รายงานวิจัยฉบับสมบูรณ์

การศึกษาการควบคุมการแสดงออกของเอ็นไซม์ไพรูเวทคาร์บอกซิเลซ และกลไกการยับยั้งการแสดงออกโดยเมตฟอร์มิน

Studies on regulation of pyruvate carboxylase and molecular inhibition by metformin

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สัญญาเลขที่ BRG5480002

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอ)

Abstract

Pyruvate carboxylase (PC) is a mitochondrial enzyme which converts pyruvate to oxaloacetate, one of important intermediates in the tricarboxylic acid cycle. As oxaloacetate is utilized as the precursor for many biosynthetic pathways including gluconeogenesis in liver and kidney, lipogenesis in liver and adipose tissue, neurotransmitter synthesis in astrocytes, dysregulation of PC expression is associated with metabolic syndrome including obesity and type 2 diabetes. Understanding regulation of PC will unravel molecular mechanisms underlying the above metabolic disorders, and may lead to the prevention of the disease as well as the development of new anti-diagetic drugs. This enzyme is also involved in the synthesis of coupling factor which supports insulin secretion in pancreatic β-cells. Here we dissected the molecular mechanisms underlying transcription control of PC in hepatocytes and in pancrease. Using AML12 hepatocyte cell line as the model for liver, we demonstrate that the proximal promoter of PC gene is directed by liver-enriched transcription factor, HNF4α which binds to the novel HNF4-specific binding motif (H4-SBM), allowing the precise control of this promoter in a liver-specific manner. Using rat insulinoma INS-1 832/13 cell line as the model for pancreatic islets, we deomonstrate that expression of PC in this cell type is regulated by complex glucose-responsive element (GRE), comprising multiple E-box located in the distal promoter of PC gene. Elevated concentration of glucose stimulates binding of three transcription factors, namely, the carbohydrate responsive element binding protein (ChREBP), Specificity protein-1 (Sp1) and upstream stimulatory factor-2 (USF2) to the GRE. Suppression of these three transcription factors impairs glucose-mediated transcriptional induction of PC gene indicating the critical role of those proteins in regulation of PC expression. We also cloned and identified the distal promoter of the human PC gene and found that the cis-acting elements and the putative binding sites of general and tissue-specific transcription factors, as well as the GRE therein, are highly homologous to those of the rat PC gene. Metformin, an anti-diabetic drug which is thought to act by lowering elevated plasma glucose in type 2 diabetic patients did not show any inhitory effect on PC expression in human hepatocyte cell line, HepG2. Our result together with the recent reports by other investigators, confirm that metformin does not lower plasma glucose through inhibition of gluconeogenic genes.

บทคัดย่อ

เอ็นไซม์ไพรูเวทคาร์บอกซิเลซ (PC) เป็นเอ็นไซม์ที่กระตุ้นปฏิกิริยาการเปลี่ยนสาร pyruvate ไปเป็น oxaloacetate ซึ่งเป็นตัวกลางในวัฏจักร์เครปส์ สาร oxaloaceatate ถูกนำไปใช้เป็นสารตั้งต้นในปฏิกิริยาทางชีวเคมีในร่างกายได้แก่ การสร้างน้ำตาลในตับ การสร้างไขมันในตับและเนื้อเยื่อไขมัน การสร้างสารสื่อประสาทในระบบประสาท รวมทั้งเกี่ยวข้องกับการสร้าง coupling factor สำหรับใช้ในการหลั่งอินซูลินจากตับอ่อน ความผิดปกติในการควบคุมการสร้างเอ็นไซม์ PC จึงมีความเกี่ยวข้องกับโรคกลุ่มเมตาบอลิกซินโดรมได้แก่ ภาวะอ้วนและเบาหวานชนิดที่ 2 ดังนั้นการเข้าใจกลไกการควบคุมการสร้างเอ็นไซม์ดังกล่าวในระดับยีนจึงมีความสำคัญในการนำไปสู่การเข้าใจพยาธิสภาพของการเกิดภาวะดังกล่าว และอาจนำไปสู่การป้องกันและพัฒนายารักษาโรคเบาหวาน ในโครงการวิจัยนี้คณะผู้วิจัยได้ศึกษากลไกการควบคุมการแสดงออกของยีนสร้างเอ็นไซม์ PC ในเซลล์ 2 ประเภทคือเซลล์ตับและตับอ่อนซึ่งเป็นอวัยวะที่ควบคุมการสร้างน้ำตาลและการลดระดับน้ำตาล จากการศึกษาโดยใช้เซลล์ตับเพาะเลี้ยง AML12 พบว่ายีน PC ถูกควบคุมให้มีการแสดงออกจำเพาะในเซลล์ตับผ่านโปรตีนควบคุมคือ hepatocyte nuclear factor 4a ซึ่งทำงานโดยการจับกับตับตอบสนอง H4-SBM ในบริเวณโปรโมเตอร์

ด้านไกล้ ของยีน PC ทำให้ยีนนี้มีการแสดงออกเฉพาะในเซลล์ตับ การทดลองยับยั้งการแสดงออกของ HNF4a ส่งผลให้มีการลดระดับการแสดงออกของ PC ในระดับ mRNA และโปรตีน จากการศึกษาในเซลล์ตับอ่อนเพาะเลี้ยง INS-1 832/13 พบว่าการแสดงออกของยีน PC ถูกควบคุมด้วยโปรตีนควบคุม 3 ตัวคือ carbohydrate responsive element binding protein (ChREBP), specificity-protein 1 (Sp1) และ upsteream stimulatory factor-2 (USF2) ทำงานร่วมกับโดยจับกับลำดับดีเอ็นเอทำหน้าที่เป็นตัวตอบสนองต่อกลูโคส (glucose-responsive element) (GRE) ประกอบด้วย E-box เรียบตัวติดกันในบริเวณโปรโมเตอร์ด้านไกล โดยคณะผู้วิจัยพบว่าระดับน้ำตาลความเข้มข้นสูงที่ 25 mM เร่งให้โปรตีนควบคุมทั้ง 3 ตัวดังกล่าวจับกับ GRE ได้ดีขึ้น นอกจากนี้ยังพบว่ายีนสังเคราะห์เอ็นไซม์ PC ในคนก็มีบริเวณควบคุมรวมถึง GRE คล้ายคลึงกับยีนของหนูเชื่อว่ากลไกการตอบสนองต่อระดับน้ำตาลในเลือดมีวิวัฒนาการเหมือนกับระหว่างคนและหนู ยารักษาเบาหวาน metformin ซึ่งเป็นยาออกฤทธิ์โดยลดระดับน้ำตาลในเลือดไม่มีผลต่อการยับยั้งการแสดงออกของเอ็นไซม์ PC ในเซลล์ตับมนุษย์ HepG2 ซึ่งผลการทดลองดังกล่าวร่วมกับผลการวิจัยโดยกลุ่มวิจัยต่างประเทศอีกสองกลุ่มก็ให้ผลสอดคล้องกันคือ metformin ไม่ได้ยับยั้งการสร้างน้ำตาลผ่านขบวนการสร้างน้ำตาลจากเซลล์ตับ

Keywords: Pyruvate carboxylase, type 2 diabetes, gluconeogenesis, insulin secretion, transcriptional regulation, metformin, hyperglycemia

Executive summary

Rationale

Among other key enzymes which regulate glucose and lipid metabolism, pyruvate carboxylase (PC) is an enzyme that plays an important role at the metabolic crossroad of glucose and lipid metabolism. PC catalyzes the ATP-dependent carboxylation of pyruvate to oxaloacetate, an important Krebs cycle intermediate that is utilized for various biochemical pathways (Jitrapakdee et al., 2006; Jitrapakdee et al., 2008). In liver and kidney, PC catalyzes one of four irreversible reactions in gluconeogenesis, providing glucose as a fuel for the brain and red blood cells during starvation or prolonged fasting. In white adipose tissue, PC provides oxaloacetate which is combined with acetyl-CoA to form citrate that is exported from the mitochondria to serve as a necessary precursor for de novo fatty acid synthesis in the cytoplasm. PC also plays a crucial role in glyceroneogeneis, a process by which glycerol is synthesized for fatty acid esterification. This process is extremely important for clearing free fatty acids from the circulation to triglyceride in adipose tissue and is one of the targets for thiazolidinedione, an antidiabetic drug. In brown adipose tissue, PC is involved in thermogenesis, providing oxaloacetate required for the continuous oxidation of fatty acids. Inhibition of PC in adipocytes impairs lipogenesis and adipogenesis (Si et al., 2009).

In pancreatic islets, PC supports glucose-induced insulin secretion. PC participates in the pyruvate-malate/pyruvate-citrate cycles between the cytoplasm and mitochondria known as “pyruvate cycling”, providing $NADPH_2$, one of the coupling factors required for glucose-induced insulin secretion (Jitrapakdee et al., 2010). Suppression of PC expression in -cells disrupts pyruvate cycling, causing impaired anaplerosis and reduced glucose-induced insulin secretion (Hasan et al., 2008; Xu et al., 2008). In neurons, PC provides oxaloacetate for further conversion to glutamate, one of several important neurotransmitter substances. Given the diverse roles of PC in various tissues, it is not surprising to see that the deficiency or abnormal expression of this enzyme affects the above processes leading to the development of pathophysiological conditions including obesity, diabetes and psychomotor retardation (review see Jitrapakdee et al., 2008: Jitrapakdee 2010; Martin-Valencia, 2010).

Regulation of PC gene expression

In mammals, PC is regulated by two distinct promoters, the proximal and the distal located upstream of the first coding exon (Jitrapakdee et al., 1997; Jitrapakdee et al. 2001). In rat and mouse, alternative transcription of these two promoters produces mRNAs that

share the same coding sequence but differ in their 5'-untranslated regions (Jitrapakdee et al., 1997). The proximal promoter (P1) generates mRNA transcripts that are expressed in gluconeogenic tissues (liver and kidney) and adipose tissue, while the distal promoter (P2) produces a transcript which is ubiquitously expressed but is particularly highly abundant in pancreatic islets where it is induced by glucose (Jitrapakdee et al., 1998). Both P1 and P2-promoters are differentially regulated by distinct sets of ubiquitous and tissue-specific promoters.

In hepatocytes the P1 promoter is regulated by glucagon via cAMP. In HepG2 hepatocytes, cAMP causes a long term increase of PC expression. Dexamethasone also further increases cAMP-induced PC mRNA expression. However cAMP or insulin alone did not affect PC expression (Thonpho et al., 2010). The molecular mechanism by which cAMP causes the increase of PC mRNA is mediated through the binding of CREB to the CRE located at -1639/-1631 of the P1 promoter of PC gene (Thonpho et al., 2010). This result was also supported by a previous study which demonstrated that transgenic mice overexpressing the dominant negative CREB mutant show down-regulation of genes encoding gluconeogenic enzymes including PC, concomitant with impaired gluconeogenesis (Herzig et al., 2001). Although sharing the same P1-promoter usage as in liver, transcriptional regulation of this promoter in adipocytes is remarkably different, The P1-promoter is regulated by TZD, an antidiabetic drug which is also a PPAR-γ agonist (Jitrapakdee et al., 2005). Both PPAR-γ1 and PPAR-γ2 up-regulate PC1-promoter activity through a classical PPAR-γ binding site (PPRE), located at –386/-374 (Jitrapakdee et al., 2005). Heterozygous PPAR-γ1 knockout mice exhibit marginally affected PC expression in white adipose tissue (Anghel et al., 2007) while the isoform specific knockout, PPAR-γ2 null mice, showed a 50% reduction of PC in white and brown adipose tissue (Jitrapakdee et al., 2005). In another mouse model, where PPARγ1 was over-expressed in the livers of PPARγ null mice, it also caused a 6-fold increase of hepatic PC (Yu et al., 2003). Collectively, these data clearly show that both isoforms of PPARγ regulate PC expression *in vitro* and *in vivo*. Furthermore the P1-promoter is also regulated by HNF4α (Rojvirat et al., 2011).

In pancreatic β-cells, basal transcription activity of P2-promoter is regulated by housekeeping transcription factors: Sp1/Sp3 and NF-Y (Sunyakumthorn et al., 2005). A pancreatic-specific transcription factor viz. forkhead transcription factor boxA2 (Foxa2/HNF3β), working in concert with upstream stimulatory factors (USF1 and USF2) has been identified as the positive regulator of this promoter (Boonsaen et al., 2007) (Fig.6).

Pederson et al. (2010) have recently identified a glucose-responsive element (GRE) located at -408-/392 in the P1-promoter. This GRE has dual binding sites for the carbohydrate-responsive element binding protein and USF1/USF2, and is required for glucose-induced transcription activation of PC gene in pancreatic β-cells. Recently MAFA, one of β-cell specific transcription factor that directs transcription of insulin gene has also been identified as a transcriptional regulator of the PC gene in β-cells. However, the molecular mechanism underlying this control is not clear (Wang et al., 2007).

PC and type 2 diabetes

As PC is involved in the metabolic crossroad of carbohydrate and lipid metabolism, regulation of its expression in various tissues must be coordinated to achieve an appropriate overall response to various physiological and pathological stimuli especially in β-cells. It appears that diabetic conditions affect PC expression patterns in various tissues in different ways. In several rodent models diabetes increases hepatic PC expression as the result of a decrease in insulin action, suggesting a regulatory role for PC in gluconeogenesis (Andrikpoulos and Proietto, 1995; Salto et al., 1996; Large and Beylot, 1999). Normalization of hyperglycemic conditions by administration of insulin results in a reduction of PC expression concomitant with the reduction of plasma glucose level.

In adipose tissue of obese rats which are hyperinsulinemic due to insulin resistance but have normal plasma glucose levels (i.e. do not develop diabetes), PC expression is abnormally high due to a high rate of adipogenesis (Lynch et al., 1992; Jitrapakdee et al., 1998). In the islets of this animal model, PC is also increased to support the pyruvate cycling activity required to increase glucose-induced insulin secretion during this metabolic overload (Liu et al., 2002; Liu et al., 2005). However, in obese rats/mice which develop severe hyperglycemia and impaired glucose-induced insulin release, PC expression in adipose tissue is down-regulated (Wilson-Fritch, et al., 2004). Similarly, PC expression in islets is severely decreased (MacDonald et al., 1996a; MacDonald et al., 1996b; Laybutt et al., 2003). Finally, loss of insulin secretion from β-cells causes the de-repression of PC expression together with other gluconeogenic enzymes resulting in an elevated level of hepatic gluconeogenesis.

MacDonald et al. (2009) compared the expression and activity of mitochondrial enzymes from the islets of normal and type 2 diabetic patients. Similar to the mouse model, PC expression and activity were decreased in individuals with type 2 diabetes. From these data it appears that the decrease of PC expression in pancreatic β-cells in both rodents and

humans may be a secondary effect due to β-cell loss caused by severe hyperglycemia. However, siRNA-mediated suppression of PC expression in β-cells results in impaired glucose-induced insulin secretion, providing supportive evidence that PC directly regulates glucose-induced insulin secretion (Hasan et al., 2008; Xu et al., 2008).

Metformin as anti-diabetes drug

Biguamides (metformin) is the most widely used antidiabetic drug and it acts principally by inhibiting hepatic glucose production. It also enhances glucose uptake in skeletal muscle. Other actions include reduction of serum triglyceride and free fatty acids. Unlike sulfonylureas and TZD, metformin does not cause weight gain. Although metformin has been used for more than 50 years, the molecular mechanisms of action are not yet fully understood. A protein called AMP-activated protein kinase (AMPK) is one of metformin's targets (Zhou et al., 2001). AMPK is a major cellular energy sensor and a master regulator of metabolic homeostasis (Hardie et al., 1998; Steinberg & Kemp, 2009). AMPK is activated by AMP, a metabolic indicator of low energy state of the cells (hypoglycemia). The rise of AMP level results in AMPK phosphorylation at Thr-172 by an upstream kinase, i.e. LKB kinase (Shaw et al., 2005). Once phosphorylated, AMPK phosphorylates several downstream effectors including acetyl-CoA carboxylase (ACC), a rate limiting step enzyme in *de novo* fatty acid synthesis thereby inhibiting lipogenesis. As ACC is inhibited, the malonyl-CoA level is decreased, and the inhibition of carnitine-palmitoyl transferase I is relieved, thereby stimulating fatty acid oxidation [see Figure 1]. AMPK is also activated by other physiological stimuli including exercise, muscle contraction and hormones such as adiponectin and leptin as well as by physiological stresses, hypoxia and oxidative stress, and osmotic shock conditions (Dzamko and Steinberg, 2009).

In addition to ACC, AMPK also phosphorylates the CREB-transcriptional coactivator 2 (TORC2/CRCT2), coactivator of cAMP-responsive element binding protein (CREB) (Koo et al., 2005; Shaw et al. 2005). During starvation, the CREB-TORC2 complex plays a regulatory role in transcription of gluconeogenic genes i.e. phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (Koo et al., 2005). Starvation triggers the release of glucagon and catecholamines. Binding of these hormones to their G-protein coupled receptors on the plasma membrane of hepatocytes causes signal transduction cascades, including the rise of cAMP which in turn activates protein kinase A (PKA) activity. PKA then phosphorylates CREB, transforming it into a transcriptionally active form. CREB

forms a complex with TORC2/CRCT2 that binds to the cAMP-responsive element (CRE) in the gene promoters of the gluconeogenic enzymes: phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) resulting in transcriptional activation of these two genes (Fig.2). During the fed condition when the level of insulin is high, insulin disrupts the CREB-TORC2 complex via SIK2 phosphorylation. Phosphorylation at Ser171 of TORC2 results in dissociation of the CREB-TORC2 complex, leading to inhibition of transcription of gluconeogenic genes (Dentin et al., 2007)

It has been known for quite some time that metformin stimulates AMPK phosphorylation in parallel with the decrease of gluconeogenesis. Koo et al. (2005) have reported that administration of metformin to hepatocytes results in a marked reduction of PEPCK and G6Pase gene expression. The molecular mechanism by which metformin inhibits the expression of these two enzymes involves the phosphorylation of TORC2/CRCT2, converting it into a transcriptionally inactive form. Therefore hepatic gluconeogenesis is inhibited (Fig.3). He et al. (2009) have also reported that metformin regulates the CREB-binding protein (CBP), another component of the CREB transcription complex through AMPK phosphorylation. Phosphorylated AMPK then activates PKC1/λ which subsequently phosphorylates Ser436 resulting in the dissociation of CREB-CBP-TORC2 complex. This in turn results in transcriptional inhibition of gluconeogenesis.

Furthermore, Berasi et al. (2006) have reported that metformin-induced AMPK phosphorylation stimulates expression of early growth response 1 (ERG1), a transcription factor required to turn on gene expression of a phosphatase, Dusp4. Dusp4 is known to regulate p38 MAP kinase, which also regulates CREB phosphoryation via PKA activation.

Canton et al. (2010) have identified SIRT1 and GCN5 as a new target of metformin action in hepatocytes. Under normal cirucumstances, TORC2 activity is promoted by acetylation while deacetylation inactivates its transcriptional activity. Hepatocytes treated with metformin are induced to express SIRT1 thereby eliminating their transcriptional ability to turn on gluconeogenic gene expression. Similarly, metformin also increases the expression of GCN5 which is an acetyltransferase that regulates acetylation of PGC1α, a transcription coactivator required for Foxo1-dependent transcriptional regulation of the PEPCK gene. Acetylation of PGC1α by GCN5 converts it from the active form to an inactive form, thereby inhibiting transcription of the PEPCK gene.

Although the above findings support the link between metformin-induced AMPK phosphorylation and TORC2/CREB-dependent transcription inactivation of gluconeogenic

genes, **several reports have argued that metformin-induced AMPK phosphorylation may operate through TORC2/CREB-independent pathways.** Inoue & Yamaguchi (2006) have reported that metformin-induced AMPK phosphorylation stimulates the expression of the AICAR-responsive element binding protein (AREB), a transcription factor that has been shown to bind to the PEPCK gene promoter and down-regulate transcription of the PEPCK gene. Kim et al. (2008) have shown that metformin-induced AMPK phosphorylation stimulates the expression of a small heterodimer partner (SHP), a member of the orphan nuclear receptor. SHP is a repressor of HNF4α and FoxA2 gene transcription. Since HNF4α and FoxA2 are known to transcriptionally regulate PEPCK and G6Pase genes (Matsumoto et al., 2007), increased expression of SHP would result in the transcriptional inhibition of PEPCK and G6Pase gene expression.

The following evidence indicates that metformin may suppress hepatic gluconeogenesis via PC expression.

1. Foretz et al. (2010) have shown that hepatocytes treated with a low concentration of metformin (0.5 mM) show more than 60% inhibition of gluconeogenesis whereas the expression of PEPCK and G6Pase gene were slightly affected by metformin. The marked reduction of PEPCK and G6Pase expression was only observed when a higher concentration of metformin (1 mM) was used, suggesting that PEPCK and G6Pase may not be targets of metformin at the lower concentration. It is possible that PC may be a target of metformin at the low concentration.
2. Kim et al. (2008) have reported that metformin increases expression of the SHP transcription factor that negatively regulates HNF4α and PGC1α. Since the PC gene promoter contains putative binding sites for HNF4α and PGC1α, it is possible that PC could be regulated via SHP-dependent transcription repression.
3. We have shown that PC expression is regulated by cAMP via the TORC2/CREB pathway (Thonpho et al., 2010). It is possible that metformin-induced AMPK phosphorylation could inhibit PC expression via a TORC2/CREB-dependent pathway.
4. Large and Beylot (1999) have reported that both PC and PEPCK fluxes are reduced in hepatocytes treated with metformin.

Objectives of this study

1. Examine whether PC is a target of metformin and if so what is the underlying mechanism
2. Identification of cis-acting element that regulate transcription of PC expression in pancreatic-b cells.
3. Identifiy glucose responsive element and transcriptional control mechanism which allow PC expression under glucose-stimulated insulin secretion
4. Identify liver-enriched transcription factor which regulate PC expression in liver.

Part 1 Studies on molecular inhibition of pyruvate carboxylase gene expression by metformin

1 x 10^6 HepG2 or AML12 cultured in DMEM supplemented with 10% fetal bovine serum were treated with 0, 0.5, 1.0 and 2.0 mM metformin (Metformin (1,1–Dimethylbiguanide, Hydrochloride) (CALBIOCHEM), for 6, 24, 48, 72 and 96 h. Similarly, 7.5 x 10^5 AML12 cells grown in DMEM : HAM's F12 (1:1) + 10%(v/v) Fetal bovine serum + 1% Insulin/Transferrin/Selenium +0.1μM dexamethasone were treated with the above concentrations of metformin. Cells were harvested and the RNA were extracted using Trizol reagent. The quality of RNA were assessed by formaldehyde gel electrophoresis. cDNA synthesis was performed in 20 μl reaction mixture containing 1 μg total RNA, 200 ng random primers (Promega) preheated at 70°C for 5 min before, 1x ImPromII reaction buffer, 3 mM $MgCl_2$, 1 mM dNTP and 1x ImPromII reverse transcriptase (Promega) were added and the reaction were cooled down at 25°C for 5 min prior to 42°C for 1 h. The reaction was terminated at 70°C for 15 min. Real time PCR analysis of expression of PC and PEPCK mRNA were conducted in 12 μl reaction mixture containing 1x Kapa probe fast qPCR mastermix (Kapa Biosystems), 1x ROX reference dye, 0.3 μM forward and reverse primers, 0.15 μM Taqman probe and 2 μl of 1/5 diluted cDNA. The thermal profiles consisted of preheating at 50°C for 2 min and 95°C for 10 min prior to 40 cycles of amplification. Each of which consisted of denaturation at 95°C for 15 sec, annealing and extension at 60°C for 1 min. For detection of expression of FBPase and HPRT mRNA were conducted in 20 μl mixture containing 1x Kapa SYBR fast qPCR master mix (Kapa Biosystems), 1x ROX reference dye, 0.42 mM forward and reverse primers and 2 μl of undiluted cDNA. The thermal profiles consisted of preheating at and 95°C for 5 min prior to 40 cycles of amplification. Each of which consisted of denaturation at 95°C for 30 sec, annealing at 59°C for 30 sec and extension at 72°C for 30 sec.

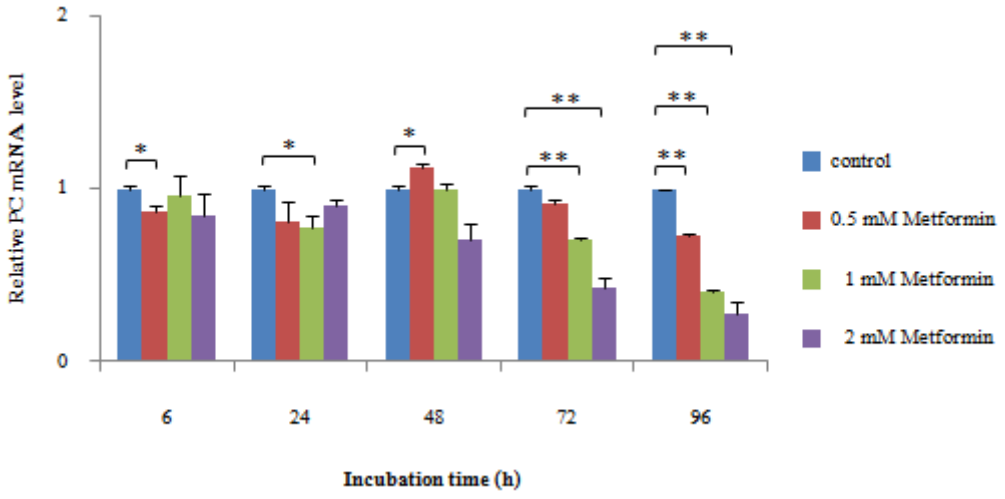
Table 1 Nucleotide sequences of primer/probed used for qRT-PCR

Primer name	**Sequence (5' to 3')**
HumanPC-F	GAT GAC TTC ACA GCC CAG
HumanPC-R	GGG CAC CTC TGT GTC CAG
PC-Probe (Human&Mouse)	[FAM] GCC CTG GTG GCC TGT ACC AAA G [TAMRA]
MousePC-F	GAT GAC CTC ACA GCC AAG CA
MousePC-R	GGG TAC CTC TGT GTC CAA AGG
FBP-F (Human&Mouse)	AGC CTT CTG AGA AGG ATG CTC

FBP-R (Human&Mouse)	GTC CAG CAT GAA GCA GTT GAC
HumanPEPCK-F	CCA CAG CGG CTG CAG AAC AT
HumanPEPCK-R	GAA GGG CCG CAT GGC AAA
HumanPEPCK-Probe	[FAM] AAG GCA AAA TCA TCA TGC ATG ACC C [TAMRA]
HPRT-F	TGT GAT GAA GGA GAT GGG AGG
HPRT-R	AAG CTT GCG ACC TTG ACC ATC T

RESULTS

Initially we examined the effect of metformin on mouse hepatocyte cell line, AML12. In this cell line, treating cells with 0.5, 1.0 and 2.0 mM metformin did not appear to affect cell viability. Quantitative real time PCR analysis performed on RNA extracted from cells grown in the presence of various concentrations of metformin showed that within the first 48 h, metformin at concentrations of 0.5 and 1.0 mM slightly affected PC expression while 2.0 mM of drug marginally reduced PC expression. However, at 72 h, 1.0 and 2.0 mM metformin lowered PC expression by 20% and 50%, respectively while this inhibitory effect was not observed at 0.5 mM metformin. At 96 h, 0.5, 1.0 and 2.0 mM metformin further reduced expression of PC by 25%, 50%, and 75%, respectively. In contrast, metformin did not appear to affect the expression of another gluconeogenic enzyme, fructose-1,6-bisphosphatase (FBPase), suggesting that the inhibitory effect of metformin was specific for PC gene.



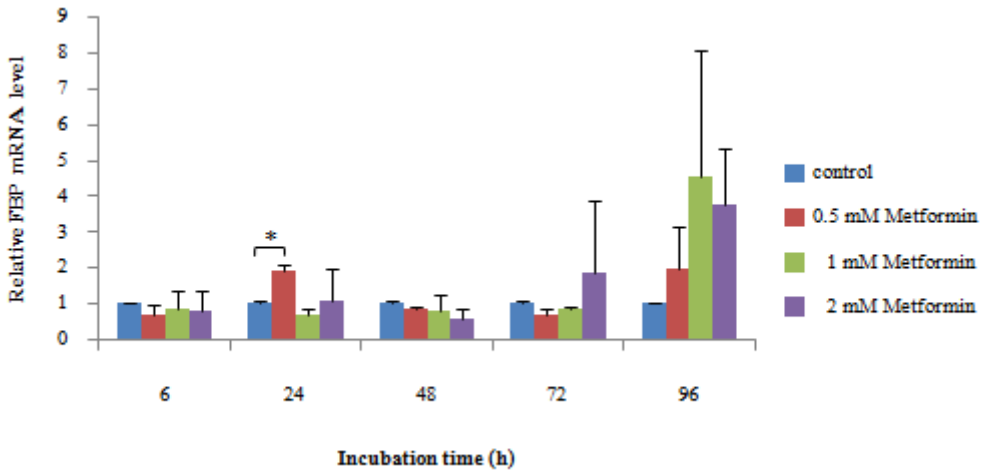
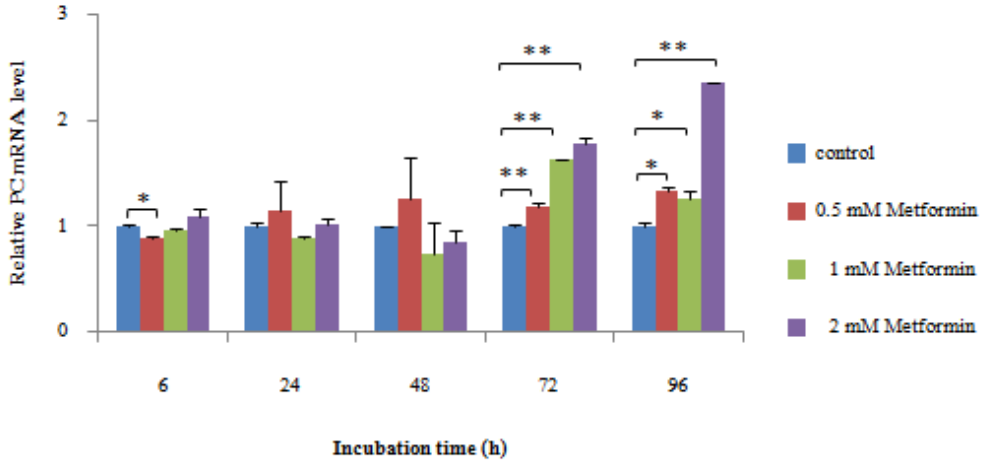


Figure 1. Effect of various concentrations (0.5, 1.0 and 2.0 mM) metformin on expression of PC (upper panel) and FBPase (lower panel) mRNA at various time points (6h, 24h, 48h, 72h and 96h) in AML12 cells.



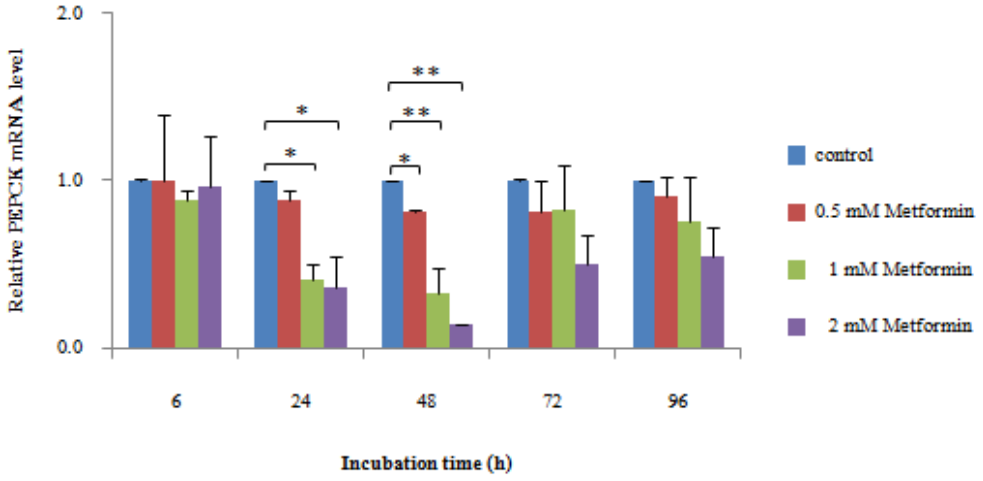


Figure 2. Effect of various concentrations (0.5, 1.0 and 2.0 mM) metformin on expression of PC (upper panel), PEPCK (middle panel) and FBPase (lower panel) mRNA at various time points (6h, 24h, 48h, 72h and 96h) in HepG2 cells.

We also examined the inhibitor effect of metformin on human hepatocyte cell line, HepG2. Treatment of HepG2 cells with 0.5, 1.0 and 2.0 mM of metformin up to 48 h did not alter expression of PC mRNA. At 72 h, 0.5 and 1.0 mM of metformin slightly increased PC mRNA expression while 2.0 mM metformin increased PC expression almost 2-fold. At 96 h, 2 mM metformin further increased PC expression to 2.5-fold while lower concentrations of metformin slightly affected PC mRNA expression. In contrast, the expression of another gluconeogenic enzyme, phosphoenolpyruvate carboxykinase (PEPCK) mRNA was obviously reduced by metformin. At 24 h, 0.5 mM metformin slightly affected PEPCK mRNA expression while 1.0 and 2.0 mM metformin lowered PEPCK mRNA expression by 60%. At 48 h, metformin at 0.5 and 1.0 mM did not further lower PEPCK mRNA expression while metformin at 2.0 mM further reduced PEPCK mRNA expression to 10%. Interestingly, at 72 and 96 h, 0.5 and 1.0 mM metformin cannot inhibit PEPCK mRNA expression while 2.0 mM metformin can inhibit PEPCK mRNA expression by only 50%. Similar to AML12 cells, all three concentrations of metformin did not appear to affect the expression of FBPase mRNA.

It is still unclear why inhibitory effect of metformin on PC expression was only observed in mouse hepatocytes (AML12) but no in human hepatoma cell line (HepG2). The fact that the expression of PEPCK gene which is also another gluconeogenic enzyme was markedly inhibited by metformin, it is possible 2-fold increase of PC expression may be a compensatory response to increase gluconeogenic rate when metformin is present at a high concentration (2 mM). **The above result suggests that hepatic PC mRNA expression is not a target of metfomrin.**

During we have performed this part of the experiment, two independent research groups have reported that metformin does not lower blood glucose by inhibiting the key gluconeogenic enzymes including PC, PEPCK or glucose-6-phosphatase (Miller et al., 2012; Madiraju et al., 2014). Therefore the study toward this part of the project was not continued. The direction of the project was then centered on another part which is the understanding the regulation of PC expression in insulin-responsive tissues including cloning and characterization of human pyruvate carboxylase gene promoter (Part 2), identification of glucose-responsive transcription factors that regulate pyruvate carboxylase expression in pancreatic β-cells (Part 3) and Characterization of liver-specific transcription factor that regulates Expression of

pyruvate carboxylase gene in hepatocytes (Part 4). Understanding precise regulation of PC expression at the transcriptional level will aid us to design transcriptional ligands which can modulate PC expression under diabetic conditions.

Part 2 Cloning and characterization of human pyruvate carboxylase gene promoter

The structural region of the human PC gene has been cloned and characterized [Carbone et al., 1998]. However, the regulatory regions of the PC gene that confer tissue-specific expression of PC in humans are not known. Recently, Wang et al., (2008) reported that unlike the rat and mouse PC genes, the human PC gene is transcribed from three promoters. Herein, we present evidence that similar to the rodent PC genes, the human PC gene is transcribed from two promoters. In addition, we identified some of the important *cis*-acting elements of the distal human PC promoter that direct transcription of PC in beta cells.

Cloning of hP2 promoter linked luciferase gene constructs.

The 1,108 bp fragment of the hP2 promoter was cloned from genomic DNA isolated from HepG2 cells using the hP2-forward primer (5'-GGTACCACTACCTACTCAGAGACATCTGC-3'; underline indicates a *Kpn*I restriction site) and the hP2-reverse primer (5'-CTCGAGGTCCTCGCCGCCGCCTCTACC-3'; underline indicates a *Xho*I restriction site). The PCR product was then ligated to the pGEM-T Easy vector (Promega) and sequenced. The clone with the correct sequence of the hP2 promoter was excised from the pGEM-T easy vector with *Kpn*I and *Xho*I sites and ligated to the equivalent sites of the pGL3-basic vector (Promega) to generate a hP2-luciferase reporter construct. 5'-truncated hP2 promoter constructs comprising 985, 640, 365, 240, 114, and 41 nucleotides of the hP2 promoter were generated by PCR using a full length hP2 promoter-luciferase construct as a template. The forward primers containing a *Kpn*I site at their 5'-ends and the reverse primer containing an *Xho*I site at the 3'-end were designed. The PCR products were then ligated into the pGEM-T Easy vector and sequenced. The correct sequences of 5'-truncated hP2 promoter were excised with *Kpn*I and *Xho*I and ligated to the equivalent sites of the pGL3-basic vector. Primers used for cloning of 5'-truncated hP2 promoters are shown in Table 1. For the construction of a 489 bp fragment of hP2 promoter, the promoter was generated by double digestion of the full length hP2 promoter-luciferase

construct with *Nhe*I and *Xho*I. The 489 bp fragment of the hP2 promoter was then re-ligated into the *Nhe*I and *Xho*I site of the pGL3-basic vector.

Site-directed mutagenesis.

Site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Agilent Technologies) was performed to generate 5, 15 and 25 nucleotide internal deletion mutants of the hP2 promoter constructs. The mutagenesis reaction was carried on in a total volume of a 50 µl-reaction mixture containing 300 ng of DNA template, 125 ng of each mutagenic oligonucleotide primer, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl pH 8.8, 2 mM $MgSO_4$, 0.1% TritonX-100 and 0.1 mg/ml nuclease-free bovine serum albumin (BSA), 200 µM dNTP mix, and 2.5 U of *Pfu*Turbo polymerase (Stratagene-Agilent Technologies). The amplification profile consisted of an initial denaturation at 95 °C for 30 sec followed by 20 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 1 min, and extension at 68 °C for 10 min. The primers used for site-directed mutagenesis are shown in Tables 1 and 2. The correct mutant constructs were verified by automated nucleotide sequencing. The corrected clones with 5, 15 or 25 nucleotide deletion were double digested with *Kpn*I and *Xho*I and re-ligated into the pGL3 basic vector digested with the same enzymes.

Cell culture and transfection.

INS-1 832/13 cells [Hohmeier et al., 2000] were maintained in RPMI 1640 supplemented with 28 mmol/l $NaHCO_3$, 1 mM sodium pyruvate (Gibco), 50 µM β-mercaptoethanol, 10% (v/v) heat-inactivated fetal bovine serum (Gibco), and 50 units/l penicillin/streptomycin at 37^oC in 5% CO_2. In the transfection experiments, 2 x 10^5 cells were seeded in 24-well plates and were cultured in 0.5 ml of antibiotic-free DMEM (Dulbecco's modified Eagle's medium; Gibco) containing 10% fetal bovine serum for 24 h before transfection. Cells were transfected with 250 ng of the luciferase reporter constructs and 250 ng of pRSV-β-gal plasmid expressing β-galactosidase using Lipofectamine^{TM} 2000 reagent (Invitrogen). For transactivation assays, 250 ng of plasmids overexpressing Sp1, Sp3 [Rojvirat et all, 2011], USF1 or USF2 [24] were also included with the luciferase reporter construct and pRSV-β-gal plasmid. The transfected cells were maintained in the antibiotic-free DMEM at 37^oC for 48 h. For the transfection of the non-beta cell line, the human embryonic kidney cell line (HEK293T) was grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum, and 50 units/L penicillin/streptomycin at 37^oC in 5% CO_2. The transfections were

carried out as described for the INS-1 832/13 cells [Boonsaen et al., 2007], except that the cells were seeded in 24-well plates at a density of 4 x 10^5 cells. The luciferase reporter assays were performed using the luciferase reporter assay system (Promega), while the β-galactosidase assay was performed using ONPG as substrate.

Electrophoretic mobility shift assay (EMSA).

1 x 10^7 of INS-1 832/13 cells were harvested for preparation of nuclear extracts. The cells were washed with PBS and resuspended in 1 ml of nuclear extraction buffer I (10 mM HEPES pH7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 1x protease inhibitor cocktail (Roche) at 4^{o}C for 1 min. The nuclei were centrifuged at 3,000 g at 4^{o}C for 1 min before resuspended in 100 μl nuclear buffer 2 (20 mM HEPES, pH7.9, 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA and 0.2 mM PMSF) and incubated on ice for 5 min. The nuclear lysate was centrifuged at 3,000 g for 5 min at 4^{o}C and the supernatant was kept at -80^{o}C and used for EMSA.

The 5'-end labeled biotinylated oligonucleotide was synthesized by BioBasic (Canada) and annealed with the unlabelled complementary strand oligonucleotide. The oligonucleotides used in EMSA are listed in Table 3. The DNA-protein binding assay was carried out in a 20 μl-reaction mixture containing 1x binding buffer (25 mM HEPES, pH7.9), 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM EDTA, 0.5 mM DTT and 0.2 mM PMSF, 10 μg of nuclear extract, 2 μg of poly dI-dC and 120 fmole of biotinylated double stranded oligonucleotide at 4^{o}C for 30 min. For supershift assays, 1 μg of anti-Sp1 (sc-59), anti-Sp3 (sc-644), anti-USF1 (sc-22) or anti-USF2 (sc-862) polyclonal antibody (SantaCruz Biotech) was included in the binding reaction. The DNA-protein complexes were analyzed by 5% non-denaturing polyacrylamide gel electrophoresis followed by electroblotting. The bands of DNA-protein interaction were detected using LightShift Chemiluminescent EMSA kit (Pierce). The image was captured using Gel Doc System (GeneTools).

RESULTS

The human PC gene is regulated by two promoters and the distal promoter is functional in pancreatic β-cells. We have previously reported two PC mRNA isoforms with distinct 5'-untranslated regions (UTR) that contain the same coding sequences have been identified in liver and kidney. These two mRNA variants are likely to be generated from alternate

transcription from two promoters [Jitrapakdee et al., 1996]. In contrast to our study, Wang et al [2008] compared the 5'-UTR sequences of three human PC mRNA variants namely, variant 1 (NM_000920.3), 2 (NM_022172.2) and 3 (BC011617.2) deposited at the NCBI database to the genomic sequence of human PC gene and concluded that these variants are alternatively spliced from four 5'-UTR exons, i.e. UE1, UE2, UE3 and UE4, respectively, with the distal, middle and proximal promoters located immediately upstream of exons UE1, UE2 and UE4, respectively [Wang et al., 2008].

However, we re-examined the alignment of those three variants and found that variants 1 and 3 share the common 83 nucleotides upstream of the first initiation codon, while variant 1 contains 11 additional nucleotides at its 5'-end (see Figure 1A). Wang et al [Wang et al., 2008] reported that this extra sequence is derived from an upstream exon, UE1. However, direct comparison of 5'-UTR sequences of variants 1 and 3 with the genomic sequence of the human PC gene clearly showed that these extra 11 nucleotides in variant 1 are located immediately upstream of UE2, thus forming part of this exon. Therefore, it is highly likely that the 11 nucleotide segment in variant 1 could easily be a truncated transcript or result from the use of multiple start sites of the TATA-less genes. In agreement with Wang et al. [2008], the 5'-UTR sequence of variant 2 is derived from a separate 5' UTR exon which is located proximal to the first coding exon. The lack of an intron between UE1 and UE2 rules out the possibility that there is a middle promoter located between these two upstream exons as proposed by Wang et al. [2008]. Based on this new information we revised the structural organization of the human PC gene as follows: the human PC gene contains only three 5'-UTR exons, i.e. UE1/UE2, UE3 and UE4, with the proximal promoter located upstream of UE4 and the distal promoter located upstream of UE1/UE2. Transcription initiated from the proximal promoter produces variant 2 while transcription from the distal promoter produces variants 1 and 3 (Figure 1B). The presence of two alternative promoters of human PC gene appears to recapitulate that of the rat [14] and mouse PC genes [Jitrapakdee et al., 1996]. This is in contrast to bovine PC gene which possesses three promoters, the proximal (P1), middle (P2) and distal (P3) promoter [20]. However, there is no report about which of these promoters is highly active in bovine pancreatic β-cells.

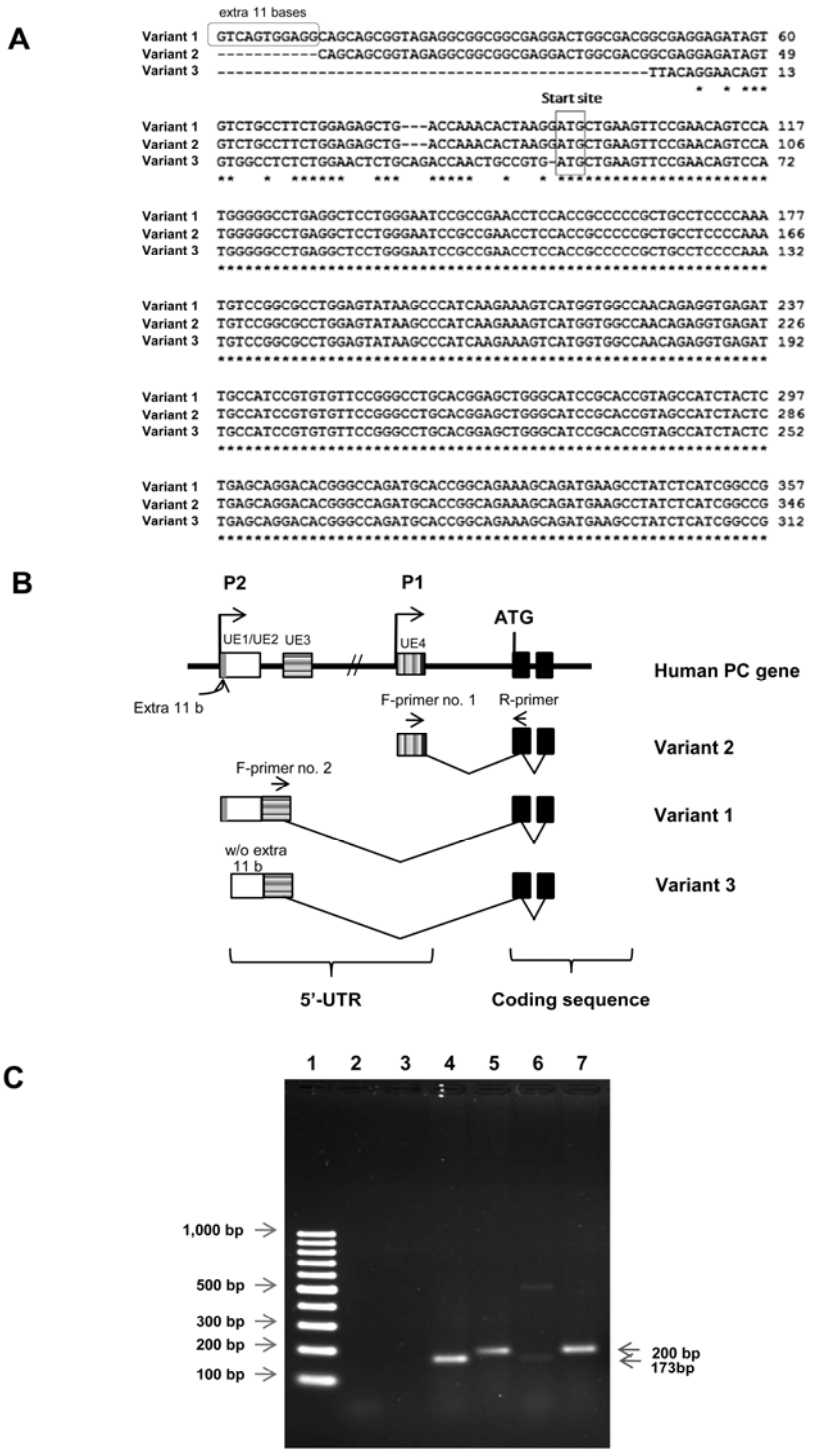


Figure 1. RT-PCR analysis of PC mRNA variants in human liver and human pancreatic islets. (A) Schematic diagram showing alignment of 3 variants of human PC mRNA (GenBank NM_000920.3, NM_022172.2, BC011617.2). (B) Schematic diagram showing the structure of the human PC gene. Two isoforms of human PC mRNA are initiated by two alternative promoters, the proximal (P1) promoter and the distal (P2) promoter. All PC mRNA variants contain the same coding sequences but differ in their 5'-untranslated regions (UTR) produced from different 5'-UTR exons (UE1/UE2, UE3 and UE4) (C) Examination of human PC mRNA in liver and pancreatic islets using RT-PCR. Two sets of primers were used to amplify two different isoforms of human PC mRNA both in human liver and human

islets. The 173 bp fragment PCR product of variant 2 and the 200 bp fragment PCR product of variant 1 were amplified by using Primers set no. 1 and primer set no.2, respectively, Lane 1; 1 kb marker, Lane 2; Negative control for primer set no.1, Lane 3; Negative control for primer set no.2, Lane 4; PCR using primer set no.1 and cDNA prepared from human liver, Lane 5; PCR using primer set no.2 and cDNA prepared from human liver, Lane 6; PCR using primer set no.1 and cDNA prepared from human islets, Lane 7; PCR using primer set no.2 and cDNA prepared from human islets.

Although the two PC mRNA isoforms have been described in liver and kidney [13, 19], it is not known which of these isoform(s) is expressed in human pancreatic islets. To address this question, we performed an RT-PCR analysis of cDNA prepared from human islets using two forward primers that specifically bind to the 5'-UTRs of variant 1 and variant 2 together with a reverse primer that binds to exon 1 (see Figure 1B). With these primers, the amplicons with sizes of 173 bp and 200 bp, representing variant 1 and variant 2 were expected. As shown in Fig. 1C, both primer sets were able to amplify the 173 bp and 200 bp PCR products representing variants 1 and 2 which are produced from both proximal and distal promoters of the human PC gene from HepG2 cDNA (lanes 4 and 5), respectively. This result indicated that both proximal and distal promoters are active in liver. In a sharp contrast, RT-PCR of cDNA prepared from human islets produced a faint band of the 173 bp PCR product amplified by primers set no.1 (lane 6) while primer set no. 2 amplified a strong band of the 200 bp PCR product (lane 7), suggesting that the distal promoter of the human PC gene primarily controls its transcription in human pancreatic islets similarly to rat islets.

Cloning and characterization of hP2 promoter. To identify the critical *cis*-acting elements that control PC transcription in pancreatic islets, we isolated approximately the 1 kb upstream sequence of exon UE1/2 of the human PC gene which would potentially serve as the distal promoter (hP2) of the PC gene using PCR with the primers designed from the human genome database [Strausberg et al., 2002]. A comparison of the nucleotide sequences of the hP2 promoter with the distal promoter of rat PC gene revealed that they are 59.6 % similar, with the highest similarity observed within the first 500 nucleotides. The hP2 promoter lacks a canonical TATA box in the first 100 nucleotides but contains two copies of CCAAT boxes and one copy of a GC box located at nucleotide positions -101/-97, -71/-67 and -54/-39, respectively. These features are the characteristic of housekeeping genes [Dynan, 1986]. Further analysis of the hP2 promoter sequence using the PROMO database [Messeguer et al., 2002] identified several putative transcription factor binding sites including USF1/USF2, Sp1

and HNF3β/FoxA2. These putative binding sites are also conserved in the rat PC gene (Figure 2).

