 30 min, and centrifuged for 30 min at 25,000 rpm. The proteins in the supernatant were precipitated using $(NH_4)_2SO_4$ (0.39 g/ml) and centrifuged for 30 min at 30,000 rpm. The pellet was dissolved in l ml of dialysis buffer (20 mM HEPES, pH 7.6, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) and dialyzed for 2 h. The dialysates were centrifuged for 10 min at 5,000 rpm. Nuclear extracts were assayed for protein concentration by the method of Bradford (1976).

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed using Box VI and Box IV sequences within rat GLUT2 promoter(UPE, sense; AGTTAACAA TCTTG ATTFCCA CATCA CAAAC-GTGCAATT, antisense; AATTGCACGTTTGTG ATGTGGAAATCAAGATT GTAACT, AP-1, sense; GCCTCTACTCTTATCTGACTCAAC AGGAGA, antisense; TCTCCTGTTG AGT CAGATAAGAGTA -GAGGC). Briefly, 50,000 cpm of [α-^{32}P] labeled probe, 5~15 μg of nuclear extracts were incubated in 10 mM HEPES, pH7.9, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 7% glycerol, and 0.1 mg/ml of poly(dI-dC) on ice for 30 min. The mixture were separated at 4% acrylamide gel containing 5% glycerol and 0.25× TBE at 200V for 2 h. Gel was dried and exposed to X-ray film.

DNase I footprinting

As a preliminary study to find out possible protein binding sites on rat GLUT2 promoter, EMSAs were carried out, and six potential protein binding sites were confirmed. These regions were used for DNase I footprinting. In this extract, the amount of nuclear extracts used were 0, 20, 40 ,60, and 100 μg for reaction. While the reactions were proceeded on the ice for 30 min, various dilutions (from 1:200 to 1:2,000) of DNase I (10 units/μl, Boehringer Mannheim, Germany) were added to reaction mixture for 2 min at room temperature. The reaction was stopped by adding 78 μl of stop solution (20 mM Tris-C1, pH 8.0, 20 mM EDTA, 250 mM NaCl, 0.5% SDS), and 4 μg of yeast tRNA (Sigma), 10 μg of proteinase K, and incubated at 45˚C for 60 min. DNA was extracted with phenol/chloroform and precipitated. The DNAs were separated at 6% polyacrylamide/8 M urea gel electrophoresis at

Figure 2. *In vitro* DNase I footprinting analysis of the rat GLUT2 promoter. RF-0, RF-1, RF-2, RF-3, and RF-4 were labeled at both ends by Klenow filling-in reaction. The labeled fragments were incubated for 30 min on ice with increasing amounts of nuclear extracts prepared from rat liver and treated with DNase I for 2 min at room temperature. The areas protected from DNase I were boxed. The corresponding nucleotide positions were determined by running a G+A chemical sequencing ladder. Roman numerals represent the putative protein binding sites distant from transcription initiation sites. Bottom and top represent the footprinting using antisense and sense strand, respectively. (-): no nuclear extract, The nuclear extracts were used at concentrations of 20, 40, and 60 μg.

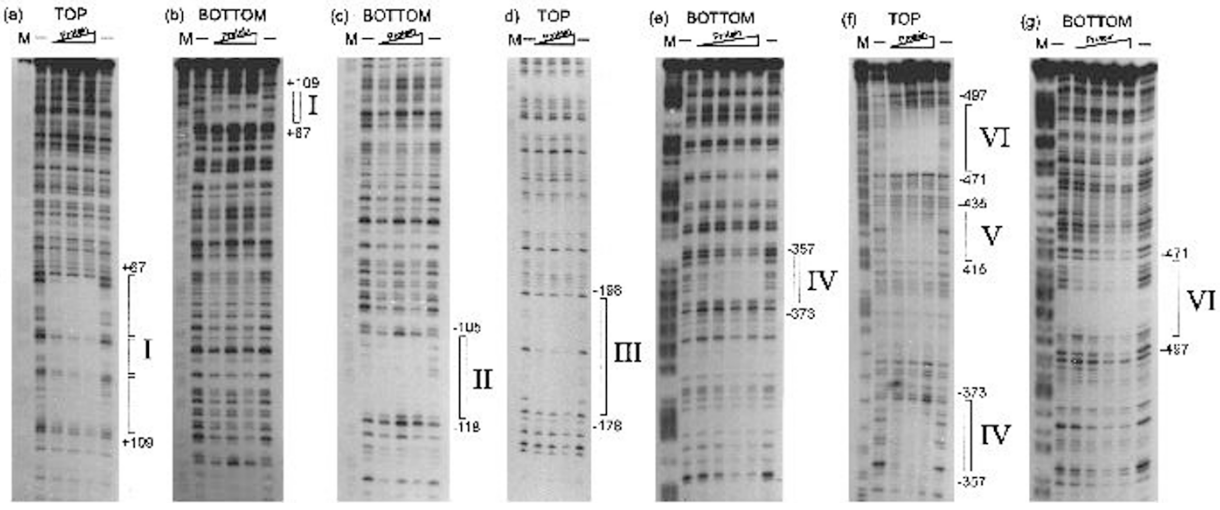

Figure 3. Summary of protein binding sites. Computer search revealed that box VI, IV, II, and I matched UPE, AP-1, HNF-5, TFIID, respectively. However, Box V, III did not match any known consensus sequences.