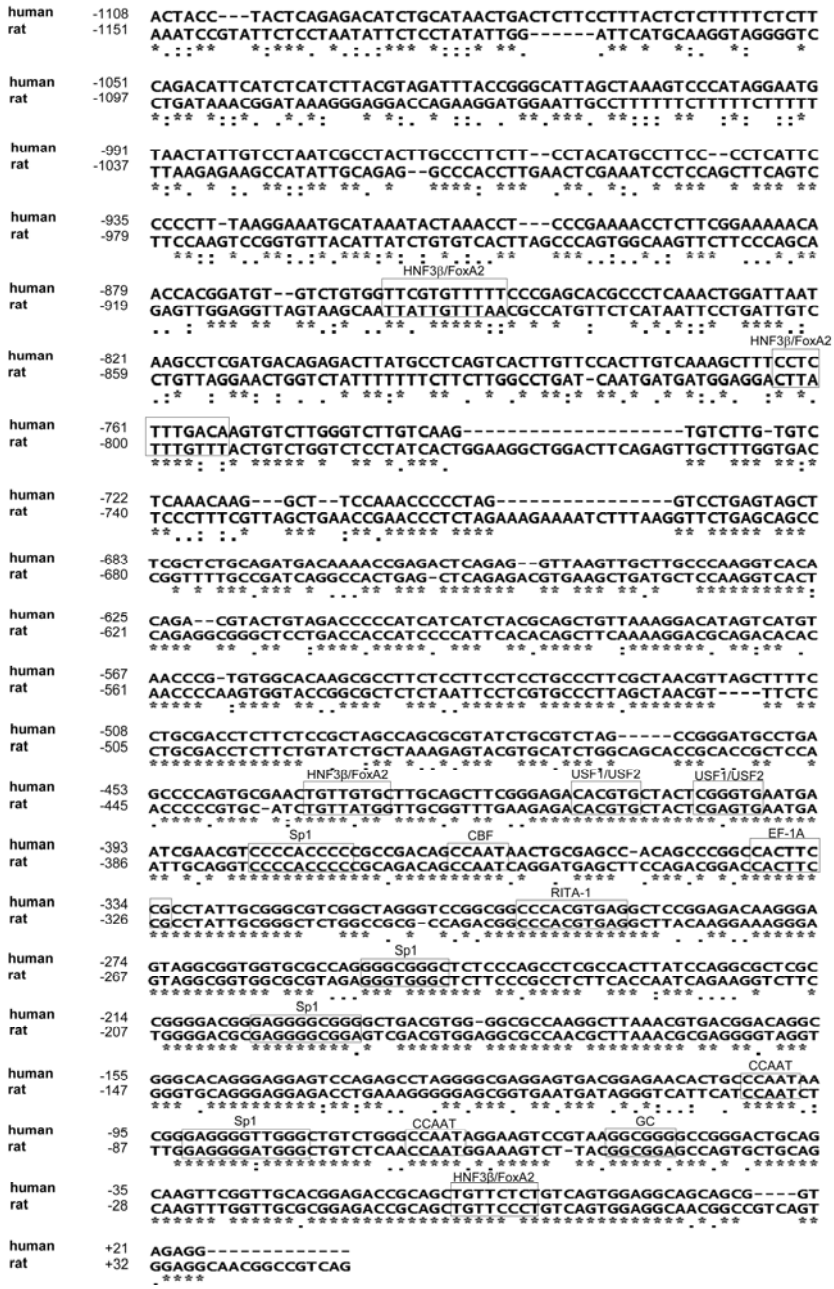


Figure 2. The human PC P2 promoter sequence and its alignment with the rat PC P2 promoter. Boxes represent the putative transcription factor binding sites for Sp1, FoxA2/HNF3β, USF1/2, and CBF. Identical nucleotides between human and rat sequence are symbolized by an asterisk

To determine the transcriptional activity of the distal promoter of the human PC gene, a series of 5'-truncated hP2 promoter constructs were generated and used in transient transfection experiments. In this study, eight constructs of the hP2 promoter were transiently transfected into INS-1 832/13 cells. As shown in Fig. 3, deletions of regions -1108 to -985, -640, and -489 did not significantly affect promoter activity. However, when the deletions were made from the region -498 to -365, this resulted in a significant increase of promoter activity, suggesting the presence of a repressor element between these regions. On the other hand, deletions from the region -365 to -240 resulted in a significant decrease in promoter activity, suggesting the presence of (a) positive regulatory element(s) in this region. Further deletion from -240 to -114 did not affect promoter activity. However, deletion to -40 resulted in a dramatic decrease of promoter activity, suggesting the presence of a second positive regulatory element between -114 and -40.

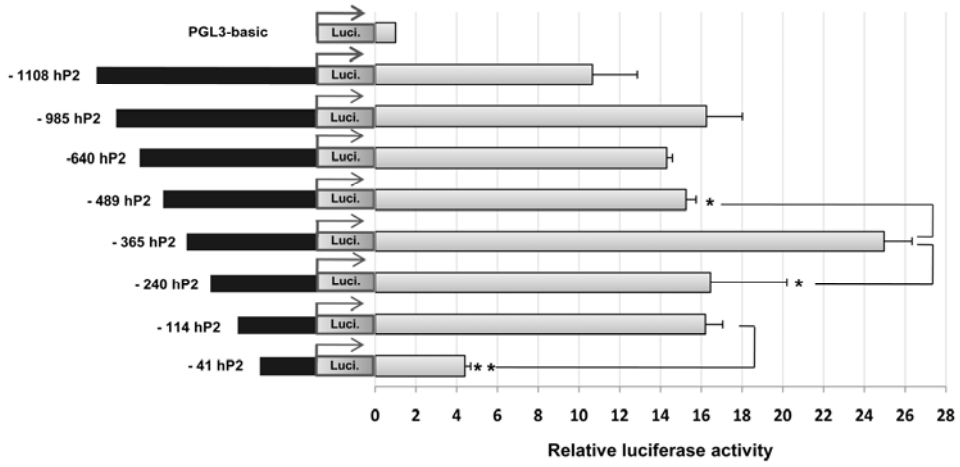


Figure 3. Localization of *cis*-acting elements of the human PC P2 promoter. Transient transfections of 8 constructs containing of the 5'-truncated hP2 promoter into INS-1 832/13 cells were performed to identify the regulatory regions of the hP2 promoter. The basal activity of each 5'-truncated hP2 promoter was calculated from the values of luciferase activity which was normalized with the values of β-galactosidase activity to control for transfection efficiency. The normalized luciferase activity of each P2 construct was compared with the activity of the pGL3-basic vector which was arbitrarily set to 1 and presented as the relative luciferase activity. *P value < 0.05, **P value < 0.01.

The -69/-54, -340/-315 regions of the hP2 promoter contain *cis*-acting elements that confer non-beta cell and beta-cell specificity, respectively. As the first *cis*-acting element which serves as an activator sequence was located between -114 and -41 of the hP2 promoter,

a series of 15 bp-internal deletions across this region were generated in order to precisely map the critical element located in this region. These mutant constructs were transiently transfected into both the INS-1 832/13 cell line and the human embryonic kidney cell line, HEK293T. A schematic diagram of 15 bp deletions of the -114/-39 region of the hP2 promoter is shown in Figure 4A. As shown in Figure 4B, transient transfections of -114/-99, -99/-84, -84/-69 deletion mutants did not significantly affect the reporter activity in either cell line. However, deletion of regions between -69 and -54 (-69/-54 hP2) resulted in a dramatic decrease in promoter activity to 35% and 25% of that seen with the INS-1 832/13 and HEK293T cell lines, respectively, suggesting that the -69 to -54 region of the hP2 promoter contains (a) critical *cis*-acting element(s) for basal transcription factors in both the INS-1 832/13 and the HEK293T cell lines. Examination of the nucleotide sequence located between the -69 and-54 of the hP2 construct identified the presence of a CCAAT box located between -71 and -67 (Figure 4B, underlined). To examine whether the dramatic decrease of the luciferase reporter activity observed from the -69/-54 hP2 mutant construct could indeed be attributed to the lack of an intact CCAAT box, we generated another mutant (-71/-67 hP2) in which the whole CCAAT box was deleted. Transient transfection of this mutant construct into INS-1 832/13 and HEK293T cells resulted in a marked reduction of promoter activity in both cell lines, similar to that of the -69/-67 hP2 mutant construct, suggesting that the -71/-67 CCAAT box is crucial for maintaining basal activity of the P2 promoter both in INS-1 832/13 and HEK293T cells. Deletion of the regions between -54 to -39 (-54/-39 hP2 construct), resulted in a marginal reduction of the reporter activity in both cell lines. Examination of the nucleotide sequence surrounding this region identified the presence of a GC-box, which is also found in the identical position in the distal promoter of the rat PC gene. This GC-rich region serves as a binding site for ubiquitous transcription factors Sp1/Sp3 [Sunyakumthorn et al., 2005]. Mutation of this similarly located GC-box in the rat gene resulted in a reduction of the reporter gene activity to a greater extent (80% reduction) than mutation of this sequence in the human gene [Sunyakumthorn et al., 2005], suggesting the rat and human PC genes are regulated differently via the GC-box.

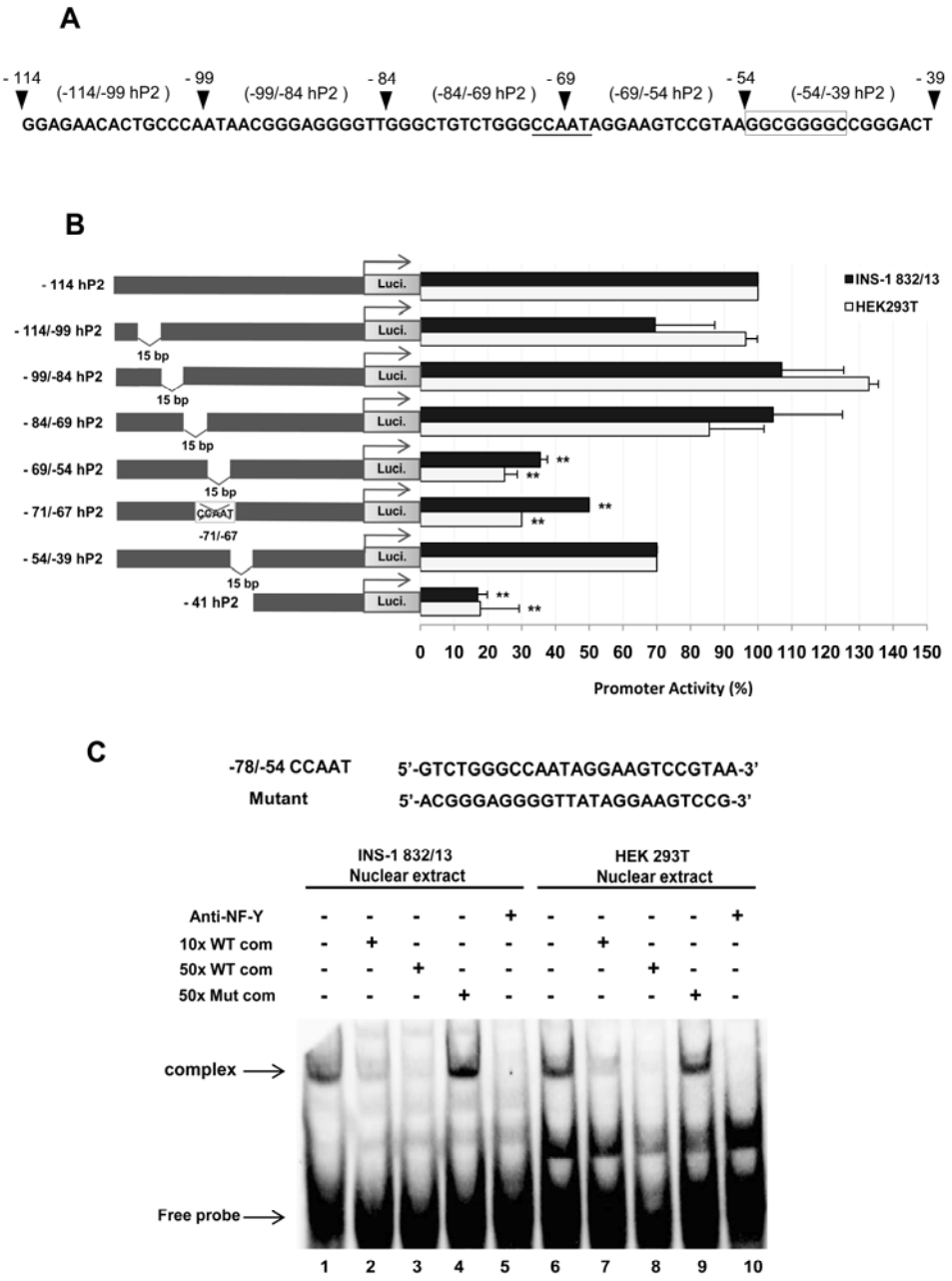


Figure 4. Identification of positive regulatory element(s) located between -114 and -39 of the human PC P2 promoter. (A) Schematic diagram of 15 bp internal deletions of -114/-39 of the human PC P2 promoter**.** (B) Transient transfections of a series of 15 bp internal deletion constructs into the INS-1 832/13 and non-beta cell HEK293T cell lines were performed to localize the positive regulatory sequence in the human PC P2 promoter. The luciferase activity of each construct was normalized with the β-galactosidase activity. The normalized reporter activity obtained from each construct is shown as a percent relative to those transfected with the wild type -365 hP2 promoter that was arbitrarily set at 100%. *P value < 0.05, **P value < 0.01. (C) Gel shift and supershift assays of biotin-labeled probe -78 to -54 region of hP2 promoter (-78/-54 CCAAT-probe) using INS-1 832/13 nuclear extract

(Lane 1-5) and non-beta cell HEK293T (Lanes 6-10). The nucleotide sequence of wild type and mutant of the hP2 promoter in the -78 to -54 regions are also shown. Lanes 1 and 5 show probes incubated with nuclear extracts from INS-1 832/13 or HEK293T cells; lanes 2 and 6, 10-fold excess wild-type unlabeled oligonucleotides were incubated with nuclear extracts and probes; lanes 3 and 7, 50-fold excess wild-type unlabeled oligonucleotides were incubated with nuclear extracts and probes; lane 4 and 9, 50-fold excess amount of mutant unlabeled oligonucleotides were incubated with nuclear extracts and probes; lanes 5 and 10, nuclear extracts were pre-incubated with anti-NF-Y antibody before the probes were added to the reactions. Arrow represents CCAAT box–NF-Y, complex.

A CCAAT box serves as a potential binding site for the nuclear factor Y (NF-Y) [25] and binding of this factor to this sequence is essential for transcriptional activation of TATA-less genes [Nicolas et al., 2003, Hou et al., 2010]. We confirmed this by performing gel shift experiments. As shown in Figure 4C, incubation of the –78/-54 probe harboring the -71/-67 CCAAT box with a nuclear extract of INS-1 832/13 cells produced a predominant DNA-protein complex (lane 1). This complex was readily competed off with 10x and 50x unlabelled WT double-stranded oligonucleotide (lanes 2-3), but was not competed off with an unrelated double stranded oligonucleotide sequence (lane 4). Incubation of anti-NF-Y polyclonal antibody prevented the formation of a DNA-protein binding complex (lane 5). A similar result was obtained when a nuclear extract of HEK293T cells was used in the experiment (lanes 6-10). These data indicate that NF-Y is a transcription factor that directs PC transcription via the -71/-67 CCAAT box in both cell lines. Although this CCAAT box appears to be conserved in the distal promoter of both the rat and human PC genes, it serves different roles in transcriptional regulation in the two genes. In the distal promoter of rat PC gene, this CCAAT box serves a repressor element, while in the human PC gene, this sequence clearly acts as an activator sequence. This dual function of NF-Y being both transcriptional activator and repressor is not totally unexpected as this depends on the promoter context [Bernadt et al., 2005]. NF-Y can possess a repressor activity if its recognition sequence is overlapped with the nearby activator binding sequence, antagonizing activator function [Papazafiri et al., 1991; Eggers et al., 1998; Shi et al., 2001]. The above data indicate that although the *cis*-acting elements including the CCAAT box and the GC-box are found in similar locations for both human and rat PC genes, their actions are substantially different. In the rat PC gene, the CCAAT box serves as a repressor element that somewhat antagonizes the GC-box function [Sunyakumthorn et al., 2005], while in the human PC gene, the CCAAT box sequence clearly acts as an activator element. As the GC-box in the human PC gene is not as strong an activator as in the rat PC gene, it appears that in the human PC gene, the upstream CCAAT box acts as an activator sequence to maximize transcription.

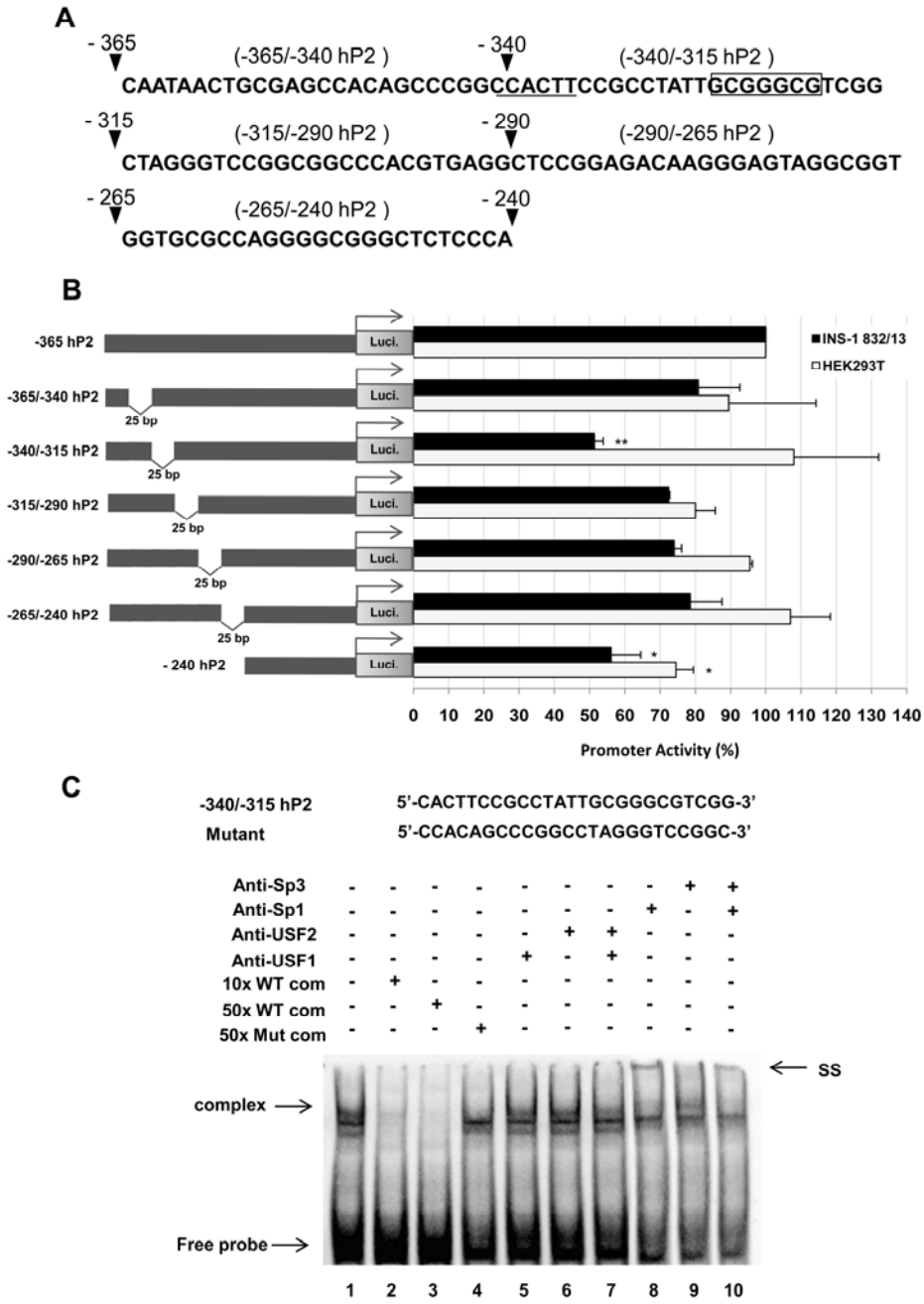


Figure 5. Identification of positive regulatory element(s) located between -365 and -240 of the human PC P2 promoter. (A) Schematic diagram of 15 bp internal deletions of -114/-39 the human PC P2 promoter**.** (B) Transient transfections of a series of 25 bp internal deletion constructs into the INS-1 832/13 cell line and non-beta cell HEK293T cell line were performed to identify the positive regulatory sequences in the hP2 promoter. The luciferase activity of each construct was normalized with β-galactosidase activity. The normalized reporter activity obtained from each construct is shown as a percent relative to those transfected with the wild type -365 hP2 promoter, which was arbitrarily set at 100%. *P value < 0.05, **P value < 0.01. (C) Gel shift and supershift assays of the biotin-labeled probe of the -78 to -54 region of the hP2 promoter (-340/-315 hP2 probe) using an INS-1 832/13 nuclear extract. The nucleotide sequences of the wild type and mutant of the hP2 promoter -78 to -54 regions are also shown. Lane 1 probes incubated with nuclear extracts from INS-1 832/13;

lanes 2-3, 10-fold or 50-fold excess wild-type unlabeled oligonucleotides were incubated with nuclear extracts and probes; lane 4, 50-fold excess amount of mutant unlabeled oligonucleotides were incubated with nuclear extracts and probes; lanes 5-7, nuclear extracts were pre-incubated with anti-USF1 or anti-USF2 or both, respectively, before the probes were added to the reactions. Lanes 8-10, nuclear extracts were pre-incubated with anti-Sp1 or anti-Sp3 or both, respectively, before the probes were added to the reactions. Arrow represents DNA-protein complex, SS = supershift band.

To more precisely localize the positive regulatory sequences between -365 and -240 of hP2, 25-bp internal deletions of the -365/-240 hP2 promoter were made. Five mutants harboring 25 bp internal deletions across the -365 to -240 regions (-365/-340, -340/-315, -315/-290, -290/-265 and -265/-240 hP2) were generated and transfected into both INS-1 832/13 and HEK293T cells. A schematic diagram of the 25 bp deletions of the -365/-240 hP2 promoter region is shown in Figure 5A. As shown in Figure 5B, transient transfection of -340/-315 hP2 mutant construct markedly reduced the reporter gene activity to 50% of the -365 hP2 promoter in INS-1 832/13 cells, while no reduction of reporter gene activity was observed in HEK293T cells. In contrast, deletion of other regions did not affect the promoter activity when compared to the wild type -365 hP2 promoter in either cell line. These data suggest the presence of a tissue specific *cis*-acting element(s) located between -340 and -315 in the hP2 promoter. To identify which transcription factors might bind to this element we performed gel shifts experiments in which double stranded oligonucleotides harboring -340/-315 were incubated with a nuclear extract of INS-1 832/13 cells. As shown in Figure 5C, a strong DNA-protein complex was observed. Examination of nucleotide sequences between -340 and -315 identifies an E-box, a binding site for USF [Ferre et al., 199], located between –341 and -336 and a GC-box and a binding site for Sp1/Sp3, located between –326 and -320, respectively. Incubation of an anti-Sp1 antibody in the binding reaction produced a weak super-shift band, while incubation in the presence of anti-Sp3, anti-USF1 or anti-USF2 antibodies had no effect on the DNA-protein complex formation, indicating that these three factors may not attribute to the binding to this sequence. To confirm the gel shift experiment, we performed a transactivation assay in which the wild type (-365 hP2) construct was co-transfected with plasmid overexpressing Sp1, Sp3, USF1 or USF2, and the luciferase activities were measured. As shown in Figure 6, co-transfection of Sp1 or Sp3 resulted in only 1.5-fold or 2-fold increase in the reporter gene activity, consistent with a poor or lack of evidence of their binding to the -340/-315 sequence shown in Figure 5C. Mutation of this sequence also had no effect on the expression of the reporter gene. The poor remaining Sp1 and Sp3-mediated transcriptional activation of the human PC promoter may be attributed to

the GC box located at -54/-39 (Figure 4A). Despite the lack of evidence of binding of USF1 or USF2 to the E-box located between -340/-315, overexpression of USF1 or USF2 resulted in approximately 5-fold or 10-fold increase in the promoter activity. However, deletion of the sequences located between -340 and -315 did not significantly affect USF1- or USF2-mediated transcriptional activation of the human PC promoter, suggesting that the transactivation by these two factors may be mediated through the downstream E-boxes.

In summary we have shown that: (i) the human PC gene possesses only two promoters, P1 and P2, which mediate transcription of the human PC gene similar to the rat and mouse genes; (ii) the P1 and P2 promoters are active in hepatocytes while only the P2 promoter is active in pancreatic β-cells; (iii) both CCAAT box and GC-boxes serve as activator sequences in β-cells; (iv) a *cis*-acting element located between -340/-315 serves as binding site for β-cell specific transcription factor.

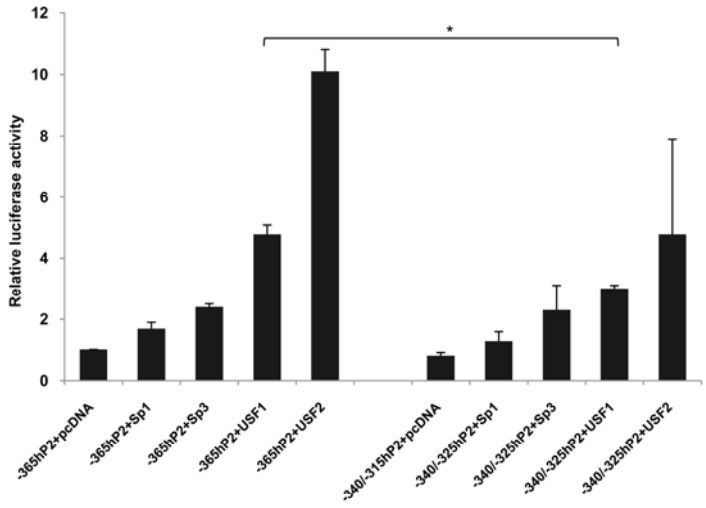


Figure 6. Transactivation of a WT -365 human PC P2 luciferase reporter construct and its mutant by Sp1, Sp3, USF1 or USF2. WT -365 hP2 or -340/-315 hP2 constructs were co-transfected with an empty vector (pcDNA3) or a plasmid overexpressing Sp1, Sp3, USF1 or USF2 into the INS-1 832/13 cell line, and the luciferase activities measured. The luciferase activity was normalized to β-galactosidase activity and expressed as relative luciferase activity. Relative luciferase values obtained from co-transfecting cells with wild type (-365 hP2) or its mutant (-340/-315 hP2) and plasmid overexpressing Sp1, Sp3, USF1 or USF2 were presented as fold change relative to those obtained from those co-transfected with WT with empty vector (pcDNA3) which was arbitrarily set at 1. *$P < 0.01$.

References

Part 3 Identification of glucose-responsive transcription factors that regulate pyruvate carboxylase expression in pancreatic β-cells

Glucose homeostasis is tightly regulated by glucagon and insulin which counteract each other to maintain the concentration of plasma glucose within a narrow range. Glucagon secreted during prolonged starvation raises the blood glucose level by stimulating glycogen breakdown and gluconeogenesis [Jiang et al., 2003] while insulin secreted during fed conditions [glucose-induced insulin secretion (GSIS)] lowers blood glucose levels by stimulating glucose uptake, glycolysis and glycogen synthesis in liver and skeletal muscle [Saltiel and Kahn, 2001]. This process occurs efficiently in the pancreatic β-cell due to the presence of GLUT2 and glucokinase [Matchinsky et al., 1990], which together acts as a sensor allowing high concentrations of glucose to enter to the cell for aerobic glycolysis and oxidative phosphorylation [Schuit et al., 1997]. These biochemical pathways raise the concentrations of cellular ATP which drive insulin secretion known as K_{ATP}-dependent GSIS [Ashcroft et al., 1984]. Although pyruvate formed after glycolysis appears to enter beta cell mitochondria through pyruvate decarboxylation catalyzed by pyruvate dehydrogenase complex (PDH) and pyruvate carboxylation by pyruvate carboxylase (PC) with similar proportions, flux toward the latter reaction is tightly associated with the glucose concentration the cells is exposed to and thus correlates with the magnitude of insulin secretion [MacDonald, 1993a; MacDonald, 1993b]. In further support of this observation, PC protein levels in rat pancreatic islets were found to be rapidly induced by exogenous glucose [MacDonald, 1995a]. Subsequent studies have clearly revealed that indeed pyruvate carboxylation by PC constitutes an important component of the "pyruvate cycling" which provides NADPH, one of coupling factors required for GSIS in pancreatic β-cells [MacDonald, 1995b; Farbari et al., 2000; Cline et al., 2004]. Suppression of PC expression in rat insulinoma cells impairs anaplerosis, concomitant with the lowered GSIS [Hasan et al., 2008; Xu et al., 2008] while overexpression of PC enhances GSIS [Xu et al., 2008], indicating the critical role of PC in supporting insulin secretion. Several studies performed in models of diabetic rats and human subjects with type 2 diabetes clearly show that down-regulation of PC expression in pancreatic islets is associated with type 2 diabetic phenotypes further supporting the role of PC in GSIS [MacDonald et al., 1996a; MacDonald et al., 1996b].

In the rat, mouse, and human, PC expression is regulated by two alternative promoters, the proximal promoter is active in liver and adipose tissue where PC is involved in

gluconeogenesis and lipogenesis, respectively while the distal promoter is active in β-cells where PC is required to support GSIS [Jitrapakdee et al., 1998; Jitraapkdee et al., 2001; Jitrapakdee et al., 2005]. Characterization of the distal promoter of rat PC gene revealed that a member of the forkhead box protein 3β (FoxA2) regulates PC expression in a β-cell specific manner [Boonsaen et al., 2007]. Although previous work has shown that PC expression is inducible by exogenous glucose, it remains largely unknown how glucose increases PC expression [MacDonald, 1995a; Jitrapakdee et al., 1998].

Plasmids construction

The construction of the 1.2 kb 5'-flanking region representing the distal promoter of rat PC gene-luciferase reporter construct (pGL-P2) and its 5'-truncated mutant constructs were described previously [Jitrapakdee et al., 1997]. The pGL-P2 reporter construct with various E-boxes mutated, namely, MuE1, MuE2, MuE3, MuE4 were constructed using pGL-P2 as a template while the double mutant MuE2&E4 was constructed using MuE4 as the template. The pGL-P2ΔKpnI mutants containing 25 bp or 23 bp internal deletions (Δ1, Δ2, Δ3, Δ4, Δ5 and Δ6) were constructed using pGL-P2ΔKpnI as template. The mutagenic reaction was performed using Quik-change XL site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions. The mutagenic primers used to generate the above constructs are shown in Table1. The nucleotide sequences of the mutagenic clones were verified by automated DNA sequencing (Macrogen Inc, USA).

The plasmids encoding USF1 and USF2 were cloned from cDNA prepared from INS-1 832/13 cells by RT-PCR using the primers (USF1CDS F/R and USF2CDS F/R) designed from published cDNA sequences [Viollet et al., 1996]. The USF1 and USF2 cDNAs were cloned into the *Hind*III and *Kpn*I sites of pcDNA3 expression vector (Invitrogen). Plasmids encoding Sp1 and Sp3 were constructed as described previously [Rojvirat et al., 2011].

Cell culture and transient transfection

INS-1 and INS-1 832/13 cell lines [Hohmeier et al., 2000] were kindly provided by C.B. Newgard, Duke University. INS-1 and INS-1 832/13 cells were maintained in RPMI 1640 medium (Gibco) supplemented with 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 10 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) heat-inactivated fetal bovine serum (Gibco), at 37°C with 5% CO_2. Cultures were maintained to 70–80% confluence before being used in the transfection experiments.

INS-1 832/13 cells were seeded at a density of $1x10^6$ cells/well in antibiotic-free medium containing 5.5 mM glucose in 6-well plates for 4 days prior to transfection. Cells were transfected with mixtures containing 5 μg of Lipofectamine 2000 transfection reagent (Invitrogen), 1 pmole of a firefly luciferase reporter construct and 2 μg of pRSV-βgal plasmid encoding *E. coli* β-galactosidase in Optimem I-reduced serum medium. The transfected cells were maintained in this medium for 24 h before it was replaced with RPMI medium containing either 5.5 mM or 25mM glucose for the next 24 h.

For transactivation assays, 1.5 μg of the luciferase reporter construct, 1.5 μg of plasmid overexpressing USF1, USF2, Sp1 (pSp1), Sp3 (pSp3) or pcDNA3 (empty vector) and 2 μg of pRSV-βgal were mixed with 5 μg of Lipofectamine2000 (Invitrogen) in Optimem I-reduced serum medium and transfected to INS-1 832/13 as described above. The luciferase and β-galactosidase assays were performed as described previously [Boonsaen et al., 2007]. The luciferase activity was normalized with the β-galactosidase activity and presented as the relative luciferase activity.

siRNA transfection

INS-1 832/13 cells were transfected with Sp1, USF1, USF2 and ChREBP siRNAs. In brief, 5 x 10^5 INS-1 832/13 cells were plated in 6-well plates 24 h before transfection in Optimem I-reduced serum medium before transfected with with 25 ng of siRNAs targeted to rat Sp1, USF1, USF2, ChREBP or scrambled siRNA (Ambion) and 2 μg of lipofectamine 2000 in the presence of 2 ml growth medium for 5 min. The transfected cells were maintained at 37^{o}C with 5% CO_2 for 48 h before being harvested for RNA extraction and real time PCR analysis.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from INS-1 832/13 cell were prepared as described previously [Boonsaen et al., 2007]. EMSA was performed using a non-radioactive EMSA. The two complementary oligonucleotides with their 3'-end labeled with biotin (BioBasic, Canada) were annealed and subjected to binding assays as described previously [Chavalit et al., 2013]. Supershift assays were performed by pre-incubating 2 μg of polyclonal antibodies against USF1 (sc-22), USF2 (sc-862), ChREBP (sc-21189), Sp1 (sc-59) or Sp3 (sc-644-G) [Santa Cruz Biotechnology] with nuclear extracts for 10 min before binding reactions were performed. The DNA-protein complexes were separated by 5% native polyacrylamide gel

electrophoresis using 0.5% TBE. The DNA-protein complexes were transferred onto Biodyne membrane (PALL) and the bands were detected using the Lightshift Chemiluminescent EMSA kit (Pierce). The sequences of oligonucleotides used in these experiments are shown in Table 1.

Chromatin immunoprecipitation assay (ChIP)

INS-1 832/13 cells were seeded at a density of $6x10^6$ cells in a 100 mm dish and maintained in RPMI medium containing 5.5 mM glucose for 4 days. The cells were cultured in RPMI medium containing 5.5 mM or 25 mM glucose for 12 h. DNA and proteins were cross-linked by adding formaldehyde to the culture medium to the final concentration of 1% (v/v) at 37°C for 5 min. ChIP was performed as described previously [Boonsaen et al., 2007] except that the pre-cleared chromatin was precipitated with 1 μg of anti-USF1, anti-USF2, anti-Sp1, anti-Sp3 or anti-ChREBP as described above. The transcription factor-bound DNA was amplified using Q-PCR with FastStart Universal SYBR Green master mix (Roche). Input (unbound DNA) of each group (5.5 or 25 mM glucose) was used to normalize the target DNA (set as 100%) and non-IgG condition was used as the reference in real time PCR. The primers used for amplifying the target site are −408 F/R (5'-GCGACCTCTTCTGTATCTGCTAA-3' and 5'-AGACCTTCTGATTGGTGAAGAGG-3') which flanked the Sp1 and USF sites of rat PC promoter [Boonsaen et al., 2007], and Ex2 F/R primers (5'-GCCCATCAAGAAAGTAATGGTA-3' and 5'-CTTGGCCACCTTAATGATGTCT-3') that are located within exon 2 of the rat PC gene [Jitrapakdee et al., 1997].

Quantitative RT-PCR (Q-PCR)

Total RNA was isolated from cells using TRI Reagent (Molecular Research Center) and its concentration was determined by spectrophotometry. cDNA synthesis was carried out in a 20 μl-reaction mixture containing 500 ng of total RNA, 200 ng of random hexamers (Promega), 1x first-strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 0.1 mM DTT, 1 mM each of dNTP and 200 units of SuperscriptIII reverse transcriptase (Invitrogen). Quantitation of PC expression was performed by real time PCR (Q-PCR) using *Taq*man probe. The Q-PCR was performed in a 12 μl-reaction mixture containing 1x *Taq*man PCR master mix (Roche), 2 μl of 1:10 diluted cDNA, 1 μM of each primer and 0.5 μM of *Taq*man probe. The thermal cycling consisted of an initial incubation at 50°C for 2

min and 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15s and annealing/extension at 60°C for 1 min using MxPro 3005 (Agilent Technologies). Expression of PC was normalized with that of 18s rRNA and the results are shown as "relative gene expression". The sequences of primers and probes for PC mRNA and 18s rRNA gene are the same as previously described [19]. Q-PCR for the detection of USF1, USF2, Sp1 and ChREBP expression in INS-1 832/13 was performed using Sybergreen. The primers used for detection of rat USF1 (PPR45083A), USF2 (PPR49799A), Sp1 (PPR49794A) and ChREBP (PPR49636B) were purchased from Qiagen.

Western blot analysis

Forty micrograms of nuclear and cytosolic extracts from INS-1 832/13 cells were subjected to 10% discontinuous SDS-PAGE before transferring onto PVDF membrane (PALL). The blots were incubated with appropriate dilution of anti-Sp1 (sc-59), anti-USF1 (sc-22), anti-USF2 (sc-862), anti-ChREBP (sc-21189) [Santa Cruz Biotechnology], anti-phospho Sp1 (ab59257), anti-tubulin (ab6046) or anti-laminB (ab16048) [Abcam], in 5% BSA in TBS-T overnight. The blots were washed with TBS-T before incubating with appropriate secondary antibodies conjugated with HRP for 3 h. The immunoreactive bands were visualized by an enhanced chemiluminescence using ECL Western substrate kit (Pierce).

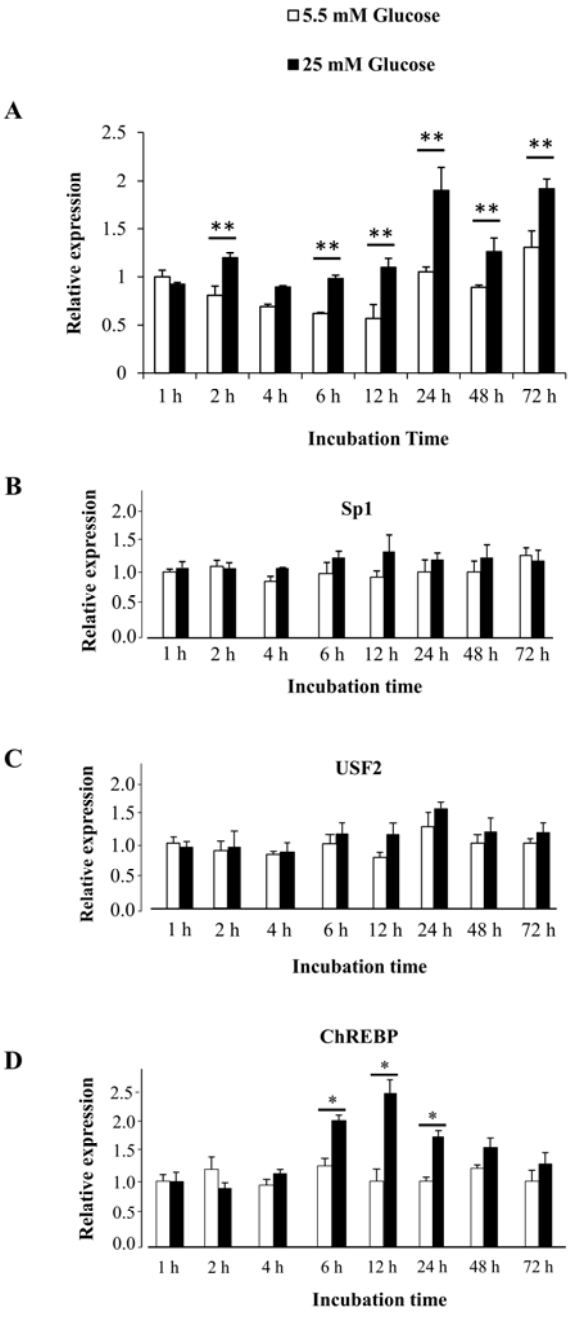
Statistical Analysis

All data are presented as the means ± SD from three independent experiments. Statistical analyses were performed using the ANOVA test. A P value < 0.05 was considered to be statistically significant.

RESULTS

Glucose induces PC mRNA expression in a time-dependent manner

Previous work has shown that incubation of rat islets with elevated concentrations of glucose resulted in the up-regulation of PC protein [9]. To examine whether the increased PC protein was due to up-regulation of PC mRNA, INS-1 cells were exposed to glucose at normal (5.5 mM) and high (25 mM) concentrations, followed by quantitative real time PCR analysis of PC mRNA expression. As shown in Figure 1A, incubation of the INS-1 cells with a high concentration of glucose resulted in a significant increase in PC mRNA expression at 2h, 6h, 12h, 24h, 48h and 72h ($P < 0.01$).


5.5 mM Glucose
25 mM Glucose
A
Relative expression
2.5
2
1.5
1
0.5
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1 h
2 h
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24 h
48 h
72 h
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Sp1
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2.0
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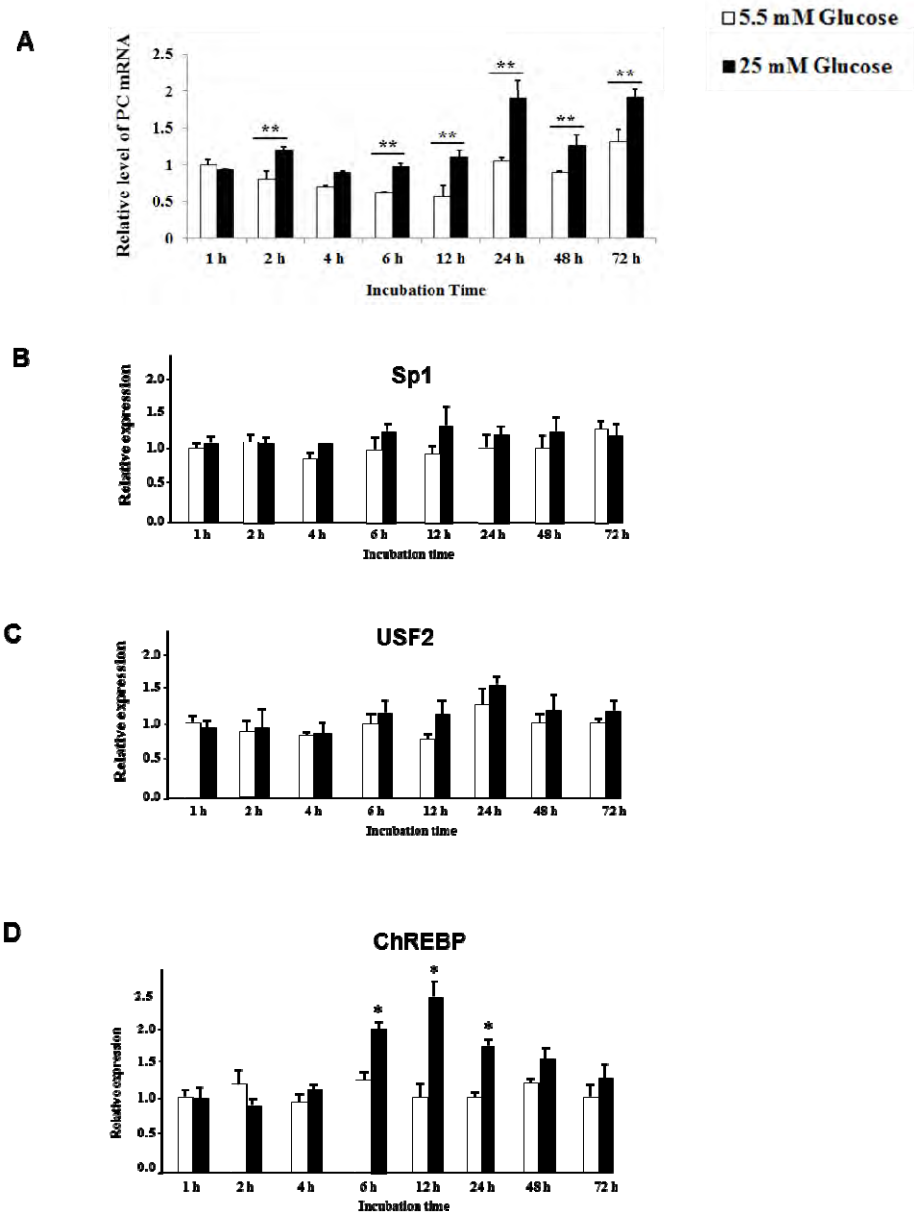


Figure 1. Glucose increases expression of PC and ChREBP mRNAs in INS-1 832/13 cells. INS-1 832/13 cells were cultured in RPMI medium containing normal (5.5 mM) or high (25 mM) glucose for 1, 2, 4, 6, 12, 24, 48 and 72 h. Total RNA was prepared from cells harvested at the indicated time points, converted to cDNA and analyzed by real time PCR. The expression of PC (A), Sp1 (B), USF2 (C) and ChREBP (D) was normalized with that of 18s rRNA and shown as relative PC expression. Relative expression values of each gene obtained from cells maintained in RPMI containing 5.5 mM and 25 mM glucose at various time points were compared with those obtained from cells cultured in RPMI containing 5.5 mM at 1 h, which was arbitrarily set as 1. The statistical analysis was conducted using ANNOVA test where *P < 0.05; **P < 0.01.

Multiple E-boxes in the distal promoter of rat PC gene function as the glucose-responsive element

Because the distal promoter of the rat PC gene is the only promoter that is transcriptionally active in rat pancreatic β-cells [Jitrapakdee et al., 1998] and mostly active in the insulinoma cell line, INS-1 832/13 [Pedersen et al., 2010] we examined whether the distal promoter of the PC gene contains a glucose-responsive element (GRE) by transfecting the 1.2 kb distal promoter-luciferase chimeric reporter constructs into the INS-1 832/13, a cell line that responds to glucose more robust than the INS-1 cell line [Hohmeier et al., 2000]. This promoter fragment has previously been characterized to contain full basal and tissue-specific cis-acting elements [Boonsaen et al., 2007]. The transfected cells were maintained in the medium containing 5.5 mM or 25 mM glucose for 24 h. As shown in Figure 2, the transfected cells maintained in medium containing 25 mM glucose possessed luciferase activity approximately 2-fold higher than those maintained at 5.5 mM glucose, suggesting that glucose exerts its stimulatory effect on PC expression via GRE located within the 1.2 kb distal promoter of PC gene. To define the GRE, we transfected a series of 5'-truncated distal promoter-luciferase reporter constructs into INS-1 832/13 cells. Truncations from nucleotides -1146 to -653 (pGL-P2ΔSacI construct) or to nucleotide -546 (pGL-P2ΔKpnI) did not affect glucose-mediated transcriptional activation of the luciferase reporter gene (Figure 2). However, truncation to nucleotide -399 (pGL-P2ΔXhoI) not only completely eliminated the glucose induction effect but this also unexpectedly increased the luciferase reporter gene under low glucose conditions. Further truncation of the P2 promoter to the -34 region (pGL-P2ΔPstI), yielded a similar result with that of the pGL-P2ΔXhoI construct, suggesting that the GRE was located between nucleotides -546 and -399.

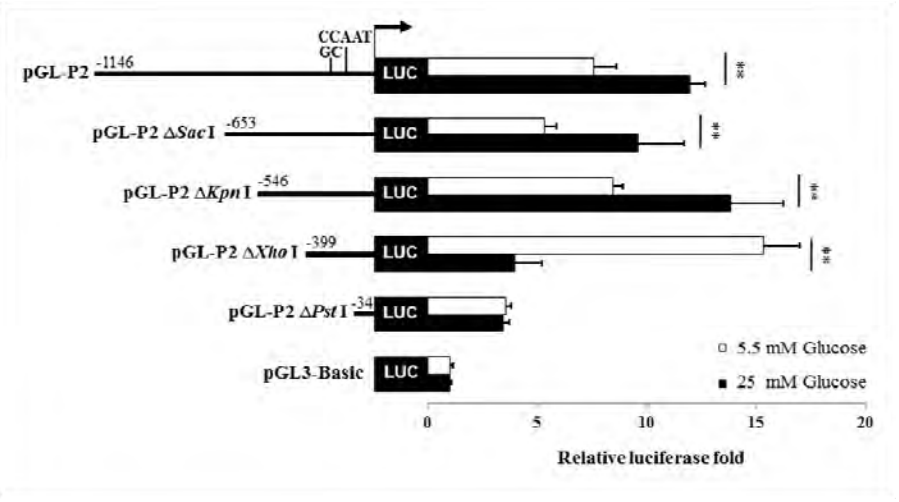


Figure 2. The P2 promoter of rat PC gene contains glucose-responsive element(s) (GRE). A series of 5'-truncated P2-luciferase reporter constructs were transfected into INS-1 832/13 cells. The transfected cells were maintained in RPMI containing normal (5.5 mM) or high (25 mM) glucose for 24 h. The luciferase activity of each construct was normalized to the β-galactosidase activity and expressed as relative luciferase activity. Relative luciferase values obtained from transfected cells maintaining in high glucose medium were presented as fold change relative to those maintaining in normal concentration of glucose, each of which was arbitrarily set as 1. The statistical analysis was conducted using ANOVA test where **P < 0.01.

To precisely localize the GRE, we generated a series of internal deletions across the -546 to -399 region and the resulting constructs [Δ1(-546/-522), Δ2(-521/-497), Δ3(-496/-472), Δ4(-471/-447), Δ5(-446/-422) and Δ6(-421/-399)] were transfected into INS-1 832/13 cells. As shown in Figure 3A, deletion of the nucleotides -546/-522 (Δ1), -521/-497 (Δ2) and -496/-472 (Δ3) did not affect the glucose response, however deletion of the nucleotides -471/-447 (Δ4) resulted in a complete loss of the glucose response. Similar results were observed when the nucleotides -446/-422 (Δ5) and -421/-399 (Δ6) were deleted. Of particular interest, the deleted nucleotides in Δ4, Δ5 and Δ6 mutant constructs corresponded to the three copies of canonical E-boxes (CANNTG), designated E1 (-465/-460), E3 (-436/-431) and E4 (-408/-403), and one E-box-like element [E2 (-442/-437), respectively (See Figure 3A). E-boxes have previously been reported to be involved in many glucose-responsive genes [Read et al., 1993; Matsukawa et al., 2001; Portois et al., 2002; Martin et al., 2003; Iynedjian, 1998; Roth et al., 2004]. To examine whether these four E-boxes confer glucose-mediated transcription induction of the PC gene, each of them was mutated. As shown in Figure 3B, mutations of E1, E2 or E3, resulted in a complete loss of glucose-mediated transcription induction of the reporter gene but had no effect on transcriptional induction under normal glucose (5.5 mM). In contrast, mutation of a whole ChoRE consisting of E4 and E-box-like (see ChoRE in

Figure 4A) not only eliminated high glucose induction effect but also resulted in a 2-fold increase in the reporter activity under low glucose condition. This result was in agreement with those of the pGL-P2ΔXhoI (Figure 2) and Δ6 mutants (Figure 3A), suggesting ChoRE functions as an activator under high glucose induction condition and as a repressor under low glucose condition.

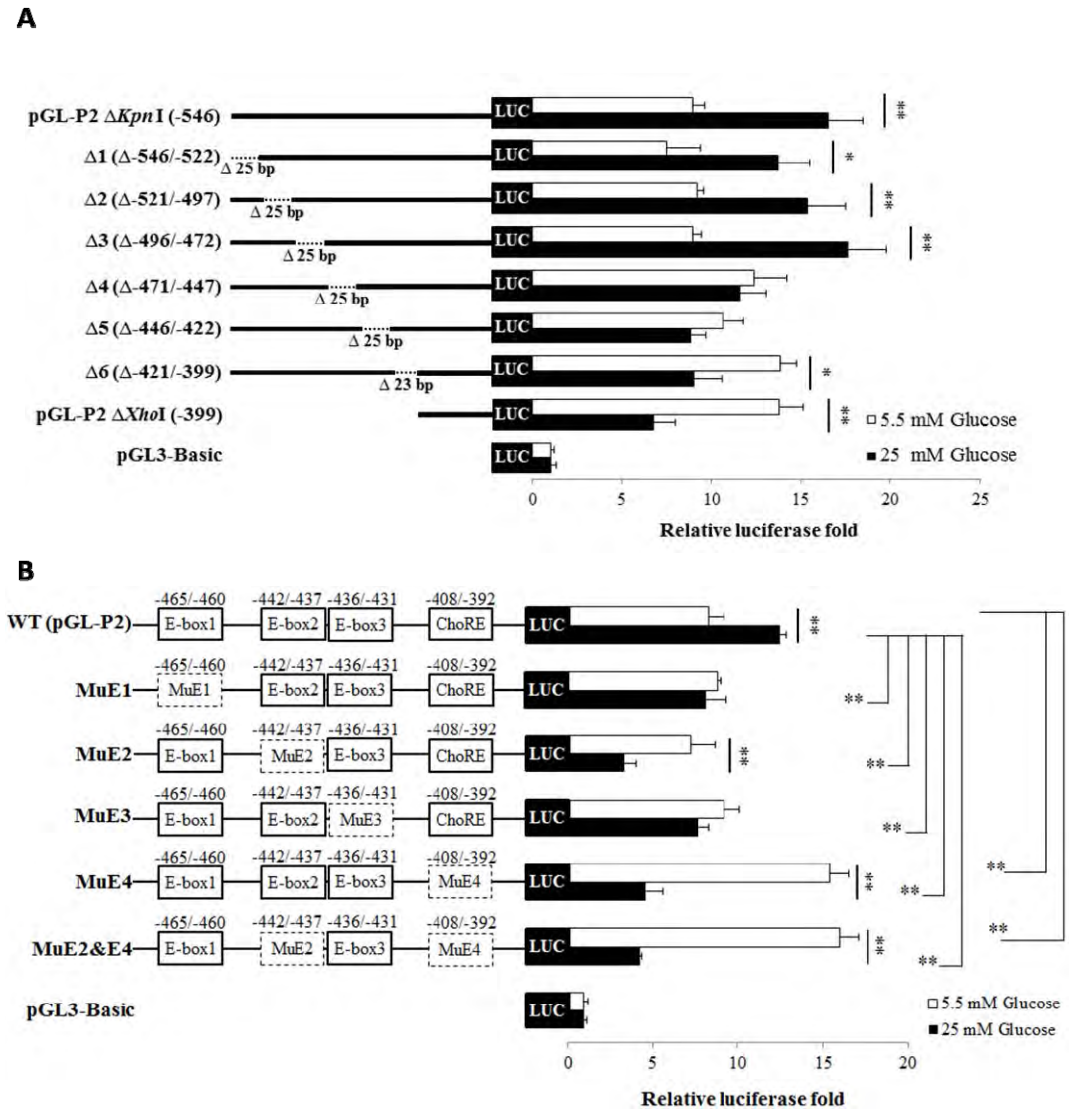


Figure 3. Multiple E-boxes in the P2 promoter of the PC gene function as GREs. **A**, A series of 25 or 23-nucleotide internal deletions were generated across the -546 to -399 of P2 promoter (Δ1, Δ2, Δ3, Δ4, Δ5 and Δ6). **B**, The WT P2 promoter construct containing mutation at E1, E2, E3 and whole ChoRE (MuE1, MuE2, MuE3, MuE4 and MuE2&E4) were generated and transiently transfected into INS-1 832/13 cells. The transfected cells were maintained in RPMI containing normal (5.5 mM) or high (25 mM) glucose for 24 h. The luciferase activity of each construct was normalized to the β-galactosidase activity and expressed as relative luciferase activity. Relative luciferase values obtained from transfected cells maintaining in high glucose medium were presented as fold change relative to those obtained from those maintaining in normal concentration of glucose, each of which was

arbitrarily set as 1. The statistical analysis was conducted using ANOVA test where *P < 0.05; **P < 0.01.

Sp1 functions as a glucose-responsive transcription factor binding to E-box like element

To identify which transcription factors might bind to these four E-boxes, we performed EMSA using two double stranded oligonucleotide probes corresponding to various E-boxes. Incubation of M4+M5 probe which covers E1, E2 and E3 (Figure 4A) with nuclear extract of INS-1 832/13 cells produced two prominent species of DNA-protein complexes (C1 and C2, respectively) (Figure 4B, lane 2) compared to control with no nuclear extract (lane 1). Since the transcription factors, USF1, USF2 and ChREBP have been reported to regulate several glucose responsive genes via binding to E-boxes in their enhancer regions, we performed a supershift assay using antibodies against these three transcription factors. As shown in Figure 4B, neither of these antibodies affected the formation of both C1 and C2 complexes, indicating that these two complexes could not be attributed to the above three transcription factors (lanes 3-5, respectively). Re-examination of the nucleotide sequence surrounding these E-boxes by the PROMO transcription factor binding site database [Messeguer et al., 2002] revealed the presence of CCCCCG (positions -444/-439), within E2. This sequence is similar to the GC-rich, a putative binding site of Sp1 and Sp3 transcription factors [Kodonaga and Tjian, 1986]. We examined if this is the case by incubating the binding reaction in the presence of anti-Sp1 or anti-Sp3 antibody. Addition of anti-Sp1, anti-Sp3 or both antibodies reduced the formation of C1 complex (lanes 7-9, respectively). Incubation of the probe with nuclear extract of INS-1 832/13 overexpressing Sp1 or Sp3 produced a predominant strong band of C1 (lanes 13 and 15, respectively) which was much stronger than that observed from nuclear extract of cells transfected with an empty vector (lane 12). Incubation of anti-Sp1 or anti-Sp3 antibody to the nuclear extract of the Sp1- or Sp3-overexpressing cells prior to the reaction markedly eliminated this strong band (lanes 14 and 16, respectively), further confirming that Sp1 and Sp3 bind to this CCCCCG within E2. To examine whether the CCCCCG motif indeed mediates C1 and C2 formation, we performed a competition EMSA in which the competitor sequence lacking this sequence (M4+5 pmut competitor) was used in the assay. In contrast to the use of WT sequence as a competitor which can eliminate the complex formation (lane 11), the competitor lacking CCCCCG sequence failed to prevent the complex formation (lane 10), suggesting that Sp1 and Sp3 bind to this CCCCCG within E2.

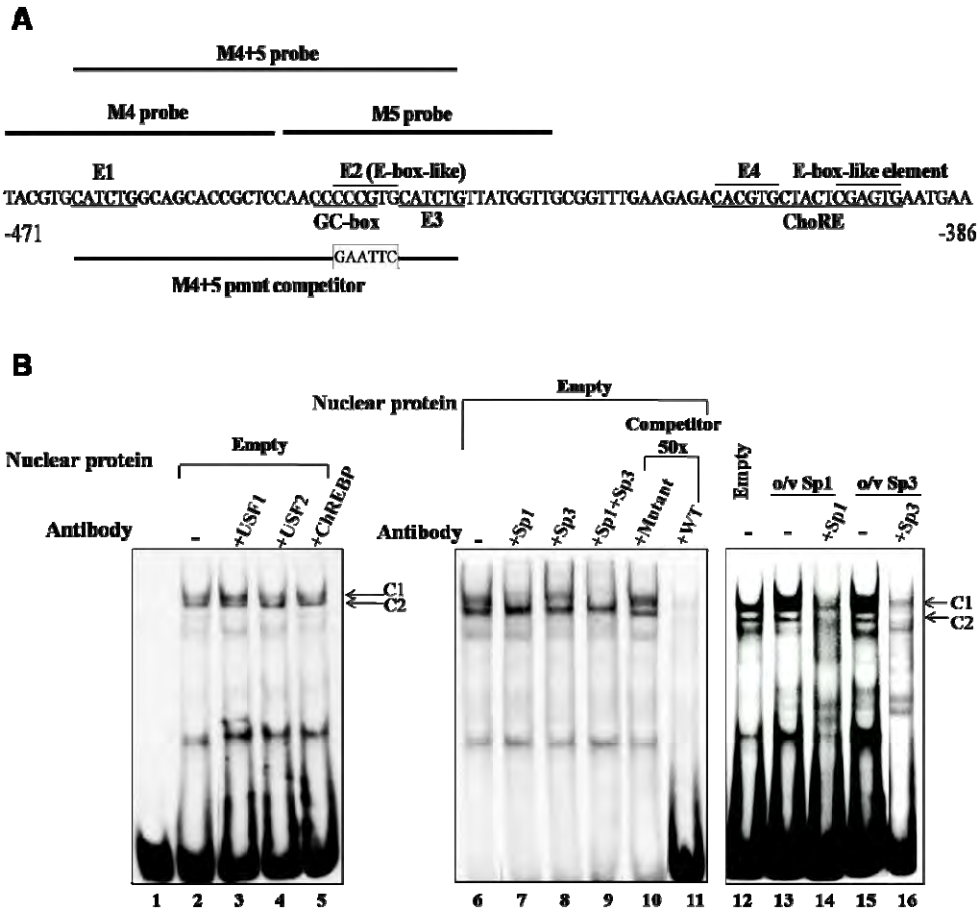


Figure 4. Sp1 and Sp3 bind E-box-like element (E2) *in vitro*. A, Nucleotide sequence of M4+M5 and location of various E-boxes [E1, E2 (E-box like), E3 and E4]. **B**, M4+M5 probe was incubated with nuclear extract of INS-1 832/13 cells and subjected to EMSA. Lanes 1, M4+5 probe alone; lanes 2 and 6, probe incubated with nuclear extract; lanes 3-5, nuclear extracts were pre-incubated with anti-USF1, anti-USF2, anti-ChREBP, respectively. Lanes 7-9, nuclear extracts incubated with anti-Sp1, anti-Sp3 antibodies or both before the probes were added into the reaction, respectively. Lanes 10-11, nuclear extracts were pre-incubated with 50-fold excess mutant or wild type unlabeled oligonucleotide before the probes were added into the reaction, respectively. Lane 12, probe incubated in nuclear extract of INS-1 832/13 cells transfected with an empty vector (Empty) or nuclear extract of INS-1 832/13 cells transfected with plasmid over-expressing Sp1 (o/v Sp1) (lane 13) or Sp3 (o/v Sp5) (lane 15). Lanes 14 and 16, nuclear extracts of INS-1 832/13 cells over-expressing Sp1 or Sp3 were pre-incubated with anti-Sp1 or anti-Sp3 antibody, respectively before probe was added into the reaction. Arrows (C1, C2) represent DNA-protein complexes.

To examine whether over-expression of Sp1 or Sp3 in INS-1 832/13 could influence PC promoter activity, we performed a reporter assay in which we co-transfected the WT PC promoter-reporter construct or promoter-reporter construct containing mutated CCCCCG with plasmids over-expressing Sp1or Sp3 in the INS-1 832/13 and cultured the transfected cells in the medium containing normal (5.5 mM) or high (25 mM) of glucose. As shown in

Figure 5A, in the presence of 5.5 mM glucose, over-expression of Sp1 or Sp3 increased the reporter activity of the WT promoter construct approximately 2-fold while in the presence of 25 mM glucose, over-expression of Sp1 or Sp3 further increased the reporter activity of the WT promoter to 5-fold. In the mutant construct, in the presence of 5.5 mM, over-expression of Sp1 or Sp3 was capable of increasing the reporter activity to the similar levels as that of the WT construct. However, further increase of reporter activity of this mutant construct mediated by Sp1 or Sp3 was lost, indicating that mutation of CCCCCG sequence blunts Sp1- and Sp3-mediated transactivation under high glucose concentration. We next confirmed the above result by performing a ChIP assay that showed in situ binding of Sp1 and Sp3 to CCCCCG in the P2 promoter of the rat PC gene in INS-1 832/13 cells maintained in medium containing either 5.5 mM or 25 mM glucose. As shown in Figure 5B, in the presence of 25 mM glucose, Sp1 appeared to bind to the CCCCCG sequence approximately 2-fold greater than in the presence of 5.5 mM glucose. Although we have shown that Sp3 also bound to E2 by EMSA, we were not able to show that Sp3 bound to E2 in the presence of either 5.5 mM or 25 mM glucose using the ChIP assay. This may suggest that Sp1 rather than Sp3 regulates PC expression via CCCCCG sequence in vivo. This enhanced binding of Sp1 to CCCCCG sequence in the presence of high glucose cannot be attributed to an increased level of Sp1 mRNA as its mRNA expression did not change after cells were exposed to high glucose from 1h to72 h. (Figure 1B). Likewise, the increased binding of Sp1 was not due to the increased entry rate of Sp1 into nucleus because the amounts of Sp1 detected by Western blot analysis in the nucleus and cytoplasm of cells maintained in the presence of 5.5 mM and 25 mM glucose at the time when ChIP was performed (12h) were no difference from one another (Figure 5C). Furthermore, most of the Sp1 was also found in the nucleus. Several reports have demonstrated that phosphorylation/dephosphorylation of Sp1 in response to elevated glucose levels contributes to its transcriptional activity [Fojas et al., 2001]. We detected total and phosphorylated Sp1 in INS-1 832/13 cells cultured in the medium containing low and high glucose. As shown in Figure 5D, INS-1 832/13 cells grown in the presence of 5.5 mM and 25 mM glucose expressed similar amounts of total Sp1. However, most Sp1 presence in the cells maintained under low glucose was detected in the phosphorylated form while only 50% of total Sp1 presence in cells maintained under high glucose was detected in the phosphorylated form (Figure 5E). It is very likely that the enhanced recruitment of Sp1 to CCCCCG sequence detected by the ChIP assay can be attributed to the dephosphorylation of this protein. The dephosphorylation form of Sp1 has been shown to be a transcriptionally

active form which contributes to the transcriptional induction of the acetyl-CoA carboxylase 1 (ACC1) gene [Daniel et al., 1996].

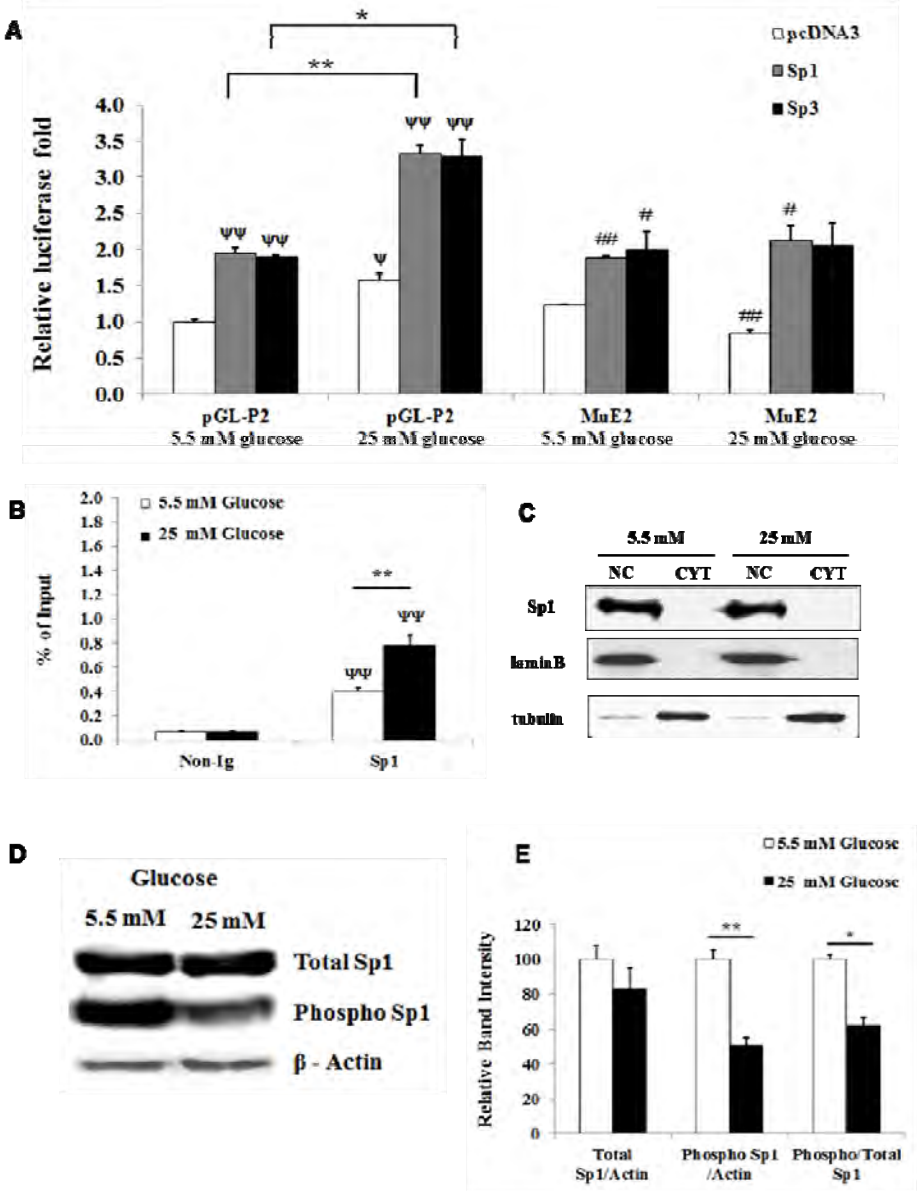


Figure 5. Sp1 regulates glucose-induced PC expression through E-box-like element (E2) in P2 promoter of the PC gene. A, The WT-P2(pGL-P2) or its mutation at E2-luciferase reporter constructs (MuE2) with plasmid overexpressing Sp1, Sp3 or empty vector (pcDNA) were transfected into INS-1 832/13 cells before they were maintained in medium containing low (5.5 mM) or high (25 mM) glucose before their luciferase activities were assayed. Relative luciferase activity of cells co-transfected with pGL-P2 (WT) or mutant promoter construct with empty vector (pcDNA), pSp1 or pSp3 maintained at 5.5 mM or 25 mM were normalized with that of cells co-transfected with pGL-P2 and empty vector maintained at 5.5 mM, which was arbitrarily set as 1. The statistical analysis was conducted using ANOVA test. Ψ ($P < 0.05$) and ΨΨ ($P < 0.01$), compared with cells transfected with pGL-P2 and pcDNA maintained at 5.5 mM glucose. # ($P < 0.05$) and ## ($P < 0.01$), compared with cells transfected with MuE2 maintained at 5.5 mM glucose. **$P < 0.01$ (transactivation of WT

promoter by Sp1 or Sp3 under 5.5 mM and 25 mM glucose). **B**, The Sp1-bound chromatin was prepared from INS-1 832/13 cells grown in low (5.5 mM) or high (25 mM) glucose, fragmented and immunoprecipitated with anti-Sp1 antibody and subjected to real time PCR. The fluorescence signals obtained from quantitation of immunoprecipitated fraction was normalized to the input levels. The input fraction was the sonicated Sp1-bound DNA before immunopreripitating with anti-Sp1 antibody. The statistical analysis was conducted by ANOVA test where *,ΨΨ $P < 0.01$. **C**, Western blot analysis of nuclear (NC) and cysolic (CYT) extracts of INS-1 832/13 cells maintained under 5.5 or 25 mM glucose with anti-Sp1 antibody. Loading controls of the cytosolic and nuclear proteins were assessed by stripping the blot and re-probed with anti-tubulin and anti-lamin B antibody, respectively. **D**, A representative of Western blot analysis of nuclear extracts of INS-1 832/13 cells maintained in the presence of 5.5 or 25 m glucose with anti-Sp1, anti-phospho Sp1. Control loading was assessed by stripping the blot and re-probed with anti-actin antibody. E, The intensity of total Sp1, phospho-Sp1 bands was quantitated and normalized with that of actin band and shown as the ratios of total Sp1/actin, phospho-Sp1/actin and total Sp1/phospho-Sp1 from three independent experiments. The statistical analysis was conducted using ANOVA test where *$P < 0.05$ **$P < 0.01$.

USF2 preferentially binds to E1 while an unknown factor(s) binds to E3

Although the EMSA shown in Figure 4B suggested that USF1, USF2 and ChREBP did not bind to the probe, this may not be the only conclusion because E1 and E3 are located close to the extreme 5'- and 3'-ends of M4+M5 probe. This may restrict the efficiency of binding of (an) additional transcription factor(s), if any, apart from Sp1 and Sp3. To rule out this possibility, we synthesized the M4 probe which contains only E1 in the middle for EMSA analysis (Figure. 6A). Incubation of the M4 probe with a nuclear extract of INS-1 832/13 revealed weak DNA-protein complexes (data not shown), suggesting the poor binding of the candidate transcription factor(s) to this probe. However, a prolonged exposure (10 min) of the blot produced two weak DNA-protein complexes (C1 and C2) (Figure 6B, lane 2). Incubation of anti-USF1 or anti-USF2 antibody in the reaction marginally affected both complexes (lanes 3 and 4). However, incubation of M4 probe with the nuclear extract prepared from INS-1 832/13 cell overexpressing USF1 produced an additional weak band corresponding to the C2 complex (lane 5, asterisk) while incubation of the same probe with a nuclear extract of INS-1 832/13 cells overexpressing USF2 also produced an additional band corresponding to the C2 complex but was much stronger (lane 6). Furthermore incubation of anti-USF1 or anti-USF2 antibodies prevented C2 formation (lanes 7 and 8), suggesting that USF2 preferentially binds to E1. Although both anti-USF1 and USF2 antibodies prevented at least one of the two complexes, anti-ChREBP antibody did not affect formation any of these complexes (lane 9), suggesting that ChREBP did not bind to E1.

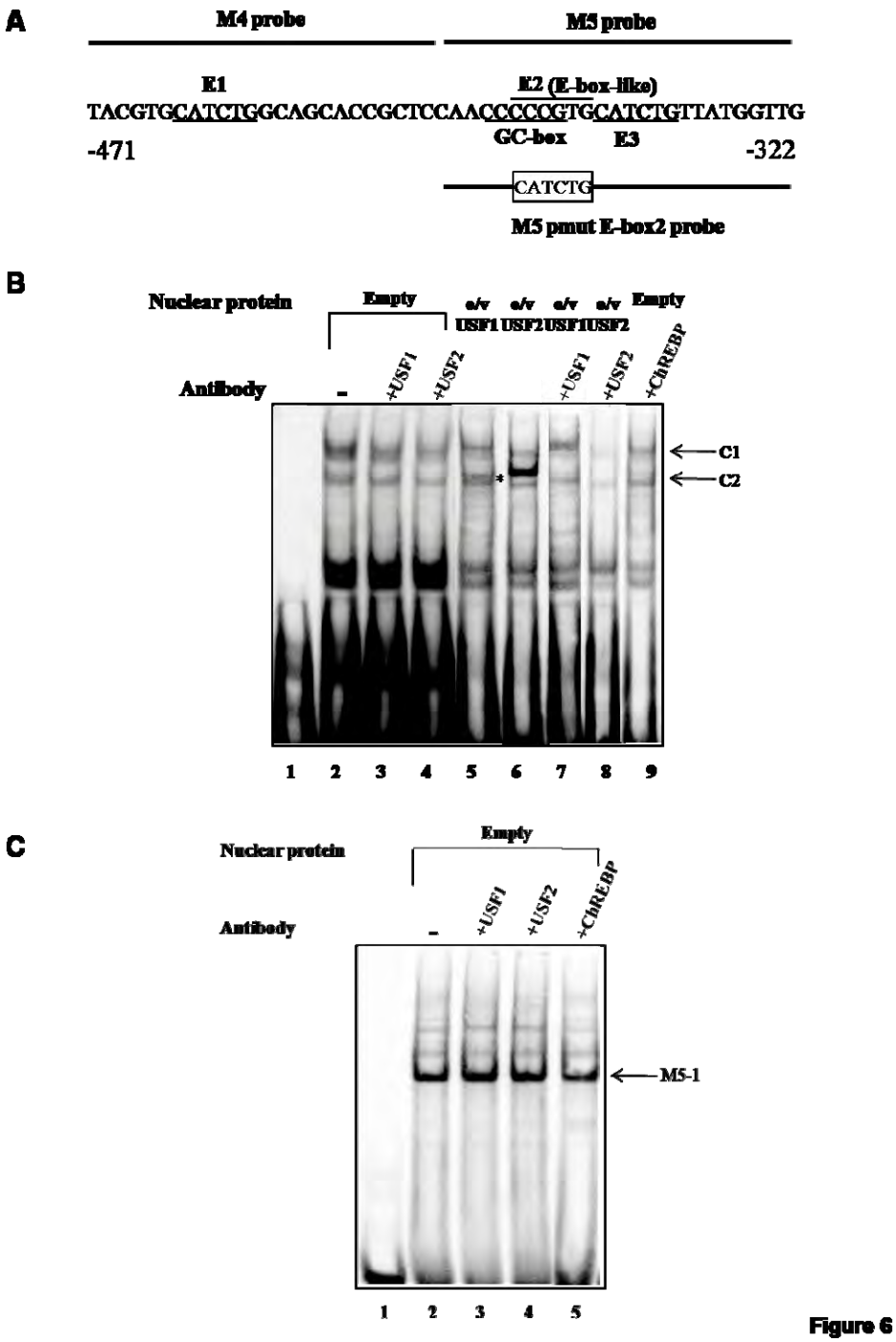


Figure 6

Figure 6. USF1 and USF2 bind to the E1 in the P2 promoter of the PC gene. A. Nucleotide sequences of M4, M5 and M5 pmut E2 probes. E-box and GC-box are underline. **B,** EMSA of M4 probe with INS-1 832/13 nuclear extract. Lane 1, M4 probe alone; lane 2, probe incubated with nuclear extract; lanes 3-4, nuclear extracts pre-incubated with anti-USF1 or anti-USF2 antibody before the probes were added into the reaction, respectively. Lanes 5-6, probe incubated with nuclear extract of INS-1 832/13 overexpressing USF1 or USF2, respectively. Lanes 7 and 8, nuclear extracts of INS-1 832/13 overexpressing USF1 pre-incubated with anti-USF1antibody or overexpressing USF2 pre-incubated with anti-USF2 antibody before the probe was added into the reaction, respectively. Lane 9, INS-1 832/13 nuclear extract pre-incubated with anti-ChREBP antibody before the probe was added into

the reaction. **C.** EMSA of M5 pmut E2 probe with an INS-1 832/13 nuclear extract. Lane 1, M5 pmut E2 probe alone; lane 2, probe incubated with nuclear extract; lanes 3-5, nuclear extracts pre-incubated with anti-USF1, anti-USF2 or anti-ChREBP antibody before the probes were added into the reaction, respectively. Arrows represent DNA-protein complexes.

We also conducted EMSA using the new probe, M5 which contains mutated CCCCCG in E2 (M5 pmut E2 probe in Figure 6A), leaving only E1 intact in the middle (M5 pmut E-box2, Figure 6A). Any binding observed in this assay would result from binding of the nuclear factor to E3 only. Incubation of this probe with a nuclear extract of non-transfected INS-1 832/13 produced one prominent band, M5-1 (Figure 6C, lane 2). Incubation of the binding reaction in the presence of anti-USF1, anti-USF2 or anti-ChREBP (lanes 3-5, respectively) did not affect this complex, suggesting other nuclear factors rather than these three proteins bind to E3.

Glucose enhances binding of USF2 and ChREBP to E4

We showed that E4 serves as a binding site for USF1 and USF2 [Boonsaen et al., 2007], however the functional importance of this binding site with respect to the glucose-induction was unknown. Pedersen et al. [2010] have previously shown that ChREBP binds relatively weakly to this site. To examine whether the recruitment of USF1, USF2 and ChREBP to this E-box is enhanced during high glucose induction, we performed a ChIP assay of this E4 with anti-USF1, anti-USF2 and anti-ChREBP antibodies in INS-1 832/13 cells maintained in the medium containing 5.5 mM and 25 mM glucose. As shown in Figure 7A, cells grown in high glucose medium did not affect the recruitment of USF1, instead recruitment of USF2 and ChREBP to E4 was increased (Figure 7A). The enhanced binding of USF2 to E4 in the presence of high glucose could not result from an increased level of USF2 mRNA expression as its abundance was unchanged after the cells were exposed to 25 mM glucose from 1h to 72 h (Figure 1C). The increased binding of USF2 to E4 was not due to the increased entry rate of this transcription factor to nucleus because the amounts of USF2 in the nucleus of cells maintained under low and high glucose concentrations were similar (Figure 7B). Also most USF2 were found in the nucleus. We failed to detect expression of the ChREBP protein in INS-1 832/13 cells grown under 5.5 mM and 25 mM glucose by Western blot analysis, suggesting that the expression of ChREBP in INS-1 832/13 may be rather low compared to other types of cells, such as liver. This rather weak expression of endogenous ChREBP in INS-1 832/13 or rat islets was also seen in the previous report [26]. Nevertheless, the increased binding of ChREBP to E4 is partly contributed by increased ChREBP mRNA

abundance. As shown in Figure 1D, expression of ChREBP mRNA was increased to 2-2.5-fold after INS-1 832/13 cells were exposed to 25 mM glucose for 6h, 12h and 24h.

Finally we confirmed the functional role of Sp1, USF1, USF2 and ChREBP in regulating glucose-mediated transcriptional induction of PC expression. We suppressed expression of these four transcription factors by their respective siRNAs in INS-1 832/13 and maintained the transfected cells in the medium containing normal or high glucose before analyzing the expression of PC mRNA by real time PCR. As shown in Figure 7C, upon transfection of siRNAs targeted to Sp1 (Sp1 KD), USF1 (USF1 KD), USF2 (USF2 KD) or ChREBP (ChREBP KD) resulted in 80%, 90%, 75% and 80% reduction in expression of their respective mRNAs. As shown in Figure 7D, suppression of USF1 did not appear to affect glucose-induced PC mRNA expression while suppression of Sp1, USF2 or ChREBP expression resulted in 50%, 40% and 30% reduction of glucose-induced PC mRNA expression. Combined knockdown of USF2, Sp1 and ChREBP expression completely prevented glucose-induced PC mRNA expression (Figure 7D). These data suggest that the glucose-mediated transcriptional activation of PC in the P2 promoter is regulated by these three transcription factors, albeit their absolute degrees of the control are varied.

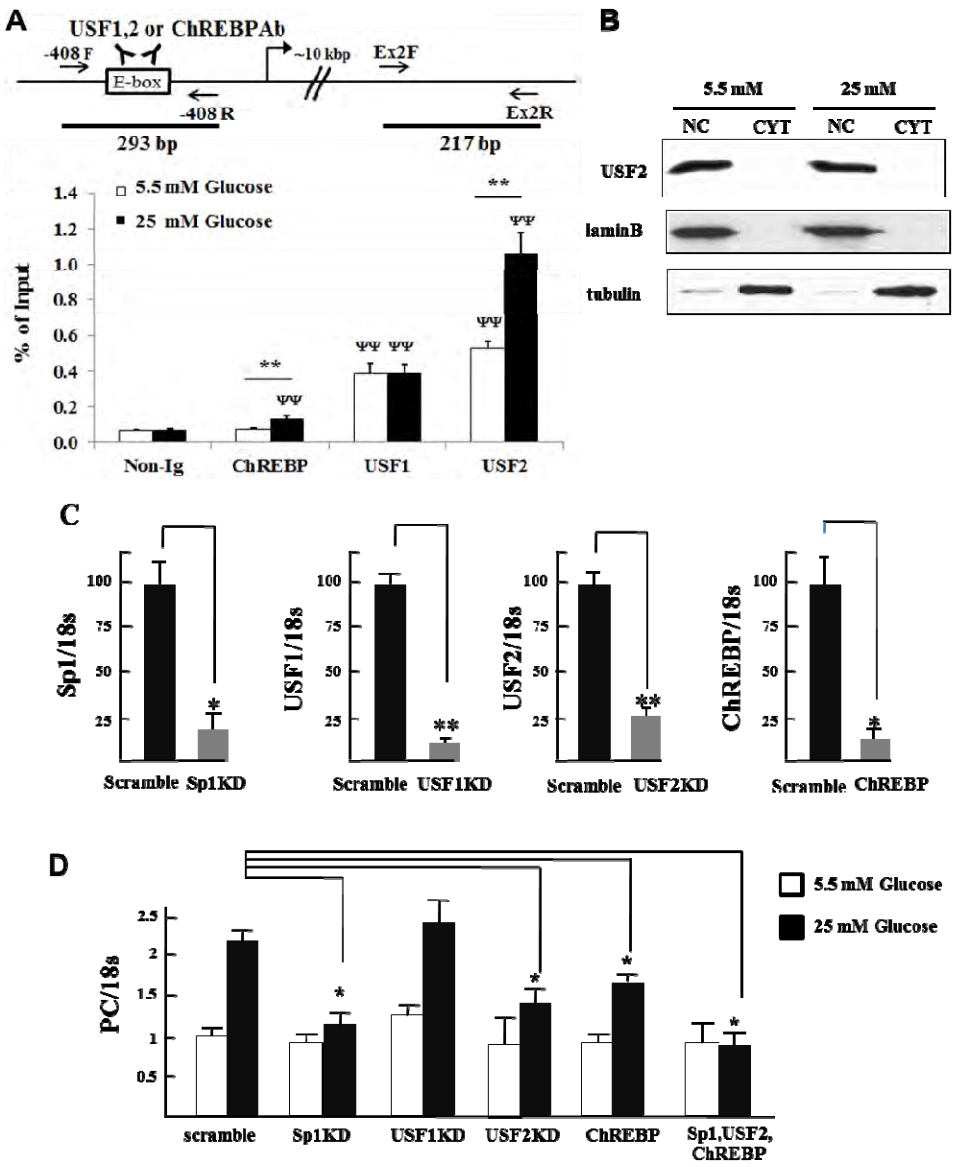


Figure 7. High glucose enhances binding of USF2 and ChREBP to E4 in the P2 promoter of the PC gene and suppression of Sp1, USF2 and ChREBP expression blunts glucose-induced expression of endogenous PC expression in INS-1 8322/13. A, *Top panel*, a schematic representation of the P2 promoter region with E-box4 and primer binding sites for quantitative real time PCR indicated. *Bottom panel*, the USF1-, USF2- or ChREBP-bound chromatin was prepared from INS-1 832/13 cells grown in low (5.5 mM) or high (25 mM) glucose was prepared, immunoprecipitated with their corresponding antibodies and subjected to real time PCR using the primers indicated above. The fluorescence signals obtained from the immunoprecipitated fractions were normalized to those obtained from the input fraction which was the sonicated transcription factor-bound DNA before immunoprecipitating with the antibodies. The statistical analysis was conducted by ANOVA test where **$P < 0.01$ compared between low and high glucose concentrations. $^{\Psi\Psi}P < 0.001$ compared with the fraction that was immunoprecipiated with no antibody at both low or high glucose concentration. **B,** Western blot analysis of nuclear (NC) and cysolic (CYT) extracts of INS-1 832/13 cells maintained under 5.5 or 25 mM glucose with anti- USF2 antibody. Loading controls of the cytosolic and nuclear proteins were assessed by stripping the blot and re-probed with anti-tubulin and anti-lamin B antibodies, respectively. **C**, INS-1 832/13 cells

were mock- or transfected with siRNAs targeted to Sp1, USF1, USF2 and ChREBP. The transfected cells were cultured in the medium containing low (5.5 mM) or high (25 mM) glucose for the next 24 h before the expression of Sp1, USF1, USF2, ChREBP and PC mRNAs was measured by quantitative real time PCR and their expression levels were normalized with that of 18s rRNA. The values obtained from scramble and each knocked down cells are expressed relative to that obtained from the mocked transfection, which was arbitrarily set as 100%. The values shown are means $\pm$ standard deviations of the three independent experiments (n = 3). The statistical analysis was conducted using ANOVA test where *P < 0.05; **P < 0.01.

Discussion

Pyruvate carboxylation has been shown to be a crucial anaplerotic reaction which regulates glucose-induced insulin secretion in pancreatic islets. PC catalyzes the above reaction and is highly abundant in this tissue where it participates in the pyruvate cycling, a process by which a coupling factor, NADPH is formed and required for glucose-induced insulin secretion (for review see [MacDonald et al., 2005; Jitrapakdee et al., 2010]). Suppression of PC expression impairs anaplerosis concomitant with the reduced glucose-induced insulin secretion [Hasan et al., 2008; Xu et al., 2008]. Previous work has shown that expression of PC in isolated rat islets is inducible by exogenous glucose and its expression is highly correlated with the flux toward pyruvate carboxylation [Lu et al., 2002]. Here we showed that induction of PC expression by glucose in the rat insulinoma cell line, INS-1 832/13 is rather slow, the levels of mRNA expression is increased to only 1.5-fold within the early hours before reaching maximum at 24 h. The slow induction of PC mRNA expression was also observed in the isolated rat islets maintained under high glucose containing medium [MacDonald, 1995a]. It is possible that the allosteric regulation of PC by acetyl-CoA may be important mechanism to increase activity of PC during this early period before transcription of this gene is activated. It is well known that equal amounts of pyruvate enter pyruvate carboxylation by PC and pyruvate decarboxylation by PDH [MacDonald 1993a; MacDonald, 1993b; MacDonald, 1995b, Khan et al., 1996]. The acetyl-CoA formed by PDH-catalyzed reaction can in turn allosterically activate PC activity during this early period.

In this report we identified a complex GRE within the P2 promoter of the rat PC gene. This GRE consists of four tandem E-boxes, locating at -465/-460 (E1), -442/-437 (E2), -436/-431(E3) and -408/-403 (E4) and as the glucose sensor. It is interesting to note that although these four E-boxes are well defined in the distal promoter of the rat PC gene, only E3 and E4 are also conserved in the distal promoter of the human PC gene, while the nucleotides corresponding to E1 and E2 contains one nucleotide difference in the distal promoter of the human PC gene [Thonpho et al., 2013]. This raises the possibility of subtle difference of

transcriptional activation of PC in response to glucose between the rat and the human. Nevertheless, mutational analysis of these sequences of distal promoter of rat PC gene showed that mutating one of these E-boxes is sufficient to eliminate glucose-mediated transcriptional activation of P2 promoter activity, suggesting that these four E-boxes are equally important. It is noted that E1 and E3 resemble the canonical E-box (CANNTG) while E4 is part of the previously reported ChoRE [Pedersen et al., 2010]. While both endogenous USF1 and USF2 can bind E1, overexpression of USF1 or USF2 in INS-1 832/13 cells revealed that USF2 preferentially binds E1 much stronger than USF1. However, ChREBP did not bind this E-box, possibly because binding of ChREBP requires two adjacent E-boxes separated by 5 nucleotides [Shih et al., 1995]. Although the E3 sequence is similar to the E1 sequence, neither USF1 nor USF2 binds to E1, it appears to bind strongly to an unknown nuclear factor whose identity remains unidentified. We have previously shown that E4 serves as a binding site of USF1 and USF2 [Boonsaen et al., 2007]. Although mutation of E4 moderately reduced P2 activity under basal conditions, its role in glucose-mediated transcriptional induction at that time was unknown [Boonsaen et al., 2007]. Pedersen et al [2010] previously reported that this E-box also serves as a binding site of ChREBP which mediates glucose-induced transcriptional induction of the P2 promoter. They have also shown that high glucose promotes the recruitment of ChREBP, but not USF1 or USF2, to this E-box. However, we have found that high glucose not only enhances binding of ChREBP, but also USF2, to this E-box. It is well known that USF2 can form a homodimer or a heterodimer with USF1, but not with ChREBP, to regulate transcription [Ma et al., 2006]. Likewise, ChREBP/Mlx heterodimer is required to activate glucose-dependent transcription. Although initial reports have clearly demonstrated that ChREBP transcriptional activity is closely associated with elevated concentrations of glucose [de Silva et al., 2006; O'Callagham et al., 2001; Wang and Wollheim, 2002] via complicated post-translational modifications [Kabashima et al., 2003; Li et al., 2006; Kawaguchi et a., 2001], growing evidence has now indicated that both USF1 and USF2 are capable of regulating transcription of several glucose-dependent genes [Portois et al., 2002; van Deursen et al., 2008; Nowak et al., 2008; Shi et al., 2008]. Although the expression levels of USF2 can be induced by elevated concentrations of glucose in rat mesangial cells [Shi et al., 2008], we did not observe a significantly increase of USF2 in INS-1 832/13 cells grown under high glucose concentration suggesting the regulation of USF2 expression may be different between cell types. Despite the lack of increased USF2 mRNA level and USF2 protein accumulated in the nucleus in INS-1 832/13 cells maintained in the presence of high glucose, we observed the enhanced binding of USF2

to E4 in the ChIP assay, suggesting that posttranslational modification of this transcription factor may be responsible for the enhanced DNA binding activity. Nowak et al. [2008] have reported that elevated concentrations of glucose stimulate expression of the apolipoprotein A5 gene through increased binding of USF1 and USF2 to their cognate E-box without changing protein abundance. Interestingly, this enhanced DNA-binding is at least in part caused by dephosphorylation of both USF1 and USF2. Therefore, it remains unclear whether USF2 and ChREBP co-regulate glucose-dependent transcriptional activation of the P2 promoter simply through competition of the same binding site.

It is also interesting to note that while all E-boxes function as glucose-responsive elements that mediate transcriptional activation of the P2 promoter under high glucose induction, E4 functions as a repressor under a normal glucose concentration as mutation of this E-box resulted in an increased reporter gene expression under a normal concentration of glucose. Jeong et al. [2011] have recently reported that ChREBP can also function as a transcriptional repressor as well as an activator, however, a molecular explanation regarding this dual role of ChREBP was not investigated. Growing evidence has also indicated that in the promoters of glucose-responsive genes, a complex ChoRE may consist of the classical core ChoRE and the nearby accessory site which allows maximal glucose-induction. The accessory sites thus far have been reported to be the binding sites of the nuclear factor-Y (NF-Y) (i.e. CCAAT-box), HNF4 or c-myc [Yu and Lou, 2009; Zhang et al., 2010]. Jeong et al. [2011] have recently employed integrated expression profiling and genome-wide analysis of ChREBP target genes and identified a guanine-rich sequence similar to a Sp1-binding site associated with ChREBP even though this sequence does not bind ChREBP. The authors suggest that this guanine-rich sequence may form part of an accessory site allowing Sp1-family transcription factors to bind and co-regulate with ChREBP. As mentioned above, we found that the CCCCCG sequence (i.e. GGGGGC on the complementary strand) coincided within the E2 and the ChREBP binding site (E4) and are in the close vicinity, which may be the case as reported by Jeong et al. [2011].

Although, Sp1 has previously been known to be a ubiquitously expressed transcription factor which controls basal transcription of a variety of “housekeeping” genes [Tan and Khachigian, 2009; Chu, 2012], growing evidence now indicates that the abundance and its transcriptional activity of this transcription factor can be modulated by nutrients and cellular metabolites [Schafer et al., 1997; Smih et al., 2002; Lee and Pedersen, 2002; Hwang and Ismail-Beigi, 2006]. We have found that the CCCCCG sequence within E2 serves as a glucose-sensor because a high concentration of glucose increases the recruitment of Sp1 to

this sequence. Also mutation of this sequence eliminated glucose-induced transcriptional induction of P2 promoter activity. We found that an elevated concentration of glucose did not affect the abundance of Sp1 mRNA or Sp1 protein in the nucleus but rather affects the phosphorylation status of the protein. We found that a high concentration of glucose increases dephosphorylation of threonine 453 of Sp1 and this might in part contribute to the enhanced transcriptional activity under this condition. Phosphorylation of this residue of Sp1 has been reported to decrease its ability to activate transcription of the cystathionine -lyase gene in pancreatic beta cells [Zhang et al., 2011]. The regulation of PC expression by glucose appears to be similar to another biotin containing enzyme, namely acetyl-CoA carboxylase (ACC). In mouse preadipocytes, Sp1 also mediates glucose activation of the ACC1 gene via the two GC-rich sequences which form part of the glucose-responsive element [Daniel and Kum, 1996]. Exposure of preadipocytes to high glucose stimulates dephosphorylation of Sp1 concomitant with an enhanced binding to its cognate binding site in the ACC1 gene promoter [Daniel et al., 1996].

Finally the functional roles of USF1, USF2, ChREBP and Sp1 in transcriptional induction of endogenous PC expression were validated by an siRNA experiment. This clearly demonstrated that silencing expression of USF2, ChREBP and Sp1, but not USF1 mRNA, individually resulted in modest reductions of glucose-induced transcriptional induction of PC mRNA expression, suggesting that these three transcription factors may act in concert allowing maximal induction of PC in response to an elevated concentration of glucose.

The findings that glucose-induced transcriptional activation of PC in pancreatic -cells is regulated by multiple transcription factors provide a complex paradigm in terms of the disease development because deregulation of some of these transcription factors in part underlies the impaired insulin secretion or hyperglycemia. For example, ChREBP was first known to link glycolysis and de novo fatty acid synthesis in liver. There is also a direct and strong association between ChREBP expression and insulin sensitivity in adipose tissue of humans with insulin resistance [Herman et al., 2012]. Growing evidence now indicates that this transcription factor also regulates transcription of -cell specific genes [Wang and Wollheim, 2002]. Suppression of ChREBP expression impairs glucose-stimulated pancreatic -cell proliferation [Metukuri et al., 2012]. Recently, glucose-induced ChREBP overexpression has been shown to play a pivotal role in mediating glucotoxicity of pancreatic beta cells, causing impaired insulin secretion [Poungvarin et al., 2012]. Likewise deregulation of Sp1 via the abundance or its posttranslational modification is well known to at least in part cause diabetes [Majumdar et al., 2004].

In summary we identified a complex glucose-responsive unit which mediates glucose-induced transcription of theP2 promoter of the rat PC gene. Although this GRU appears to provide a platform for binding of at least four transcription factors, namely USF1, USF2, ChREBP and Sp1, only the latter three are functionally relevant to glucose-induced transcription of P2 promoter of rat PC gene.

Part 4 Characterization of liver-specific transcription factor that regulates Expression of pyruvate carboxylase gene in hepatocytes

In the rat and mouse, the PC gene is regulated by two alternative promoters: the proximal (P1) and distal (P2) promoters [Jitrapakdee et al., 1997; Jitrapakdee et al., 2001]. The P2-promoter is active in a variety of tissues, but is most highly active in the pancreatic β-cells [Jitrapakdee et al., 1998] where PC participates in the pyruvate cycling that supports GSIS [MacDonald, 1995a; Hason et al., 2008]. In contrast, the P1-promoter is active in hepatocytes where it is regulated by the ubiquitous transcription factors Sp1 and Sp3 [Rojvirat et al., 2011], and by the cAMP-responsive element binding protein (CREB) [Thonpho et al., 2010]. Interestingly, adipocytes, where PC is involved in *de novo* fatty acid synthesis and glyceroneogenesis, employ the same promoter usage as the liver. This transcriptional activation in adipocytes is mediated through binding of the peroxisome proliferator activated receptor gamma (PPARγ) to the PPAR-responsive element (PPRE) [Jitrapakdee et al., 2005]. More specifically, in mouse, genetic ablation of the PPARγ2 isoform in adipose tissue markedly down-regulates PC expression in adipose tissue but not in liver, confirming the specific control of PC in adipose tissue by this nuclear receptor (NR) [Jitrapakdee et al., 2005]. Although PPARγ2 was identified as the main transcriptional regulator of the P1-promoter in adipocytes, the transcription factor(s) that drive PC expression in hepatocytes remain unknown.

Here we show that the P1-promoter contains three classical and non-classical HNF4α binding sites, which are not functionally equivalent. We also demonstrate that siRNA mediated suppression of HNF4α expression in AML12 cells results in reduced expression of PC and G6Pase, suggesting that PC is a direct target of HNF4α. This study highlights the link between the MODY1 gene (HNF4α) and the first regulatory enzyme of the gluconeogenic pathway, and thus leads to the concept that HNF4α regulates the overall program of hepatic gluconeogenesis through modulation of PC, PEPCK and G6Pase gene expression.

Generation of reporter constructs

Chimeric reporter constructs comprising 603 (pGL-P1ΔDraI) or 166 (pGL-P1ΔDelA) [Rojvirat et al., 2011] nucleotides of the mouse PC gene P1-promoter ligated 5' to the luciferase reporter gene were used as templates to generate other reporter constructs. The putative HNF4α binding sites in the above constructs were mutated, singly and in combination, using the Quik change site-directed mutagenesis kit (Stratagene-Agilent Technology). Mutagenesis was carried out in a 50 μl reaction mixture containing 1x cloned *Pfu* polymerase buffer (100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris HCl pH 8.8, 20 mM $MgSO_4$, 1% Triton® X-100 and 1 mg/ml BSA), 0.2 mM dNTP, 125 ng of each primer, 100 ng of template, and 2.5 units of *Pfu* Turbo polymerase. PCR profiles consisted of an initial denaturation at 95°C for 30 s, followed by 20 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min and extension at 68°C for 8 min, before a final extension at 68°C for 8 min. 10 units of *Dpn*I were then added to the PCR mixture and, following overnight digestion at 37°C, 5 μl were transformed into *E. coli* DH5α. The mutagenic primers used to produce the above constructs are shown in Table 1. The inserts from the clones with mutated nucleotides were excised and used to replace the equivalent wild type fragments in the parental plasmid.

Cell culture, transient transfection and reporter assays

The mouse hepatoma cell line, AML12 (ATCC: CRL254), was maintained in a 1:1 (v/v) mixture of Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium (Gibco) supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 40 ng/ml dexamethasone, 10% (v/v) fetal bovine serum (Gibco), 100 units/ml penicillin, 100 μg/ml streptomycin (Gibco), at 37°C in a 5% CO_2 atmosphere. One day before transfection, 1 x 10^5 cells were plated in 24-well plates in the above medium without antibiotics for 1 day. In 100 μl Opti-MEM® I reduced serum medium with 2 μg of lipofectamine2000™, cells were transfected with 250 ng of luciferase reporter construct and 250 ng of pRSV-βGal plasmid, with or without 250 ng of plasmid encoding human HNF4α (pcDNA-hHNF4α). The transfected cells were maintained at 37°C in the complete medium for 48 h. Cells were scraped from dishes, harvested by centrifugation, suspended in 1x reporter lysis buffer (Promega) and subjected to 3 cycles of freezing/thawing. 50 μg of protein lysates were subjected to luciferase assay using luciferase assay reagent (Promega) in a GloMax20/20

luminometer (Promega). β-galactosidase activity, measured using ONPG as the substrate, was used to normalize for transfection efficiency.

For the transfection of AML12 with siRNA, 1 x 10^6 cells were plated in 6-well plates in medium without antibiotics for 24 h, and then transfected with 100 ng of validated mouse HNF4α siRNA (Ambion). The transfected cells were maintained in complete medium at 37^{o}C with 5% CO_2 for 48 h before being harvested for quantitative real time PCR.