2,000 V. The gel was exposed to X-ray film overnight. The region of protein binding sites were confirmed by A+G sequencing reaction of Maxam-Gilbert chemical sequencing (Sambrook *et al*., 1989).

Results

Using liver nuclear extracts *in vitro* EMSA and DNase I footprinting were performed to search for DNA-binding protein factors interacting with the promoter region (-600/+190) of rat GLUT2. Six protected regions were detected from DNA-protein binding analysis (Figure 2). The Box VI extends from nucleotide -497 to -471 (Figure 2g), which shows homology with UPE region. Box IV corresponds to potential AP-1 site (Figure 2e) (Neuberg *et al*, 1989). In these two consensus sequences, UPE and AP-I draw a special attention. The UPE sequence is well known cis-element (Magnuson and Jetton, 1993) controlling the tissue specific expression of gene in pancreatic β-cell. Another interesting consensus sequence in this region is AP-1 site. AP-1 protein is a Jun-Fos heterodimer and known as an oncoprotein involved in cellular carcinogenesis. Figure 2 b, c and d show the footprinting pattern of Box I, II, and III. Box II (-118/-105) matched the HNF-5. Box I (+67/+85, +87/+95, +98/+109) occupied the exon 1 region and considered to be a potential binding site for transcription initiation factor IID. Although the Box III represented a footprint (-198/-178), there is no known consensus sequence for the region. Figure 3 summarizes the binding sites of trans-acting factors possibly involved in GLUT2 gene expression.

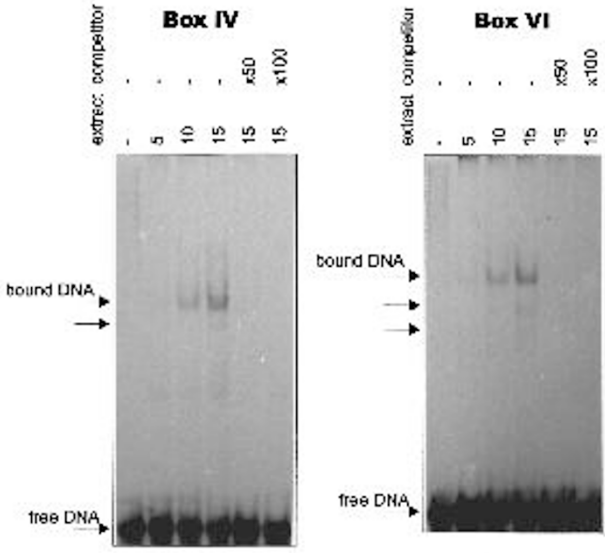

Figure 4. Mobility shift assay of UPE and AP-1 binding sites. Box IV and Box VI sequence (sense and antisense strand) were synthesized and labeled using polynucleotide kinase. [^{32}P]-labeled probe (50,000 cpm), nuclear extracts (5-15 μg) were incubated on ice for 30 min. The reaction mixtures were separated at 4% polyacrylamide gel electrophoresis. For specificity of binding, cold Box IV and Box VI were used, respectively.

To further characterize the Box IV and VI, oligonucleotides covering the UPE and AP-1 site were synthesized and subjected to mobility shift assay. In accordance with the DNase I footprinting experiment, the oligonucleotides containing UPE and AP-1 binding sites specifically retarded the mobilities, respectively (Figure 4).

To further characterize the role of Box VI in differential expression of GLUT2 in hepatocyte and β-cell, deletion studies were performed. The deletion of the region between -585 bp and -146 bp resulted in dramatic changes in