Cloning and production of recombinant human HNF4α

The human HNF4α (hHNF4α) cDNA was cloned from HepG2 cells. Total RNA was isolated from 1 x 10^6 HepG2 cells using Trizol reagent (Invitrogen). cDNA synthesis was performed in a 20 μl reaction mixture containing 2 μg of heat denatured total RNA, 200 ng random hexamer, 1 mM dNTP, 1x reverse transcriptase buffer (50 mM Tris pH8.3, 75 mM KCl, 1 mM DTT) and 200 units of SuperscriptIII reverse transcriptase (Invitrogen) at 50^{o}C for 1 h. hHNF4α cDNA was cloned by PCR using forward and reverse primers designed from the published sequence [Chartier et al., 1994]. PCR was carried out in a 50 μl reaction mixture containing 1x PCR buffer (20 mM Tris, pH 8.4, 50 mM $MgCl_2$, 1.5 mM, $MgCl_2$), 0.2 mM dNTP, 5 ng cDNA, 0.5 μM HNF-F (5'-AAGCTTATGCGACTCTCCAAAACCTCG-3', underline indicates *Hind*III restriction site) and HNF-R (5'-GGTACCCTAGATAACTTCCTGCTTGTG-3', underline indicates *Kpn*I restriction site) primers, and 2.5 units of *Taq* DNA polymerase (Invitrogen). PCR consisted of an initial denaturation at 94^{o}C for 5 min, followed by 35 cycles of denaturation at 94^{o}C for 30 s, annealing at 55^{o}C for 45 s and extension at 72^{o}C for 1 min. The PCR product was digested with *Hind*III and *Kpn*I, and ligated to the equivalent sites of pcDNA3 (Invitrogen). The resulting construct, pcDNA-hHNF4α was sequenced and used for transactivation assays. To obtain purified hHNF4α, the protein was recombinantly expressed in a bacterial expression system [Oxombre et al., 2004]. The hHNF4α cDNA was modified to encode an N-terminal hexahistidine tag. This was achieved by PCR using pcDNA-hHNF4α as the template and 6xHis-forward primer (5'-CAT ATG $(CAT)_6$ GGA GGT CGA CTC TCC AAA ACC CTC GTC-3') and reverse primer (5'-GCG GCC GC TAG ATA ACT TCC TGC TTG GTG-3') which incorporate *Nde*I and *Not*I sites at their 5'-ends, respectively. The PCR product was digested with *Nde*I and *Not*I and ligated to the equivalent sites in the polylinker region of pET17b vector (Merck), forming the pET-6xHis hHNF4α construct. This construct was validated by sequencing before transformation into *E. coli* BL21(DE3) for expression. 5 ml of

an overnight culture of *E. coli* BL21(DE3) harboring the pET-6xHis hHNF4α clone were subcultured in 250 mL of LB broth containing 100 μg/ml ampicillin. Cultures were grown at 37^{o}C until the OD_{600} reached 0.8, at which time the culture was induced with 0.1 mM IPTG and transferred to 30^{o}C for overnight incubation. The culture was centrifuged at 3,000 x g for 10 min and the resulting cell pellet resuspended in 5 ml lysis buffer (300 mM NaCl, 50 mM NaH_2PO_4, 10 mM imidazole, pH 8.0, 1 mM PMSF and 1 mg/mL lysozyme). Cell lysates were sonicated on ice for 3 min, centrifuged at 10,000 x g for 20 min, and the supernatant loaded onto a 0.5 ml NiNTA agarose column (Qiagen). The column was washed with 4 column volumes of wash buffer (300 mM NaCl, 50 mM NaH_2PO_4, 20 mM imidazole, pH 8.0) before proteins were eluted with 2 ml of elution buffer (300 mM NaCl, 50 mM NaH_2PO_4, 250 mM imidazole, pH 8.0) and collected. The elution buffer was exchanged with protein storage buffer (20 mM Tris-HCl, 1mM DTT, 0.1 EDTA, 100 mM KCl, 20% (v/v) glycerol) using an Amicon concentrator (10 kDa cut off).

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed using the Lightshift EMSA kit (Thermo Scientific). AML12 nuclear extracts were prepared as described previously (15). 3'-biotinylated oligonucleotides (10μM) (Biobasic, Canada) were annealed with their complementary strands in 1x annealing buffer (10 mM Tris pH7.4, 100 mM NaCl and 1 mM EDTA). Binding reactions were performed in 20 μl reaction mixtures containing 1x binding buffer (10 mM Tris, pH 7.4, 50 mM KCl, 1 mM DTT, 5% (v/v) glycerol), 2 μg poly(dI-dC), 1% (v/v) NP-40, 120 pmol annealed probe, and 5 μg nuclear extract or 0.25 μg purified 6xHis hHNF4α, at 4^{o}C for 20 min. For the competition assays, excess amounts of unlabeled double stranded oligonucleotide probes were included in the binding reactions. For supershift assays, 1 μg of anti-HNF4α polyclonal antibody (H-171) (SantaCruz Biotech) was included in the binding reaction. The DNA-protein complexes were subjected to 4% native polyacrylamide gel electrophoresis using 0.5x TBE (44.5 mM Tris-HCl, pH8.0, 44.5 mM boric acid and 1.25 mM EDTA) as the running buffer at 100 V for 2 h. The gel was electroblotted onto a Biodyne B nylon membrane (Pall) using a semi-dry transfer unit (Hoeffer). The DNA was crosslinked on the membrane by UV-crosslinker (UVP). The shifted bands were visualized using non-radioactive nucleic acid detection kit (Pierce). The image was captured with fluorescence image capture system (GeneTools).

Chromatin immunoprecipation (ChIP) assay

In brief, 2 x 10^6 AML12 cells were plated in 1 cm dishes and maintained in DMEM/Ham's F12 medium at 37^{o}C for 24 h. The DNA and proteins were crosslinked by adding formaldehyde to a final concentration of 1% (v/v) to the culture medium at 37^{o}C for 10 min. The cells were scraped from the plate and centrifuged at 1,000 x g for 5 min. The pellet was suspended in 200 μl of lysis buffer (1% (w/v) SDS, 50 mM Tris pH 8.1, 10 mM EDTA and 1x protease inhibitor (Roche) and sonicated for 15 x 30 s before centrifugation at 5,000 g for 10 min. The lysate was then diluted in 1x ChIP dilution buffer (0.01% (w/v) SDS, 167 mM NaCl, 16.7 mM Tris, pH8.1, 1.2 mM EDTA and 1.1% (v/v) Triton X-100). 0.5 ml lysate was immunoprecipitated with 5 μl of anti-HNF4α or anti-actin polyclonal antibody at 4^{o}C overnight. The immune complexes were captured by adding 20 μl of 50% (w/v) protein A agarose beads (upstate biotech) and centrifuged at 3,000 x g for 1 min. The captured immune complexes were washed with immune wash buffer (0.1% (v/v) SDS, 2 mM EDTA, 1% (v/v) Triton X-100, 20 mM Tris-HCl, pH 8.1 and 500 mM NaCl) and eluted from the beads with 1% (w/v) SDS, 0.1M $NaHCO_3$. Protein was removed from the DNA by digestion with 10 μg/ml proteinase K at 50^{o}C for 1 h. The DNA from input and immunoprecipitated fractions were purified with the Nucleospin Extract II kit (Macherey-Nagel GmbH) and eluted with 50 μl TE buffer. 2 μl of the eluate was subjected to Q-PCR using –108/-90 HNF4α-F and +72/+92 HNF4α-R primers that flank the HNF4α3 site (-26/-14). A similar set of PCR was performed using downstream primers which flank exon 13 of the mouse PC gene [Jitrapakdee et al., 2001]. Q-PCR was carried out as described below except SYBR® Green master mix (Kapa Biosystems) was used in the assays.

Suppression of HNF4α by siRNA

AML12 cells were transfected with HNF4α siRNA using the reverse transfection method. In brief, 1 x 10^6 AML12 cells were mixed with 50 ng of HNF4α siRNA or scramble siRNA (Ambion) and 2 μg of FuGene 6 (Roche) in the presence of 1 ml growth medium for 10 min. The mixture was plated in a 6-well plate and an extra 1 ml of growth medium added. The transfected cells were maintained at 37^{o}C with 5% CO_2 for 48 h before being harvested for RNA extraction and real time RT-PCR analysis.

Quantitative real time RT-PCR(Q-PCR)

Total RNA was extracted from AML12 cells using the RNAeasy miniprep kit (Qiagen). cDNA synthesis was carried out at 50^{o}C for 1 h in a 20 μl reaction mixture containing 1 μg of total RNA, 100 ng random hexamer primers (Invitrogen), 1x reverse transcriptase buffer, 1 mM dNTP and 100 units of superscriptIII reverse transcriptase (Invitrogen). To detect the expression of PC and G6Pase, PCR was carried out in 20 μl reaction mixture containing 1x *Taq*man universal master mix (Applied Biosystems), 2 μl of 1/10 dilution of cDNA, and 0.125 mM primer/probe set described previously [16, 17]. PCR was carried out in an MxPro3000^{TM} thermal cycler (Agilent Technology). The thermal cycling consisted of an initial incubation at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. The expression of PC and G6Pase was normalized to the expression of 18s rRNA and presented as relative gene expression. For ChIP assays, PCR was carried out as described above except the *Taq*man master mix was replaced with SYBR® Green master mix.. All statistical analyses were performed using Student's t-test.

Western blot analysis

Total protein lysates of AML12 cells transfected with siRNA were lysed in 100 μl of RIPA buffer [150 mM NaCl, 10 mM Tris pH7.4, 0.25% (w/v) sodium deoxycholate, 1% (v/v) NP-40, 1 mM EDTA and 1x protein inhibitor cocktail (Sigma)]. 20 μg of protein lysate was subjected to 10% reducing SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane using semi dry blotting. PC bands were detected by rabbit anti-PC polyclonal antibody as described previously [Jitrapakdee et al., 2005], while HNF4α bands were detected using the same antibody that was used in the EMSA. To normalize protein loading, anti-actin polyclonal antibody (Cell signaling) was used to detect β-actin on the blot. The immunoreactive bands were detected using an enhanced chemiluminescence detection system (GE Health Sciences).

3. RESULTS

3.1 The classical PPRE/HNF4α site binds HNF4α but is not required for transcriptional induction by HNF4α

We previously identified the peroxisome proliferator activated receptor responsive element (PPRE), AGGGCAAGGGTCA (-386/-374, antisense strand), as a critical element for PPARγ2-mediated transcriptional activation of PC in adipocytes [Jitrapakdee et al., 2005].

This PPRE resembles the classical direct repeat known as DR1, which is a binding site for HNF4α and several other NRs [Cotnoir-White et al., 2012]. To assess whether HNF4α can bind this PPRE, we performed EMSAs using the nuclear extracts of HEK293T cells overexpressing HNF4α, and two hepatocyte cell lines, namely, human hepatoma HepG2 and mouse hepatoma AML12. As shown in Fig. 1A, incubation of the PPRE/HNF4α dual binding site (designated HNF4α1 site) with HepG2 nuclear extracts produced a clear band shift (lane 2) which was supershifted by the addition of anti-HNF4α antibody (lane 3). A similar, but much stronger, DNA-protein complex was observed when the nuclear extract of HEK293T cells overexpressing human HNF4α was used in the assay (lane 4). The addition of anti-HNF4α antibody to this reaction also produced a strong band shift (lane 5) whereas the addition of anti-PPARγ antibody had no effect (lane 6). Incubation of the HNF4α1 site probe with the nuclear extract of HEK293T cells overexpressing PPARγ2 also produced a clear band shift (lane 7) which was supershifted by an anti-PPARγ antibody (lane 8). A similar result was observed when this probe was incubated with the nuclear extract of AML12 cells (Fig. 1B, lane 1). Incubation of the HNF4α1 site oligonucleotide probe with increasing amounts (5x, 10x and 25x) of unlabeled oligonucleotide caused a gradual decrease in formation of the DNA-protein complex (Fig. 1B, lanes 2-4). The inclusion of anti-HNF4α antibody in the binding reaction markedly inhibited formation of the complex (lane 5), while an anti-PPARγ antibody had no effect (lane 6). The results of these competition experiments indicate that the DNA-protein complex is highly specific and consists mainly of HNF4α.

Having shown that HNF4α binds the HNF4α1 site, we next performed transactivation assays to assess the functional significance of the interaction. A chimeric reporter construct, pGL-P1ΔDraI, comprising the luciferase reporter gene fused to the 603 nucleotides upstream of the P1-promoter's transcription start site (Rojvirat et al., 2011), was co-transfected into AML12 cells with plasmid overexpressing HNF4α. Overexpression of HNF4α resulted in a 4-fold increase in reporter gene activity (Fig. 1C), indicating that the P1-promoter is regulated by HNF4α. Unexpectedly, however, mutation of the HNF4α1 site had no effect on HNF4α-induced transcriptional activation of the promoter. Furthermore, removal of the HNF4α1 site, by deletion of the 5'-flanking sequence to nucleotide position -335, did not significantly affect HNF4α-mediated transcriptional activation of reporter activity. These results point to the presence of an alternative HNF4α binding site(s) within the first 335 nucleotides upstream of the P1-promoter's transcription start site.

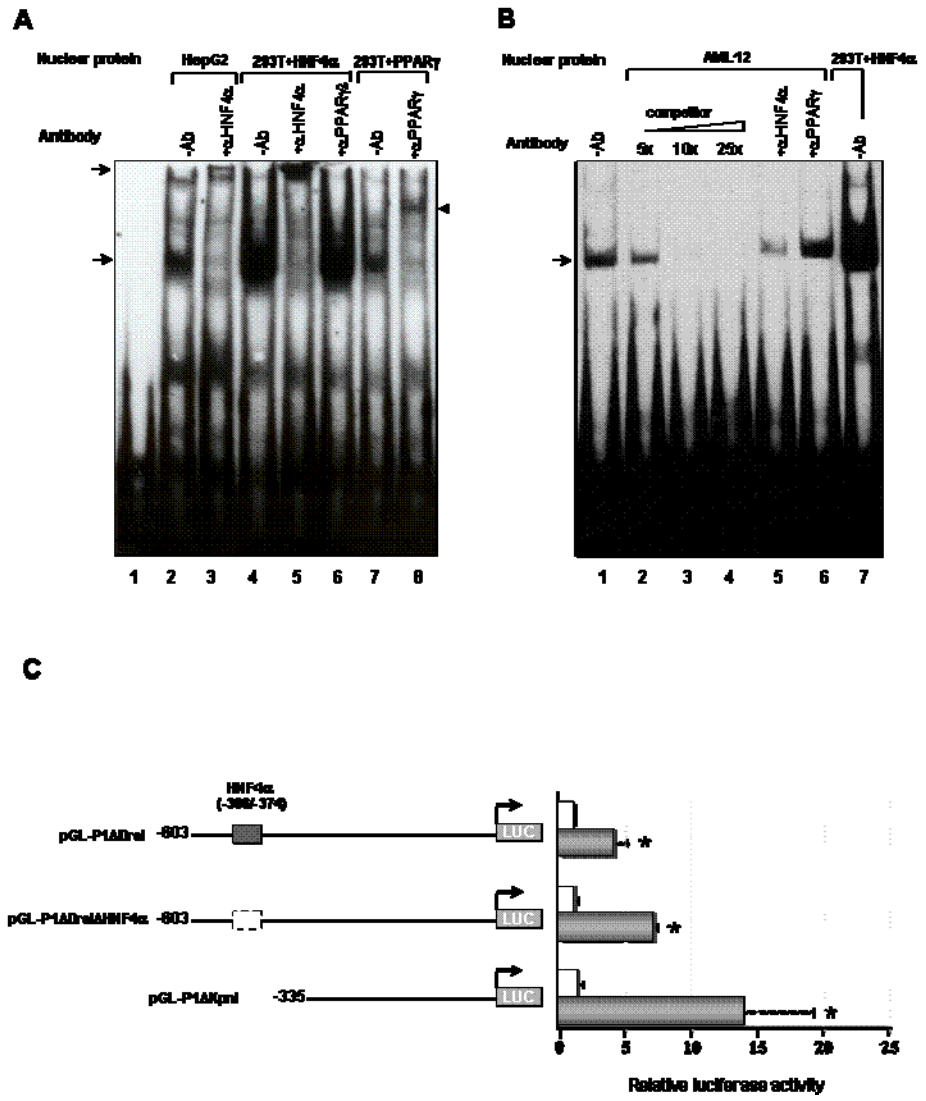


Fig. 1. Binding of HNF4α to the HNF4α1 site and transcriptional induction of the P1-promoter by HNF4α. A, EMSA of biotin-labeled double stranded oligonucleotide probe alone (lane 1), and in the presence of HepG2 nuclear extract with or without anti-HNF4α antibody (lanes 2 and 3). Probe was also incubated with nuclear extract of HEK293T cells overexpressing HNF4α in the absence (lane 4) or presence of anti-HNF4α antibody (lane 5), anti-PPARγ antibody (lane 6) or incubated with nuclear extract of HEK293T overexpressing PPARγ2 in the absence (lane 7) or presence of anti-PPARγ antibody (lane 8). **B**, EMSA of biotin-labeled double stranded oligonucleotide harboring HNF4α1 site (-386/-374) dual site probe in the presence of AML12 nuclear extract (lane 1) with excess amounts of unlabeled double stranded oligonucleotide competitor (5x, 10x, 25x) (lanes 2-4). Lanes 5 and 6, probe incubated with nuclear extract in the presence of anti-HNF4α antibody or anti-PPARγ antibody, respectively. Lane 7, probe incubated with nuclear extract of HEK293 overexpressing HNF4α. **C**, The 603 nucleotides of the P1-promoter luciferase reporter construct (pGL-P1ΔDraI) or its mutants were co-transfected with empty plasmid (white bars) or plasmid overexpressing HNF4α (grey bars) into AML12 cells. Promoter activity obtained from various constructs is presented as fold change relative to the value obtained from the

cells which were transfected with pGL-P1ΔDraI and empty vector, which was arbitrarily set as 1. *$p < 0.05$.

3.2 Non-perfect DR1 is required for basal transcription

Computer assisted analysis - using the PROMO software [Messeguer et al., 2002] - of the 335 nucleotides 5' of the P1-promoter's transcription start site revealed two potential binding sites for HNF4α, located at -118/-106 (sense strand) and -26/-14 (antisense strand) (Fig. 2A). The -118/-106 (HNF4α2) site possesses a non-perfect DR1 (5'-AGCCAGTGGCCCA-3'), while the -26/-14 (HNF4α3) site (5'-NNNNCAAAGTTCT-3') resembles the recently reported HNF4-specific binding motif (H4-SBM), 5'-NNNNCAAAGTCCA-3' [Fang et al., 2012].

To examine whether these potential HNF4α binding sites, together with the HNF4α1 site, contribute to transcriptional regulation of the P1-promoter under basal conditions (HNF4α expression is limited in the cell), we introduced single, double and triple HNF4α binding site mutations into the pGL-P1ΔDraI reporter construct. The wild type and mutant constructs were transiently transfected into AML12 cells and the relative luciferase activities measured. As shown in Fig. 2B, mutation of the HNF4α1 and HNF4α3 sites had no effect on reporter activity. Mutation of the HNF4α2 site, however, reduced promoter activity by approximately 50%. No further loss of reporter activity was observed when the HNF4α2 site mutation was combined with mutation of HNF4α1 (ΔHNF4α1ΔHNF4α2) or HNF4α3 (ΔHNF4α2ΔHNF4α3) binding sites, or when all three sites were mutated together (ΔHNF4α1ΔHNF4α2ΔHNF4α3). Similar results were obtained when the HNF4α2 and HNF4α3 site mutations were introduced, singly and together, into a 5' truncated reporter construct that lacks the HNF4α1 site (pGL-P1ΔDelA). Mutation of the HNF4α2 site reduced basal promoter activity by 50%, while the double mutation decreased the promoter activity only slightly further (Fig. 2B). Taken together, the above results suggest that only the HNF4α2 site contributes to transcriptional regulation of the P1-promoter under basal conditions.

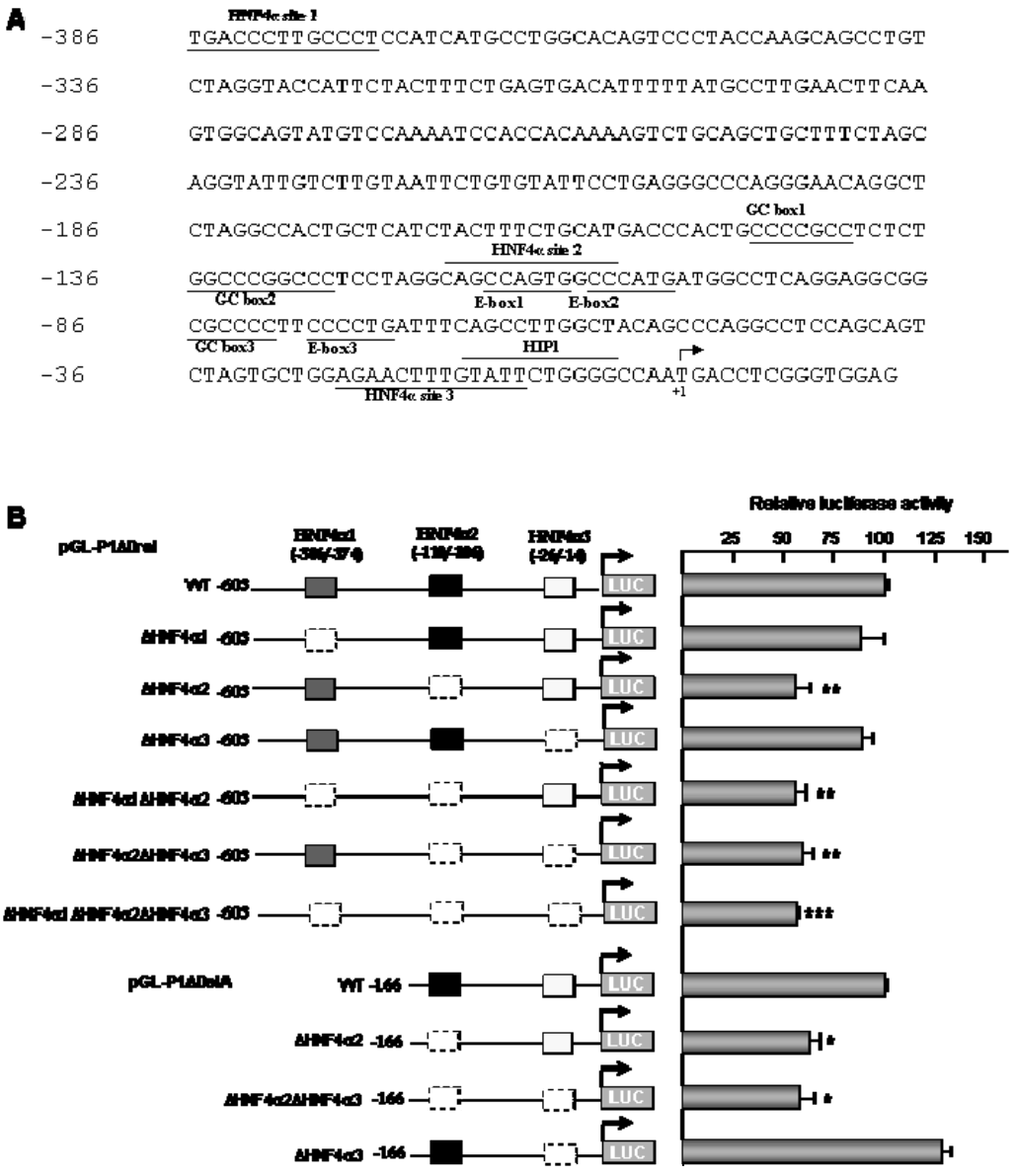


Fig. 2. Effect of mutations of HNF4α binding sites on P1-promoter activity under basal conditions. **A**, Nucleotide sequence of the 398 nucleotides proximal to the transcription start site of the P1-promoter. The HNF4α1, HNF4α2, HNF4α3 sites and E-boxes are underlined. Also shown are the HIP1, transcription start site (+1) and three GC boxes that are required for basal transcription (15). **B**, Mutations of the HNF4α1, HNF4α2 and HNF4α3 sites were introduced, singly and in combination, into pGL-P1ΔDraI or pGL-P1ΔDelA reporter constructs and transiently transfected into AML12 cells. The luciferase activity of each construct was normalized to β-galactosidase activity and expressed as relative luciferase activity. The values obtained from mutated constructs are expressed relative to the corresponding parent (WT) construct which was arbitrarily set as 100%. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3 H4-SBM proximal to the transcription start site is required for HNF4α-mediated transcriptional activation

Although the HNF4α2 site appears to regulate P1-promoter activity under basal conditions, it is unknown which of the three sites function in HNF4α-mediated transcriptional activation. To address this question, the wild type P1-promoter construct (pGL-P1ΔDraI) and plasmid overexpressing HNF4α were transiently co-transfected into AML12 cells and the reporter activities measured. As shown in Fig. 3, overexpression of HNF4α caused a 4-fold increase in promoter activity. Mutation of the HNF4α1 site did not affect HNF4α-mediated transcriptional activation of the reporter gene; unexpectedly, neither did mutation of the HNF4α2 site. Mutation of the HNF4α3 site, however, reduced HNF4α-mediated reporter gene activity by approximately 65%. Combining the HNF4α3 site mutation with HNF4α1 or HNF4α2 site mutations did not further decrease HNF4α-mediated transcriptional activation of the P1-promoter, nor did the combined mutation of all three sites. To confirm the above results, we conducted a parallel series of experiments with the pGL-P1ΔDelA reporter construct that lacks the HNF4α1 site. As shown in Fig. 3, HNF4α produced a 13-fold increase in activity of the pGL-P1ΔDelA reporter gene – an effect several fold greater effect than that observed with the full-length reporter. Similar to the full-length construct, mutation of the HNF4α2 site did not significantly affect reporter activity; however, mutation of the HNF4α3 site reduced activity by 75%. The double mutation of HNF4α2 and HNF4α3 sites again caused no further decrease in reporter activity. These data indicate that HNF4α mediates transactivation of the P1-promoter via the HNF4α3 site, although the degree of transactivation by HNF4α seems variable, depending on the promoter length.

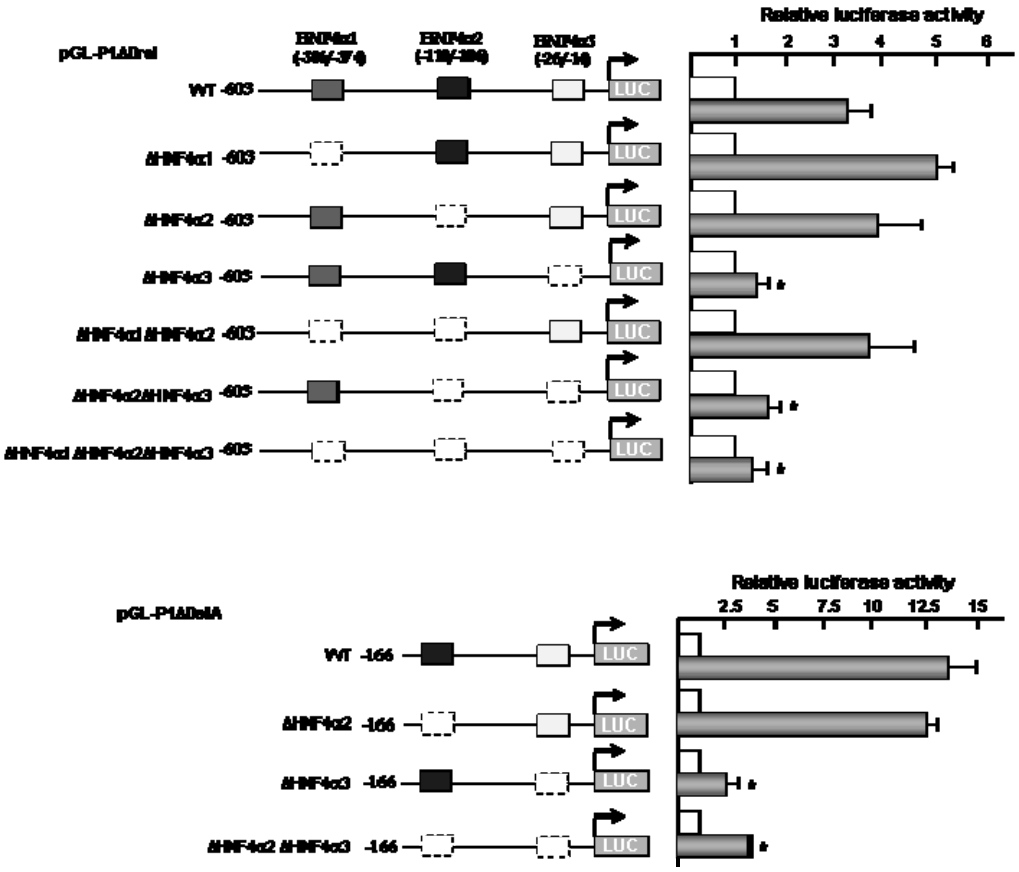


Fig. 3. The HNF4α3 site is required for HNF4α-mediated transcriptional activation of the P1- promoter. pGL-P1ΔDraI, pGLP1ΔDelA or their mutants harboring disrupted HNF4α binding sites were co-transfected into AML12 cells with plasmid overexpressing empty vector (pcDNA3, white bars) or HNF4α (grey bars). The luciferase activity of each construct was normalized to the β-galactosidase activity and expressed as relative luciferase activity. Relative luciferase values obtained from co-transfecting cells with WT (pGL-P1ΔDraI or pGL-P1ΔDelA) or their mutants and HNF4α plasmid were presented as fold change relative to those obtained from those co-transfected with WT (pGL-P1ΔDraI and pGL-P1ΔDelA), each of which was arbitrarily set as 1. *P < 0.05.

3.4 HNF4α solely binds HNF4α3 site while USFs bind HNF4α2 site and regulate P1-activity

With mutational analysis of the HNF4α2 and HNFα3 sites revealing only the latter to be involved in HNF4α-mediated induction of the P1-promoter, we next examined their abilities to bind HNF4α. To this end, EMSAs were performed with AML12 cell nuclear extract and double stranded oligonucleotide probes harboring either the HNF4α2 or HNF4α3 site. Incubation of AML12 cell nuclear extract with the HNF4α3 site probe (Fig. 4A) produced a single strong DNA-protein complex (lane 2). Formation of the complex was competitively inhibited by excess amounts of the equivalent unlabeled oligonucleotide probe (lanes 3-5), but not by oligonucleotide lacking the core HNF4α3 sequence (lane 6). Inclusion

of anti-HNF4α antibody in the binding reaction completely eliminated complex formation (lane 7). Similar results were obtained when the HNF4α3 probe was incubated with purified HNF4α, although the DNA-protein complex formed was stronger (data not shown). These results show that the HNF4α3 site binds HNF4α, and are consistent with the involvement of this site in HNF4α-mediated induction of the P1-promoter.

Although mutation of the HNFα2 site had no effect on induction of the P1-promoter by HNF4α, EMSA with the HNF4α2 site probe indicated that HNF4α is capable of binding to this site. Incubation of the HNF4α2 site probe with AML12 cell nuclear extract produced a single DNA-protein complex (Fig. 4B, lane 2), although this was observed after extended exposure of the blot and, therefore, probably reflects relatively weak binding of nuclear protein(s) to the site. Nevertheless, formation of the complex was competitively inhibited by unlabeled HNF4α2 probe in a concentration dependent manner (lanes 3-5), while a 50-fold excess of unrelated oligonucleotide did not affect the complex formation (lane 6). As shown in Fig. 4C, the inclusion of anti-HNF4α antibody in the binding reaction only partially inhibited formation of the complex (lane 3), thereby indicating that this complex is not entirely HNF4α, and that one or more other factors bind the HNF4α2 site. A disruption of the interaction of these alternative factor(s) with the HNF4α2 site could explain the reduced basal promoter activity of the HNF4α2 site mutant P1-promoter. Re-examination of the surrounding sequence revealed two E-boxes that overlap the 5' (E-box 1, -116/-111) and 3' (E-box 2, -109/-104) ends of the HNF4α2 site (-118/-106) [Fig.2A]. As the E-box motif is a potential binding site for upstream stimulatory factor (USF) 1 and 2 transcription factors [23], we explored the possibility of USF1/USF2 binding to the HNF4α2 site. In EMSAs with the HNF4α2 probe, inclusion of antibodies to USF1 (lane 4), USF2 (lane 5), or both (lane 6) markedly reduced the amount of complex formed; combining either of the anti-USF1 or anti-USF2 with anti-HNF4α antibody caused a slight further reduction (lanes 7 and 8). These results indicate that, in addition to HNF4α, USF1 and USF2 can bind the HNF4α2 site in the P1-promoter. We, therefore, went on to examine the potential for USF-mediated regulation of the P1-promoter through this site.

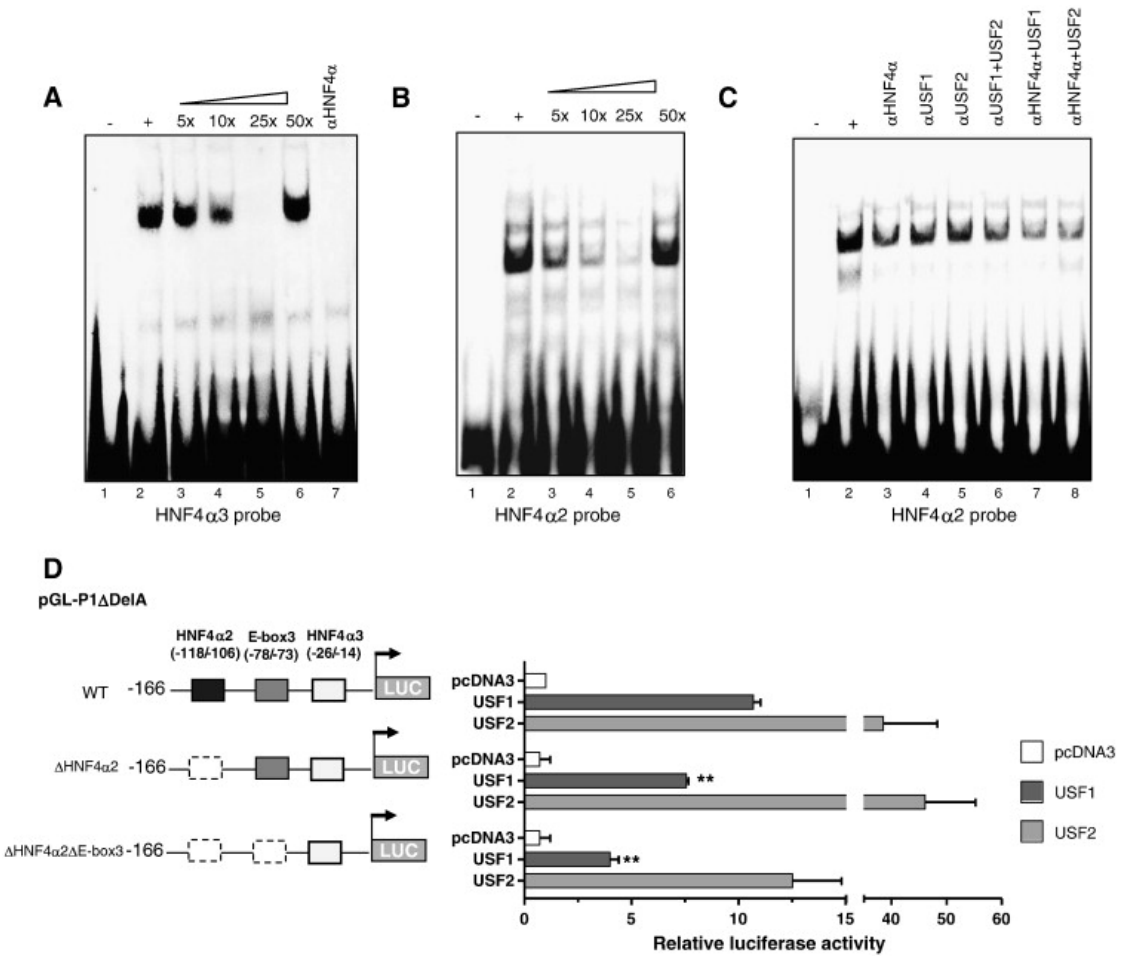


Fig. 4. EMSA of HNF4α2 and HNF4α3 sites with AML12 nuclear extract and transactivation of pGL-P1ΔDelA by USF1 and USF2. EMSAs of biotin-labeled double stranded oligonucleotides harboring HNF4α3 site **(A)** or HNF4α2 probes **(B, C)** with AML12 cell nuclear extracts**. A**, HNF4α3 site probe alone (lane 1), or in the presence of AML12 nuclear extract alone (lane 2) or together with excess (5x, 10x and 25x) unlabeled double stranded oligonucleotide competitor (lanes 3-5) or excess (50x) unrelated double stranded oligonucleotide competitor (lane 6), or with anti-HNF4α antibody (lane 7). **B,** HNF4α2 site probe alone (lane 1), or probe in the presence of nuclear extract alone (lane 2) or together with excess (5x, 10x and 25x) unlabeled double stranded competitor (lanes 3-5) or excess 50x unrelated double stranded oligonucleotide competiror (lane 6). **C**, HNF4α2 probe alone (lane 1) and in the presence of AML12 nuclear extract alone (lane 2) or together with anti-HNF4α (lane 3), anti-USF1 (lane 4), anti-USF2 (lane 5), anti-USF1 and anti-USF2 (lane 6), anti-HNF4α and anti-USF1 (lane 7), or anti-HNF4α and anti-USF2 (lane 8) antibodies. Blots shown in B and C were obtained from the extended exposure. **D**, The 166 nucleotide P1-promoter luciferase reporter construct (pGL-P1ΔDelA) with an intact (WT) or mutated HNF4α2 (ΔHNF4α2) site was co-transfected with empty plasmid or plasmid overexpressing USF1 or USF2 into AML12 cells. Promoter activity is presented as fold change relative to the value obtained from cells transfected with pGL-P1ΔDelA and empty vector, which was arbitrarily set as 1. **P < 0.01

USF1/2-mediated regulation of the P1-promoter was demonstrated in AML12 cells co-transfected with the pGL-P1ΔDelA reporter construct (containing the HNF4α2 and HNF4α3 sites) and plasmid overexpressing USF1 or USF2. As shown in Fig. 4D, overexpression of USF1 led to a 10-fold increase in P1-promoter activity, while overexpression of USF2 caused an even greater 40-fold increase. Mutation of the HNF4α2 site resulted in a 25% reduction in USF1-mediated induction of P1-promoter activity, but did not affect USF2-mediated activation. The remaining reporter activity could easily be achieved through E-box 3 which is located at -78/-73. To examine the functional importance of E-box 3, a P1-promoter with combined mutation of the HNF4α2 site and E-box 3 was generated and co-transfected into AML12 cells with plasmid overexpressing USF1 or USF2. The double mutant resulted in 50% and 70% loss of USF1- and USF2-mediated transcriptional activation of P1-promoter activity, respectively. This result suggests that while USF2 exerts its transactivation ability through E-box3, USF1 exerts its transactivation activity through both the HNF4α2 site and E-box 3.

3.5 HNF4α binds the three HNF4α binding sites with different affinities

As EMSAs showed that all three sites are able to bind HNF4α, we looked to their affinities to explain the lack of function of the HNF4α1 and HNF4α2 sites in HNF4α-induction of the P1-promoter. Affinities for HNF4α were compared in binding assays in which increasing concentrations (60, 120, 240, 360 and 480 fmoles) of HNF4α1, HNF4α2 or HNF4α3 site oligonucleotide probes were incubated with a limited amount (100 ng) of purified HNF4α (Fig. 5A). Quantification of the DNA-protein complex bands in Fig. 5A (same exposure times) revealed that HNF4α bound the HNF4α1 and HNF4α3 sites with similar affinity, while its affinity for HNF4α2 was markedly lower (Fig. 5B). This poor binding of HNF4α to the HNF4α2 site is consistent with the transactivation experiment showing that mutation of the HNF4α2 site had no effect on HNF4α-mediated activity (Fig. 3). The similar affinity of HNF4α for the HNF4α1 and HNF4α3 sites, however, does not lend itself to a simple explanation for the lack of involvement of the former in HNF4α induction of the promoter.

Although the HNF4α1 and HNF4α3 sites bind HNF4α with similar affinities, their sequences are notably distinct: the HNF4α3 site resembles the recently described H4-SBM (viz. NNNNCAAAGTCCA) [Fang et al., 2012], while the HNF4α1 site resembles the classic

DR1 motif (viz. AGGTCAxAGGTCA). As only mutation of the HNF4α3 site affected HNF4α-mediated transactivation of the P1-promoter, we hypothesized that the H4-SBM sequence was specifically required for the effect. We therefore substituted the -26/-14 H4-SBM with the consensus DR1 sequence (AGGTCAGAGGTCA) (H4-SBM:DR1 mutant) and measured the effect on HNF4α-induced P1-promoter activity. The chimeric reporter construct was effectively transactivated by HNF4α despite the substitution; however, the magnitude of the response was approximately 25% less than that observed with the parent reporter, as was its basal transcriptional activity (Fig. 5C).

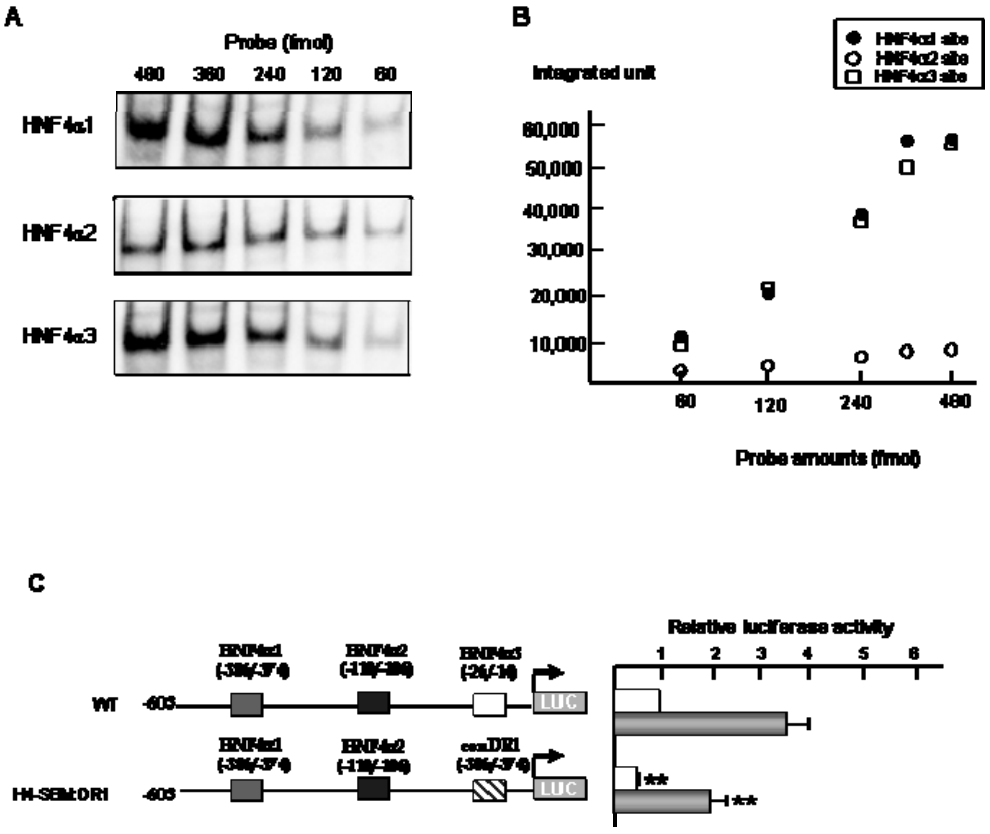
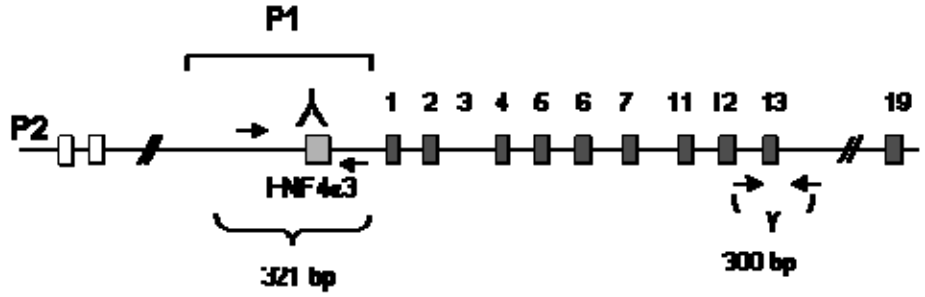


Fig. 5. HNF4α binds to HNF4α1, HNF4α2 and HNF4α3 sites with different affinities and DR1 and H4-SBM cannot be functionally substituted. **A**, Various amounts of biotin-labeled double stranded oligonucleotides harboring HNF4α1, HNF4α2 and HNF4α3 sites were incubated with 100 ng of purified HNF4α. **B**, The intensities of the HNF4α-bound complexes were plotted against the concentration of oligonucleotide probe (fmol). **C**, Relative luciferase expression of the pGL-P1ΔDraI construct (WT) in which the HNF4α3 site was converted to a conserved DR site (H4-SBM:DR1) co-transfected with HNF4α. Relative luciferase values obtained from AML12 cells transfected with WT or H4-SBM:DR1 mutant with HNF4α plasmid (grey bar) were presented as fold change relative to those obtained from transfecting the with WT or mutants with empty vector (white bar), which was arbitrarily set as 1. **P < 0.01.

3.6 HNF4α binds to the P1-promoter in vivo

We also performed chromatin immunoprecipitation (ChIP) assays to confirm the binding of HNF4α to the HNF4α3 site. Transcription factor-bound soluble chromatin was prepared from AML12 cells. The HNF4α-bound chromatin fragments were immunoprecipitated with anti-HNF4α antibodies and amplified by PCR using a primer pair that flanks the HNF4α3 site (see Fig. 6A). An anti-β-actin antibody was used to prepare the negative control sample. Compared with the input fraction, approximately 2.2% of the HNF4α-bound HNF4α3 site was detected in samples immunoprecipitated with anti-HNF4α antibody (Fig. 6B). In marked contrast, only 0.05% of the HNF4α-bound HNF4α3 site was detected in samples immunoprecipitated with anti-β-actin antibody (Fig. 6B). There was also no detectable signal generated from a second primer pair located several nucleotides downstream of the enhancer region in the samples which were immunoprecipitated with anti-HNF4α and anti β-actin antibodies. It is noted that although we were able to detect HNF4α binding to HNF4α3 sites, given the close proximity of the three sites, we cannot rule out the possibility that the immunoprecipitated chromatin fragments also contained binding of HNF4α to the HNF4α1 and HNF4α2 sites. This close proximity (approximately 370 nucleotides between HNF4α1 and HNF4α3) makes it technically impossible to immunoprecipitate chromatin which contains only HNF4α3 site3-bound HNF4α.

A



B

Antibody	HNF4α-F/R primers	Exon 13-F/R primers
HNF4α	2.2±0.05%	0.1±0.07
Actin	0.05±0.01%	ND

Fig. 6. Chromatin immunoprecipitation assay of HNF4α binding to HNF4α3 site. **A**, Schematic representation of the P1-promoter region with the HNF4α3 site and primer binding sites indicated. **B**, The HNF4α-bound chromatin was prepared from AML12 cells and precipitated with anti-HNF4α antibody. The HNF4α-DNA fragments were amplified by Q-PCR using primers that flank the HNF4α3 site (HNF4α-F/R), or primers that locate between exon 13 (Exon13-F/R) (15) and normalized to the input levels. The input was the sonicated and HNF4α-bound DNA cross-linked before immunoprecipitating with antibody. ND, not detectable.

3.7 Suppression of HNF4α down-regulates PC expression

Finally, the functional relevance of HNF4α in the regulation of PC expression was examined by siRNA knockdown. AML12 cells were transiently transfected with siRNA targeted to HNF4α, and the level of PC expression monitored by real time RT-PCR and Western blot. Transient transfection of HNF4α siRNA reduced the levels of both HNF4α mRNA and HNF4α protein expression by approximately 90% (Fig. 7A and 7B). This resulted in the down-regulation of PC mRNA and PC protein by 60% and 50%, respectively (Fig. 7A and 7B). We also found that suppression of HNF4α lowered G6Pase mRNA expression by 50%. The latter finding is consistent with the 70% reduction in hepatic G6Pase mRNA expression in HNF4α null mice (Rhee et al. (2003).

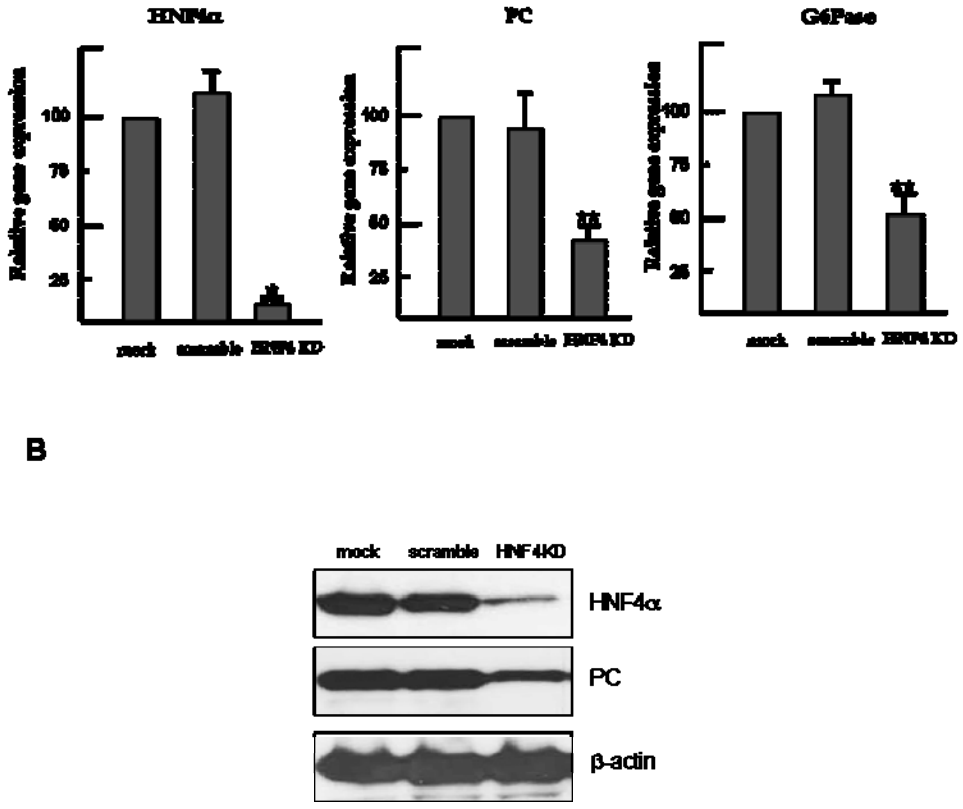


Fig. 7. Suppression of HNF4α down-regulates PC expression in AML12 cells. AML12 cells were mock-transfected or transfected with HNF4α siRNA (HNF4 KD) or non-specific siRNA (scramble) control. (A) The expression of HNF4α, PC and G6Pase mRNAs in these and mock-transfected cells (mock) were measured by quantitative real time PCR and normalized to 18s rRNA expression. The values obtained from scramble and HNF4 KD transfected cells are expressed relative to that obtained from the mock transfection which was arbitrarily set as 100%. The values shown are means ± standard deviation (n = 3). *P < 0.01; **p < 0.05. (B) Western blot analysis of the expression of HNF4α and PC proteins in mock- and siRNA-transfected AML12 cells. Membrane was stripped and re-probed with anti-β-actin antibody as a loading control.

4. Discussion

HNF4α is a member of the non-steroid nuclear receptor superfamily of ligand-dependent transcription factors (NR2A1) [Bookout et al., 2006; Sladek, 2011]. HNF4α regulates liver development and the maintenance of liver function [Odom et al., 2004]. The latter role involves the transcriptional regulation of several enzymes in key metabolic pathways, such as glucose metabolism, lipoprotein metabolism, cholesterol metabolism and bile acids biosynthesis [Kaestner, 2010]. The functional importance of HNF4α in the control of glucose homeostasis is evidenced by the finding that mutation of this gene results in the

development of a specific form of diabetes known as maturity onset diabetes of the young 1 (MODY1) [Yamagata et al., 1996].

The mouse and rat PC genes possess two alternative promoters to regulate its transcription. While the distal (P2) promoter is active in the pancreatic β-cells, the proximal (P1) promoter is active in both gluconeogenic (liver) and lipogenic (adipose) tissues [Jitrapakdee et al., 1997; Jitrapakdee et al., 1998]. This suggests that the liver and adipose tissues employ different tissue-specific factors to direct PC transcription. PC expression in adipocytes is largely regulated by PPARγ2 which activates the P1-promoter via PPRE binding; little, however, is known about the transcriptional regulator(s) of PC expression in the liver. The -386/-374 PPRE located in the P1-promoter of the mouse PC gene resembles the classical DR1 motif – a binding site for several nuclear receptors (NRs), including HNF4α. This prompted us to suspect the involvement of HNF4α in the regulation of PC expression. Supportive of such a role, overexpression of HNF4α in AML12 cells resulted in a significant increase in the activity of a P1-promoter reporter construct. Unexpectedly, however, the PPRE (HNF4α1 site) was not required for this effect (nor for basal promoter activity), despite EMSA confirming its ability to bind both endogenous and exogenous HNF4α. Having ruled out the PPRE as the HNF4α-responsive element, the computer-assisted identification of potential HNF4α binding sites at -118/-106 (HNF4α2) and -26/-14 (HNF4α3) provided two alternative candidates. Although both of these sequences bound HNF4α in EMSA, only the HNF4α3 site was functional with respect to HNF4α-mediated transcriptional activation. The HNF4α3 site resembles the recently described HNF4-specific binding motif (H4-SBM), NNNNCAAAGTCCA [Fang et al., 2012]. While not affecting basal promoter activity, mutation of the HNF4α3 site caused a decrease in HNF4α-mediated P1-promoter activity; the opposite was true for the HNF4α2 site.

It is unclear why mutation of the HNF4α1 site did not affect HNF4α-mediated transcriptional activation of the P1-promoter despite binding purified HNF4α with an almost identical affinity as the HNF4α3 site (Fig. 5). An explanation might lie in the relative specificities of the two binding sites. The HNF4α1 site resembles the DR1 motif that, unlike the H4-SBM, is recognized by multiple NRs. Therefore, while unlikely at the H4-SBM-like HNF4α3 site, it is reasonable to expect competition between HNF4α and other NRs for binding to the HNF4α1 site. While such competition could explain the HNF4α1 site's lack of responsiveness to HNF4α [Mangelsdorf and Evans, 1995], it is perhaps an unlikely

explanation in the context of exogenous HNF4α-overexpression (used in the present study). Promoter context, therefore, might better explain the difference between the HNFα1 and HNFα3 site's abilities to mediate HNF4α transactivation. Promoter context is known to influence the specific transcriptional response to different NRs. Nakshatri and Bhat-Nakshatri (1998) have shown that the DR1 of the acyl-CoA oxidase (ACO) gene promoter acts as a PPRE, allowing robust transcriptional induction by PPARγ. However, when placed in the enhancer region of cellular retinol-binding protein II (CRBPII) promoter, the same DR1 sequence acts as an HNF4α-responsive element. The distal location of the HNF4α1 (PPRE) site compared to the proximal location of the HNF4α3 site may also restrict HNF4α interplay with the basal transcriptional complex that facilitates activation of the P1-promoter. Whatever the mechanism, our results suggest that the functional importance of the HNF4α1 (PPRE) site is restricted to PPARγ induction in adipocytes.

As suggested above, the proximal location of the HNF4α3 site to the transcription start site may facilitate interaction of HNF4α with RNA polymerase II to assist initiation of PC gene transcription. It is also noted that the HNF4α3 site is close to GC box 3, a binding site for Sp1 and Sp3 [Rojvirat et al., 2011]. This site has previously been shown to be an important binding site for basal transcription as well as for Sp1- and Sp3-mediated activation of the P1-promoter [Rojvirat et al., 2011]. The close proximity of the HNF4α binding site and GC box may allow interplay between Sp1, Sp3 and HNF4α that in turn stimulates transcription from the P1-promoter. In the promoters of the human sterol 27-hydroxylase [Takahashi et al., 2002] and haem oxygenase-1 genes [Garuti et al., 2002], Sp1 and Sp3 cooperate with HNF4α to enhance both basal and HNF4α-mediated transcription. Synergistic transactivation of the apolipoprotein CIII gene promoter by Sp1 and HNF4α is also mediated through a direct interaction [Kardassis et al., 2002]. It is interesting to note that the H4-SBM found in the HNF4α3 site appears to overlap with the initiator site [Jitrapakdee et al., 1997] (see Fig. 2) which resembles the initiator of many housekeeping genes [Means et al., 1990]. This element is known to bind to housekeeping initiator protein 1 (HIP1). The 25% reduction in promoter activity observed in the H4-SBM:DR1 mutant under basal conditions is likely due to loss of the HIP-1 binding site resulting from substitution mutation. Several studies have shown that in TATA-less genes, mutation of this initiator sequence reduces the efficiency of transcription, possibly due to improper positioning of RNA polymerase II [Means et al., 1990; Javahery et al., 1994; Li et al., 1996]. The ability to respond to HNF4α

was retained in the chimeric H4-SBM:DR1 promoter, with the magnitude of HNF4α mediated transactivation reduced compared to wild type by a similar extent as basal activity. This result suggests that DR1 might, to some extent, be functionally substituted with the H4-SBM in this promoter context.

The affinity of purified HNF4α for the HNF4α2 site, which possesses a non-perfect DR1, was relatively poor compared to the HNFα1 and HNFα3 sites. Consistent with this observation, mutation of the HNFα2 site had no effect on HNF4α-mediated transcription of the P1-promoter; it did, however, significantly reduce basal promoter activity. This is at least in part attributed to the presence of two adjacent E-boxes that co-localize with the HNF4α2 site and serve as a binding site for the non-tissue specific factors, USF1 and USF2 [Ferre-D'Amare et al., 1992]. These two transcription factors have been shown to play a role in the regulation of glucose responsive genes such as L-type pyruvate kinase and glucokinase [Girard et al., 1997]. The reduction in basal P1-promoter activity caused by the HNF4α2 site mutation is likely due to a disruption of the interaction with USF1. This conclusion comes from two pieces of evidence. Firstly, as shown by EMSA using anti-USF1 antibody, USF1 was capable of binding to an HNF4α2 site probe. Secondly, the 10-fold increase in wild type P1-promoter activity caused by overexpression of USF-1 was reduced by 25% by disruption of the HNF4α2 site, and by 50% when the HNF4α2 site was disrupted together with the downstream E-box (E-box 3). Interestingly, overexpression of USF2 caused a 50-fold increase in P1-promoter activity and this activation was mediated solely through E-box 3. These data indicate that USF1 rather than USF2 stimulates P1-promoter activity through the HNF4α2 site. In the rat PC gene, USF1 and USF2 have previously been implicated in the regulation of distal (P2) promoter activity in pancreatic β-cells via interplay with the β-cell specific factor, FoxA2 [Boonsaen et al., 2007].

Based on the results of the present study and that already known about PC regulation in the adipocyte, we propose the following model (illustrated in Fig. 8) for the tissue-specific regulation of the PC gene. Adipocytes are abundant in PPARγ2 which binds the PPRE (HNF4α1 site) and turns on PC expression [Jitrapakdee et al., 2005]. In contrast, the liver expresses high levels of HNF4α to regulate PC expression via binding to the H4-SBM (HNFα3 site). This is supported by Western blot analysis demonstrating that PPARγ2 expression is extremely low while HNF4α is highly abundant in AML12 cells (data not shown). Other ubiquitously expressed transcription factors, including Sp1 and Sp3 via GC box 3 [Rojvirat et al., 2011], USF1 via HNF4α2 site, and USF2 via other E-boxes, cooperate

with HNF4α to stimulate P1-activity in a liver-specific manner. Furthermore, CREB regulates P1-promoter activity via the distal CRE [Thonpho et al., 2010].

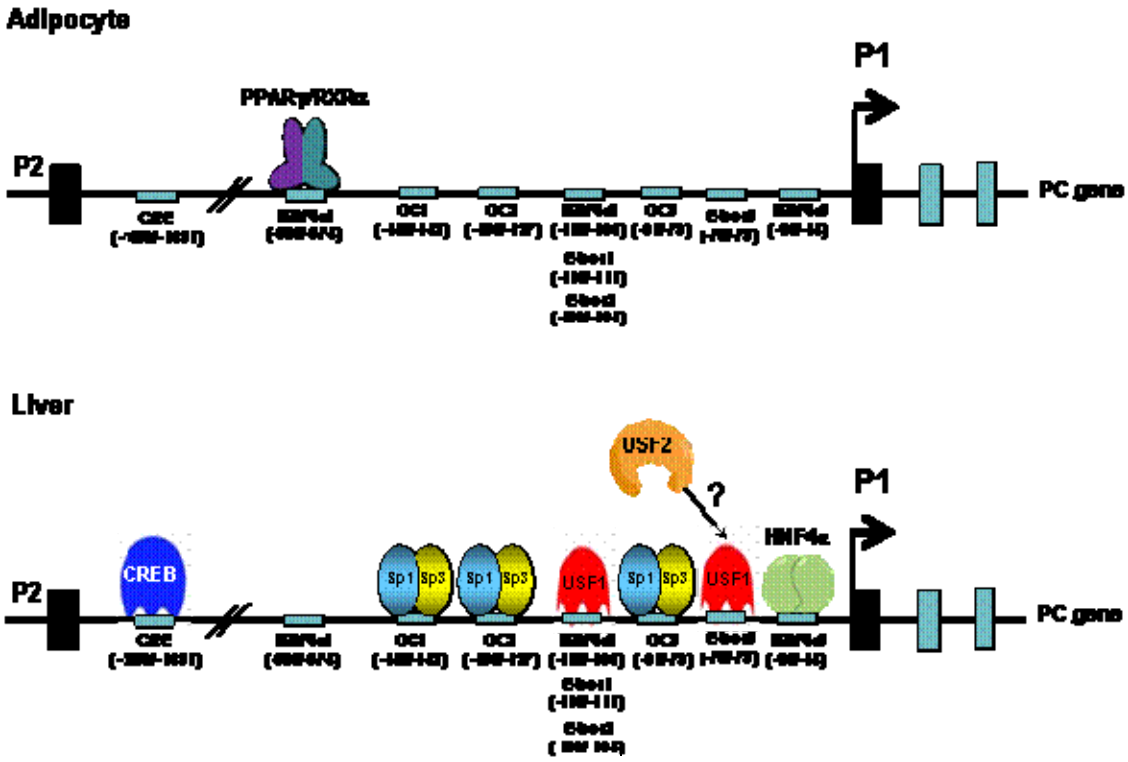


Fig. 8. Schematic diagram showing differential regulation of PC in the adipocyte and liver. The PC gene is regulated by P1 (proximal) and P2 (distal) promoters. P1 is active in the liver and adipocytes while P2 is active in pancreatic β-cells. In adipocytes, P1 is solely regulated by PPARγ via the -386/-374 HNF4α1 site (DR1). In the liver, PC is regulated by an interplay of transcription factors at the P1-promoter: HNF4α binding to the -26/-14 HNF4α3 site (H4-SBM), Sp1/Sp3 binding to GC box 1 (GC1), GC box 2 (GC2), and GC box 3 (GC3), and USF1 binding to E-box 1 (-116/-111), E-box 2 (-109/-104) and E-box 3 (-78/-73) The -1639/-1631 CRE serves as a cAMP-responsive binding protein (CREB) during cAMP-mediated transcriptional activation.

HNF4α also regulates the gluconeogenic G6Pase and PEPCK genes, but by a substantially different mechanism than we have shown here for the PC gene. In the promoters of G6Pase and PEPCK genes, HNF4α binding sites are part of larger regulatory units that mediate glucocorticoid and cAMP induction of gene expression. The rat G6Pase gene promoter contains a single HNF4α binding site (located at -455/-431) that acts as an accessory factor binding site (AF1) in a complex glucocorticoid responsive unit (GRU) [Imai et al., 1990]. Binding of HNF4α to this site facilitates glucocorticoid binding to the glucocorticoid responsive element and, thereby, the induction of PEPCK gene expression; deletion of the site lowers glucocorticoid-mediated induction of the PEPCK gene by approximately 50% [Hall et al., 1995; Stafford et al., 2001]. In the G6Pase gene, four HNF4α

binding sites, located at -672/-667, -516/-511, -76/-64 and +9/+15, form part of a GRU [vander Kooi et al., 2005] and a protein kinase A/cAMP-responsive unit (CRU) [Gautier-Stein et al., 2005].

Finally, the functional importance of HNF4α in the regulation of the PC gene was validated in a cellular context. siRNA mediated suppression of HNF4α expression resulted in a 60% reduction in PC mRNA expression, a 50% reduction in PC protein, and approximately halved the expression of G6Pase mRNA. While total ablation of HNF4α in mice is embryonic lethal [Chen et al., 1994], support for these findings can be found in the liver-specific HNF4α knockout mice which are viable but display abnormal metabolism of cholesterol, glucose, and fatty and bile acids [Hayhurst et al., 2001; Hanniman et al., 2006; Inoue et al., 2006; Miura et al., 2006]. Holloway et al. (2008) examined the effect of liver-specific HNF4α ablation on sex-specific genes by microarray analysis and found that PC mRNA was 85% lower in both male and female knockout mice [Holloway et al., 2008]. Although not reporting on PC expression, Rhee et al. (2003) reported reduced expression of both PEPCK and G6Pase genes in these mice. The present report on the transcriptional regulation of PC by HNF4α provides novel molecular insight into how ablation of the HNF4α gene affects PC expression. It appears that HNF4α regulates an overall gluconeogenesis program through the regulation of at least three out of four gluconeogenic enzymes, namely, PC, PEPCK and G6Pase.

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SUMMARY AND IMPLICATIONS

Also the discovery that anti-diabetic drug, metformin which is usually prescribed for type 2 diabeteic patients clearly demonstrates that this drug does not reduced hyperglycemia via inhibition of key gluconeogenic enzyme but it rather exerts its effect through other targets. Nevertheless, this study unravels the complicated regulation of pyruvate carboxylase expression at the transcriptional level, which is mediated through selective activation of two promoters, P1 (proximal) and P2 (distal promoter) in two major tissues, i.e. liver and pancreatic-β cells. These two tissues are important for glucose homeostasis. Identifying the key transcription or regulatory proteins which are HNF4α, Sp1, USF2 and ChREBP as the main regulators which control expression of pyruvate carboxylase will lead to the delveopment of the transcriptional antagonist(s) which would down-regulate expression of this enzyme in liver, resulting in reduction of overt hyperglycemia in the patients.

Output

Publications

1. ***Jitrapakdee, S.** (2012) Transcription factors and coactivators controlling nutrient and hormonal regulation of hepatic gluconeogenesis. *Int J. Biochem. Cell. Biol*. **44**, 33-45. (Q1, Impact factor = 4.907)
2. Thonpho, A., Rojvirat, P., Brown, L.J., Hasan, N.M., ***Jitrapakdee, S** MacDonald, M.J., (2013) Characterization of the distal promoter of the human pyruvate carboxylase gene in pancreatic beta cells. *PLoS One* **8**, e55139. (Q1, impact factor = 3.73)
3. Chavalit, T., Rojvirat, P., Muangsawad, S., ***Jitrapakdee, S.** (2013) Hepatocyte nuclear factor4α regulates the expression of the murine pyruvate carboxylase gene through the HNF4-specific binding motif located in its proximal promoter. *Biochim Biophys Acta (Gene regulatory mechanisms)* **1829**, 987-999. (Q1, impact factor = 5.456).
4. Wutthisatapornchai A, Vongpipatana T, Muangsawat S, MacDonald MJ, ***Jitrapakdee S.** (2014) Multiple E-boxes in the Distal Promoter of the Rat Pyruvate Carboxylase Gene Function as a Glucose-Responsive Element: Coordinate regulation by Sp1, USF2 and ChREBP. *PLoS One* (Accepted) (Q1, impact factor = 3.73)

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Award

TRF-CHE Scopus Researcher Award (Life Science Category), 2011

Students' theses

1. Wutthisathapornchai A. (2014) Identification of b-cell specific and glucose-sensing elements that mediate transcriptional regulation of pyruvate carboxylase in insulinoma cells. PhD thesis, Faculty of Graduate Studies, Mahidol University.
2. Vongpipatana T. (2012) Interation of USF1 and USF2 with glucose-responsive element in the distal promoter of pyruvate carboxylase gene, M.Sc.thesis, Faculty of Graduate Studies, Mahidol University.
3. Chavalit T. (2012) Transcriptional regulation of the proximal promoter of the pyruvate carboxylase gene by Sp1, Sp3 and HNF4a in hepatocytes. M.Sc.thesis, Faculty of Graduate Studies, Mahidol University.

ภาคผนวก

The International Journal of Biochemistry & Cell Biology 44 (2012) 33–45

Contents lists available at SciVerse ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Review

Transcription factors and coactivators controlling nutrient and hormonal regulation of hepatic gluconeogenesis

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ARTICLE INFO

Article history:
Received 5 August 2011
Received in revised form
30 September 2011
Accepted 4 October 2011
Available online 8 October 2011

Keywords:
Coactivator
Gluconeogenesis
Liver
Metabolism
Transcription factor

ABSTRACT

Hepatic gluconeogenesis is a major pathway that maintains normal plasma glucose levels during prolonged starvation. The aim of this review is to provide insights into the integration of transcriptional regulation of gluconeogenic enzyme genes in response to nutritional and hormonal changes. The roles of transcription factors/co-regulators in response to those factors will be discussed. Overall, glucagon and glucocorticoids are positive regulators of gluconeogenesis. Glucagon, via cAMP, promotes the interaction of cAMP-responsive binding protein with CREB-regulated transcription coactivator 2 which facilitates its binding to cAMP-responsive elements (CREs). The response to glucocorticoids is mediated by the glucocorticoid receptor that binds to glucocorticoid responsive elements (GREs) in the promoters of gluconeogenic genes. These CREs and GREs may be arranged as distinct elements or combined to form a "unit" to ensure the maximal transcriptional response to these hormones. The hepatocyte nuclear factors, forkhead O box, and the peroxisome proliferator-activated receptor-γ coactivator 1α can also synergistically increase transcription of gluconeogenic genes. Surtuin 1, an energy sensor can also modify the transcriptional activity of some of these transcription factors. In contrast, insulin secreted during fed conditions acts to repress transcription of gluconeogenic enzymes. This is achieved via activation of Akt/PKB and the consequent disruption of interactions between certain transcription factors/coactivators and their positive response elements in the promoters of those genes. Hypothalamic signaling via the insulin/leptin axis also regulates hepatic gluconeogenesis. Mice lacking the above transcription factors/coactivators show impaired gluconeogenesis, indicating their essential roles in the control of this vital metabolic process.

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Abbreviations: Akt/PKB, acutely transforming retrovirus AKT8 in rodent T cell lymphoma/protein kinase B; AMPK, AMP-activated protein kinase; CBP, CREB-binding protein; C/EBP, CCAAT enhancer binding protein; CNS, central nervous system; CREB, cAMP-responsive binding protein; CRE, cAMP-responsive element; CRU, cAMP-responsive unit; CRTC2/TORC2, CREB-regulated transcription coactivator 2; DAX-1, dosage-sensitive sex reversal adrenal hypoplasia congenital critical region on the X chromosome gene 1; DUSP4, dual specificity protein phosphatase 4; EGR1, early growth response 1; FBPase, fructose-1,6-bisphosphatase; FoxO, forkhead O box; G6Pase, glucose-6-phosphatase; GCN5, histone acetyltransferase GCN5; GR, glucocorticoid receptor; GREs, glucocorticoid responsive elements; GRU, glucocorticoid responsive unit; HNF, hepatocyte nuclear factor; NR4A1, nuclear receptor subfamily 4 group A member 1; PPARγ, peroxisome proliferator-activated receptor-γ; PC, pyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; PGC1α, PPARγ coactivator 1α; PFK2/FBPase2, phosphofructokinase 2/fructose-2,6-bisphosphatase; PKA, protein kinase A; PKC1/λ, protein kinase C 1/λ; SHP, small heterodimer partner; SIK2, serine/threonine-protein kinase SIK2; SIRT1, NAD-dependent deacetylase sirtuin-1; SRC-1, steroid receptor coactivator 1; T2DM, type 2 diabetes mellitus.

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doi:10.1016/j.biocel.2011.10.001

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1. Introduction

Glucose homeostasis is tightly regulated to meet energy demands during the starvation/feeding cycle in animals. Although fat and protein can be mobilized from adipose tissue and muscle, respectively, to supply energy for various organs during prolonged starvation, red blood cells and the brain primarily rely on glucose as their fuel source. During fed conditions, insulin is secreted from pancreatic β-cells in response to the elevated level of plasma glucose. Pancreatic β-cells possess the machinery that senses the rapid increase in plasma glucose during the postprandial period. This machinery includes the expression of glucokinase, high rates of anaplerosis and pyruvate cycling, and an abundance of ATP-sensitive potassium channels which provide "coupling factors" that trigger insulin exocytosis (Maechler and Wollheim, 2001). Binding of insulin to its receptor on the plasma membrane of target cells, i.e., muscle, adipose tissue and liver, results in the rapid uptake of glucose into these cells (Saltiel and Kahn, 2001). In skeletal muscle, glucose is stored as glycogen through glycogenesis, while in adipose tissue glucose is converted to triglycerides through *de novo* fatty acid synthesis (Fig. 1). In the liver, insulin inhibits glycogenolysis and gluconeogenesis while stimulating glycolysis and the synthesis of glycogen and triglycerides (Saltiel and Kahn, 2001). Therefore, the outcome of insulin action is the reduction of plasma glucose levels in the body. During starvation (Fig. 1), when the level of glucose is low, pancreatic α-cells secrete glucagon which stimulates the mobilization of fat from the adipose tissue to the skeletal muscle for β-oxidation. In the liver, glucagon and glucocorticoids stimulate glycogenolysis to supply glucose to red blood cells and the brain while the long term effect of these hormones is the stimulation of hepatic gluconeogenesis (Jiang and Zhang, 2003) (Fig. 1).

2. Gluconeogenesis

Gluconeogenesis is the formation of glucose from non-carbohydrate precursors, namely, pyruvate, lactate, glycerol and glucogenic amino acids (Fig. 2). This biochemical pathway occurs principally in the liver and is essential for survival during prolonged starvation. It also plays a major role in the disposal of lactate and the maintenance of glucose during exercise (Corssmit et al., 2001). Gluconeogenesis requires four additional enzymes which bypass the three irreversible reactions catalyzed by the glycolytic enzymes pyruvate kinase, phosphofructokinase and hexokinase. These four gluconeogenic enzymes are pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase and glucose-6-phosphatase.

2.1. Pyruvate carboxylase (PC)

PC catalyzes the first committed step of gluconeogenesis by converting pyruvate (derived from alanine via alanine aminotransferase or from lactate via lactate dehydrogenase) to oxaloacetate (Utter and Keech, 1960). Oxaloacetate is then converted to phosphoenolpyruvate by mitochondrial or cytosolic phosphoenolpyruvate carboxykinase (see Section 2.2). PC from most sources is allosterically activated by acetyl-CoA (Scrutton et al., 1965). PC also plays non-gluconeogenic roles including lipogenesis and glyceroneogenesis in white adipose tissue (Reshef et al., 2003), glutamate synthesis in astrocytes (Hertz et al., 1999) and glucose-induced insulin secretion in pancreatic β-cells (Hasan et al., 2008) (for review see Jitrapakdee et al., 2006, 2008).

In rat and mouse, the PC gene is regulated by two alternative promoters (Jitrapakdee et al., 1997, 2001), while in bovine (Hazelton et al., 2008a) and human (Wang et al., 2008) PC gene is regulated by three promoters. The use of alternative promoters results in the production of alternative transcripts with different 5′-untranslated regions (Jitrapakdee et al., 1997, 2001). In rat and bovine, these mRNA variants possess different translational efficiencies, possibly through the tendency to form secondary structures at their 5′-untranslated regions which obstruct ribosome entry (Jitrapakdee et al., 1998; Hazelton et al., 2008b). In rat, the proximal promoter is activated under gluconeogenic and lipogenic conditions (in the liver and adipose, respectively), while the distal promoter is activated during glucose-induced insulin secretion in pancreatic β-cells (Jitrapakdee et al., 1998). In contrast, the physiological role of three alternative promoters of the PC gene in bovine (White et al., 2011a,b) and human (Wang et al., 2008) is not clearly understood.

2.2. Phosphoenolpyruvate carboxykinase (PEPCK)

PEPCK catalyzes the GTP-dependent conversion of oxaloacetate to phosphoenolpyruvate (Hanson and Reshef, 1997). There are two forms of PEPCK, cytosolic and mitochondrial, which are encoded by two different nuclear genes (Hanson and Reshef, 1997). Mitochondrial PEPCK readily converts the oxaloacetate formed in the mitochondria to phosphoenolpyruvate. In order for oxaloacetate to enter the cytosol where it is a substrate for cytosolic PEPCK, it is first converted to malate by mitochondrial malate dehydrogenase. Malate is then exported to the cytosol where it is converted back to oxaloacetate by cytosolic malate dehydrogenase (Fig. 2). Mitochondrial PEPCK is proposed to support gluconeogenesis from lactate while cytosolic PEPCK is proposed to support gluconeogenesis from glucogenic amino acids (Hanson and Reshef, 1997). Unlike most metabolic enzymes, PEPCK is not subject to allosteric or other post-translational regulation but is instead multi-hormonally controlled at the transcription level as evidenced by the presence of insulin-, glucocorticoid receptor-, cAMP- and thyroid hormone-responsive elements in its promoter (Hanson and Reshef, 1997; Yang et al., 2009a,b). In contrast to the cytosolic PEPCK, the transcriptional regulation of mitochondrial PEPCK is largely unknown.

2.3. Fructose-1,6-bisphosphatase (FBPase)

FBPase catalyzes the Mg^{2+}-dependent hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate. Two isoforms of FBPase, FBPase-1 and FBPase-2, have been identified and each is encoded by separate genes. The function of FBPase-2, which is expressed in

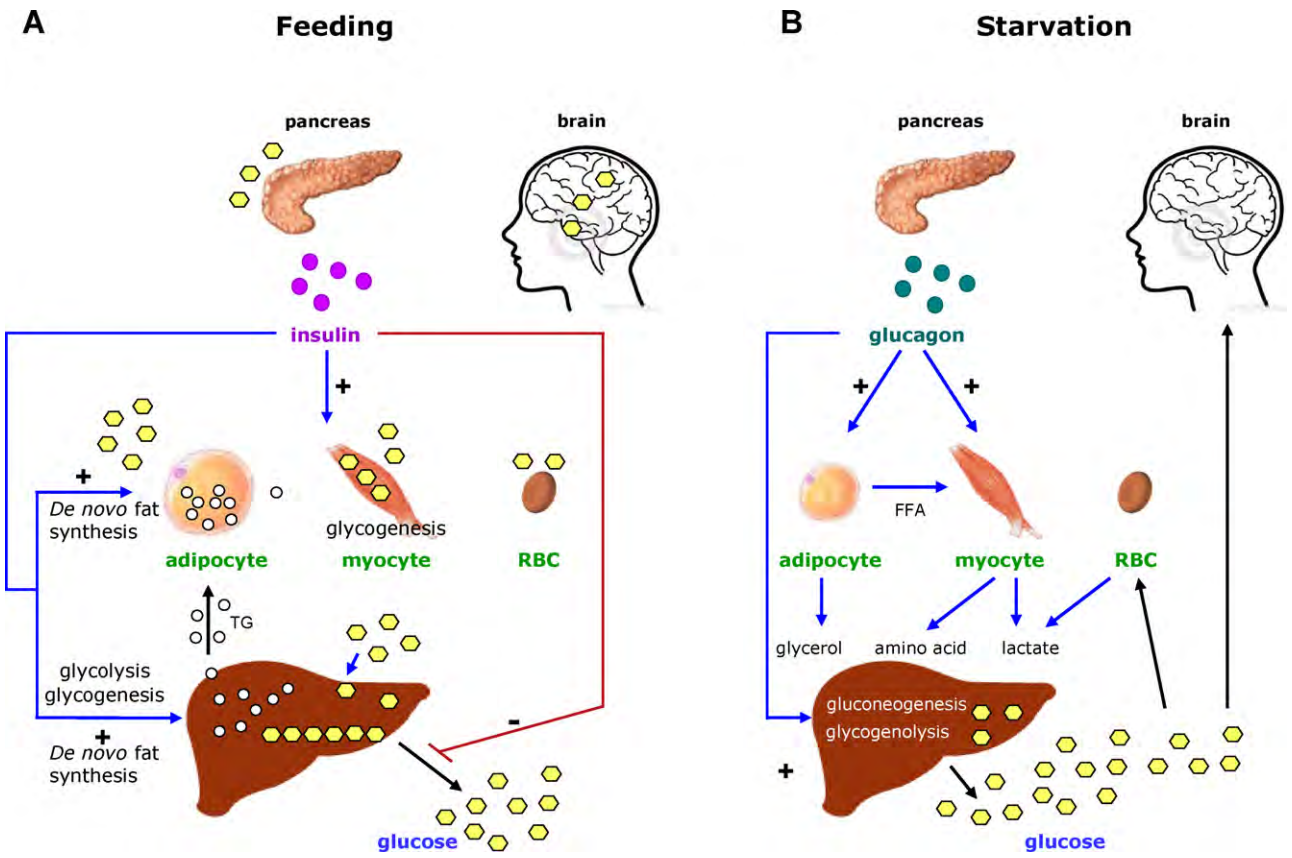

Fig. 1. Hormonal regulation of fuel during starvation and feeding. (A) Elevated levels of glucose during the postprandial period stimulate insulin release from pancreatic β-cells. Once it reaches its target tissues, insulin stimulates glycolysis and glycogenesis in muscle and liver while inhibiting glycogenolysis and hepatic gluconeogenesis to preventing hyperglycemia. Insulin also stimulates *de novo* fatty acid synthesis in liver and adipose tissue. The mobilization of triglycerides from liver to adipose tissue is also stimulated by insulin. (B) During starvation, pancreatic α-cells release glucagon in response to low levels of glucose. Glucagon stimulates fat mobilization from adipose tissue to skeletal muscle for β-oxidation to release energy. Glycerol released from lipolysis, amino acids from muscle protein breakdown, and lactate generated from muscle and red blood cells (RBCs) are transported through the circulation to the liver where they serve as substrates for gluconeogenesis. Glucagon stimulates short term production of glucose via glycogenolysis while it also stimulates long term production of glucose via gluconeogenesis. The glucose produced by these two pathways provides fuel for RBCs and the brain. FFA, free fatty acids; RBC, red blood cell; TG, triglycerides. Yellow hexagons, glucose; white circles, triglycerides (TG); pink circles, insulin; green circles, glucagon.

muscle, is largely unknown. FBPase-1 is mainly expressed in the liver and is allosterically inhibited by fructose-2,6-bisphosphate which is produced by the bifunctional enzyme phosphofructokinase 2/fructose-2,6-bisphosphatase (PFK2/FBPase2) (O'Brien and Granner, 1996). Although the promoter and enhancer regions of the FBPase-1 gene have been cloned, limited information is available regarding the *cis*-acting elements that mediate transcriptional regulation of this enzyme under various physiological conditions (El-Maghrabi et al., 1991; Herzog et al., 2000).

2.4. Glucose-6-phosphatase (G6Pase)

The G6Pase enzyme system comprises G6Pase, the glucose-6-phosphate transporter (T1), the glucose transporter (T2) and the inorganic phosphate transporter (T3), all of which are endoplasmic reticulum (ER) bound (Mithieux, 1997). The glucose-6-phosphate transporter functions in transporting glucose-6-phosphate from the cytosol to the ER. G6Pase catalyzes the Mg^{2+}-dependent hydrolysis of glucose-6-phosphate to glucose and inorganic phosphate in what is the terminal step of gluconeogenesis. Glucose is then transported across the ER and plasma membranes through the glucose T2 transporter and the GLUT2 transporter, respectively, and released into the circulation. G6Pase is expressed predominantly in the liver and kidney but is also expressed at a much lower level in the intestines. Like PEPCK, G6Pase is regulated at the transcriptional level but not post-translationally. The G6Pase promoter contains multiple binding sites for hormonally responsive transcription factors/coactivators (Hutton and O'Brien, 2009).

Regulation of hepatic gluconeogenesis can be controlled via pre- and post- translational mechanisms. Post-translational controls include the regulation of substrate supply and allosteric regulation of enzyme activity, while pre-translational control is achieved through transcription factor mediated activation and repression of genes encoding gluconeogenic enzymes (Corssmit et al., 2001; Klover and Mooney, 2004). The expression of mRNAs encoding these various transcription factors and their activities are controlled by insulin and glucagon which explain how gluconeogenesis is hormonally regulated. Binding sites for multiple transcription factors have been identified in the promoter/enhancer regions of genes encoding gluconeogenic enzymes, suggesting the cooperative interaction of transcription factors during the starvation-induced increase in gluconeogenic gene expression.

3. Transcription factors controlling gluconeogenesis

3.1. 3.1 cAMP-responsive element binding protein (CREB)

CREB is a helix–loop–helix leucine zipper transcription factor that is ubiquitously expressed in many tissues and activated during prolonged starvation (Mayr and Montminy, 2001). During the starvation period, pancreatic α-cells release glucagon in response to low levels of plasma glucose. Once it reaches

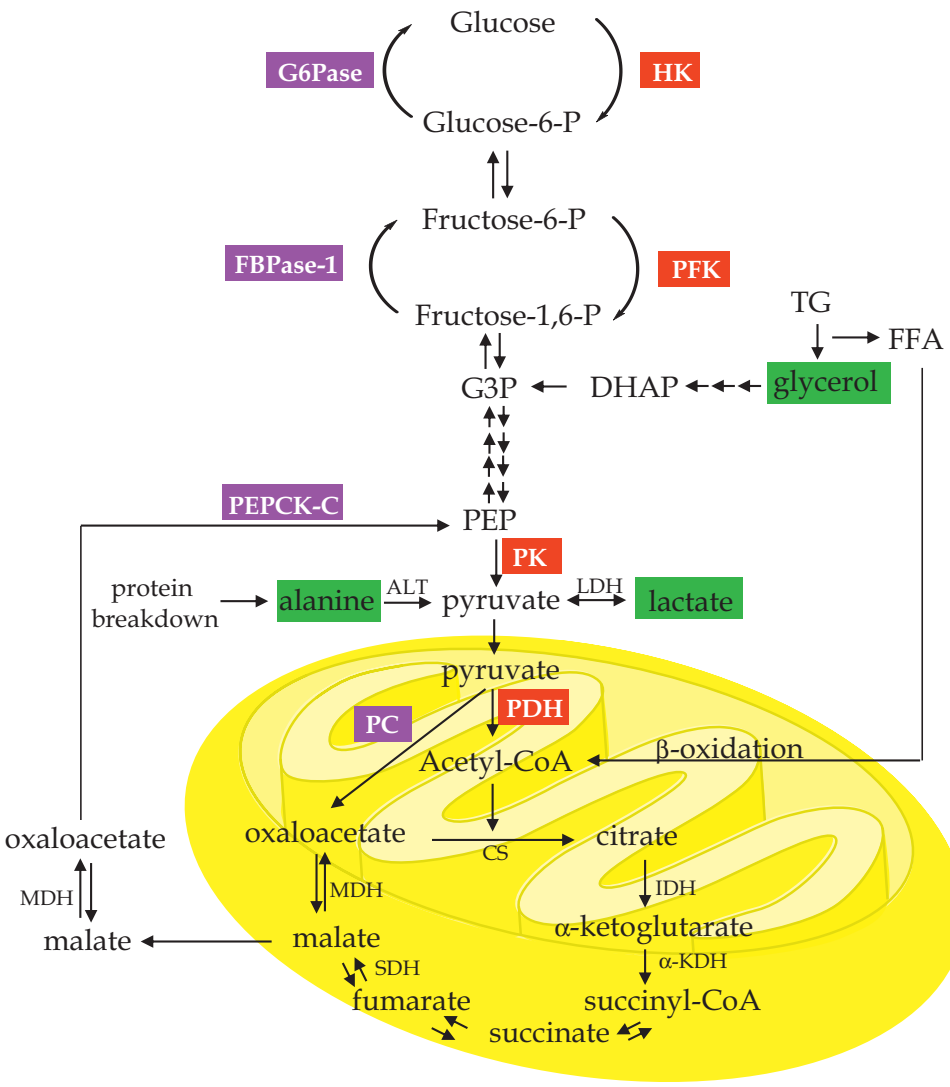

Fig. 2. Gluconeogenic/glycolytic pathways and TCA cycle. Lactate and alanine, the products of anaerobic glycolysis and protein breakdown, respectively are converted to pyruvate which is then carboxylated by PC to oxaloacetate. Oxaloacetate is then converted to malate by mitochondrial malate dehydrogenase (MDH) in the TCA cycle. Malate exits mitochondria to the cytoplasm where it is oxidized to oxaloacetate by cytosolic NAD-dependent MDH. Malate is finally converted to phosphoenolpyruvate (PEP) by PEPCK-C. The multi-step conversion of phosphoenolpyruvate to glucose proceeds via reversal of the reactions of glycolysis but requires the gluconeogenic enzymes, fructose-1,6-bisphosphatase (FBPase-1) and glucose-6-phosphatase (G6Pase), to bypass the irreversible glycolytic reactions catalyzed by phosphofructokinase (PFK) and hexokinase (HK). The glycerol obtained from the hydrolysis of triglycerides (TG) can also enter the gluconeogenic pathway via conversion to dihydroxyacetonephosphate (DHAP). ALT, alanine aminotransferase; CS, citrate synthase; G3P, glyceraldehyde-3-phosphate; IDH, isocitrate dehydrogenase; α-KDH, α-ketoglutarate dehydrogenase; LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase complex; PK, pyruvate kinase; SDH, succinate dehydrogenase. Gluconeogenic enzymes (purple) and glycolytic enzymes (red) are highlighted.

the hepatocytes, glucagon binds its cognate trimeric G-protein coupled receptor and causes GDP/GTP exchange. This in turn stimulates the activity of the adjacent membrane bound adenylyl cyclase to catalyze the conversion of ATP to cAMP. The rise in intracellular cAMP stimulates the dissociation of the catalytic and regulatory subunits of protein kinase A (PKA) (Authier and Desbuquois, 2008). The catalytic subunit of PKA can then enter the nucleus where it phosphorylates CREB at Ser133 and converts it into the transcriptionally active form. Phospho-CREB is then able to bind to the cAMP-responsive element (CRE), TGACGTCA, located in the promoter of its target genes (Mayr and Montminy, 2001) (Fig. 3A). However, CREB alone cannot produce the maximal effect on transcriptional activation of its target genes – it requires the binding of its coactivator, CREB-regulated transcription coactivator 2 (CRTC2/TORC2). CRTC2/TORC2 has been shown to be a key regulator of glucose homeostasis during fasting (Koo et al., 2005). During fasting CRTC2/TORC2 is localized exclusively in the nucleus where it binds to phospho-CREB and enhances transcription of gluconeogenic genes (Fig. 3). However, when the level of insulin is high, CRTC2/TORC2 undergoes rapid phosphorylation by serine/threonine-protein kinase SIK2 (Dentin et al., 2007) which results in its sequestration in the cytoplasm via interaction with 14-3-3 protein. This extra-nuclear localization of phospho-CRTC2/TORC2 disrupts the transcriptional activity of CREB and results in transcriptional repression of gluconeogenic genes. In addition to phosphorylation, CRTC2/TORC2 activity is also modulated by glycosylation. Exposure of hepatocytes to high concentrations of glucose or glucosamine (an intermediate in the hexosamine biosynthetic pathway) promotes *O*-glycosylation of CRTC2/TORC2 (Dentin et al., 2008). *O*-glycosylation at Ser70 and Ser171 by *O*-glycosyl transferase prevents phosphorylation of these two residues by SIK2, which in turn disrupts interaction of CRTC2/TORC2 with 14-3-3 proteins and promotes CRTC2/TORC2 to remain in the nucleus and stimulate transcription of the G6Pase

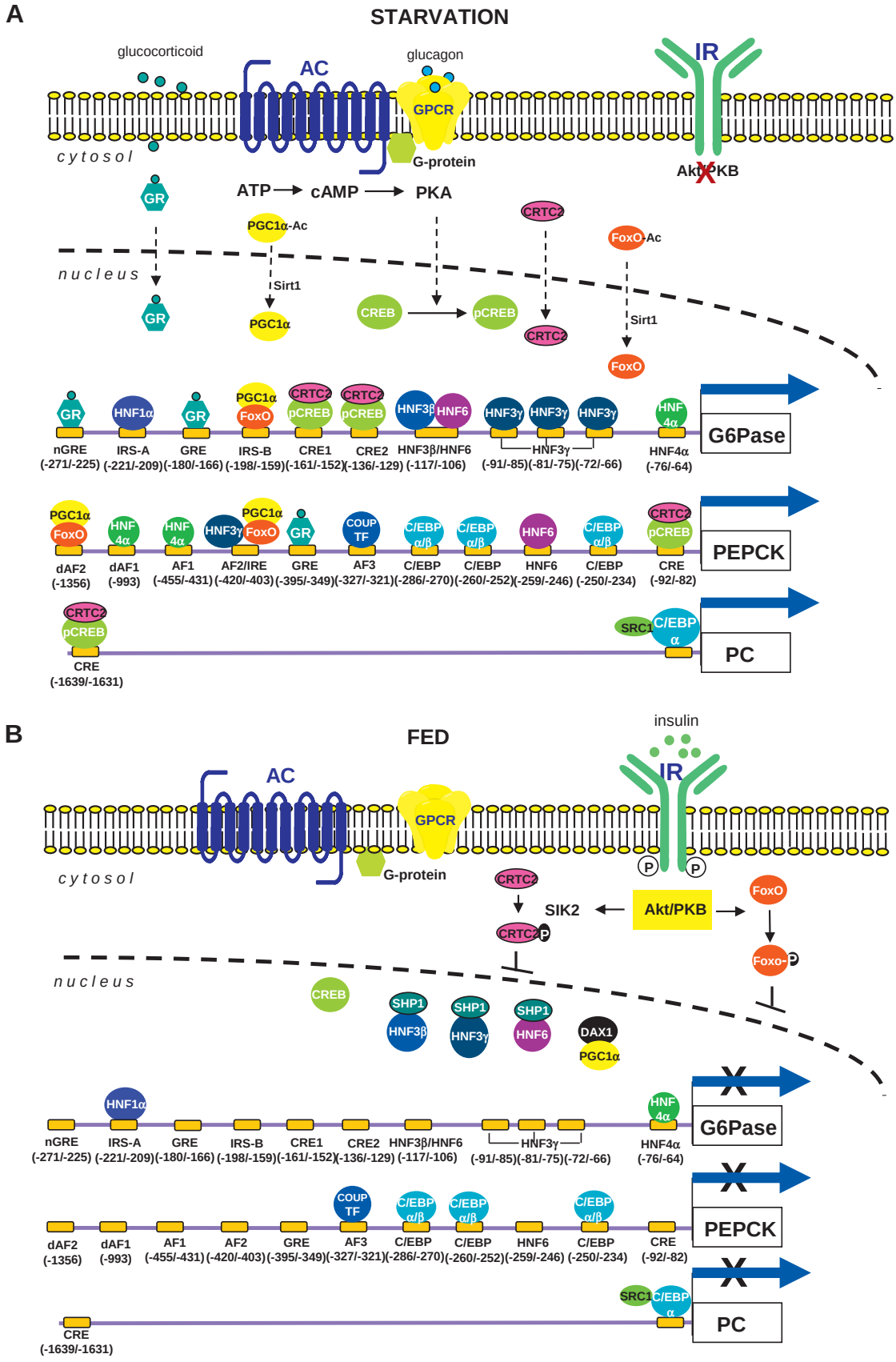

Fig. 3. Transcriptional regulation of gluconeogenic enzymes, G6Pase, PEPCK and PC in liver. (A) During starvation, glucocorticoids and glucagon are released from the adrenal glands and α-cells of the pancreas, respectively, in response to the hypoglycemic condition. Glucocorticoids bind the glucocorticoid receptor (GR) and the binary complex is translocated from the cytoplasm to the nucleus where it binds to glucocorticoid responsive elements (GREs) in the promoters of mouse G6Pase and rat PEPCK genes. In the PC promoter region, C/EBPα interacts with coactivator SRC-1 and activates transcription of the mouse PC gene (Louet et al., 2010). Binding of glucagon to its cognate G-protein coupled receptor stimulates activity of adenylyl cyclase (AC) which converts ATP to cAMP. The cAMP in turn stimulates protein kinase A (PKA) to phosphorylate the

gene. This modification is proposed to be a factor in the development of type 2 diabetes (Dentin et al., 2008).

Recent work has also shown that there is a coactivator switch controlling CRTC2/TORC2 activity (Liu et al., 2008). Within 6 h of exposure to cAMP, CRTC2/TORC2 in hepatocytes is acetylated by histone acetyltransferase p300 and rendered resistant to ubiquitination. However, when the cAMP level is low, another coactivator – the NAD-dependent deacetylase sirtuin-1 (SIRT1) – deacetylates CRTC2/TORC2 and thereby promotes its degradation (Liu et al., 2008). cAMP has been shown to directly or indirectly increase the abundance of PC, PEPCK, FBPase-1 and G6Pase mRNAs via the CRE/CREB axis in several hepatocyte cell lines.

Glucagon and cAMP increase PC mRNA levels in HepG2 cells (Thonpho et al., 2010), however, this stimulatory effect occurs rather slowly compared to other gluconeogenic enzymes (i.e., the maximal effect is observed 72 h after exposure). The CRE located at −1639/−1631 in the gluconeogenic/lipogenic promoter of the mouse PC gene binds CREB *in vitro* and *in vivo* (Thonpho et al., 2010).

cAMP sharply increases PEPCK expression in hepatocytes within the first few hours of exposure and the maximum is reached by 24 h (Lamers et al., 1982; Chrapkiewicz et al., 1982). The CRE unit identified in the rat PEPCK promoter is complicated (Fig. 3). It comprises one CRE located at −92/−82 providing the binding site for CREB; three copies of the CCAAT enhancer binding protein (C/EBP) binding site for C/EBPα or C/EBPβ; and one AP-1 binding site located at −330/−230 (Quinn et al., 1988; Roesler, 2000; Park et al., 1993). These elements are required for the maximum response to cAMP.

Two CREs (CRE1, −161/−152 and CRE2, −136/−129) have been identified in the promoter of the human G6Pase gene (Lin et al., 1997; Schmoll et al., 1999; Thiel et al., 2005). These two regions are required for cAMP-mediated transcriptional activation of a chimeric reporter gene in HepG2 and H4IIE hepatocyte cell lines. Two similarly located CREs were later identified in the mouse G6Pase gene (Streeper et al., 2000). Consistent with the presence of CREs in the promoters of PC, PEPCK and G6Pase genes, Herzig et al. (2001) showed that transgenic mice carrying a dominant negative mutant of CREB display impaired gluconeogenesis concomitant with down-regulation of the mRNAs of these enzymes in hepatocytes. Disruption of the CRTC2/TORC2 gene in mice also markedly decreases the expression of PC, PEPCK and G6Pase mRNAs and results in the reduction of hepatic glucose production (Koo et al., 2005; Le Lay et al., 2009).

Unlike other gluconeogenic enzymes, cAMP does not induce expression of the FBPase-1 gene directly but instead exerts its effect through the up-regulation of a nuclear receptor, NR4A1 (Pei et al., 2006). Ectopic expression of NR4A1 in primary mouse hepatocytes increases expression of the FBPase-1 gene. Similarly, adenovirus mediated expression of NR4A1 in mouse liver increases expression of FBPase-1 concomitant with increased hepatic gluconeogenesis. Furthermore, overexpression of a dominant negative NR4A1 mutant in mouse hepatocytes decreases cAMP-mediated transcriptional induction of endogenous NR4A1 expression (Pei et al., 2006). Ectopic expression of this NR4A1 mutant in the livers of type 2 diabetic mice reduces FBPase-1 gene expression, concomitant with lowered levels of plasma glucose, indicating that NR4A1 controls plasma glucose via FBPase-1 expression (Pei et al., 2006). Although the above data clearly indicate that the FBPase-1 gene is a target of NR4A1, the NR4A1-responsive element in the FBPase-1 promoter is unknown. A putative NR4A1-response element located at −3031 relative to the transcription start site has been identified bioinformatically (Pei et al., 2006), but remains to be validated.

3.2. Forkhead O box 1 (FoxO1)

The forkhead O box (FoxO) proteins are members of the forkhead (Fox) family of transcription factors. FoxO regulates various biological and metabolic processes including cell proliferation, longevity, energy expenditure and intermediary metabolism (Barthel et al., 2005; Nakae et al., 2008). The FoxO family comprises four members – FoxO1, FoxO3, FoxO4 and FoxO6 – with FoxO1 being the predominant isoform that regulates hepatic gluconeogenesis (Quinn and Yeagley, 2005). The FoxO proteins share a C-terminal transactivation domain and a common N-terminal forkhead domain (also known as the "winged helix" domain) that recognizes a DNA motif referred to as the insulin-responsive element (IRE). FoxO also binds several transcriptional coactivators including CREB-binding protein (CBP), C/EBPα and PGC1α. These interactions are required for the cooperative association with other transcription factors at the promoters of target genes (Barthel et al., 2005). FoxO proteins are also subject to post-translational modification by acetylation and phosphorylation. Acetylation of FoxO at Lys242, Lys245 and Lys246 by CBP/p300 reduces its ability to bind the promoters of its target genes (Matsuzaki et al., 2005); this modification is enhanced when insulin levels are high (Perrot and Rechler, 2005). Interestingly, acetylation increases FoxO's sensitivity to phosphorylation by Akt/PKB at Ser253 (Matsuzaki et al., 2005). Phosphorylation of FoxO at Ser253, Ser316 and Thr24 promotes binding of 14-3-3 and results in its cytoplasmic sequestration (Hannenhalli and Kaestner, 2009; Daitoku et al., 2011). Conversely, during fasting when insulin levels are low, the Akt/PKB phosphorylation signal is attenuated and FoxO1 is deacetylated by SIRT1 (Brunet et al., 2004; Daitoku et al., 2004; Frescas et al., 2005). These modifications result in the nuclear retention of FoxO1 and enhanced transcriptional activation of PEPCK and G6Pase genes (Fig. 3). Similar to CRTC2/TORC2, *O*-glycosylation of FoxO1 converts it into a transcriptionally active form which is responsible for the overexpression of PEPCK and G6Pase (Housley et al., 2008).

The mouse G6Pase gene promoter contains two IREs (IRS-A and IRS-B) which form the insulin response unit (IRU). IRS-A is located between nucleotides −221 and −209 while IRS-B is located between −198 and −159 (Fig. 2). IRS-B is absolutely required for insulin-mediated suppression of G6Pase while IRS-A acts as an accessory element that enhances the action of insulin and is recognized by hepatocyte nuclear factor 1 (HNF1) (Streeper et al., 1997, 1998). IRS-B is composed of three copies of a T(G/A)TTTT motif, designated IRS1-3, which bind FoxO1 with different affinities. Only IRS1 and IRS2 are required for the insulin response (Vander Kooi et al., 2003). Overexpression of FoxO1 promotes its binding to IRS-B and results in transcriptional activation of the G6Pase gene (Vander Kooi et al., 2003; Schmoll et al., 2000). This interaction is disrupted by insulin and overexpression of Akt/PKB, resulting in transcriptional repression of G6Pase (Schmoll et al., 2000). Insulin also inhibits cAMP/dexamethasone-induced G6Pase expression (Schmoll et al., 2000). Although gluconeogenesis also occurs in the kidney cortex, the inhibitory effect of insulin on G6Pase expression is not observed in this tissue due to the limited expression of FoxO1 (Nakae et al., 2001). IRS-B, arranged similarly to that of the mouse gene described above, is also present in the human G6Pase gene (Ayala et al., 1999).

An early study demonstrated that FoxO1 interacts with the IRE (TGTTT) located at −416/−407 in the promoter of the rat PEPCK gene (Durham et al., 1999). This IRE is located within the glucocorticoid accessory unit (see Section 3.4). A PEPCK reporter gene study performed in the rat hepatocyte cell line H4IIE revealed that overexpression of FoxO1 increases PEPCK promoter activity and this stimulatory effect is repressed by insulin (Hall et al., 2000). A later study showed that overexpression of FoxO1 in H4IIE cells increases G6Pase but not PEPCK gene expression (Barthel et al., 2001). Although this cell culture model reveals an uncertain role for FoxO1 in the transcriptional regulation of the PEPCK gene, several studies using mouse models have demonstrated that FoxO1 indeed regulates hepatic gluconeogenesis through both PEPCK and G6Pase.