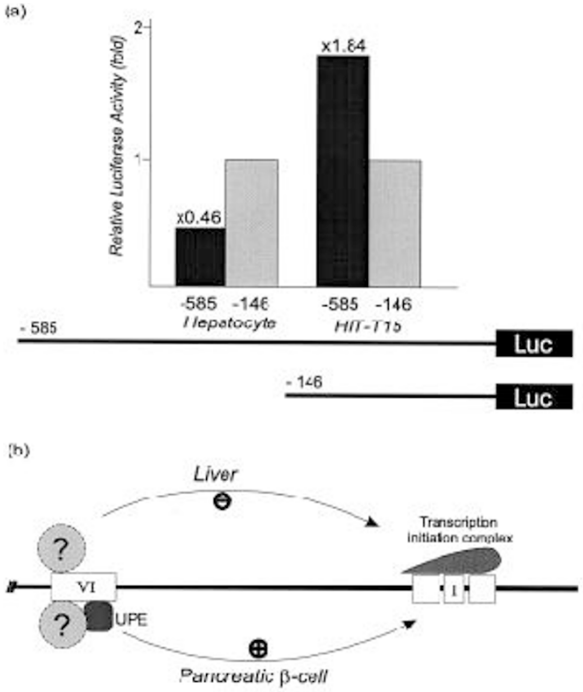

Figure 5. Effect of UPE region of rat GLUT2 promoter on luciferase activity. **(a)** The region containing UPE binding sites (-585/-146) were deleted for testing the role on rat GLUT2 expression. The DNAs [-585/ +190 (black bar) and -146/+190 (grey bar) constructs] were cotransfected with pRSV-gal into cultured hepatocytes or HIT-T15, a β-cell line. Relative luciferase activities were normalized to β-galactosidase activity. Values represent average of at least three independent experiments expressed as folds of the activities of cells transfected with -146/+190 GLUT2 constructs. **(b)** Models illustrating tissue specific transcriptional control of rat GLUT2 gene in liver and β-cell. Assuming that Box VI binding protein is expressed both in hepatocyte and β-cell and that it acts as a negative regulator of GLUT2 gene, UPE binding protein expressed in β-cell may overcome the inhibition imposed by Box VI binding protein.

promoter activity when they were expressed in hepatocyte and β-cell line. The activity was descreased to 46% in hepatocytes transfected with the -585/+190 construct, whereas in the transfected b-cell line, HIT-T15 cell, the activity increased by 84% (Figure 5a).

Discussion

Several potential protein binding sites have been detected by sequence analysis of rat GLUT2 promoter region (Ahn *et al.*, 1995). A putative UPE consensus sequence (Magnuson and Jetton, 1993) at -497 to -471, AP-I site (Neuberg *et al.*, 1989) at -373 to -357, HNF-5 at -118 to -105, C/EBP (Landschulz *et al.*, 1988) at -273 to -266, Sp1 site(Biggs *et al.*, 1988) at -239 to -230, and CAAT box (Mitchell and Tjian, 1988) at -81 to -84 were observed. *In vitro* EMSA and DNase I footprinting revealed 6 protected regions. Of these boxes, UPE and AP-1 site seemed important in regulating GLUT2 activity. Especially, UPE may act as a negative regulator in liver whereas it could be a positive gene regulator in the β-cells. In this respect, it may be one of possible regulator elements to control the differential expression of GLUT2 between liver and pancreas in the hyperglycemia induced by diabetes. Another site, AP-1 binding site is known to bind AP-1 protein, which plays an important role in cellular transformation.

To date, two main regulators of GLUT2 gene expression are glucose and transformation. The expression of GLUT2 is increased by high glucose concentration in liver and decreased by cellular transformation. Therefore, it is quite possible that the AP-1 consensus sequence may act as a negative regulator of GLUT2 expression in cellular transformation. The exact role of this region in the carcinogenesis is under intensive study. In order to further characterize the role of UPE in the rat GLUT2 expression in hepatocyte and β-cell, the promoter region containing UPE binding site was deleted and its effect was studied in hepatocyte and β-cell line. The UPE sequences are known to be responsible for the specific expression of certain genes in pancreatic β-cell (Magnuson and Jeffon, 1993). On this experimental background, we have attempted to test the role of UPE in liver and β-cell.

To test the hypothesis that UPE may act as a negative or a positive regulator in hepatocyte and pancreatic β-cell line, we have truncated the region and fused to luciferase reporter genes. Figure 5b shows the hypothetical regulation mechanism mediated by UPE. There may be a common regulator inhibiting the expression of GLUT2 promoter in hepatocyte and β-cell. Because the UPE is not expressed in liver, the inhibition imposed by a common regulator is not relieved. In pancreatic β-cell, the presence of UPE caused the physical contact between a hypothetical negative regulator and leads to the activation of the GLUT2 gene. The UPE-negative regulator complex binding to UPE in pancreatic β-cell acts as a positive regulator of Box I, which binds the transcription initiation complex. However, it should be taken into consideration that HIT-T15 cell is a type of cell expressing low level of GLUT2. The question of how the cells activate the GLUT2 promoter is yet to be answered.

In conclusion, our analysis on rat GLUT2 promoter region revealed the possible presence of a common negative elements for GLUT2 expression in liver and pancreatic β-cell. Considering that GLUT2 expression is cell specific, it is quite likely that a specific mechanism of regulation may operate in these cells. In this regard, a direct approach to identify the common negative regulator binding to UPE consensus sequence should be performed. Further studies are necessary to explore the significance of the unique cis-activating elements in the transcriptional control of GLUT2 as well as in the transcriptional signal

system of coordinate genes of expression working both in liver and pancreatic β-cell.

Acknowledgement

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