Transgenic mice carrying a dominant negative FoxO1 mutant in the liver show an 80% reduction in hepatic G6Pase mRNA and a 50% reduction in PEPCK mRNA concomitant with impaired fasting and non-fasting glucose levels (Altomonte et al., 2003). Ectopic expression of this FoxO1 mutant in the livers of *db*/*db* mice, which display increased G6Pase and PEPCK expression and increased fasting glucose levels, results in the reduction of G6Pase and PEPCK expression and the lowering of fasting glucose (Altomonte et al., 2003). Similarly, antisense mediated FoxO1 knockout mice display reduced expression of G6Pase and PEPCK mRNAs by 65% and 50%, respectively, as well as decreased hepatic gluconeogenesis (Samuel et al., 2006). Administration of FoxO1 antisense oligonucleotides to the livers of diet-induced obese mice also reduces hepatic gluconeogenesis (Samuel et al., 2006). Matsumoto et al. (2007) showed that liver-specific FoxO1 knockout mice have impaired cAMP-mediated transcriptional induction of G6Pase and PEPCK expression. Furthermore, crossing of the FoxO1 knockout mouse with the insulin receptor (IR) knockout rescues the impaired fasting blood glucose observed in the IR knockout. This strongly indicates that insulin regulates hepatic gluconeogenesis via FoxO1 activity (Matsumoto et al., 2007). Consistent with these findings, mice overexpressing a constitutively active form of FoxO1 display increased PEPCK expression and hepatic gluconeogenesis (Zhang et al., 2006).

3.3. Hepatocyte nuclear factors (HNFs)

Hepatocyte nuclear factors (HNFs) are liver enriched transcription factors that control liver development and regulate hepatocyte specific expression of genes in adults. There are four HNF subfamilies whose prototypes are HNF1 (homeodomain family), HNF3 (forkhead family), HNF4 (orphan nuclear receptor family) and HNF6 (onecut family) (Costa et al., 2003). All four have been implicated in the regulation of hepatic gluconeogenesis. Mutations of HNF4α and HNF1 disrupt glucose homeostasis, causing a specific form of diabetes known as maturity-onset diabetes of the young (MODY): MODY1 and MODY3, respectively (Bell and Polonsky, 2001).

The HNF1 binding site on the G6Pase promoter was first identified by a gel-shift assay and transactivation study (Lin et al., 1997). It was later found that this HNF1α binding site (referred to as IRS-A) acts as an accessory element within the insulin responsive unit (IRU) of the G6Pase promoter (see Section 3.2). Binding of HNF1 to this accessory site was required for maximal insulin-mediated repression of a G6Pase reporter gene (Streeper et al., 1998). HNF1α binding to its cognate binding site (−205/−155) in the PEPCK promoter appears to be involved in its transcriptional regulation in the kidney, especially during metabolic acidosis, but not in the liver (Cassuto et al., 1997, 2003).

In the G6Pase gene promoter, Lin et al. (1997) identified five binding sites for HNF3γ located at −180/−174, −139/−133, −91/−85, −81/−75 and −72/−66. However, only the latter three sites were found to be essential for both basal promoter activity and HNF3γ-induced expression of G6Pase (Lin et al., 1997). Although HNF3γ appears to bind these sites and regulates G6Pase transcription, the expression of the G6Pase gene is not impaired in the hepatocytes of HNF3γ knockout mice. This is likely due to compensation by HNF3α and/or HNF3β in the liver (Kaestner et al., 1998).

The essential role of HNF4α in controlling gluconeogenesis has been demonstrated using liver-specific HNF4α knockout mice whose livers show markedly reduced PEPCK and G6Pase expression (Rhee et al., 2003). HNF4α and HNF3β are also necessary for efficient glucocorticoid-induced expression of the PEPCK gene (see Section 3.4). An HNF4 binding site is located at −76/−64 in the promoter of the mouse G6Pase gene (Boustead et al., 2003) whereas four additional HNF4α binding sites, −718/−713, −672/−667, −511/−516 and +9/+15, are present in the promoter of the rat G6Pase gene (Rajas et al., 2002). For the PEPCK gene, a single HNF4α binding site has been identified at −455/−431 in its promoter region (Hall et al., 1995).

HNF6 binding sites were identified at −114/−99 and −259/−246 in the promoters of G6Pase and PEPCK genes, respectively. HNF4α and HNF6 are both required for PKA-mediated induction of G6Pase expression. Deletion of the HNF6 binding site (−114/−99) abolished PKA-mediated transcriptional induction of a mouse G6Pase promoter reporter gene (Streeper et al., 2001). Similarly, deletion of any of the HNF4α binding sites located at −672/−667, −76/−64 or +9/+15 in the rat G6Pase promoter, abolished PKA-mediated induction of that reporter gene (Gautier-Stein et al., 2005). It appears that these binding sites for HNF4α provide a tissue-specific platform that is tightly associated with the cAMP-responsive unit (CRU). Both tissue-specific binding sites and the CRU must be present to mediate the PKA response (Gautier-Stein et al., 2005).

The transcriptional activity of HNFs can also be modulated by repressor and corepressor proteins, namely, the small heterodimer partner (SHP) and the dosage-sensitive sex reversal adrenal hypoplasia congenital critical region on the X chromosome gene 1 (DAX-1). Both SHP and DAX-1 are atypical nuclear receptors that lack the classical DNA binding domain but contain the nuclear receptor binding domain at the N-terminus and the ligand binding domain at the C-terminus (Bavner et al., 2005). The *bona fide* ligands for SHP and DAX-1 are, however, unknown. It appears that these two proteins function as corepressors rather than transcription factors. Both SHP1 and DAX-1 are expressed in several tissues, including the liver, and their expression is reciprocal with that of the gluconeogenic enzymes, that is, their expression is high in the fed condition when plasma insulin is high (Nedumaran et al., 2009). SHP interferes with the binding of HNF3 and HNF6 to their cognate sequences in the promoters of G6Pase and PEPCK genes, and thereby represses transcription of those genes (Kim et al., 2004; Lee et al., 2008). Similarly, SHP inhibits HNF4α-mediated transcription by interfering with its interaction with the transcriptional coactivator CBP (Yamagata et al., 2004). In contrast, DAX-1 interferes with HNF4α binding to G6Pase and PEPCK gene promoters by competing for binding to PGC1α, and thereby inhibits PEPCK and G6Pase expression during fasting (Nedumaran et al., 2009). Adenovirus mediated overexpression of DAX-1 in mice and isolated primary hepatocytes prevents cAMP-induced expression of PEPCK and G6Pase genes in parallel with reduced hepatic gluconeogenesis.

With regard to FBPase-1, HNF4α is capable of transactivating a FBPase-1 promoter reporter construct in HepG2 cells (Yamagata et al., 2004). Similar to PEPCK and G6Pase genes, SHP represses HNF4α-mediated transactivation of the FBPase-1 promoter in these cells possibly by disrupting HNF4α-coactivator complexes (Yamagata et al., 2004).

3.4. Glucocorticoid receptor: tight control with tissue-specific transcription factors

Glucocorticoids have long been known to stimulate hepatic gluconeogenesis. These steroid hormones acutely stimulate transcription of PEPCK and G6Pase genes, but not PC and FBPase-1 genes. In general, glucocorticoids exert their stimulatory effects by binding to the glucocorticoid receptor (GR). Once bound, the glucocorticoid-GR complex is translocated to the nucleus where it binds to the glucocorticoid responsive elements (GREs) in target genes. The consensus GRE is (T/G)GTACnnnTGTTCT (Schoneveld et al., 2004). In the promoters of the PEPCK and G6Pase genes, the GRE is complex. Their GREs appear to be surrounded by tissue-specific or cAMP-responsive accessory binding sites which combine to form the glucocorticoid responsive unit (GRU) (see Fig. 2).

In the G6Pase gene promoter, the proximal GRU is composed of three GRE sites (−180/−166) flanked upstream by the HNF1α

binding site (IRS-A, −221/−209) and the IRE (IRS-B, −198/−159), and downstream by two CREs (CRE1, −161/−152 and CRE2, −136/−129) and binding sites for HNF3β (−117/−106) and HNF4α (−64/−76) (Vander Kooi et al., 2005). Mutation of any one of the three GREs resulted in 50–75% loss of glucocorticoid-stimulated induction of a chimeric GP6ase reporter. Similarly, mutation of any of the accessory factor binding sites resulted in a 40–75% reduction in the reporter gene activity. Interestingly, the distal region of the promoter (i.e., −271 to −225) contains a negative GRU comprising a negative GRE and negative accessory factor binding sites that confer glucocorticoid-mediated inhibition of G6Pase promoter activity (Vander Kooi et al., 2005).

The GRU in the PEPCK gene promoter is as complicated as that of the G6Pase gene. The PEPCK GRU consists of two distantly situated GRUs the proximal GRU and the distal GRU. The proximal GRU, located at −455/−321, consists of two GREs (GR1 and GR2 at −395/−349) flanked by accessory factor binding sites AF1 (−455/−431) and AF2 (−420/−403) (Imai et al., 1990) at the 5′ end, and AF3 (−327/−321) (Scott et al., 1996) and CRE (−92/−82) (Imai et al., 1993) at the 3′ end. The chicken ovalbumin upstream promoter transcription factor (COUP-TF) and HNF4α bind to AF1 (Hall et al., 1992; 1995) and AF3 (Scott et al., 1996), while HNF3β preferentially binds AF2 (Wang et al., 1996, 1999). Mutations of GR1 and GR2 caused a marked reduction in glucocorticoid-mediated transcription of a chimeric PEPCK reporter. With GR1 and GR2 intact, mutations of the accessory factor binding sites also caused significant reductions (50–75%) in expression from the PEPCK reporter (Imai et al., 1990, 1993; Scott et al., 1996). These studies indicate cooperation between GR and accessory factors in glucocorticoid-mediated PEPCK gene induction. The positioning of AF1 and AF2 appears to be specific, as swapping these two sites in this GRU reduced the maximal response of the PEPCK reporter gene (Wang et al., 1999). Binding of glucocorticoid receptors to GR1 and GR2 was also greatly facilitated by binding of HNF4α and HNF3β to AF1 and AF2, respectively (Stafford et al., 2001). Ectopic expression of an HNF3β mutant lacking its C-terminal activation domain reduces basal and glucocorticoid-induced expression of the PEPCK gene (Vallet et al., 1995; Wang et al., 2000). Recently, two additional accessory factor binding sites, termed dAF1 (−993) and dAF2 (−1356), were identified by Reshef's group (Cassuto et al., 2005). These dAF1 and dAF2 sites provide binding sites for HNF4α and FoxO1, respectively, and were shown to function cooperatively with the proximal GRU to promote maximal glucocorticoid-mediated induction of the PEPCK gene (Cassuto et al., 2005). Cassuto et al. (2005) also found that binding of FoxO1, rather than HNF3β, to AF2 is responsible for glucocorticoid-mediated induction of PEPCK. This is consistent with a previous report (Wolfrum et al., 2004).

3.5. Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α)

Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α) was first identified as the transcriptional coactivator of peroxisome proliferator-activated receptor-γ (PPARγ). PGC-1α consists of four domains, from N- to C-terminus: the transactivation domain which also possesses the nuclear hormone receptor interacting motif; the repression domain which contains phosphorylation sites; the RNA processing domain; and the RNA polymerase interacting domain (Puigserver and Spiegelman, 2003). Although PGC1α plays an important role in mitochondrial biogenesis, β-oxidation, and adaptive thermogenesis in skeletal muscle and brown adipose tissue, it is also involved in the regulation of glucose homeostasis (Puigserver and Spiegelman, 2003). Similar to FoxO, acetylation is a key post-translational modification that regulates the transcriptional activity of PGC1α. Acetylation of PGC1α by coactivators possessing acetylase activity, including CBP/p300 and GCN5, reduces its transcriptional activity, while deacetylation by SIRT1 increases its activity (Rodgers et al., 2008).

In mice, starvation rapidly induces PGC1α expression in the liver concomitantly with up-regulation of PEPCK and G6Pase expression (Yoon et al., 2001; Rodgers et al., 2005). The rise in PGC1α levels is also correlated with increased activity of SIRT1, which is stimulated by the high concentrations of NAD^+ and pyruvate associated with periods of starvation (Nemoto et al., 2005; Rodgers et al., 2005). Disruption of the SIRT1 gene in the mouse liver concomitantly reduces gluconeogenic gene expression and impairs glucose homeostasis (Rodgers and Puigserver, 2007). Furthermore, SIRT1 knockdown in the liver lowers diabetes-induced excessive hepatic gluconeogenesis (Erion et al., 2009), indicating the critical role of SIRT1 in the regulation of PGC1α during fasting and the development of diabetes. Ectopic expression of PGC1α in mouse hepatocytes results in increased expression of PEPCK, FBPase-1 and G6Pase mRNAs in parallel with increased hepatic gluconeogenesis, suggesting a role for PGC1α in the regulation of hepatic glucose production (Yoon et al., 2001; Puigserver et al., 2003). While cAMP and dexamethasone increase PGC1α expression, insulin inhibits its expression as demonstrated in three mouse models: (i) mice with streptozotocin-induced β-cell loss, (ii) genetically obese (*ob*/*ob*) mice and, (iii) liver-specific insulin receptor knockout mice, all of which show overexpression of PGC-1α and increased expression of PEPCK (Yoon et al., 2001). In a later study, Puigserver et al. (2003) showed that the inhibitory effect of insulin on PGC1α is in fact mediated by the counter hormone glucagon: PGC1α mRNA levels were unaltered in mice subjected to hyperinsulinemic-euglycemic clamps.

Puigserver et al. (2003) proposed that insulin regulates PGC1α through the modulation of FoxO1 activity. PGC1α has been shown to physically interact with FoxO1 and synergistically activate G6Pase promoter activity (Puigserver et al., 2003). When insulin is present, it stimulates a signal transduction cascade leading to phosphorylation and activation of Akt/PKB. This in turn results in the phosphorylation and nuclear exclusion of FoxO1, thus inhibiting PEPCK and G6Pase expression (Puigserver et al., 2003). However, Schilling et al. (2006) re-evaluated the PGC-1α/FoxO1 interaction hypothesis. They showed that FoxO1 is not required for synergistic activation of the G6Pase gene by PGC-1α since deletions of all possible FoxO binding sites in the G6Pase promoter did not eliminate this synergistic action. Deletion of the HNF4α binding site, however, dramatically reduced the synergistic effect of PGC-1α on transcriptional activation of the G6Pase promoter (Boustead et al., 2003; Schilling et al., 2006). This argues against the previous conclusion that PGC-1α regulates hepatic gluconeogenesis via an interaction with FoxO1. By showing that PGC-1α fails to induce G6Pase and PEPCK gene expression during fasting conditions in HNF4α knockout mice, Rhee et al. (2003) confirmed that the interaction of PGC1α with HNF4α is required for the induction of hepatic gluconeogenesis. Although the *bona fide* interaction partner of PGC-1α in the control of glucose homeostasis is still debated, Li et al. (2007) demonstrated that Akt/PKB phosphorylates Ser570 of PGC-1α and thereby converts it into a transcriptionally inactive form that is unable to interact with either FoxO1 or HNF4α. In contrast, PGC1α has been shown to interact with the glucocorticoid receptor to enhance PEPCK gene expression (Yoon et al., 2001).

Although global disruption of PGC1α in several mouse models does not affect the expression of genes encoding gluconeogenic enzymes (Lin et al., 2004; Leone et al., 2005; Burgess et al., 2006), suppression of PGC1α by siRNA in hepatocytes (Koo et al., 2004) or by liver-specific ablation in mice has been shown to impair fasting induced expression of PEPCK and G6Pase genes (Handschin et al., 2005). These latter studies provide evidence of a critical role for PGC1α in controlling fasting-induced gluconeogenesis.

There are conflicting reports regarding PGC1α regulation of FBPase-1 gene expression. Yoon et al. (2001) have shown that adenovirus mediated expression of PGC1α in primary mouse hepatocytes increases both basal and dexamethasone-induced expression of FBPase-1. In contrast, Pei et al. (2006) reported that neither ectopic expression nor suppression of PGC1α in primary hepatocytes affects endogenous levels of FBPase-1 mRNA. These conflicting results may result from different titers of the adenoviruses carrying the PGC1α gene used to infect the primary hepatocytes in these two studies.

3.6. CCAAT enhancer-binding protein (C/EBP)

CCAAT enhancer-binding proteins (C/EBPs) are liver enriched transcription factors that function as central regulators of energy production. There are four C/EBP subtypes: C/EBPα, C/EBPβ, C/EBPδ and C/EBPε (Ramji and Foka, 2002). All four isoforms share a common N-terminal basic region and a leucine zipper DNA binding domain that recognizes specific DNA sequences in the promoters of their target genes (Ramji and Foka, 2002). C/EBPs regulate PEPCK gene transcription in a liver-specific manner by binding to three specific sites upstream of the CRE. In fact, these three C/EBP binding sites (−286/−270, −260/−252 and −250/−234) and the downstream CRE (−92/−82) form the structure known as the cAMP-responsive unit (CRU) in the PEPCK promoter (Park et al., 1990). Both CRE and C/EBPα binding sites are required for cAMP-mediated induction of PEPCK gene expression as deletion of either CRE or any one of the C/EBP binding sites abolished cAMP-induced expression of a chimeric PEPCK reporter gene (Liu et al., 1991). Transactivation studies and DNase I footprint analyses of the PEPCK promoter's CRU revealed that not only CREB, but also C/EBPα and C/EBPβ, can bind to the CRE and mediate the response to cAMP, suggesting that CREB and C/EBPs can be functionally substituted during cAMP-induced expression of the PEPCK gene (Park et al., 1990, 1993; Roesler et al., 1998). C/EBPα and C/EBPβ also bind the three C/EBP binding sites on the PEPCK promoter (Park et al., 1993). The alternative binding of C/EBPα and C/EBPβ to these C/EBP binding sites could influence the degree of responsiveness of the PEPCK gene to cAMP (Roesler, 2000). Although the above experiments point to a functional redundancy between C/EBPα and C/EBPβ, deletion of either results in profound effects on gluconeogenesis. The C/EBPα knockout mice have undetectable levels of liver PEPCK mRNA and die within a few hours of birth due to severe hypoglycemia (Wang et al., 1995). While not lethal, the conditional knockout of C/EBPα in the livers of adult mice also results in strongly reduced PEPCK mRNA expression (Lee et al., 1997). In another conditional knockout model, disruption of the C/EBPα gene in the liver, spleen, adipose, pancreas, lung and kidney produces hypoglycemic mice with severely reduced levels of hepatic PEPCK and G6Pase mRNAs (Yang et al., 2005). Despite the markedly reduced basal levels of PEPCK and G6Pase mRNAs, cAMP is still capable of stimulating the expression of both genes in this mouse model. This result indicates that C/EBPα is required for expression of the PEPCK gene under basal conditions but not for cAMP-mediated induction of this gene. These knockout mice die about a month after ablation of the C/EBPα gene due to hypoglycemia, hypophagia, lipodystrophy and other complications (Yang et al., 2005). In the *db*/*db* mouse, in which PEPCK mRNA expression is up-regulated, knocking down the level of C/EBP in the liver results in a reduction of PEPCK and G6Pase mRNA expression in parallel with reduced hepatic glucose production (Qiao et al., 2006). Similar to C/EBPα knockout mice, C/EBPβ ablated mice have a very low level of PEPCK mRNA expression and die within a few hours of birth due to severe hypoglycemia (Croniger et al., 1997, 2001). Taken together these observations indicate that C/EBPα and C/EBPβ are critical for postnatal gluconeogenesis.

Steroid receptor coactivator-1 (SRC-1, also known as nuclear receptor coactivator 1, NCOA1), a transcriptional coactivator, has recently been shown to regulate transcription of gluconeogenic genes. SRC-1 ablated mice are hypoglycemic secondary to a defect in hepatic gluconeogenesis caused by down-regulation of FBPase-1 and PC but not G6Pase and PEPCK genes (Louet et al., 2010). Despite unaltered PEPCK expression in the intact animal, the isolated hepatocytes of SRC-1 knockout mice show markedly reduced PEPCK mRNA, suggesting that hormonal effects may mask the transcriptional regulation of this gene by SRC-1. SRC-1 interacts with C/EBPα and modulates transcription of the PC gene and this is thought to be a key mechanism to control hepatic gluconeogenesis during periods of prolonged fasting (Louet et al., 2010). Although a transactivation study and chromatin immunoprecipitation assay revealed that C/EBPα and SRC-1 interact within the first 600 nucleotides proximal to the transcription start site of the mouse PC gene, the precise binding site of C/EBPα in this enhancer region remains to be determined (Louet et al., 2010). The molecular mechanism by which SRC-1 regulates FBPase-1 expression likewise awaits further study (Louet et al., 2010).

In summary, signaling through glucagon during starvation results in the increased levels of cAMP which stimulates PKA activity and thereby promotes phosphorylation of CREB. Phospho-CREB interacts with CRTC2/TORC2 and binds to the CRE in the promoters of gluconeogenic enzyme genes. CREB also binds to the promoter of the PGC1α gene and stimulates its transcription. As the level of SIRT1 protein and activity are also increased during this period, PGC1α is deacetylated and in turn interacts with FoxO1 and HNF4α to enhance transcription of gluconeogenic genes. Furthermore, high glucocorticoid concentrations during starvation promote transcription of gluconeogenic genes via binding of the GR to GRE's in their promoter regions. This GR-mediated transcription also requires various HNFs to maximize transcriptional activation of gluconeogenic genes. In contrast, during feeding when the insulin level is high, the attenuation of PKA signaling combines with high protein phosphatase 1 activity to promote the dephosphorylation and transcriptional inactivation of CREB. Insulin signaling through Akt/PKB drives the phosphorylation and nuclear exclusion of CRTC2/TORC2 and FoxO1. The nuclear exclusion of CRTC2/TORC2 results in the transcriptional attenuation of the PGC1α gene and thereby limits PGC1α protein abundance. High levels of insulin also decrease the abundance and activity of SIRT1, and therefore, promote conversion of PGC1α into a transcriptionally inactive (i.e., deacetylated) form. These events lead to the transcriptional repression of gluconeogenic genes and the suppression of hepatic glucose production.

4. Regulation of gluconeogenesis via brain action

The central nervous system (CNS) has an integral role in the regulation of glucose homeostasis. Studies have shown that insulin signaling in the hypothalamus plays an important role in the regulation of hepatic gluconeogenesis (Demuro and Silvana, 2006; Sandoval et al., 2009). Mice carrying insulin receptors specifically disrupted in the brain are hyperglycemic and do not suppress hepatic gluconeogenesis during hyperinsulinemic clamp (Brüning et al., 2000). Obici et al. (2002) showed that in the presence of fixed and basal concentrations of plasma insulin, infusion of insulin into the hypothalamus (third cerebral ventricle) suppresses hepatic gluconeogenesis, while infusion of inhibitors of insulin signaling blocks this inhibitory effect of insulin in rats. This effect is mediated through the activation of K_{ATP}-sensitive potassium channels – rats infused with K_{ATP}-sensitive potassium channel blockers and mice lacking the SUR1 subunit of the K_{ATP} channel are resistant to the central insulin-mediated suppression of hepatic

gluconeogenesis (Pocai et al., 2005a). Interestingly, inhibition of carnitine palmitoyl transferase I in the brain shows a similar inhibitory effect on hepatic gluconeogenesis and is accompanied by reduced expression of PEPCK and G6Pase genes (Pocai et al., 2005b). The precise mechanism of communication between the hypothalamus and liver is unclear, but it certainly requires efferent signals from the brain to liver (Pocai et al., 2005a, b). Inoue et al. (2006) showed that suppression of hepatic gluconeogenesis by centrally administrated insulin involves the activation of signal transducer and activator of transcription 3 (STAT3), which has previously been shown to inhibit PEPCK expression (Inoue et al., 2004). Infusion of insulin into the cerebral ventricle in mice induces phosphorylation of STAT3 in the liver and results in decreases in PEPCK expression and hepatic glucose production. This inhibitory effect on gluconeogenesis mediated by insulin action in the brain is eliminated in liver-specific STAT3 knockout mice, confirming the role of STAT3 as a hepatic effector of brain-insulin action (Inoue et al., 2006).

Leptin is an adipocyte derived hormone with important regulatory roles in energy homeostasis. Among the more established actions of leptin is the regulation of food intake via the stimulation and suppression of the hypothalamic anorexigenic and orexigenic neurons, respectively (Cheung et al., 1997; Rossi et al., 1998). This effect is mediated by the binding of leptin to the leptin receptor (LRb) on these neurons, and the subsequent activation of the JAK2/STAT3 signal transduction pathway (Vaisse et al., 1996; Banks et al., 2000). Hypothalamic leptin signaling is also implicated in the control of hepatic gluconeogenesis (Cohen et al., 1996; Rossetti et al., 1997; Raman et al., 2003; Toyoshima et al., 2005). CNS-administration of leptin to MKR mice, a model of T2DM caused by defective insulin signaling in the skeletal muscle, normalizes plasma glucose and reduces the expression of the G6Pase gene, mimicking the insulin effect (Toyoshima et al., 2005). German et al. (2009) reported a similar response to the restoration of leptin signaling in another model of T2DM, the LR-deficient Koletsky (fa^k/fa^k) rat. Adenovirus-mediated expression of the LR in the hypothalamic arcuate nucleus of these rats promotes insulin-mediated suppression of hepatic gluconeogenesis and reduced G6Pase and PEPCK gene expression. The precise mechanism for this leptin-mediated enhancement of hepatic insulin action is still unclear; however, increased signal transduction through PI3K in the liver appears to play a role (German et al., 2009). Interestingly, the effects of leptin on hepatic gluconeogenesis appear to differ between normal and diabetic animals. In contrast to the studies described above, systemic or intracerebroventricular administration of leptin to normal rats during hyperinsulinemic clamp increases PEPCK gene expression and the rate of hepatic gluconeogenesis, but due to a compensatory increase in the rate of glycogenolysis, does not affect overall hepatic glucose production (Rossetti et al., 1997; Liu et al., 1998). The molecular mechanism by which leptin exerts its stimulatory effect on hepatic gluconeogenesis in these animals is not clearly understood, although the activations of β3-adrenergic and α-MSH receptors (melanocortin receptor 3 or 4) in the hypothalamus have been reported to recapture the above results (Liu et al., 1998; Gutiérrez-Juárez et al., 2004). Progress in this area is all but assured as the potential for leptin-based therapies in the treatment of diabetes continues to be explored.

5. Hepatic gluconeogenesis is a target of an anti-diabetic drug

Immense efforts have gone into unraveling the transcriptional regulation of gluconeogenesis as this biochemical process is critical for maintaining fuel homeostasis. Based on current understandings of the pathophysiology of type 2 diabetes mellitus (T2DM), multiple pharmacological interventions have been developed with the aim of improving glycemic control and slowing disease progression. These efforts have been paid off by the recent studies which explain the mechanism of action of the anti-diabetic drug, metformin. Although it has been known for quite some time that it stimulates phosphorylation of the cellular sensor kinase AMP-activated protein kinase (AMPK), the molecular mechanism by which metformin inhibits hepatic gluconeogenesis was unknown until recently. Shaw et al. (2005) reported that metformin, acting via AMPK, disrupts the interaction between CREB and CRTC2/TORC2, and thereby prevents binding of the CREB–CRTC2/TORC2 complex to the promoters of PEPCK and G6Pase genes (Shaw et al., 2005; He et al., 2009).

He et al. (2009) later reported that metformin regulates the CREB-binding protein (CBP), another component of the CREB transcription complex, through AMPK phosphorylation. Phosphorylated AMPK activates protein kinase C 1/λ (PKC1/λ) which subsequently phosphorylates CREB at Ser436 and results in the dissociation of CREB–CBP–CRTC2/TORC2 complexes. This in turn results in transcriptional repression of gluconeogenesis. Metformin also acts through SHP to interfere with binding of HNF4 and FoxO to their binding sites in the promoters of G6Pase and PEPCK genes (Kim et al., 2008). Furthermore, Berasi et al. (2006) showed that metformin-induced AMPK phosphorylation stimulates expression of early growth response 1 (EGR1), a transcription factor required to turn on expression of the dual specificity protein phosphatase 4 (DUSP4) gene. DUSP4 is known to regulate p38 MAP kinase, which regulates CREB phosphorylation via PKA activation. Caton et al. (2010) recently identified SIRT1 and histone acetyltransferase GCN5 (also know as KAT2A) as new targets of metformin action in hepatocytes. Under normal circumstances, CRTC2/TORC2 activity is promoted by acetylation whereas deacetylation inactivates its transcriptional activity. Hepatocytes treated with metformin are induced to express SIRT1, an NAD^+-dependent deacetylase, which thereby eliminates their ability to activate transcription of gluconeogenic genes. Metformin also increases the expression of the acetyltransferase GCN5. Acetylation of PGC1α by GCN5 converts it to an inactive form and thereby inhibits transcription of the PEPCK gene.

Although the above studies convincingly show that metformin suppresses gluconeogenesis via the CREB–CRTC2/TORC2 axis, this idea has recently been re-examined (Foretz et al., 2010). Foretz et al. (2010) have shown that suppression of hepatic gluconeogenesis is not eliminated in AMPK ablated mice. They have also shown that inhibition of hepatic gluconeogenesis by metformin is operated independently of the AMPK pathway via a decrease in hepatic energy state.

6. Conclusion and future directions

The recent studies summarized in this review have greatly advanced our understanding of the transcriptional regulation of gluconeogenesis by various hormones including insulin, glucagon, glucocorticoids and leptin. Insulin and glucagon modulate the transcriptional activities of FoxO, CREB, CRTC2/TORC2, PGC1α and acetylases/deacetylases which in turn cooperate with C/EBPs and HNFs to regulate expression of gluconeogenic genes during the fasting/feeding cycle. As uncontrolled hepatic glucose production is a hallmark of T2DM, identification of key transcriptional regulators that turn these genes on and off will aid the development of drugs able to inhibit episodic increases in hepatic glucose in patients with this disorder. Although there are growing data on the identification of transcription factors that control transcription of PEPCK and G6Pase genes, limited information is available regarding

regulation of PC and FBPase-1 genes. Progress in the development of new anti-diabetic drugs will require additional insights into the transcriptional regulation of these two enzymes. Importantly, although the transcriptional studies of gluconeogenesis are both satisfying and thrilling, they raise new questions about the relative contribution of each gluconeogenic enzyme to overall gluconeogenesis. Burgess et al. (2007) recently reported the surprising observation that PEPCK-deficient mice show only a 40% reduction in gluconeogenic flux despite a 90% reduction in PEPCK protein. A more recent study has also shown that fasting hyperglycemia is not associated with overexpression of PEPCK and G6Pase in patients with poorly controlled T2DM (Samuel et al., 2009). The authors also pointed out that PC and FBPase-1, rather than PEPCK and G6Pase, may contribute to the regulation of gluconeogenesis based on the fact that both PC and FBPase-1 are both allosterically regulated. Several earlier studies performing flux and metabolomic analyses also reported that PC rather than PEPCK is a rate-limiting step in controlling overall hepatic gluconeogenesis (Groen et al., 1986; Argaud et al., 1991; Yang et al., 2006). These recent pharmacological inventions also indicate that inhibitors of FBPase1 are potential drugs for inhibiting hepatic gluconeogenesis in patients with T2DM (Dang et al., 2011; Yoshida et al., 2011; Hebeisen et al., 2011). These observations challenge the long held paradigm that PEPCK and G6Pase are the most important gluconeogenic enzymes and highlight the importance of looking at the roles and regulation of the lesser studied PC and FBPase-1 in hepatic gluconeogenesis.

Acknowledgements

Research in S. Jitrapakdee's laboratory is supported by the Thailand Research Fund (BRG548002), the Commission of Higher Education and the Faculty of Science, Mahidol University. The author thanks Professor John Wallace and Clair Alvino for critical reading of the manuscript.

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Characterization of the Distal Promoter of the Human Pyruvate Carboxylase Gene in Pancreatic Beta Cells

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Abstract

Pyruvate carboxylase (PC) is an enzyme that plays a crucial role in many biosynthetic pathways in various tissues including glucose-stimulated insulin secretion. In the present study, we identify promoter usage of the human PC gene in pancreatic beta cells. The data show that in the human, two alternative promoters, proximal and distal, are responsible for the production of multiple mRNA isoforms as in the rat and mouse. RT-PCR analysis performed with cDNA prepared from human liver and islets showed that the distal promoter, but not the proximal promoter, of the human PC gene is active in pancreatic beta cells. A 1108 bp fragment of the human PC distal promoter was cloned and analyzed. It contains no TATA box but possesses two CCAAT boxes, and other putative transcription factor binding sites, similar to those of the distal promoter of rat PC gene. To localize the positive regulatory region in the human PC distal promoter, 5′-truncated and the 25-bp and 15-bp internal deletion mutants of the human PC distal promoter were generated and used in transient transfections in INS-1 832/13 insulinoma and HEK293T (kidney) cell lines. The results indicated that positions −340 to −315 of the human PC distal promoter serve as (an) activator element(s) for cell-specific transcription factor, while the CCAAT box at −71/−67, a binding site for nuclear factor Y (NF-Y), as well as a GC box at −54/−39 of the human PC distal promoter act as activator sequences for basal transcription.

Citation: Thonpho A, Rojvirat P, Jitrapakdee S, MacDonald MJ (2013) Characterization of the Distal Promoter of the Human Pyruvate Carboxylase Gene in Pancreatic Beta Cells. PLoS ONE 8(1): e55139. doi:10.1371/journal.pone.0055139

Editor: Bridget Wagner, Broad Institute of Harvard and MIT, United States of America

Received December 5, 2012; **Accepted** December 24, 2012; **Published** January 30, 2013

Funding: This work was supported by the Thailand Research Fund (BRG5480002) and the Faculty of Science, Mahidol University to SJ and NIH grant DK28348 to MJM. AT was supported by a Royal Golden Jubilee-PhD program from the Thailand Research Fund. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Pyruvate carboxylase (PC) is an important anaplerotic enzyme that catalyzes the ATP-driven carboxylation of pyruvate to oxaloaceate. This reaction is not only the first important committed step of hepatic gluconeogenesis but also crucial for cataplerosis as Krebs cycle intermediates are withdrawn for various biosynthetic purposes including *de novo* fatty acid synthesis in liver and adipose tissue, glyceroneogenesis in adipose tissue and glutamate production in astrocytes (for review see [1–3]). PC also plays an important role in normal glucose-stimulated insulin secretion (GSIS) in pancreatic β-cells [4–6]. Dysregulation of PC expression in liver, adipose tissue or islets is also associated with obesity and type 2 diabetes [7–11]. PC deficiency is a rare autosomal recessive phenotype characterized by mild to severe lactic acidemia associated with delayed psychomotor development and death within the first year of life in about one-half the cases [12].

PC is allosterically activated by acetyl-CoA, a signaling molecule that is produced by increased fatty acid oxidation during prolonged starvation. In mammals, the PC gene is transcriptionally regulated by alternate promoters which mediate the production of multiple mRNA isoforms which differ in their 5′-untranslated regions. The PC genes from rat and mouse are well characterized and they are controlled by two promoters namely the proximal and the distal promoters [13–15]. The proximal promoter is responsible for production of PC mRNA in the gluconeogenic tissues including liver and kidney, as well as the lipogenic tissues including liver and adipose tissues. The presence of a cAMP-responsive element (CRE) [16]) and a peroxisome proliferator activated receptor response element (PPRE) [17] in the proximal promoter allows liver and adipose tissue, respectively, to produce more PC during prolonged fasting. In contrast, the distal promoter is linked to anaplerosis especially in pancreatic β-cells. The structural region of the human PC gene has been cloned and characterized [18]. However, the regulatory regions of the PC gene that confer tissue-specific expression of PC in humans are not known. Recently, Wang et al [19] reported that unlike the rat and mouse PC genes, the human PC gene is transcribed from three promoters. Herein, we present evidence that similar to the rodent PC genes, the human PC gene is transcribed from two promoters. In addition, we identified some of the important *cis*-acting elements of the distal human PC promoter that direct transcription of PC in beta cells.

A

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                extra 11 bases
Variant 1  GTCAGTGGAGGCAGCAGCGGTAGAGGCGGCGGCGAGGACTGGCGACGGCGAGGAGATAGT 60
Variant 2  ----------CAGCAGCGGTAGAGGCGGCGGCGAGGACTGGCGACGGCGAGGAGATAGT 49
Variant 3  ------------------------------------------------TTACAGGAACAGT 13
                                                             *  * ***
                                        Start site
Variant 1  GTCTGCCTTCTGGAGAGCTG---ACCAAACACTAAGGATGCTGAAGTTCCGAACAGTCCA 117
Variant 2  GTCTGCCTTCTGGAGAGCTG---ACCAAACACTAAGGATGCTGAAGTTCCGAACAGTCCA 106
Variant 3  GTGGCCTCTCTGGAACTCTGCAGACCAACTGCCGTG-ATGCTGAAGTTCCGAACAGTCCA 72
           **   *  ******   ***   *****   *   * ************************

Variant 1  TGGGGGCCTGAGGCTCCTGGGAATCCGCCGAACCTCCACCGCCCCCGCTGCCTCCCCAAA 177
Variant 2  TGGGGGCCTGAGGCTCCTGGGAATCCGCCGAACCTCCACCGCCCCCGCTGCCTCCCCAAA 166
Variant 3  TGGGGGCCTGAGGCTCCTGGGAATCCGCCGAACCTCCACCGCCCCCGCTGCCTCCCCAAA 132
           ************************************************************

Variant 1  TGTCCGGCGCCTGGAGTATAAGCCCATCAAGAAAGTCATGGTGGCCAACAGAGGTGAGAT 237
Variant 2  TGTCCGGCGCCTGGAGTATAAGCCCATCAAGAAAGTCATGGTGGCCAACAGAGGTGAGAT 226
Variant 3  TGTCCGGCGCCTGGAGTATAAGCCCATCAAGAAAGTCATGGTGGCCAACAGAGGTGAGAT 192
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Variant 1  TGCCATCCGTGTGTTCCGGGCCTGCACGGAGCTGGGCATCCGCACCGTAGCCATCTACTC 297
Variant 2  TGCCATCCGTGTGTTCCGGGCCTGCACGGAGCTGGGCATCCGCACCGTAGCCATCTACTC 286
Variant 3  TGCCATCCGTGTGTTCCGGGCCTGCACGGAGCTGGGCATCCGCACCGTAGCCATCTACTC 252
           ************************************************************

Variant 1  TGAGCAGGACACGGGCCAGATGCACCGGCAGAAAGCAGATGAAGCCTATCTCATCGGCCG 357
Variant 2  TGAGCAGGACACGGGCCAGATGCACCGGCAGAAAGCAGATGAAGCCTATCTCATCGGCCG 346
Variant 3  TGAGCAGGACACGGGCCAGATGCACCGGCAGAAAGCAGATGAAGCCTATCTCATCGGCCG 312
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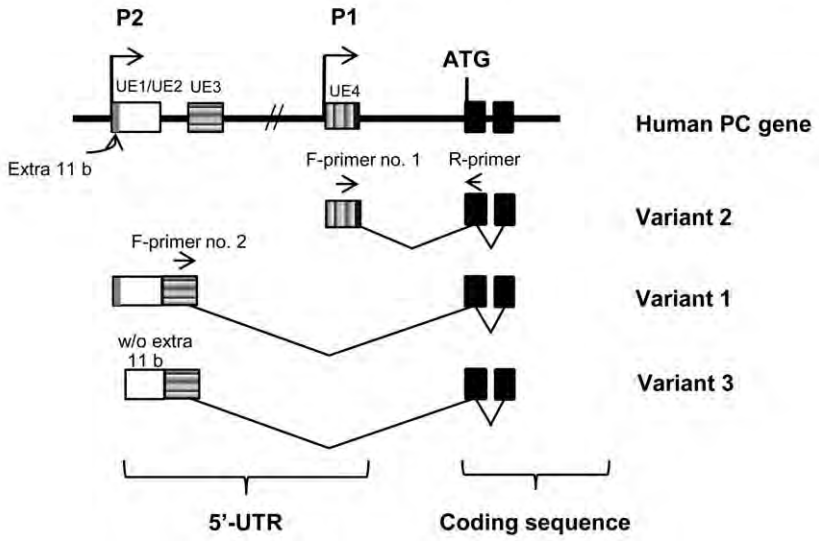
B



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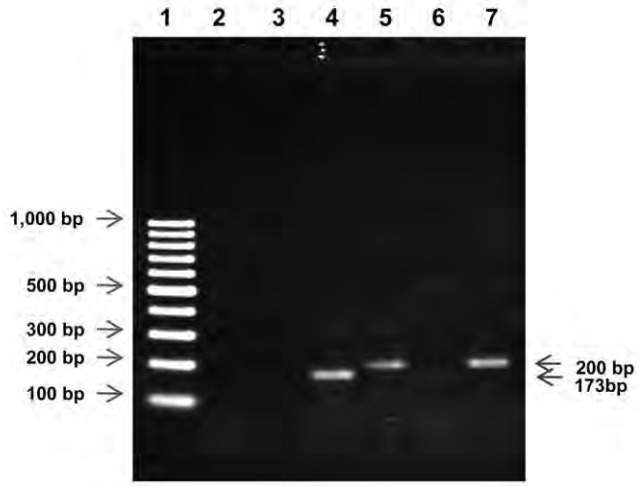

Figure 1. RT-PCR analysis of PC mRNA variants in human liver and human pancreatic islets. (A) Schematic diagram showing alignment of 3 variants of human PC mRNA (GenBank NM_000920.3, NM_022172.2, BC011617.2). (B) Schematic diagram showing the structure of the human PC gene. Two isoforms of human PC mRNA are initiated by two alternative promoters, the proximal (P1) promoter and the distal (P2) promoter. All PC mRNA variants contain the same coding sequences but differ in their 5′-untranslated regions (UTR) produced from different 5′-UTR exons (UE1/UE2, UE3 and UE4) (C) Examination of human PC mRNA in liver and pancreatic islets using RT-PCR. Two sets of primers were used to amplify two different isoforms of human PC mRNA both in human liver and human islets. The 173 bp fragment PCR product of variant 2 and the 200 bp fragment PCR product of variant 1 were amplified by using Primers set no. 1 and primer set no.2, respectively, Lane 1; 1 kb marker, Lane 2; Negative control for primer set no.1, Lane 3; Negative control for primer set no.2, Lane 4; PCR using primer set no.1 and cDNA prepared from human liver, Lane 5; PCR using primer set no.2 and cDNA prepared from human liver, Lane 6; PCR using primer set no.1 and cDNA prepared from human islets, Lane 7; PCR using primer set no.2 and cDNA prepared from human islets.
doi:10.1371/journal.pone.0055139.g001

Results and Discussion

The Human PC Gene is Regulated by Two Promoters and the Distal Promoter is Functional in Pancreatic β-cells

We have previously reported two PC mRNA isoforms with distinct 5′-untranslated regions (UTR) that contain the same coding sequences have been identified in liver and kidney. These two mRNA variants are likely to be generated from alternate transcription from two promoters [13]. In contrast to our study, Wang et al [19] compared the 5′-UTR sequences of three human PC mRNA variants namely, variant 1 (NM_000920.3), 2 (NM_022172.2) and 3 (BC011617.2) deposited at the NCBI database to the genomic sequence of human PC gene and concluded that these variants are alternatively spliced from four 5′-UTR exons, i.e. UE1, UE2, UE3 and UE4, respectively, with the distal, middle and proximal promoters located immediately upstream of exons UE1, UE2 and UE4, respectively [19].

However, we re-examined the alignment of those three variants and found that variants 1 and 3 share the common 83 nucleotides upstream of the first initiation codon, while variant 1 contains 11 additional nucleotides at its 5′-end (see Figure 1A). Wang et al [19] reported that this extra sequence is derived from an upstream exon, UE1. However, direct comparison of 5′-UTR sequences of variants 1 and 3 with the genomic sequence of the human PC gene clearly showed that these extra 11 nucleotides in variant 1 are located immediately upstream of UE2, thus forming part of this exon. Therefore, it is highly likely that the 11 nucleotide segment in variant 1 could easily be a truncated transcript or result from the use of multiple start sites of the TATA-less genes. In agreement with Wang et al [19], the 5′-UTR sequence of variant 2 is derived from a separate 5′ UTR exon which is located proximal to the first coding exon. The lack of an intron between UE1 and UE2 rules out the possibility that there is a middle promoter located between these two upstream exons as proposed by Wang et al [19]. Based on this new information we revised the structural organization of the human PC gene as follows: the human PC gene contains only three 5′-UTR exons, i.e. UE1/UE2, UE3 and UE4, with the proximal promoter located upstream of UE4 and the distal promoter located upstream of UE1/UE2. Transcription initiated from the proximal promoter produces variant 2 while transcription from the distal promoter produces variants 1 and 3 (Figure 1B). The presence of two alternative promoters of human PC gene appears to recapitulate that of the rat [14] and mouse PC genes [14]. This is in contrast to bovine PC gene which possesses three promoters, the proximal (P1), middle (P2) and distal (P3) promoter [20]. However, there is no report about which of these promoters is highly active in bovine pancreatic β-cells.

Although the two PC mRNA isoforms have been described in liver and kidney [13,19], it is not known which of these isoform(s) is expressed in human pancreatic islets. To address this question, we performed an RT-PCR analysis of cDNA prepared from human islets using two forward primers that specifically bind to the 5′-UTRs of variant 1 and variant 2 together with a reverse primer that binds to exon 1 (see Figure 1B). With these primers, the amplicons with sizes of 173 bp and 200 bp, representing variant 1 and variant 2 were expected. As shown in Fig. 1C, both primer sets were able to amplify the 173 bp and 200 bp PCR products representing variants 1 and 2 which are produced from both proximal and distal promoters of the human PC gene from HepG2 cDNA (lanes 4 and 5), respectively. This result indicated that both proximal and distal promoters are active in liver. In a sharp contrast, RT-PCR of cDNA prepared from human islets produced a faint band of the 173 bp PCR product amplified by primers set no.1 (lane 6) while primer set no. 2 amplified a strong band of the 200 bp PCR product (lane 7), suggesting that the distal promoter of the human PC gene primarily controls its transcription in human pancreatic islets similarly to rat islets.

Cloning and Characterization of hP2 Promoter

To identify the critical *cis*-acting elements that control PC transcription in pancreatic islets, we isolated approximately the 1 kb upstream sequence of exon UE1/2 of the human PC gene which would potentially serve as the distal promoter (hP2) of the PC gene using PCR with the primers designed from the human genome database [21]. A comparison of the nucleotide sequences of the hP2 promoter with the distal promoter of rat PC gene revealed that they are 59.6% similar, with the highest similarity observed within the first 500 nucleotides. The hP2 promoter lacks a canonical TATA box in the first 100 nucleotides but contains two copies of CCAAT boxes and one copy of a GC box located at nucleotide positions −101/−97, −71/−67 and −54/−39, respectively. These features are the characteristic of housekeeping genes [22]. Further analysis of the hP2 promoter sequence using the PROMO database [23] identified several putative transcription factor binding sites including USF1/USF2, Sp1 and HNF3β/FoxA2. These putative binding sites are also conserved in the rat PC gene (Figure 2).

To determine the transcriptional activity of the distal promoter of the human PC gene, a series of 5′-truncated hP2 promoter constructs were generated and used in transient transfection experiments. In this study, eight constructs of the hP2 promoter were transiently transfected into INS-1 832/13 cells. As shown in Fig. 3, deletions of regions −1108 to −985, −640, and −489 did not significantly affect promoter activity. However, when the deletions were made from the region −498 to −365, this resulted in a significant increase of promoter activity, suggesting the presence of a repressor element between these regions. On the other hand, deletions from the region −365 to −240 resulted in a significant decrease in promoter activity, suggesting the presence of (a) positive regulatory element(s) in this region. Further deletion from −240 to −114 did not affect promoter activity. However, deletion to −40 resulted in a dramatic decrease of promoter activity, suggesting the presence of a second positive regulatory element between −114 and −40.

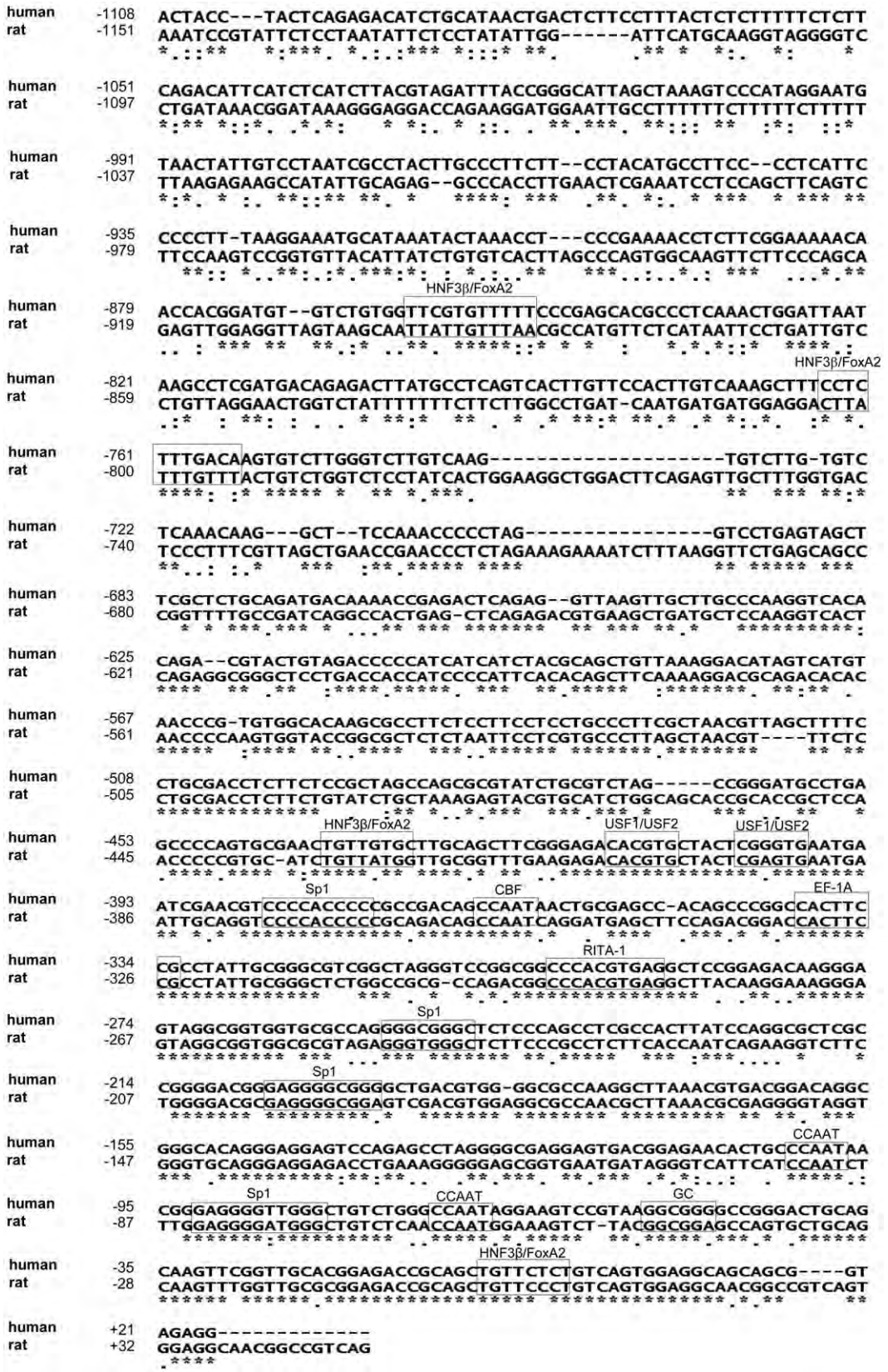

Figure 2. The human PC P2 promoter sequence and its alignment with the rat PC P2 promoter. Boxes represent the putative transcription factor binding sites for Sp1, FoxA2/HNF3β, USF1/2, and CBF. Identical nucleotides between human and rat sequence are symbolized by an asterisk.

doi:10.1371/journal.pone.0055139.g002

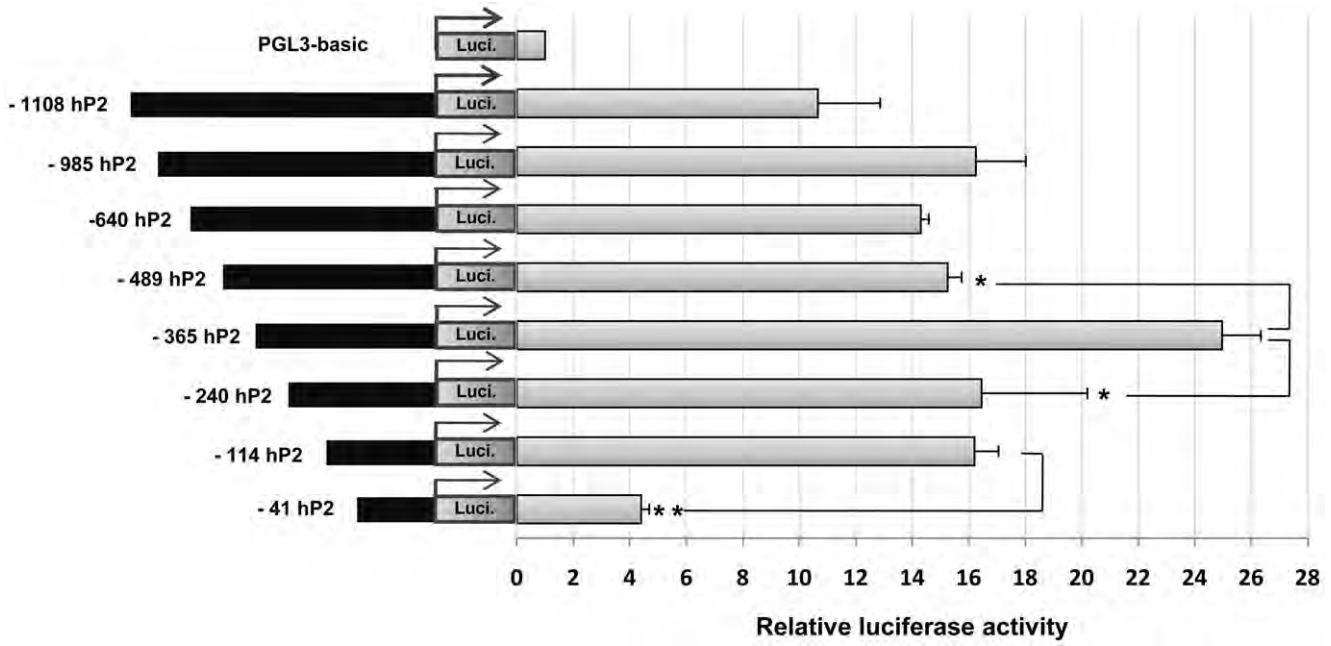

Figure 3. Localization of *cis*-acting elements of the human PC P2 promoter. Transient transfections of 8 constructs containing of the 5′-truncated hP2 promoter into INS-1 832/13 cells were performed to identify the regulatory regions of the hP2 promoter. The basal activity of each 5′-truncated hP2 promoter was calculated from the values of luciferase activity which was normalized with the values of β-galactosidase activity to control for transfection efficiency. The normalized luciferase activity of each P2 construct was compared with the activity of the pGL3-basic vector which was arbitrarily set to 1 and presented as the relative luciferase activity. *P value <0.05, **P value <0.01.
doi:10.1371/journal.pone.0055139.g003

The −69/−54, −340/−315 Regions of the hP2 Promoter Contain *cis*-acting Elements that Confer Non-beta Cell and Beta-cell Specificity, Respectively

As the first *cis*-acting element which serves as an activator sequence was located between −114 and −41 of the hP2 promoter, a series of 15 bp-internal deletions across this region were generated in order to precisely map the critical element located in this region. These mutant constructs were transiently transfected into both the INS-1 832/13 cell line and the human embryonic kidney cell line, HEK293T. A schematic diagram of 15 bp deletions of the −114/−39 region of the hP2 promoter is shown in Figure 4A. As shown in Figure 4B, transient transfections of −114/−99, −99/−84, −84/−69 deletion mutants did not significantly affect the reporter activity in either cell line. However, deletion of regions between −69 and −54 (−69/−54 hP2) resulted in a dramatic decrease in promoter activity to 35% and 25% of that seen with the INS-1 832/13 and HEK293T cell lines, respectively, suggesting that the −69 to −54 region of the hP2 promoter contains (a) critical *cis*-acting element(s) for basal transcription factors in both the INS-1 832/13 and the HEK293T cell lines. Examination of the nucleotide sequence located between the −69 and-54 of the hP2 construct identified the presence of a CCAAT box located between −71 and −67 (Figure 4B, underlined). To examine whether the dramatic decrease of the luciferase reporter activity observed from the −69/−54 hP2 mutant construct could indeed be attributed to the lack of an intact CCAAT box, we generated another mutant (−71/−67 hP2) in which the whole CCAAT box was deleted. Transient transfection of this mutant construct into INS-1 832/13 and HEK293T cells resulted in a marked reduction of promoter activity in both cell lines, similar to that of the −69/−67 hP2 mutant construct, suggesting that the −71/−67 CCAAT box is crucial for maintaining basal activity of the P2 promoter both in INS-1 832/13 and HEK293T cells. Deletion of the regions between −54 to −39 (−54/−39 hP2 construct), resulted in a marginal reduction of the reporter activity in both cell lines. Examination of the nucleotide sequence surrounding this region identified the presence of a GC-box, which is also found in the identical position in the distal promoter of the rat PC gene. This GC-rich region serves as a binding site for ubiquitous transcription factors Sp1/Sp3 [24]. Mutation of this similarly located GC-box in the rat gene resulted in a reduction of the reporter gene activity to a greater extent (80% reduction) than mutation of this sequence in the human gene [24], suggesting the rat and human PC genes are regulated differently via the GC-box.

A CCAAT box serves as a potential binding site for the nuclear factor Y (NF-Y) [25] and binding of this factor to this sequence is essential for transcriptional activation of TATA-less genes [26,27]. We confirmed this by performing gel shift experiments. As shown in Figure 4C, incubation of the –78/−54 probe harboring the −71/−67 CCAAT box with a nuclear extract of INS-1 832/13 cells produced a predominant DNA-protein complex (lane 1). This complex was readily competed off with 10x and 50x unlabelled WT double-stranded oligonucleotide (lanes 2–3), but was not competed off with an unrelated double stranded oligonucleotide sequence (lane 4). Incubation of anti-NF-Y polyclonal antibody prevented the formation of a DNA-protein binding complex (lane 5). A similar result was obtained when a nuclear extract of HEK293T cells was used in the experiment (lanes 6–10). These data indicate that NF-Y is a transcription factor that directs PC transcription via the −71/−67 CCAAT box in both cell lines. Although this CCAAT box appears to be conserved in the distal promoter of both the rat and human PC genes, it serves different roles in transcriptional regulation in the two genes. In the distal promoter of rat PC gene, this CCAAT box serves a repressor element, while in the human PC gene, this sequence clearly acts as

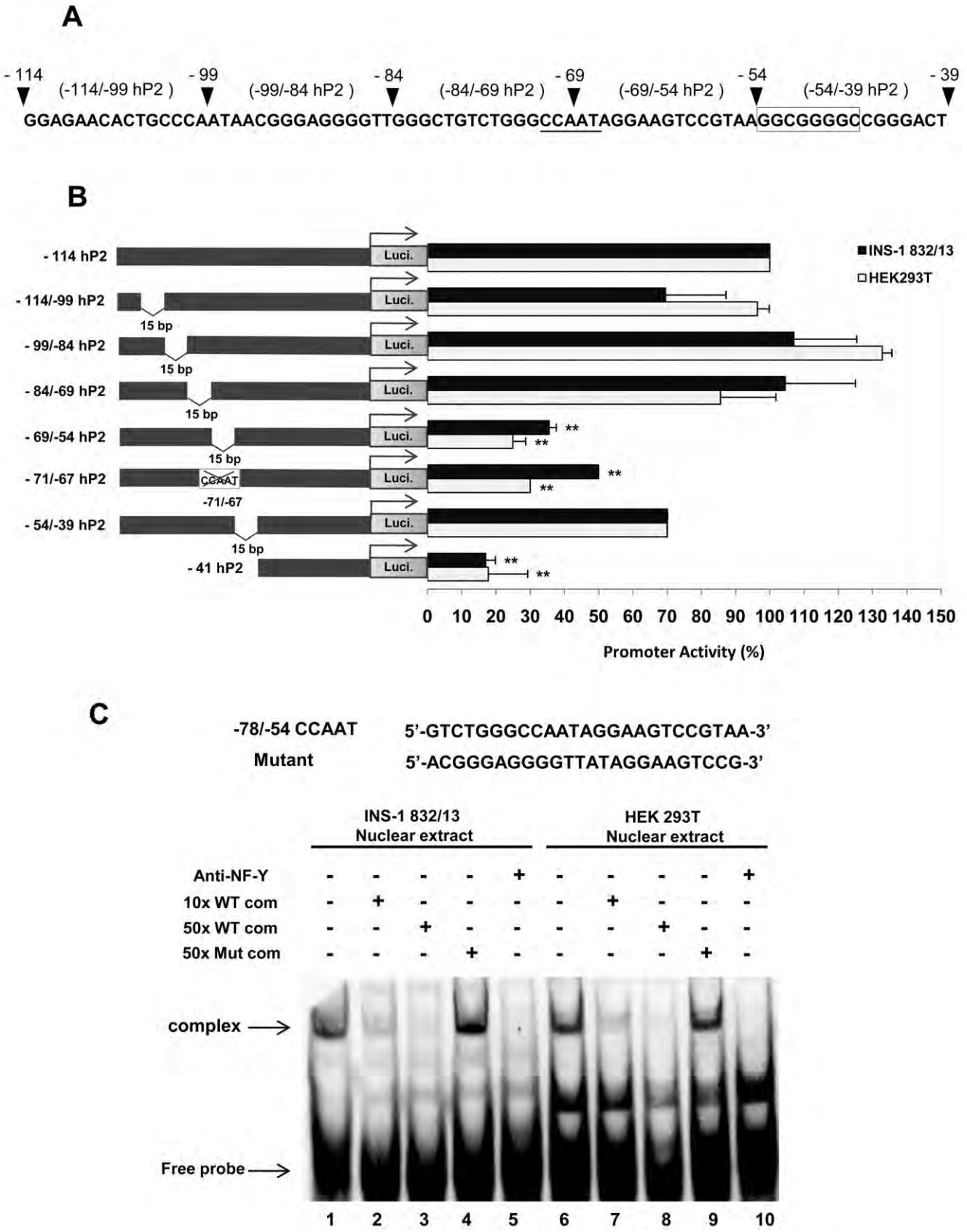

Figure 4. Identification of positive regulatory element(s) located between −114 and −39 of the human PC P2 promoter. (A) Schematic diagram of 15 bp internal deletions of −114/−39 of the human PC P2 promoter. (B) Transient transfections of a series of 15 bp internal deletion constructs into the INS-1 832/13 and non-beta cell HEK293T cell lines were performed to localize the positive regulatory sequence in the

human PC P2 promoter. The luciferase activity of each construct was normalized with the β-galactosidase activity. The normalized reporter activity obtained from each construct is shown as a percent relative to those transfected with the wild type −365 hP2 promoter that was arbitrarily set at 100%. *P value <0.05, **P value <0.01. (C) Gel shift and supershift assays of biotin-labeled probe −78 to −54 region of hP2 promoter (−78/−54 CCAAT-probe) using INS-1 832/13 nuclear extract (Lane 1–5) and non-beta cell HEK293T (Lanes 6–10). The nucleotide sequence of wild type and mutant of the hP2 promoter in the −78 to −54 regions are also shown. Lanes 1 and 5 show probes incubated with nuclear extracts from INS-1 832/13 or HEK293T cells; lanes 2 and 6, 10-fold excess wild-type unlabeled oligonucleotides were incubated with nuclear extracts and probes; lanes 3 and 7, 50-fold excess wild-type unlabeled oligonucleotides were incubated with nuclear extracts and probes; lane 4 and 9, 50-fold excess amount of mutant unlabeled oligonucleotides were incubated with nuclear extracts and probes; lanes 5 and 10, nuclear extracts were pre-incubated with anti-NF-Y antibody before the probes were added to the reactions. Arrow represents CCAAT box–NF-Y, complex.
doi:10.1371/journal.pone.0055139.g004

an activator sequence. This dual function of NF-Y being both transcriptional activator and repressor is not totally unexpected as this depends on the promoter context [28]. NF-Y can possess a repressor activity if its recognition sequence is overlapped with the nearby activator binding sequence, antagonizing activator function [29–31]. The above data indicate that although the *cis*-acting elements including the CCAAT box and the GC-box are found in similar locations for both human and rat PC genes, their actions are substantially different. In the rat PC gene, the CCAAT box serves as a repressor element that somewhat antagonizes the GC-box function [24], while in the human PC gene, the CCAAT box sequence clearly acts as an activator element. As the GC-box in the human PC gene is not as strong an activator as in the rat PC gene, it appears that in the human PC gene, the upstream CCAAT box acts as an activator sequence to maximize transcription.

To more precisely localize the positive regulatory sequences between −365 and −240 of hP2, 25-bp internal deletions of the −365/−240 hP2 promoter were made. Five mutants harboring 25 bp internal deletions across the −365 to −240 regions (−365/−340, −340/−315, −315/−290, −290/−265 and −265/−240 hP2) were generated and transfected into both INS-1 832/13 and HEK293T cells. A schematic diagram of the 25 bp deletions of the −365/−240 hP2 promoter region is shown in Figure 5A. As shown in Figure 5B, transient transfection of −340/−315 hP2 mutant construct markedly reduced the reporter gene activity to 50% of the −365 hP2 promoter in INS-1 832/13 cells, while no reduction of reporter gene activity was observed in HEK293T cells. In contrast, deletion of other regions did not affect the promoter activity when compared to the wild type −365 hP2 promoter in either cell line. These data suggest the presence of a tissue specific *cis*-acting element(s) located between −340 and −315 in the hP2 promoter. To identify which transcription factors might bind to this element we performed gel shifts experiments in which double stranded oligonucleotides harboring −340/−315 were incubated with a nuclear extract of INS-1 832/13 cells. As shown in Figure 5C, a strong DNA-protein complex was observed. Examination of nucleotide sequences between −340 and −315 identifies an E-box, a binding site for USF [32], located between –341 and −336 and a GC-box and a binding site for Sp1/Sp3, located between –326 and −320, respectively. Incubation of an anti-Sp1 antibody in the binding reaction produced a weak supershift band, while incubation in the presence of anti-Sp3, anti-USF1 or anti-USF2 antibodies had no effect on the DNA-protein complex formation, indicating that these three factors may not attribute to the binding to this sequence. To confirm the gel shift experiment, we performed a transactivation assay in which the wild type (−365 hP2) construct was co-transfected with plasmid overexpressing Sp1, Sp3, USF1 or USF2, and the luciferase activities were measured. As shown in Figure 6, co-transfection of Sp1 or Sp3 resulted in only 1.5-fold or 2-fold increase in the reporter gene activity, consistent with a poor or lack of evidence of their binding to the −340/−315 sequence shown in Figure 5C. Mutation of this sequence also had no effect on the expression of the reporter gene. The poor remaining Sp1 and Sp3-mediated transcriptional activation of the human PC promoter may be attributed to the GC box located at −54/−39 (Figure 4A). Despite the lack of evidence of binding of USF1 or USF2 to the E-box located between −340/−315, overexpression of USF1 or USF2 resulted in approximately 5-fold or 10-fold increase in the promoter activity. However, deletion of the sequences located between −340 and −315 did not significantly affect USF1- or USF2- mediated transcriptional activation of the human PC promoter, suggesting that the transactivation by these two factors may be mediated through the downstream E-boxes.

In summary we have shown that: (i) the human PC gene possesses only two promoters, P1 and P2, which mediate transcription of the human PC gene similar to the rat and mouse genes; (ii) the P1 and P2 promoters are active in hepatocytes while only the P2 promoter is active in pancreatic β-cells; (iii) both CCAAT box and GC-boxes serve as activator sequences in β-cells; (iv) a *cis*-acting element located between −340/−315 serves as binding site for β-cell specific transcription factor.

Materials and Methods

Reverse Transcriptase-polymerase Chain Reaction (RT-PCR)

To identify the predominant isoform of the human PC mRNA in pancreatic beta cells, RT-PCR using human cDNA prepared from human islets and liver was performed. In this experiment, two sets of primers directed to various 5′-UTR exons of the PC gene (GenBank NM_000920.3, NM_022172.2, BC011617.2) were designed and used in RT-PCR. Both primer sets consisted of the same sequence of the reverse primer (R-primer) and a different sequence of the forward primer (F-primer). The F-primer set no. 1 (5′-ACCAACTGCCGTGATGCTGA-3′) was designed to bind to the 5′-UTR of variant 2 of human PC mRNA which is transcribed by the proximal promoter while the F-primer set no. 2 (5′-GATAGTGTCTGCCTTCTGGAGAGC-3′) was designed to bind to the 5′-UTR region of variant 3 of the human PC mRNA which is transcribed by the distal promoter. The R-primer (5′-ACACACGGATGGCAATCTCACC-3′) was designed to bind to exon 1 of human PC mRNA [33]. Tissues were homogenized with a Qiashredder (Qiagen) (islets) or using a Potter–Elvehjem homogenizer (liver) and RNA was prepared using the RNeasy Mini kit (Qiagen). On-column DNase digestion was performed using the Qiagen RNase-Free DNase Set. cDNA was made with randomized primers with the Retroscript kit (AM1710) (Applied Biosystems). Quantitative PCR was performed on a BioRad MyIQ Real Time Detection System with SYBR Premix Ex Taq (RR041Q) (Takara). Human liver RNA was from a 51-year old male (Clontech, catalog number 636531) and a liver surgical specimen from a person (of unknown age and gender due to privacy protection) [34]. The PCR was carried out in a 20 μl-reaction mixture containing 2 μl of cDNA, 1x PCR reaction buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 0.2 μM of each primer, 100 μM of each dNTP, 2 mM $MgCl_2$, and 1 unit *Taq* DNA

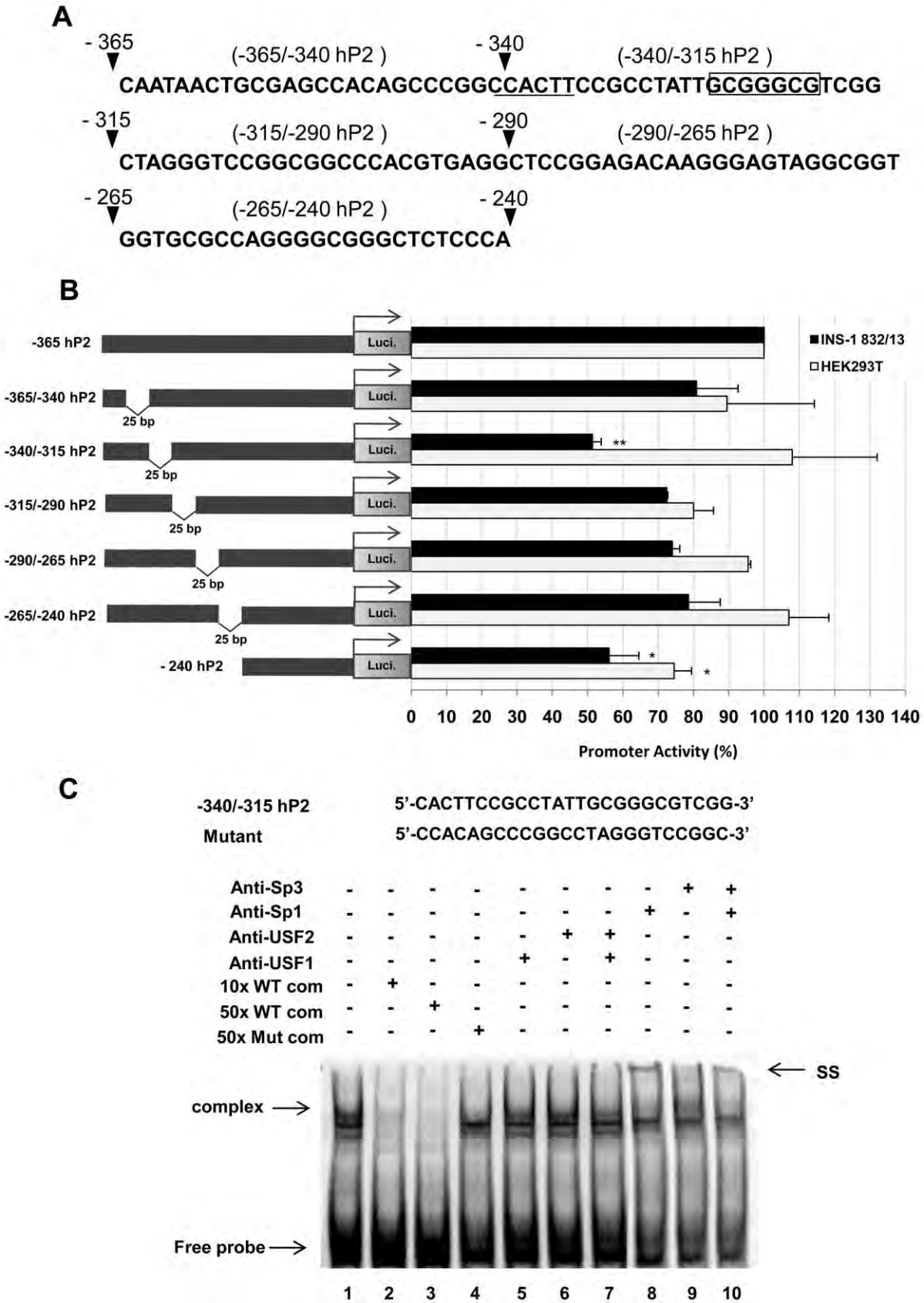

Figure 5. Identification of positive regulatory element(s) located between −365 and −240 of the human PC P2 promoter. (A) Schematic diagram of 15 bp internal deletions of −114/−39 the human PC P2 promoter. (B) Transient transfections of a series of 25 bp internal deletion constructs into the INS-1 832/13 cell line and non-beta cell HEK293T cell line were performed to identify the positive regulatory sequences in

the hP2 promoter. The luciferase activity of each construct was normalized with β-galactosidase activity. The normalized reporter activity obtained from each construct is shown as a percent relative to those transfected with the wild type −365 hP2 promoter, which was arbitrarily set at 100%. *P value <0.05, **P value <0.01. (C) Gel shift and supershift assays of the biotin-labeled probe of the −78 to −54 region of the hP2 promoter (−340/−315 hP2 probe) using an INS-1 832/13 nuclear extract. The nucleotide sequences of the wild type and mutant of the hP2 promoter −78 to −54 regions are also shown. Lane 1 probes incubated with nuclear extracts from INS-1 832/13; lanes 2–3, 10-fold or 50-fold excess wild-type unlabeled oligonucleotides were incubated with nuclear extracts and probes; lane 4, 50-fold excess amount of mutant unlabeled oligonucleotides were incubated with nuclear extracts and probes; lanes 5–7, nuclear extracts were pre-incubated with anti-USF1 or anti-USF2 or both, respectively, before the probes were added to the reactions. Lanes 8–10, nuclear extracts were pre-incubated with anti-Sp1 or anti-Sp3 or both, respectively, before the probes were added to the reactions. Arrow represents DNA-protein complex, SS = supershift band.
doi:10.1371/journal.pone.0055139.g005

polymerase. The PCR profile consisted of an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 45 sec, and final extension at 72°C for 10 min.

Cloning of hP2 Promoter Linked Luciferase Gene Constructs

The 1,108 bp fragment of the hP2 promoter was cloned from genomic DNA isolated from HepG2 cells using the hP2-forward primer (5′-GGTACCACTACCTACTCAGAGACATCTGC-3′; underline indicates a *Kpn*I restriction site) and the hP2-reverse primer (5′-CTCGAGGTCCTCGCCGCCGCCTCTACC-3′; underline indicates a *Xho*I restriction site). The PCR product was then ligated to the pGEM-T Easy vector (Promega) and sequenced. The clone with the correct sequence of the hP2 promoter was excised from the pGEM-T easy vector with *Kpn*I and *Xho*I sites and ligated to the equivalent sites of the pGL3-basic vector (Promega) to generate a hP2-luciferase reporter construct. 5′-truncated hP2 promoter constructs comprising 985, 640, 365, 240, 114, and 41 nucleotides of the hP2 promoter were generated by PCR using a full length hP2 promoter-luciferase construct as a template. The forward primers containing a *Kpn*I site at their 5′-ends and the reverse primer containing an *Xho*I site at the 3′-end were designed. The PCR products were then ligated into the pGEM-T Easy vector and sequenced. The correct sequences of 5′-truncated hP2 promoter were excised with *Kpn*I and *Xho*I and ligated to the equivalent sites of the pGL3-basic vector. Primers used for cloning of 5′-truncated hP2 promoters are shown in Table 1. For the construction of a 489 bp fragment of hP2 promoter, the promoter was generated by double digestion of the full length hP2 promoter-luciferase construct with *Nhe*I and *Xho*I. The 489 bp fragment of the hP2 promoter was then re-ligated into the *Nhe*I and *Xho*I site of the pGL3-basic vector.

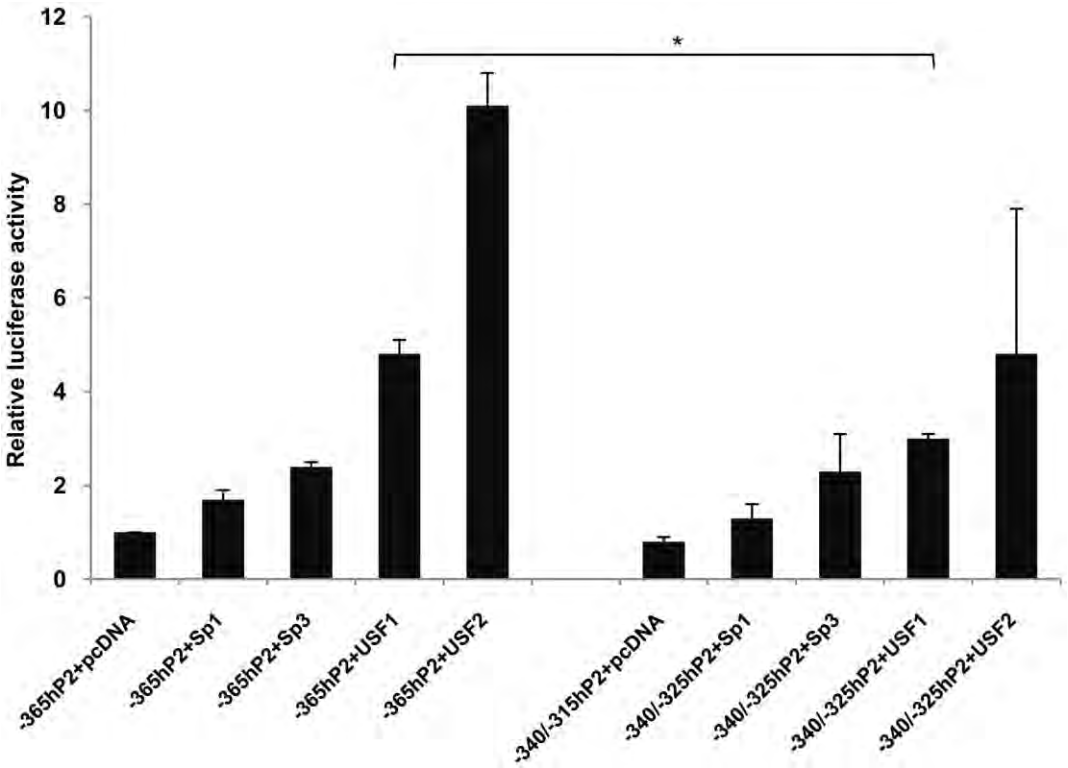

Figure 6. Transactivation of a WT −365 human PC P2 luciferase reporter construct and its mutant by Sp1, Sp3, USF1 or USF2. WT −365 hP2 or −340/−315 hP2 constructs were co-transfected with an empty vector (pcDNA3) or a plasmid overexpressing Sp1, Sp3, USF1 or USF2 into the INS-1 832/13 cell line, and the luciferase activities measured. The luciferase activity was normalized to β-galactosidase activity and expressed as relative luciferase activity. Relative luciferase values obtained from co-transfecting cells with wild type (−365 hP2) or its mutant (−340/−315 hP2) and plasmid overexpressing Sp1, Sp3, USF1 or USF2 were presented as fold change relative to those obtained from those co-transfected with WT with empty vector (pcDNA3) which was arbitrarily set at 1. *p≤0.01.
doi:10.1371/journal.pone.0055139.g006

Table 1. Oligonucleotides used for construction of 5′-trucated hP2 promoter.

Primer name	Sequences (5′ to 3′)	Length (bp)
−985 bp hP2-F	GGTACCTTGTCCTAATCGCCTACTTGC	27
−640 bp hP2-F	GGTACCTTGCCCAAGGTCACACAGACG	27
−365 bp hP2-F	GGTACCCAATAACTGCGAGCCACAGC	26
−240 bp hP2-F	GGTACCGCCTCGCCACTTATCCAGGCG	27
−114 bp hP2-F	GGTACCGGAGAACACTGCCCAATAACG	27
−41 bp hP2-F	GGTACCCTGCAGCAAGTTCGGTTGCACG	28
−39 bp hP2-R	CTCGAGGTCCTCGCCGCCGCCTCTACC	27

*Restriction enzyme recognition sites are underlined.
doi:10.1371/journal.pone.0055139.t001

Site-directed Mutagenesis

Site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Agilent Technologies) was performed to generate 5, 15 and 25 nucleotide internal deletion mutants of the hP2 promoter constructs. The mutagenesis reaction was carried on in a total volume of a 50 µl-reaction mixture containing 300 ng of DNA template, 125 ng of each mutagenic oligonucleotide primer, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl pH 8.8, 2 mM $MgSO_4$, 0.1% TritonX-100 and 0.1 mg/ml nuclease-free bovine serum albumin (BSA), 200 µM dNTP mix, and 2.5 U of *Pfu*Turbo polymerase (Stratagene-Agilent Technologies). The amplification profile consisted of an initial denaturation at 95°C for 30 sec followed by 20 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 1 min, and extension at 68°C for 10 min. The primers used for site-directed mutagenesis are shown in Tables 1 and 2. The correct mutant constructs were verified by automated nucleotide sequencing. The corrected clones with 5, 15 or 25 nucleotide deletion were double digested with *Kpn*I and *Xho*I and re-ligated into the pGL3 basic vector digested with the same enzymes.

Cell Culture and Transfection

INS-1 832/13 cells [35] were maintained in RPMI 1640 supplemented with 28 mmol/l $NaHCO_3$, 1 mM sodium pyruvate (Gibco), 50 µM β-mercaptoethanol, 10% (v/v) heat-inactivated fetal bovine serum (Gibco), and 50 units/l penicillin/streptomycin at 37°C in 5% CO_2. In the transfection experiments, 2×10^5 cells were seeded in 24-well plates and were cultured in 0.5 ml of antibiotic-free DMEM (Dulbecco's modified Eagle's medium; Gibco) containing 10% fetal bovine serum for 24 h before transfection. Cells were transfected with 250 ng of the luciferase reporter constructs and 250 ng of pRSV-β-gal plasmid expressing β-galactosidase using Lipofectamine^{TM} 2000 reagent (Invitrogen). For transactivation assays, 250 ng of plasmids overexpressing Sp1, Sp3 [36], USF1 or USF2 [24] were also included with the luciferase reporter construct and pRSV-β-gal plasmid. The transfected cells were maintained in the antibiotic-free DMEM at 37°C for 48 h. For the transfection of the non-beta cell line, the human embryonic kidney cell line (HEK293T) was grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum, and 50 units/L penicillin/streptomycin at 37°C in 5% CO_2. The transfections were carried out as described for the INS-1 832/13 cells [37], except that the cells were seeded in 24-well plates at a density of 4×10^5 cells. The luciferase reporter assays were performed using the luciferase reporter assay system (Promega), while the β-galactosidase assay was performed using ONPG as substrate.

Table 2. Oligonucleotides used for generation of 25 bp deletion of −365/−240 hP2, 15 bp deletion of −114/−39 hP2 and 5 bp deletion of −114/−39 hP2 promoter constructs.

Primer name	Sequences (5′ to 3′)	Construct name
−365/−340 hP2-F	TCGATTGGTACCCACTTCCGCCTA	−365/−340 hP2
−365/−340 hP2-R	TAGGCGGAAGTGGGTACCAATCGA	
−340/−315 hP2-F	CCACAGCCCGGCCTAGGGTCCGGC	−340/−315 hP2
−340/−315 hP2-R	GCCGGACCCTAGGCCGGGCTGTGG	
−315/−290 hP2-F	TGCGGGCGTCGGCTCCGGAGACAA	−315/−290 hP2
−315/−290 hP2-R	TTGTCTCCGGAGCCGACGCCCGCA	
−290/−265 hP2-F	GCCCACGTGAGGGGTGCGCCAGGG	−290/−265 hP2
−290/−265 hP2-R	CCCTGGCGCACCCCTCACGTGGGC	
−265/−240 hP2-F	GGAGTAGGCGGTGCCTCGCCACTT	−265/−240 hP2
−265/−240 hP2-R	AAGTGGCGAGGCACCGCCTACTCC	
−114/−99 hP2-F	TCGATAGGTACCATAACGGGAGGG	−114/−99 hP2
−114/−99 hP2-R	CCCTCCCGTTATGGTACCTATCGA	
−99/−84 hP2-F	GAACACTGCCCAGGGCTGTCTGGG	−99/−84 hP2
−99/−84 hP2-R	CCCAGACAGCCCTGGGCAGTGTTC	
−84/−69 hP2-F	ACGGGAGGGGTTATAGGAAGTCCG	−84/−69 hP2
−84/−69 hP2-R	CGGACTTCCTATAACCCCTCCCGT	
−69/−54 hP2-F	CTGTCTGGGCCAGGCGGGGCCGGG	−69/−54 hP2
−69/−54 hP2-R	CCCGGCCCCGCCTGGCCCAGACAG	
−71/−67 hP2-F	GGGCTGTCTGGGAGGAAGTCCGTA	−71/−67 hP2
−71/−67 hP2-R	TACGGACTTCCTCCCAGACAGCCC	
−54/−39 hP2-F	GGAAGTCCGTAAGCAGCAAGTTCG	−54/−39 hP2
−54/−39 hP2-R	CGAACTTGCTGCTTACGGACTTCC	

doi:10.1371/journal.pone.0055139.t002

Electrophoretic Mobility Shift Assay (EMSA)

1×10^7 of INS-1 832/13 cells were harvested for preparation of nuclear extracts. The cells were washed with PBS and resuspended in 1 ml of nuclear extraction buffer I (10 mM HEPES pH7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 1x protease inhibitor cocktail (Roche) at 4°C for 1 min. The nuclei were centrifuged at 3,000 g at 4°C for 1 min before resuspended in 100 µl nuclear buffer 2 (20 mM HEPES, pH7.9, 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA and 0.2 mM PMSF) and incubated on ice for 5 min. The nuclear lysate was centrifuged at 3,000 g for 5 min at 4°C and the supernatant was kept at −80°C and used for EMSA.

The 5′-end labeled biotinylated oligonucleotide was synthesized by BioBasic (Canada) and annealed with the unlabelled complementary strand oligonucleotide. The oligonucleotides used in EMSA are listed in Table 3. The DNA-protein binding assay was carried out in a 20 µl-reaction mixture containing 1x binding buffer (25 mM HEPES, pH7.9), 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM EDTA, 0.5 mM DTT and 0.2 mM PMSF, 10 µg of nuclear extract, 2 µg of poly dI-dC and 120 fmole of biotinylated double stranded oligonucleotide at 4°C for 30 min. For supershift assays, 1 µg of anti-Sp1 (sc-59), anti-Sp3 (sc-644), anti-USF1 (sc-22) or anti-USF2 (sc-862) polyclonal antibody (SantaCruz Biotech) was included in the binding reaction. The DNA-protein complexes were analyzed by

Table 3. Oligonucleotides used in EMSA.

Oligonucleotide	Sequence (5′- 3′)	Probe/competitor
−78/−54 CCAAT-F*	GTCTGGGCCAATAGGAAGTCCGTAA	Probe/WT competitor
−78/−54 CCAAT-R	TTACGGACTTCCTATTGGCCCAGAC	
−78/−54 CCAAT-MuF	ACGGGAGGGGTTATAGGAAGTCCG	Mutant competitor
−78/−54 CCAAT-MuR	CGGACTTCCTATAACCCCTCCGT	
−340/−315 hP2-F*	CACTTCCGCCTATTGCGGGCGTCGG	Probe/WT competitor
−340/−315 hP2-R	CCGACGCCCGCAATAGGCGGAAGTG	
−340/−315 hP2-MuF	CCACAGCCCGGCCTAGGGTCCGGC	Mutant competitor
−340/−315 hP2-MuR	GCCGGACCCTAGGCCGGGCTGG	

*3′ labeled with biotin.
doi:10.1371/journal.pone.0055139.t003

5% non-denaturing polyacrylamide gel electrophoresis followed by electroblotting. The bands of DNA-protein interaction were detected using LightShift Chemiluminescent EMSA kit (Pierce). The image was captured using Gel Doc System (GeneTools).

Acknowledgments

The authors thank Mindy A. Kendrick and Melissa J. Longacre for technical assistance.

Author Contributions

Conceived and designed the experiments: AT PR SJ MJM. Performed the experiments: AT PR. Analyzed the data: AT PR SJ MJM. Contributed reagents/materials/analysis tools: SJ MJM. Wrote the paper: AT PR SJ MJM.

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Biochimica et Biophysica Acta 1829 (2013) 987–999

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagrm

Hepatocyte nuclear factor 4α regulates the expression of the murine pyruvate carboxylase gene through the HNF4-specific binding motif in its proximal promoter☆

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ARTICLE INFO

Article history:
Received 4 March 2013
Received in revised form 18 April 2013
Accepted 2 May 2013
Available online 9 May 2013

Keywords:
Pyruvate carboxylase
Hepatocyte nuclear factor 4alpha
Transcription
Gluconeogenesis
Biotin-containing enzyme
Upstream stimulatory factor

ABSTRACT

Pyruvate carboxylase (PC) is the first regulatory enzyme of gluconeogenesis. Here we report that the proximal promoter of the murine PC gene contains three binding sites for hepatocyte nuclear factor 4α (HNF4α). These sites include the classical direct repeat 1 (DR1) (−386/−374), non-perfect DR1 (−118/−106) and HNF4α-specific binding motif (H4-SBM) (−26/−14). Under basal conditions, mutation of the non-perfect DR1 decreased promoter activity by 50%, whereas mutation of neither the DR1 nor the H4-SBM had any effect. In marked contrast, only mutation of the H4-SBM decreased HNF4α-transactivation of the promoter activity by 65%. EMSA revealed that HNF4α binds to the DR1site and H4-SBM with similar affinity while it binds poorly to the non-perfect DR1. Interestingly, this non-perfect DR1 also coincides with two E-boxes. Mutation of the non-perfect DR1 together with the nearby E-box reduced USF1- but not USF2-transactivation of promoter activity, suggesting that USF1 partly contributes to the basal activity of the promoter. Substitution of the H4-SBM with the DR1 marginally reduced the basal promoter activity but did not eliminate HNF4α-transactivation, suggesting that HNF4α can exert its effect via DR1 within this promoter context. ChIP-assay confirmed that HNF4α is associated with the H4-SBM. Suppression of HNF4α expression in AML12 cells down-regulated PC mRNA and PC protein by 60% and 50%, respectively, confirming that PC is a target of HNF4α. We also propose a model for differential regulation of P1 promoter of PC gene in adipose tissue and liver.

1. Introduction

Metabolic imbalance of glucose uptake and production results in the development of non-insulin dependent diabetes mellitus (NIDDM, or type 2 DM) which is characterized by insulin resistance in peripheral tissues, and eventually in failure of the insulin producing β-cells to compensate. Insulin exerts its metabolic effect on glucose metabolism by stimulating glucose uptake in skeletal muscle and adipose tissue, glycogen storage in skeletal muscle and liver, and by inhibiting hepatic glucose production (gluconeogenesis). Therefore, the failure of insulin action - known as insulin resistance - can result in overt hyperglycemia caused by the failure of peripheral tissues to uptake glucose and by the over-stimulation of hepatic gluconeogenesis. The latter pathway generates glucose as a major fuel for the brain and red blood cells during prolonged fasting [1]. Gluconeogenesis is regulated by four enzymes: pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (FBPase) and glucose-6-phosphatase (G6Pase). These enzymes are regulated by substrate availability and by hormones including insulin, cAMP and glucocorticoids [2]. While the transcriptional regulation of PEPCK and G6Pase expression is well understood, little is known about transcriptional control of the PC gene [3].

PC catalyzes the ATP-driven carboxylation of pyruvate to oxaloacetate which is subsequently converted to glucose in the pathway of gluconeogenesis. The oxaloacetate generated by PC is also utilized in other biosynthetic pathways, including *de novo* fatty acid synthesis and glyceroneogenesis in adipose tissue, and glutamate synthesis in astrocytes [see [4] for review]. In pancreatic β-cells, PC is also involved in the pyruvate cycling which provides coupling factors required for glucose-induced insulin secretion (GSIS) (for review see [5,6]). Native PC comprises four identical subunits arranged as a tetramer. Each subunit folds into four distinct domains: the biotin carboxylase, carboxyl

Abbreviations: ChIP, chromatin immunoprecipitation; DR1, direct repeat 1; EMSA, electrophoretic mobility shift assay; FBPase, fructose-1,6-bisphosphatase; G6Pase, glucose-6-phosphatase; HIP1, housekeeping initiator protein 1; HNF4α, hepatocyte nuclear factor 4 alpha; MODY, maturity onset diabetes of the young; PC, pyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; PPARγ, peroxisome proliferator activated receptor gamma; PPRE, peroxisome proliferator activated receptor-responsive element; P1, proximal promoter; P2, distal promoter; NR, nuclear receptor; USF, upstream stimulatory factor

☆ Conflict of interest: The authors have declared that no competing interests exist.

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http://dx.doi.org/10.1016/j.bbagrm.2013.05.001

transferase, biotin carboxyl carrier, and the allosteric domains [7]. PC is subject to both pre- and post-translational regulation. Post-translational control is mediated through an allosteric regulator, namely, acetyl-CoA. Pre-translational control involves the use of tissue-specific promoters that mediate the production of multiple mRNA with distinct 5′-untranslated regions which appear to regulate translational activity [8–10].

In the rat and mouse, the PC gene is regulated by two alternative promoters: the proximal (P1) and distal (P2) promoters [11,12]. The P2-promoter is active in a variety of tissues, but is most highly active in the pancreatic β-cells [8] where PC participates in the pyruvate cycling that supports GSIS [13,14]. In contrast, the P1-promoter is active in hepatocytes where it is regulated by the ubiquitous transcription factors Sp1 and Sp3 [15], and by the cAMP-responsive element binding protein (CREB) [16]. Interestingly, adipocytes, where PC is involved in *de novo* fatty acid synthesis and glyceroneogenesis, employ the same promoter usage as the liver. This transcriptional activation in adipocytes is mediated through binding of the peroxisome proliferator activated receptor gamma (PPARγ) to the PPAR-responsive element (PPRE) [17]. More specifically, in mouse, genetic ablation of the PPARγ2 isoform in adipose tissue markedly down-regulates PC expression in adipose tissue but not in liver, confirming the specific control of PC in adipose tissue by this nuclear receptor (NR) [17]. Although PPARγ2 was identified as the main transcriptional regulator of the P1-promoter in adipocytes, the transcription factor(s) that drive PC expression in hepatocytes remain unknown.

Here we show that the P1-promoter contains three classical and non-classical HNF4α binding sites, which are not functionally equivalent. We also demonstrate that siRNA mediated suppression of HNF4α expression in AML12 cells results in reduced expression of PC and G6Pase, suggesting that PC is a direct target of HNF4α. This study highlights the link between the MODY1 gene (HNF4α) and the first regulatory enzyme of the gluconeogenic pathway, and thus leads to the concept that HNF4α regulates the overall program of hepatic gluconeogenesis through modulation of PC, PEPCK and G6Pase gene expression.

2. Materials and methods

2.1. Generation of reporter constructs

Chimeric reporter constructs comprising 603 (pGL-P1ΔDraI) or 166 (pGL-P1ΔDelA) [15] nucleotides of the mouse PC gene P1-promoter ligated 5′ to the luciferase reporter gene were used as templates to generate other reporter constructs. The putative HNF4α binding sites in the above constructs were mutated, singly and in combination, using the Quik change site-directed mutagenesis kit (Stratagene-Agilent Technology). Mutagenesis was carried out in a 50 μl reaction mixture containing 1× cloned *Pfu* polymerase buffer (100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris HCl pH 8.8, 20 mM $MgSO_4$, 1% Triton® X-100 and 1 mg/ml BSA), 0.2 mM dNTP, 125 ng of each primer, 100 ng of template, and 2.5 units of *Pfu* Turbo polymerase. PCR profiles consisted of an initial denaturation at 95 °C for 30 s, followed by 20 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min and extension at 68 °C for 8 min, before a final extension at 68 °C for 8 min. 10 units of *Dpn*I were then added to the PCR mixture and, following overnight digestion at 37 °C, 5 μl were transformed into *Escherichia coli* DH5α. The mutagenic primers used to produce the above constructs are shown in Table 1. The inserts from the clones with mutated nucleotides were excised and used to replace the equivalent wild type fragments in the parental plasmid.

2.2. Cell culture, transient transfection and reporter assays

The mouse hepatoma cell line, AML12 (ATCC: CRL254), was maintained in a 1:1 (v/v) mixture of Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium (Gibco) supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 40 ng/ml dexamethasone, 10% (v/v) fetal bovine serum (Gibco), 100 units/ml penicillin, 100 μg/ml streptomycin (Gibco), at 37 °C in a 5% CO_2 atmosphere. One day before transfection, 1×10^5 cells were plated in 24-well plates in the above medium without antibiotics for 1 day. In 100 μl Opti-MEM® I reduced serum medium with 2 μg of lipofectamine2000™, cells were transfected with 250 ng of luciferase reporter construct and 250 ng of pRSV-βGal plasmid, with or without 250 ng of plasmid encoding human HNF4α (pcDNA-hHNF4α). The transfected cells were maintained at 37 °C in the complete medium for 48 h. Cells were scraped from dishes, harvested by centrifugation, suspended in 1× reporter lysis buffer (Promega) and subjected to 3 cycles of freezing/thawing. 50 μg of protein lysates were subjected to luciferase assay using luciferase assay reagent (Promega) in a GloMax20/20 luminometer (Promega). β-galactosidase activity, measured using ONPG as the substrate, was used to normalize for transfection efficiency.

For the transfection of AML12 with siRNA, 1×10^6 cells were plated in 6-well plates in medium without antibiotics for 24 h, and then transfected with 100 ng of validated mouse HNF4α siRNA (Ambion). The transfected cells were maintained in complete medium at 37 °C with 5% CO_2 for 48 h before being harvested for quantitative real time PCR and Western blot analysis.

2.3. Cloning and production of recombinant human HNF4α

The human HNF4α (hHNF4α) cDNA was cloned from HepG2 cells. Total RNA was isolated from 1×10^6 HepG2 cells using Trizol reagent (Invitrogen). cDNA synthesis was performed in a 20 μl reaction mixture containing 2 μg of heat denatured total RNA, 200 ng random hexamer, 1 mM dNTP, 1× reverse transcriptase buffer (50 mM Tris pH 8.3, 75 mM KCl, 1 mM DTT) and 200 units of SuperscriptIII reverse transcriptase (Invitrogen) at 50 °C for 1 h. hHNF4α cDNA was cloned by PCR using forward and reverse primers designed from the published sequence [18]. PCR was carried out in a 50 μl reaction mixture containing 1× PCR buffer (20 mM Tris, pH 8.4, 50 mM $MgCl_2$, 1.5 mM, $MgCl_2$), 0.2 mM dNTP, 5 ng cDNA, 0.5 μM HNF-F (5′-AAGCTTATGCGAC TCTCCAAAACCTCG-3′, underline indicates *Hind*III restriction site) and HNF-R (5′-GGTACCCTAGATAACTTCCTGCTTGTG-3′, underline indicates

Table 1
Oligonucleotides used for generating mutant reporter constructs.

Oligonucleotide	Sequence (5′–3′)	Mutant construct
HNF4α mu1(−392/−368) F	TTCAGGCTAATGCCATCATGCCTG	ΔHNF4α1
HNF4α mu1(−392/−368) R	CAGGCATGATGGCATTAGCCTGAA	(Δ −386 to −374)
HNF4α mu2(−116/−92) F	CCCTCCTAGGCAGAGGCGGCGCCC	ΔHNF4α2
HNF4α mu2(−116/−92) R	GGGCGCCGCCTCTGCCTAGGAGGG	(Δ −118 to −106)
HNF4α mu3(−26/−14) F	AGTCTAGTGCTGCTGGGGCCAATG	ΔHNF4α3
HNF4α mu3(−26/−14) R	CATTGGCCCCAGCAGCACTAGACT	(Δ −26 to −14)
ΔE-box3-F	GGCGGCGCCCTTATTTCAGCCTTG	ΔHNF4α2ΔE-box3
ΔE-box3`R	CAAGGCTGAAATAAGGGCGCCGCC	(Δ −78 to −73)
HNF4α −26/−14DR1 F	TCTAGTGCTGGAGGTCATAGGTCACTG GGGCCAATGA	H4-SBM:DR1
HNF4α −26/−14 DR1 R	TCATTGGCCCCAGTGACCTATGACCTC CAGCACTAGA	(Substitution from −26/−14 to AGGTCATAGGTCA)

*Kpn*I restriction site) primers, and 2.5 units of *Taq* DNA polymerase (Invitrogen). PCR consisted of an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s and extension at 72 °C for 1 min. The PCR product was digested with *Hind*III and *Kpn*I, and ligated to the equivalent sites of pcDNA3 (Invitrogen). The resulting construct, pcDNA-hHNF4α was sequenced and used for transactivation assays. To obtain purified hHNF4α, the protein was recombinantly expressed in a bacterial expression system [19]. The hHNF4α cDNA was modified to encode an N-terminal hexahistidine tag. This was achieved by PCR using pcDNA–hHNF4α as the template and 6×His-forward primer (5′-CAT ATG $(CAT)_6$ GGA GGT CGA CTC TCC AAA ACC CTC GTC-3′) and reverse primer (5′-GCG GCC GC TAG ATA ACT TCC TGC TTG GTG-3′) which incorporate *Nde*I and *Not*I sites at their 5′-ends, respectively. The PCR product was digested with *Nde*I and *Not*I and ligated to the equivalent sites in the polylinker region of pET17b vector (Merck), forming the pET-6xHis hHNF4α construct. This construct was validated by sequencing before transformation into *E. coli* BL21(DE3) for expression. 5 ml of an overnight culture of *E. coli* BL21(DE3) harboring the pET-6×His hHNF4α clone were subcultured in 250 ml of LB broth containing 100 μg/ml ampicillin. Cultures were grown at 37 °C until the OD_{600} reached 0.8, at which time the culture was induced with 0.1 mM IPTG and transferred to 30 °C for 6 h incubation. The culture was centrifuged at 3,000 ×*g* for 10 min and the resulting cell pellet resuspended in 5 ml lysis buffer (300 mM NaCl, 50 mM NaH_2PO_4, 10 mM imidazole, pH 8.0, 1 mM PMSF and 1 mg/ml lysozyme). Cell lysates were sonicated on ice for 3 min, centrifuged at 10,000 ×*g* for 20 min, and the supernatant loaded onto a 0.5 ml NiNTA agarose column (Qiagen). The column was washed with 4 column volumes of wash buffer (300 mM NaCl, 50 mM NaH_2PO_4, 20 mM imidazole, pH 8.0) before proteins were eluted with 2 ml of elution buffer (300 mM NaCl, 50 mM NaH_2PO_4, 250 mM imidazole, pH 8.0) and collected. The elution buffer was exchanged with protein storage buffer (20 mM Tris–HCl, 1 mM DTT, 0.1 mM EDTA, 100 mM KCl, 20% (v/v) glycerol) using an Amicon concentrator (10 kDa cut off).

2.4. Electrophoretic mobility shift assay (EMSA)

EMSAs were performed using the Lightshift EMSA kit (Thermo Scientific). AML12 nuclear extracts were prepared as described previously (15). 3′-Biotinylated oligonucleotides (10 μM) (Biobasic, Canada) were annealed with their complementary strands (Table 2) in 1× annealing buffer (10 mM Tris pH7.4, 100 mM NaCl and 1 mM EDTA). Binding reactions were performed in 20 μl reaction mixtures containing 1× binding buffer (10 mM Tris, pH 7.4, 50 mM KCl, 1 mM DTT, 5% (v/v) glycerol), 2 μg poly(dI–dC), 1% (v/v) NP-40, 120 pmol annealed probe, and 5 μg nuclear extract or 0.25 μg purified 6×His hHNF4α, at 4 °C for 20 min. For the competition assays, excess amounts of unlabeled double stranded oligonucleotide probes were included in the binding reactions. For supershift assays, 1 μg of anti-HNF4α polyclonal antibody (H-171) (SantaCruz Biotech) was included in the binding reaction. The DNA–protein complexes were subjected to 4% native polyacrylamide gel electrophoresis using 0.5× TBE (44.5 mM Tris–HCl, pH 8.0, 44.5 mM boric acid and 1.25 mM EDTA) as the running buffer at 100 V for 2 h. The gel was electroblotted onto a Biodyne B nylon membrane (Pall) using a semi-dry transfer unit (Hoeffer). The DNA was crosslinked on the membrane by UV-crosslinker (UVP). The shifted bands were visualized using non-radioactive nucleic acid detection kit (Pierce). The image was captured with fluorescence image capture system (GeneTools).

2.5. Chromatin immunoprecipation (ChIP) assay

In brief, 2×10^6 AML12 cells were plated in 1 cm dishes and maintained in DMEM/Ham's F12 medium at 37 °C for 24 h. The DNA and proteins were crosslinked by adding formaldehyde to a final concentration of 1% (v/v) to the culture medium at 37 °C for 10 min. The cells were scraped from the plate and centrifuged at 1000 ×*g* for 5 min. The pellet was suspended in 200 μl of lysis buffer (1% (w/v) SDS, 50 mM Tris pH 8.1, 10 mM EDTA and 1× protease inhibitor (Roche) and sonicated for 15 × 30 s before centrifugation at 5000 *g* for 10 min. The lysate was then diluted in 1× ChIP dilution buffer (0.01% (w/v) SDS, 167 mM NaCl, 16.7 mM Tris, pH 8.1, 1.2 mM EDTA and 1.1% (v/v) Triton X-100). 0.5 ml lysate was immunoprecipitated with 5 μl of anti-HNF4α or anti-actin polyclonal antibody at 4 °C overnight. The immune complexes were captured by adding 20 μl of 50% (w/v) protein A agarose beads (upstate biotech) and centrifuged at 3000 ×*g* for 1 min. The captured immune complexes were washed with immune wash buffer (0.1% (v/v) SDS, 2 mM EDTA, 1% (v/v) Triton X-100, 20 mM Tris–HCl, pH 8.1 and 500 mM NaCl) and eluted from the beads with 1% (w/v) SDS, 0.1 M $NaHCO_3$. Protein was removed from the DNA by digestion with 10 μg/ml proteinase K at 50 °C for 1 h. The DNA from input and immunoprecipitated fractions were purified with the Nucleospin Extract II kit (Macherey–Nagel GmbH) and eluted with 50 μl TE buffer. 2 μl of the eluate was subjected to Q-PCR using −108/−90 HNF4α-F and +72/+92 HNF4α-R primers that flank the HNF4α3 site (−26/−14). A similar set of PCR was performed using downstream primers which flank exon 13 of the mouse PC gene [12]. Q-PCR was carried out as described below except SYBR® Green master mix (Kapa Biosystems) was used in the assays.

2.6. Suppression of HNF4α by siRNA

AML12 cells were transfected with HNF4α siRNA using the reverse transfection method. In brief, 1×10^6 AML12 cells were mixed with 50 ng of HNF4α siRNA or scramble siRNA (Ambion) and 2 μg of FuGene 6 (Roche) in the presence of 1 ml growth medium for 10 min. The mixture was plated in a 6-well plate and an extra 1 ml of growth medium

Table 2
Oligonucleotides used in EMSA.

Oligonucleotide	Sequence (5′–3′)	Probe/competitor
HNF4α (−392/−368)-F[a]	CTAATGTGACCCTTGCCCTCCATCA	Probe/WT competitor
HNF4α (−392/−368)-R	TGATGGAGGGCAAGGGTCACATTAG	
HNF4α (−392/−368)-MuF	TTCAGGCTAATGCCATCATGCCTG	Mutant competitor
HNF4α (−392/−368)-MuR	CAGGCATGATGGCATTAGCCTGAA	
HNF4α (−116/−92)-F[a]	GGCAGCCAGTGGCCCATGGTGGCCT	Probe/WT competitor
HNF4α (−116/−92)-R	AGGCCACCATGGGCCACTGGCTGCC	
HNF4α (−116/−92)-MuF	CCCTCCTAGGCAGAGGCGGCGCCC	Mutant competitor
HNF4α (−116/−92)-MuR	GGGCGCCGCCTCTGCCTAGGAGGG	
HNF4α (−26/−14)-F[a]	TGCTGGAGAACT TTGTATTCTGGG	Probe/WT competitor
HNF4α (−26/−14)-R	CCCAGAATACAAAGTTCTCCAGCA	
HNF4α (−26/−14) MuF	AGTCTAGTGCTGCTGGGGCCAATG	Mutant competitor
HNF4α (−26/−14)- MuR	CATTGGCCCCAGCAGCACTAGACT	

[a] 3′ labeled with biotin.

added. The transfected cells were maintained at 37 °C with 5% CO_2 for 48 h before being harvested for RNA extraction and real time RT-PCR analysis.

2.7. Quantitative real time RT-PCR(Q-PCR)

Total RNA was extracted from AML12 cells using the RNAeasy miniprep kit (Qiagen). cDNA synthesis was carried out at 50 °C for 1 h in a 20 μl reaction mixture containing 1 μg of total RNA, 100 ng random hexamer primers (Invitrogen), 1× reverse transcriptase buffer, 1 mM dNTP and 100 units of superscriptIII reverse transcriptase (Invitrogen). To detect the expression of PC and G6Pase, PCR was carried out in 20 μl reaction mixture containing 1× *Taq*man universal master mix (Applied Biosystems), 2 μl of 1/10 dilution of cDNA, and 0.125 mM primer/probe set described previously [16,17]. PCR was carried out in an MxPro3000 ™ thermal cycler (Agilent Technology). The thermal cycling consisted of an initial incubation at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. The expression of PC and G6Pase was normalized to the expression of 18s rRNA and presented as relative gene expression. For ChIP assays, PCR was carried out as described above except the *Taq*man master mix which was replaced with SYBR® Green master mix. All statistical analyses were performed using Student's t-test.

2.8. Western blot analysis

Total protein lysates of AML12 cells transfected with siRNA were lysed in 100 μl of RIPA buffer [150 mM NaCl, 10 mM Tris pH 7.4, 0.25% (w/v) sodium deoxycholate, 1% (v/v) NP-40, 1 mM EDTA and 1× protein inhibitor cocktail (Sigma)]. 20 μg of protein lysate was subjected to 10% reducing SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane using semi dry blotting. PC bands were detected by rabbit anti-PC polyclonal antibody as described previously [17], while HNF4α bands were detected using the same antibody that was used in the EMSA. To normalize protein loading, anti-actin polyclonal antibody (cell signaling) was used to detect β-actin on the blot. The immunoreactive bands were detected using an enhanced chemiluminescence detection system (GE Health Sciences).

3. Results

3.1. The classical PPRE/HNF4α site binds HNF4α but is not required for transcriptional induction by HNF4α

We previously identified the peroxisome proliferator activated receptor responsive element (PPRE), AGGGCAAGGGTCA (−386/−374, antisense strand), as a critical element for PPARγ2-mediated transcriptional activation of PC in adipocytes [17]. This PPRE resembles the classical direct repeat known as DR1, which is a binding site for HNF4α and several other NRs [20]. To assess whether HNF4α can bind this PPRE, we performed EMSAs using the nuclear extracts of HEK293T cells overexpressing HNF4α, and two hepatocyte cell lines, namely, human hepatoma HepG2 and mouse hepatoma AML12. As shown in Fig. 1A, incubation of the PPRE/HNF4α dual binding site (designated HNF4α1 site) with HepG2 nuclear extracts produced a clear band shift (lane 2) which was supershifted by the addition of anti-HNF4α antibody (lane 3). A similar, but much stronger, DNA–protein complex was observed when the nuclear extract of HEK293T cells overexpressing human HNF4α was used in the assay (lane 4). The addition of anti-HNF4α antibody to this reaction also produced a strong band shift (lane 5) whereas the addition of anti-PPARγ antibody had no effect (lane 6). Incubation of the HNF4α1 site probe with the nuclear extract of HEK293T cells overexpressing PPARγ2 also produced a clear band shift (lane 7) which was supershifted by an anti-PPARγ antibody (lane 8). A similar result was observed when this probe was incubated with the nuclear extract of AML12 cells (Fig. 1B, lane 1). Incubation of the HNF4α1 site oligonucleotide probe with increasing amounts (5×, 10× and 25×) of unlabeled oligonucleotide caused a gradual decrease in formation of the DNA–protein complex (Fig. 1B, lanes 2–4). The inclusion of anti-HNF4α antibody in the binding reaction markedly inhibited formation of the complex (lane 5), while an anti-PPARγ antibody had no effect (lane 6). The results of these competition experiments indicate that the DNA–protein complex is highly specific and consists mainly of HNF4α.

Having shown that HNF4α binds the HNF4α1 site, we next performed transactivation assays to assess the functional significance of the interaction. A chimeric reporter construct, pGL-P1ΔDraI, comprising the luciferase reporter gene fused to the 603 nucleotides upstream of the P1-promoter's transcription start site (15), was co-transfected into AML12 cells with plasmid overexpressing HNF4α. Overexpression of HNF4α resulted in a 4-fold increase in reporter gene activity (Fig. 1C), indicating that the P1-promoter is regulated by HNF4α. Unexpectedly, however, mutation of the HNF4α1 site had no effect on HNF4α-induced transcriptional activation of the promoter. Furthermore, removal of the HNF4α1 site, by deletion of the 5′-flanking sequence to nucleotide position −335, did not significantly affect HNF4α-mediated transcriptional activation of reporter activity. These results point to the presence of an alternative HNF4α binding site(s) within the first 335 nucleotides upstream of the P1-promoter's transcription start site.

3.2. Non-perfect DR1 is required for basal transcription

Computer assisted analysis – using the PROMO software [21] – of the 335 nucleotides 5′ of the P1-promoter's transcription start site revealed two potential binding sites for HNF4α, located at −118/−106 (sense strand) and −26/−14 (antisense strand) (Fig. 2A). The −118/−106 (HNF4α2) site possesses a non-perfect DR1 (5′-AGCCAGTGGCCCA-3′), while the −26/−14 (HNF4α3) site (5′-NNNNCAAAGTTCT-3′) resembles the recently reported HNF4-specific binding motif (H4-SBM), 5′-NNNNCAAAGTCCA-3′ [22].

To examine whether these potential HNF4α binding sites, together with the HNF4α1 site, contribute to transcriptional regulation of the P1-promoter under basal conditions (HNF4α expression is limited in the cell), we introduced single, double and triple HNF4α binding site mutations into the pGL-P1ΔDraI reporter construct. The wild type and mutant constructs were transiently transfected into AML12 cells and the relative luciferase activities measured. As shown in Fig. 2B, mutation of the HNF4α1 and HNF4α3 sites had no effect on reporter activity. Mutation of the HNF4α2 site, however, reduced promoter activity by approximately 50%. No further loss of reporter activity was observed when the HNF4α2 site mutation was combined with mutation of HNF4α1 (ΔHNF4α1ΔHNF4α2) or HNF4α3 (ΔHNF4α2ΔHNF4α3) binding sites, or when all three sites were mutated together (ΔHNF4α1ΔHNF4α2ΔHNF4α3). Similar results were obtained when the HNF4α2 and HNF4α3 site mutations were introduced, singly and together, into a 5′ truncated reporter construct that lacks the HNF4α1 site (pGL-P1ΔDelA). Mutation of the HNF4α2 site reduced basal promoter activity by 50%, while the double mutation decreased the promoter activity only slightly further (Fig. 2B). Taken together, the above results suggest that only the HNF4α2 site contributes to transcriptional regulation of the P1-promoter under basal conditions.

3.3. H4-SBM proximal to the transcription start site is required for HNF4α-mediated transcriptional activation

Although the HNF4α2 site appears to regulate P1-promoter activity under basal conditions, it is unknown which of the three sites function in HNF4α-mediated transcriptional activation. To address this question, the wild type P1-promoter construct (pGL-P1ΔDraI) and plasmid overexpressing HNF4α were transiently co-transfected into AML12 cells and the reporter activities measured. As shown in Fig. 3,

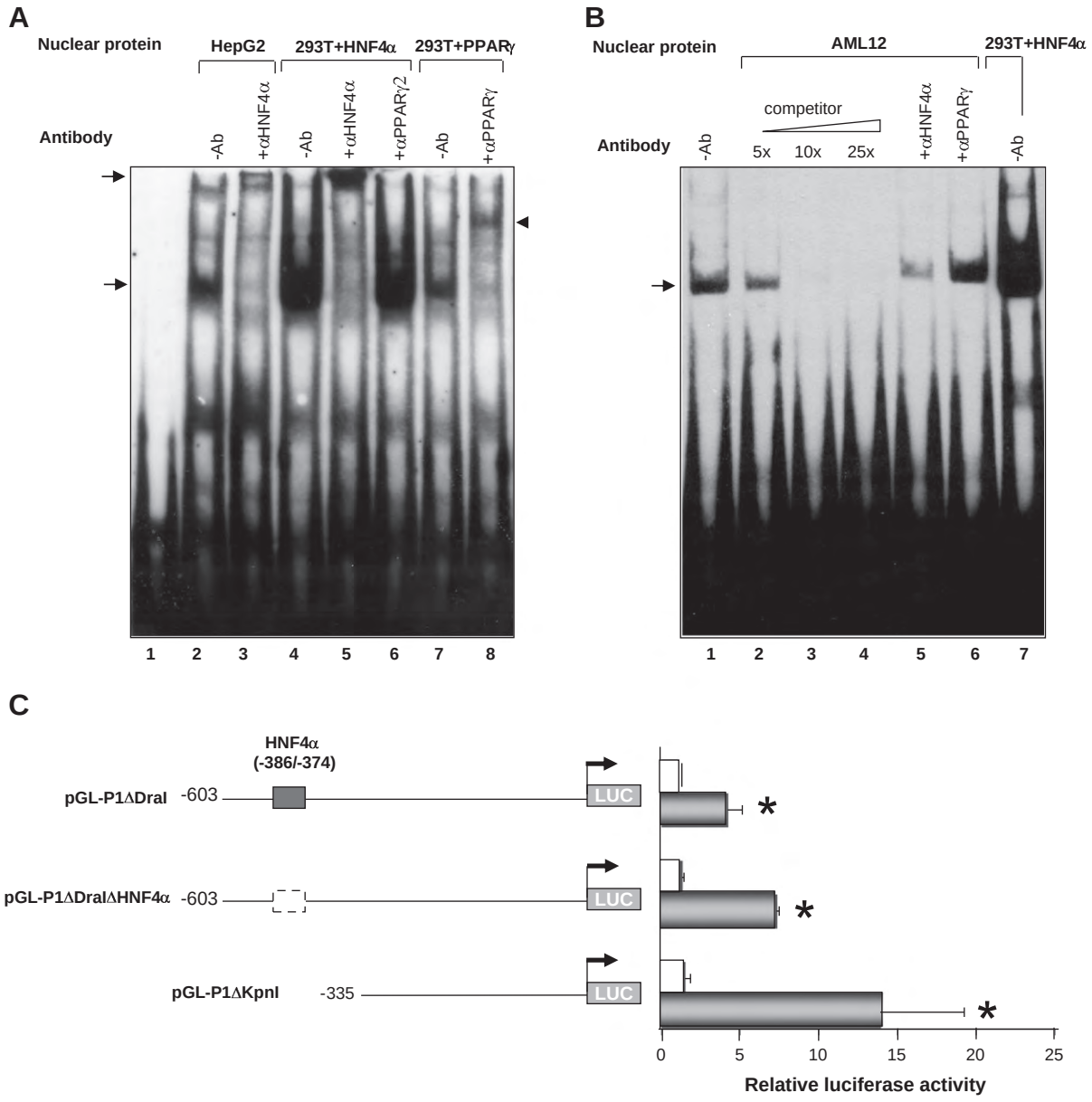


Fig. 1. Binding of HNF4α to the HNF4α1 site and transcriptional induction of the P1-promoter by HNF4α. A, EMSA of biotin-labeled double stranded oligonucleotide probe alone (lane 1), and in the presence of HepG2 nuclear extract with or without anti-HNF4α antibody (lanes 2 and 3). Probe was also incubated with nuclear extract of HEK293T cells overexpressing HNF4α in the absence (lane 4) or presence of anti-HNF4α antibody (lane 5), anti-PPARγ antibody (lane 6) or incubated with nuclear extract of HEK293T overexpressing PPARγ2 in the absence (lane 7) or presence of anti-PPARγ antibody (lane 8). B, EMSA of biotin-labeled double stranded oligonucleotide harboring HNF4α1 site (−386/−374) dual site probe in the presence of AML12 nuclear extract (lane 1) with excess amounts of unlabeled double stranded oligonucleotide competitor (5×, 10×, 25×) (lanes 2–4). Lanes 5 and 6, probe incubated with nuclear extract in the presence of anti-HNF4α antibody or anti-PPARγ antibody, respectively. Lane 7, probe incubated with nuclear extract of HEK293 overexpressing HNF4α. C, The 603 nucleotides of the P1-promoter luciferase reporter construct (pGL-P1ΔDraI) or its mutants were co-transfected with empty plasmid (white bars) or plasmid overexpressing HNF4α (grey bars) into AML12 cells. Promoter activity obtained from various constructs is presented as fold change relative to the value obtained from the cells which were transfected with pGL-P1ΔDraI and empty vector, which was arbitrarily set as 1. *$p \leq 0.05$.

overexpression of HNF4α caused a 4-fold increase in promoter activity. Mutation of the HNF4α1 site did not affect HNF4α-mediated transcriptional activation of the reporter gene; unexpectedly, neither did mutation of the HNF4α2 site. Mutation of the HNF4α3 site, however, reduced HNF4α-mediated reporter gene activity by approximately 65%. Combining the HNF4α3 site mutation with HNF4α1 or HNF4α2 site mutations did not further decrease HNF4α-mediated transcriptional activation of the P1-promoter, nor did the combined mutation of all three sites. To confirm the above results, we conducted a parallel series of experiments with the pGL-P1ΔDelA reporter construct that lacks the HNF4α1 site. As shown in Fig. 3, HNF4α produced a 13-fold increase in activity of the pGL-P1ΔDelA reporter gene — an effect several fold greater effect than that observed with the full-length reporter. Similar to the full-length construct, mutation of the HNF4α2 site did not significantly affect reporter activity; however, mutation of the HNF4α3 site reduced activity by 75%. The double mutation of HNF4α2 and HNF4α3 sites again caused no further decrease in reporter activity. These data indicate that HNF4α mediates transactivation of the P1-promoter via the HNF4α3 site, although the degree of transactivation by HNF4α seems variable, depending on the promoter length.

3.4. HNF4α solely binds HNF4α3 site while USFs bind HNF4α2 site and regulate P1-activity

With mutational analysis of the HNF4α2 and HNFα3 sites revealing only the latter to be involved in HNF4α-mediated induction of the

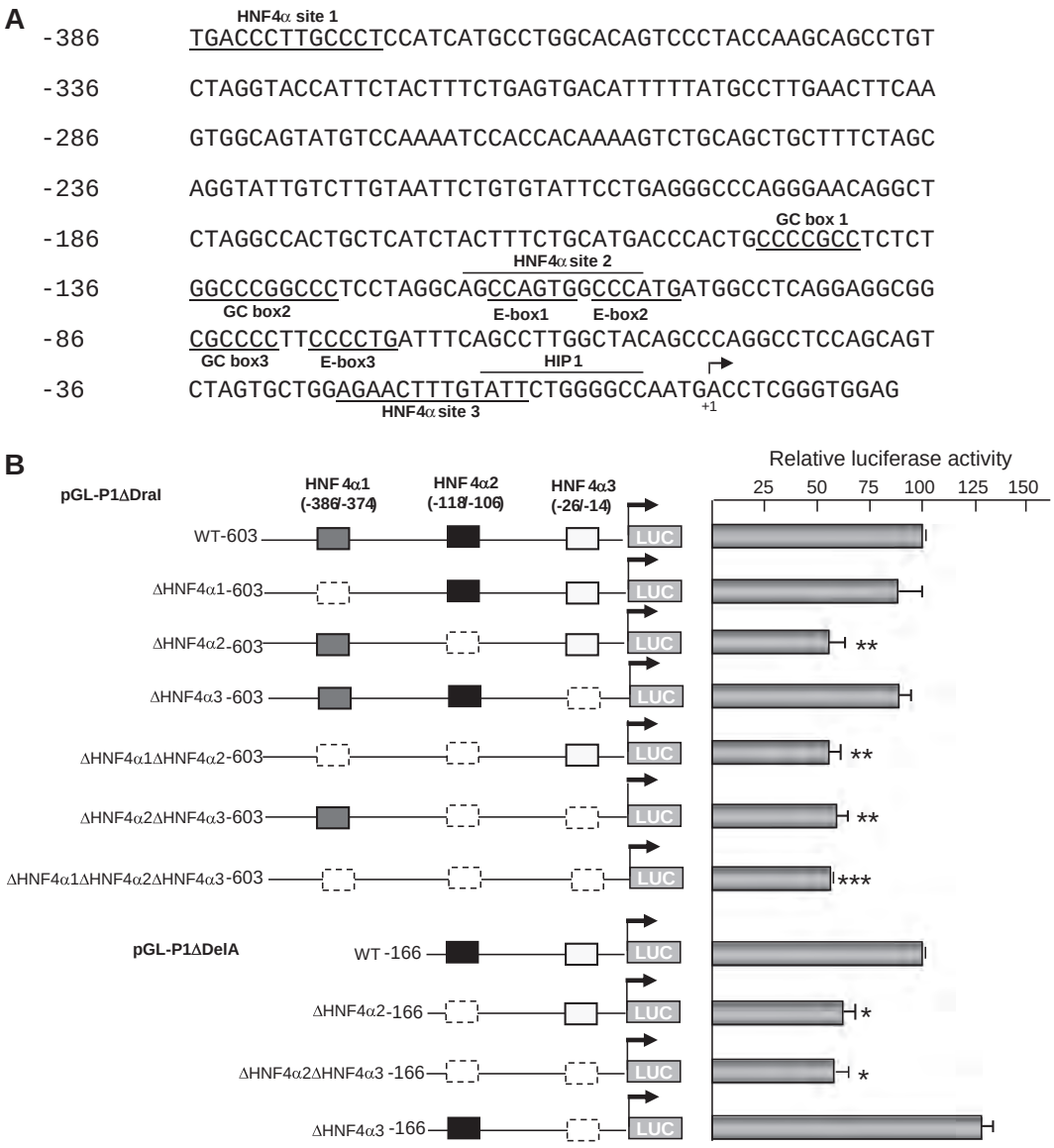


Fig. 2. Effect of mutations of HNF4α binding sites on P1-promoter activity under basal conditions. A, Nucleotide sequence of the 398 nucleotides proximal to the transcription start site of the P1-promoter. The HNF4α1, HNF4α2, HNF4α3 sites and E-boxes are underlined. Also shown are the HIP1, transcription start site (+1) and three GC boxes that are required for basal transcription (15). B, Mutations of the HNF4α1, HNF4α2 and HNF4α3 sites were introduced, singly and in combination, into pGL-P1ΔDraI or pGL-P1ΔDelA reporter constructs and transiently transfected into AML12 cells. The luciferase activity of each construct was normalized to β-galactosidase activity and expressed as relative luciferase activity. The values obtained from mutated constructs are expressed relative to the corresponding parent (WT) construct which was arbitrarily set as 100%. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

P1-promoter, we next examined their abilities to bind HNF4α. To this end, EMSAs were performed with AML12 cell nuclear extract and double stranded oligonucleotide probes harboring either the HNF4α2 or HNF4α3 site. Incubation of AML12 cell nuclear extract with the HNF4α3 site probe (Fig. 4A) produced a single strong DNA–protein complex (lane 2). Formation of the complex was competitively inhibited by excess amounts of the equivalent unlabeled oligonucleotide probe (lanes 3–5), but not by oligonucleotide lacking the core HNF4α3 sequence (lane 6). Inclusion of anti-HNF4α antibody in the binding reaction completely eliminated complex formation (lane 7). Similar results were obtained when the HNF4α3 probe was incubated with purified HNF4α, although the DNA–protein complex formed was stronger (data not shown). These results show that the HNF4α3 site binds HNF4α, and are consistent with the involvement of this site in HNF4α-mediated induction of the P1-promoter.

Although mutation of the HNFα2 site had no effect on induction of the P1-promoter by HNF4α, EMSA with the HNF4α2 site probe indicated that HNF4α is capable of binding to this site. Incubation of the HNF4α2 site probe with AML12 cell nuclear extract produced a single DNA–protein complex (Fig. 4B, lane 2), although this was observed after extended exposure of the blot and, therefore, probably reflects relatively weak binding of nuclear protein(s) to the site. Nevertheless, formation of the complex was competitively inhibited by unlabeled HNF4α2 probe in a concentration dependent manner (lanes 3–5), while a 50-fold excess of unrelated oligonucleotide did not affect the complex formation (lane 6). As shown in Fig. 4C, the inclusion of anti-HNF4α antibody in the binding reaction only partially inhibited formation of the complex (lane 3), thereby indicating that this complex is not entirely HNF4α, and that one or more other factors bind the HNF4α2 site. A disruption of the interaction of these alternative factor(s) with the HNF4α2 site could explain the reduced basal promoter activity of the HNF4α2 site mutant P1-promoter. Re-examination of the surrounding sequence revealed two E-boxes that overlap the 5′ (E-box 1, −116/−111) and 3′ (E-box 2, −109/−104) ends of the HNF4α2 site (−118/−106)

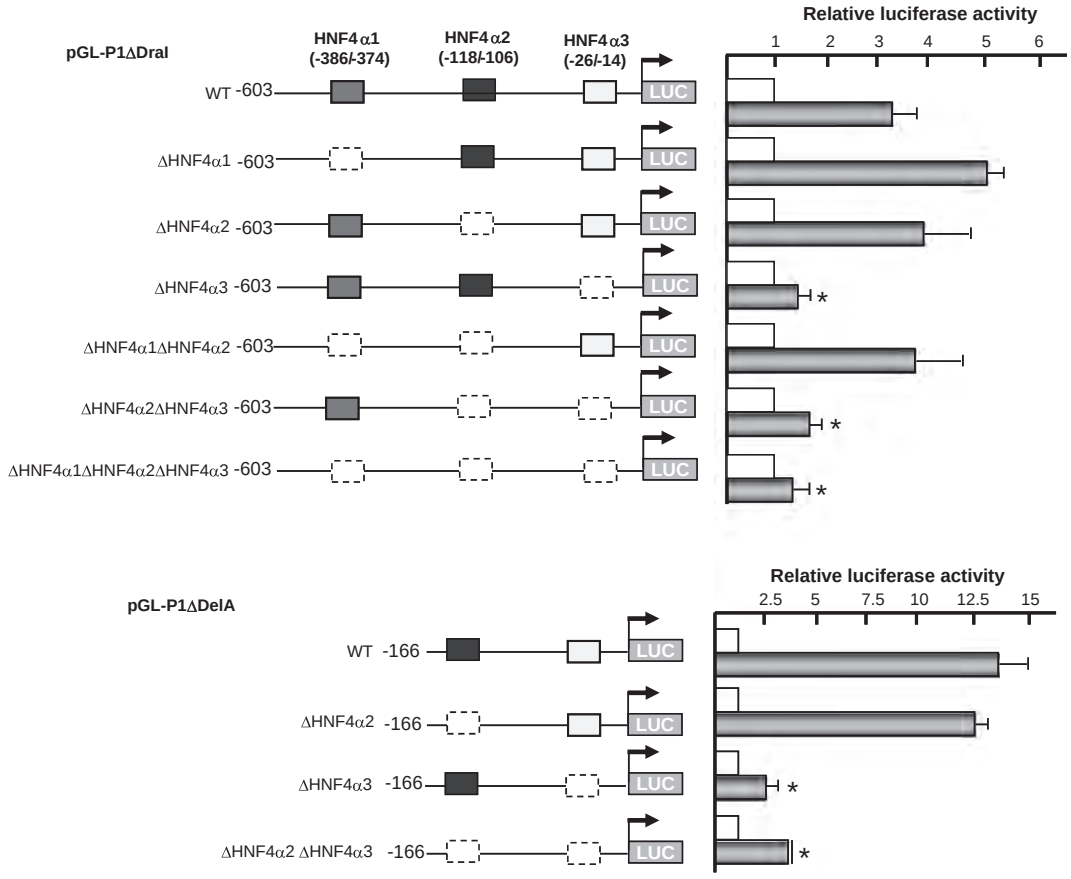


Fig. 3. The HNF4α3 site is required for HNF4α-mediated transcriptional activation of the P1- promoter. pGL-P1ΔDraI, pGLP1ΔDelA or their mutants harboring disrupted HNF4α binding sites were co-transfected into AML12 cells with plasmid overexpressing empty vector (pcDNA3, white bars) or HNF4α (grey bars). The luciferase activity of each construct was normalized to the β-galactosidase activity and expressed as relative luciferase activity. Relative luciferase values obtained from co-transfecting cells with WT (pGL-P1ΔDraI or pGL-P1ΔDelA) or their mutants and HNF4α plasmid were presented as fold change relative to those obtained from those co-transfected with WT (pGL-P1ΔDraI and pGL-P1ΔDelA), each of which was arbitrarily set as 1. *$p \leq 0.05$.

(Fig. 2A). As the E-box motif is a potential binding site for upstream stimulatory factor (USF) 1 and 2 transcription factors [23], we explored the possibility of USF1/USF2 binding to the HNF4α2 site. In EMSAs with the HNF4α2 probe, inclusion of antibodies to USF1 (lane 4), USF2 (lane 5), or both (lane 6) markedly reduced the amount of complex formed; combining either of the anti-USF1 or anti-USF2 with anti-HNF4α antibody caused a slight further reduction (lanes 7 and 8). These results indicate that, in addition to HNF4α, USF1 and USF2 can bind the HNF4α2 site in the P1-promoter. We, therefore, went on to examine the potential for USF-mediated regulation of the P1-promoter through this site.

USF1/2-mediated regulation of the P1-promoter was demonstrated in AML12 cells co-transfected with the pGL-P1ΔDelA reporter construct (containing the HNF4α2 and HNF4α3 sites) and plasmid overexpressing USF1 or USF2. As shown in Fig. 4D, overexpression of USF1 led to a 10-fold increase in P1-promoter activity, while overexpression of USF2 caused an even greater 40-fold increase. Mutation of the HNF4α2 site resulted in a 25% reduction in USF1-mediated induction of P1-promoter activity, but did not affect USF2-mediated activation. The remaining reporter activity could easily be achieved through E-box 3 which is located at −78/−73. To examine the functional importance of E-box 3, a P1-promoter with combined mutation of the HNF4α2 site and E-box 3 was generated and co-transfected into AML12 cells with plasmid overexpressing USF1 or USF2. The double mutant resulted in 50% and 70% loss of USF1- and USF2-mediated transcriptional activation of P1-promoter activity, respectively. This result suggests that while USF2 exerts its transactivation ability through E-box3, USF1 exerts its transactivation activity through both the HNF4α2 site and E-box 3.

3.5. HNF4α binds the three HNF4α binding sites with different affinities

As EMSAs showed that all three sites are able to bind HNF4α, we looked to their affinities to explain the lack of function of the HNF4α1 and HNF4α2 sites in HNF4α-induction of the P1-promoter. Affinities for HNF4α were compared in binding assays in which increasing concentrations (60, 120, 240, 360 and 480 fmol) of HNF4α1, HNF4α2 or HNF4α3 site oligonucleotide probes were incubated with a limited amount (100 ng) of purified HNF 4α (Fig. 5A). Quantification of the DNA–protein complex bands in Fig. 5A (same exposure times) revealed that HNF4α bound the HNF4α1 and HNF4α3 sites with similar affinity, while its affinity for HNF4α2 was markedly lower (Fig. 5B). This poor binding of HNF4α to the HNF4α2 site is consistent with the transactivation experiment showing that mutation of the HNF4α2 site had no effect on HNF4α-mediated activity (Fig. 3). The similar affinity of HNF4α for the HNF4α1 and HNF4α3 sites, however, does not lend itself to a simple explanation for the lack of involvement of the former in HNF4α induction of the promoter.

Although the HNF4α1 and HNF4α3 sites bind HNF4α with similar affinities, their sequences are notably distinct: the HNF4α3 site resembles the recently described H4-SBM (viz. NNNNCAAAGTCCA) [22], while the HNF4α1 site resembles the classic DR1 motif (viz.

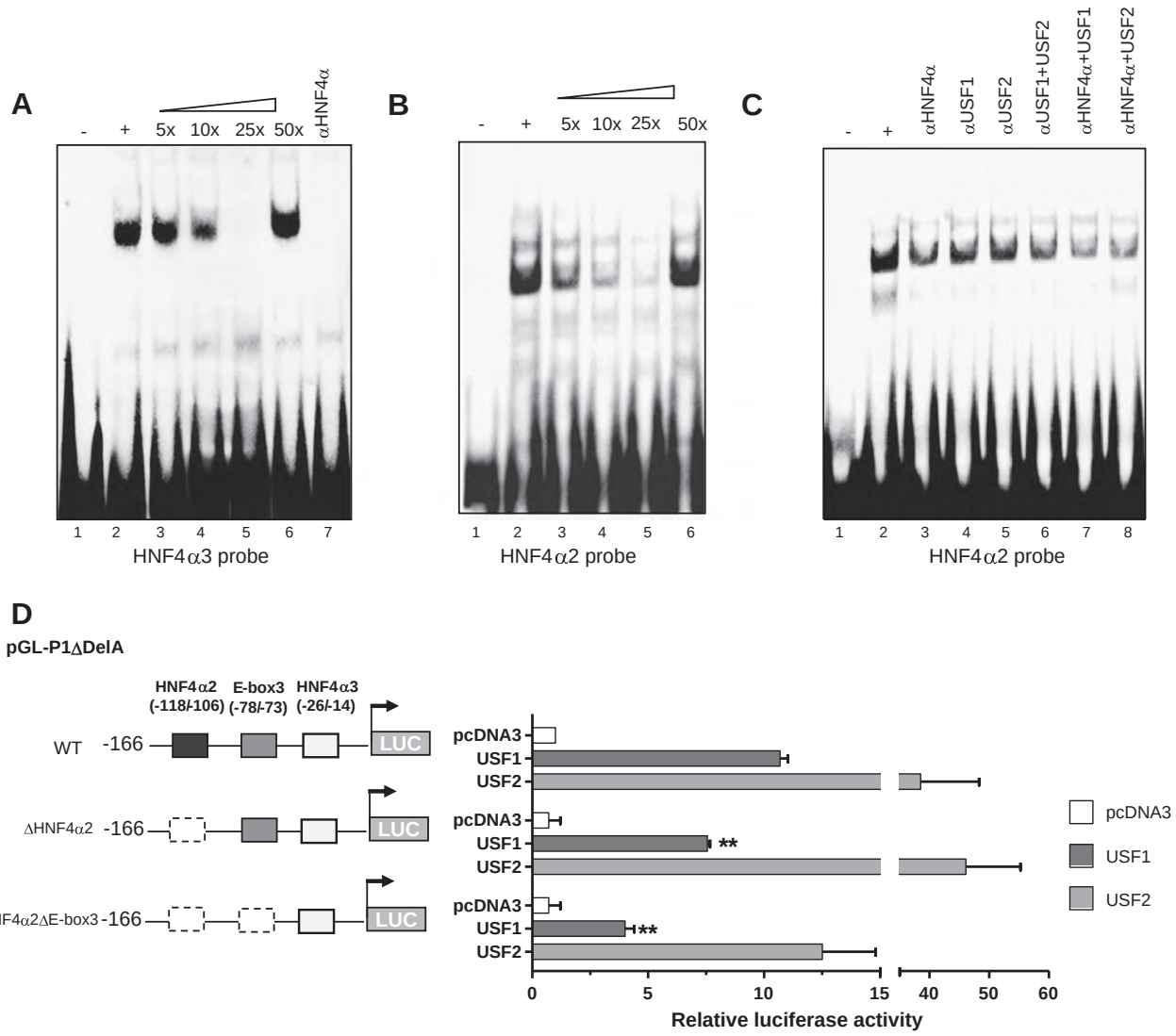


Fig. 4. EMSA of HNF4α2 and HNF4α3 sites with AML12 nuclear extract and transactivation of pGL-P1ΔDelA by USF1 and USF2. EMSAs of biotin-labeled double stranded oligonucleotides harboring HNF4α3 site (A) or HNF4α2 probes (B, C) with AML12 cell nuclear extracts. A, HNF4α3 site probe alone (lane 1), or in the presence of AML12 nuclear extract alone (lane 2) or together with excess (5×, 10×and 25×) unlabeled double stranded oligonucleotide competitor (lanes 3–5) or excess (50×) unrelated double stranded oligonucleotide competitor (lane 6), or with anti-HNF4α antibody (lane 7). B, HNF4α2 site probe alone (lane 1), or probe in the presence of nuclear extract alone (lane 2) or together with excess (5×, 10×and 25×) unlabeled double stranded competitor (lanes 3–5) or excess 50× unrelated double stranded oligonucleotide competitor (lane 6). C, HNF4α2 probe alone (lane 1) and in the presence of AML12 nuclear extract alone (lane 2) or together with anti-HNF4α (lane 3), anti-USF1 (lane 4), anti-USF2 (lane 5), anti-USF1 and anti-USF2 (lane 6), anti-HNF4α and anti-USF1 (lane 7), or anti-HNF4α and anti-USF2 (lane 8) antibodies. Blots shown in B and C were obtained from the extended exposure. D, The 166 nucleotide P1-promoter luciferase reporter construct (pGL-P1ΔDelA) with an intact (WT) or mutated HNF4α2 (ΔHNF4α2) site was co-transfected with empty plasmid or plasmid overexpressing USF1 or USF2 into AML12 cells. Promoter activity is presented as fold change relative to the value obtained from cells transfected with pGL-P1ΔDelA and empty vector, which was arbitrarily set as 1. $^{**}p \leq 0.01$.

AGGTCAxAGGTCA). As only mutation of the HNF4α3 site affected HNF4α-mediated transactivation of the P1-promoter, we hypothesized that the H4-SBM sequence was specifically required for the effect. We therefore substituted the −26/−14 H4-SBM with the consensus DR1 sequence (AGGTCAGAGGTCA) (H4-SBM:DR1 mutant) and measured the effect on HNF4α-induced P1-promoter activity. The chimeric reporter construct was effectively transactivated by HNF4α despite the substitution; however, the magnitude of the response was approximately 25% less than that observed with the parent reporter, as was its basal transcriptional activity (Fig. 5C).

3.6. HNF4α binds to the P1-promoter in vivo

We also performed chromatin immunoprecipitation (ChIP) assays to confirm the binding of HNF4α to the HNF4α3 site. Transcription factor-bound soluble chromatin was prepared from AML12 cells. The HNF4α-bound chromatin fragments were immunoprecipitated with anti-HNF4α antibodies and amplified by PCR using a primer pair that flanks the HNF4α3 site (see Fig. 6A). An anti-β-actin antibody was used to prepare the negative control sample. Compared with the input fraction, approximately 2.2% of the HNF4α-bound HNF4α3 site was detected in samples immunoprecipitated with anti-HNF4α antibody (Fig. 6B). In marked contrast, only 0.05% of the HNF4α-bound HNF4α3 site was detected in samples immunoprecipitated with anti-β-actin antibody (Fig. 6B). There was also no detectable signal generated from a second primer pair located several nucleotides downstream of the enhancer region in the samples which were immunoprecipitated with anti-HNF4α and anti β-actin antibodies. It is noted that although we were able to detect HNF4α binding to HNF4α3 sites, given the close proximity of the three sites, we cannot

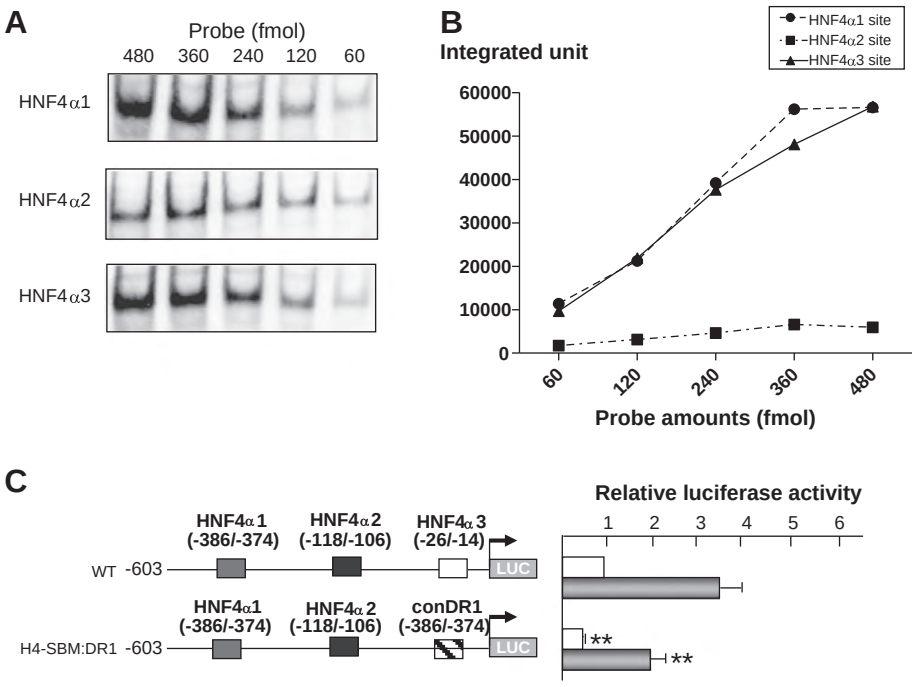


Fig. 5. HNF4α binds to HNF4α1, HNF4α2 and HNF4α3 sites with different affinities and DR1 and H4-SBM cannot be functionally substituted. A, Various amounts of biotin-labeled double stranded oligonucleotides harboring HNF4α1, HNF4α2 and HNF4α3 sites were incubated with 100 ng of purified HNF4α. B, The intensities of the HNF4α-bound complexes were plotted against the concentration of oligonucleotide probe (fmol). C, Relative luciferase expression of the pGL-P1ΔDraI construct (WT) in which the HNF4α3 site was converted to a conserved DR site (H4-SBM:DR1) co-transfected with HNF4α. Relative luciferase values obtained from AML12 cells transfected with WT or H4-SBM:DR1 mutant with HNF4α plasmid (grey bar) were presented as fold change relative to those obtained from transfecting with WT or mutants with empty vector (white bar), which was arbitrarily set as 1. $^{**}p \leq 0.01$.

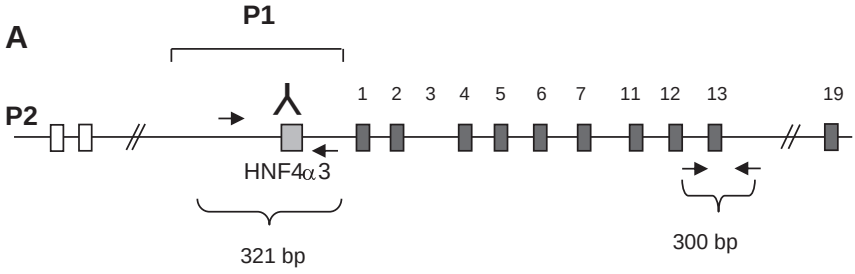
rule out the possibility that the immunoprecipitated chromatin fragments also contained binding of HNF4α to the HNF4α1 and HNF4α2 sites. This close proximity (approximately 370 nucleotides between HNF4α1 and HNF4α3) makes it technically impossible to immunoprecipitate chromatin which contains only HNF4α3 site3-bound HNF4α.

3.7. Suppression of HNF4α down-regulates PC expression

Finally, the functional relevance of HNF4α in the regulation of PC expression was examined by siRNA knockdown. AML12 cells were transiently transfected with siRNA targeted to HNF4α, and the level of PC expression monitored by real time RT-PCR and Western blot. Transient transfection of HNF4α siRNA reduced the levels of both HNF4α mRNA and HNF4α protein expression by approximately 90% (Fig. 7A and B). This resulted in the down-regulation of PC mRNA and PC protein by 60% and 50%, respectively (Fig. 7A and B). We also found that suppression of HNF4α lowered G6Pase mRNA expression by 50%. The latter finding is consistent with the 70% reduction in hepatic G6Pase mRNA expression in HNF4α null mice (Rhee et al. [24]).

4. Discussion

HNF4α is a member of the non-steroid nuclear receptor superfamily of ligand-dependent transcription factors (NR2A1) [25,26]. HNF4α



B

Antibody	HNF4α-F/R primers	Exon 13-F/R primers
HNF4α	2.2±0.05%	0.1±0.007
Actin	0.05+0.01%	ND

Fig. 6. Chromatin immunoprecipitation assay of HNF4α binding to HNF4α3 site. A, Schematic representation of the P1-promoter region with the HNF4α3 site and primer binding sites indicated. B, The HNF4α-bound chromatin was prepared from AML12 cells and precipitated with anti-HNF4α antibody. The HNF4α-DNA fragments were amplified by Q-PCR using primers that flank the HNF4α3 site (HNF4α-F/R), or primers that locate between exon 13 (Exon13-F/R) (15) and normalized to the input levels. The input was the sonicated and HNF4α-bound DNA cross-linked before immunoprecipitating with antibody. ND, not detectable.

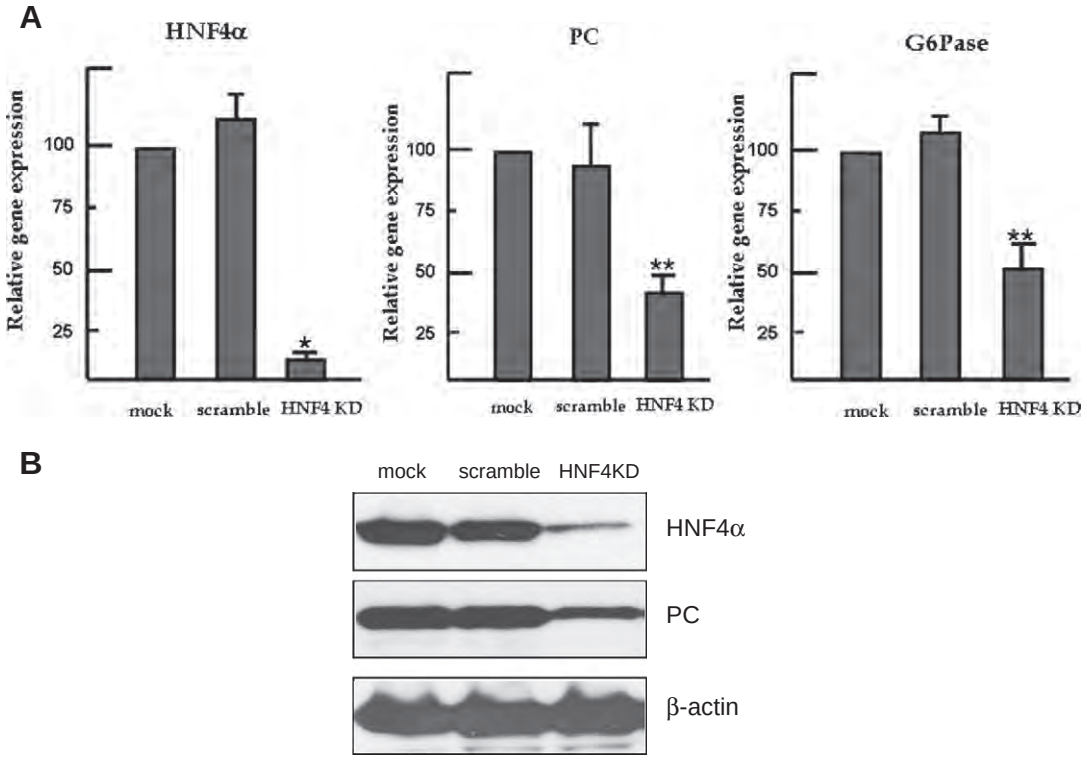


Fig. 7. Suppression of HNF4α down-regulates PC expression in AML12 cells. AML12 cells were mock-transfected or transfected with HNF4α siRNA (HNF4 KD) or non-specific siRNA (scramble) control. (A) The expression of HNF4α, PC and G6Pase mRNAs in these mock-transfected cells (mock) was measured by quantitative real time PCR and normalized to 18 s rRNA expression. The values obtained from scramble and HNF4 KD transfected cells are expressed relative to that obtained from the mock transfection which was arbitrarily set as 100%. The values shown are means ± standard deviation ($n = 3$). *$p \leq 0.01$; **$p \leq 0.05$. (B) Western blot analysis of the expression of HNF4α and PC proteins in mock- and siRNA-transfected AML12 cells. Membrane was stripped and re-probed with anti-β-actin antibody as a loading control.

regulates liver development and the maintenance of liver function [27]. The latter role involves the transcriptional regulation of several enzymes in key metabolic pathways, such as glucose metabolism, lipoprotein metabolism, cholesterol metabolism and bile acids biosynthesis [28]. The functional importance of HNF4α in the control of glucose homeostasis is evidenced by the finding that mutation of this gene results in the development of a specific form of diabetes known as maturity onset diabetes of the young 1 (MODY1) [29].

The mouse and rat PC genes possess two alternative promoters to regulate its transcription. While the distal (P2) promoter is active in the pancreatic β-cells, the proximal (P1) promoter is active in both gluconeogenic (liver) and lipogenic (adipose) tissues [8,11]. This suggests that the liver and adipose tissues employ different tissue-specific factors to direct PC transcription. PC expression in adipocytes is largely regulated by PPARγ2 which activates the P1-promoter via PPRE binding; little, however, is known about the transcriptional regulator(s) of PC expression in the liver. The −386/−374 PPRE located in the P1-promoter of the mouse PC gene resembles the classical DR1 motif — a binding site for several nuclear receptors (NRs), including HNF4α. This prompted us to suspect the involvement of HNF4α in the regulation of PC expression. Supportive of such a role, overexpression of HNF4α in AML12 cells resulted in a significant increase in the activity of a P1-promoter reporter construct. Unexpectedly, however, the PPRE (HNF4α1 site) was not required for this effect (nor for basal promoter activity), despite EMSA confirming its ability to bind both endogenous and exogenous HNF4α. Having ruled out the PPRE as the HNF4α-responsive element, the computer-assisted identification of potential HNF4α binding sites at −118/−106 (HNF4α2) and −26/−14 (HNF4α3) provided two alternative candidates. Although both of these sequences bound HNF4α in EMSA, only the HNF4α3 site was functional with respect to HNF4α-mediated transcriptional activation. The HNF4α3 site resembles the recently described HNF4-specific binding motif (H4-SBM), NNNNCAAAGTCCA [22]. While not affecting basal promoter activity, mutation of the HNF4α3 site caused a decrease in HNF4α-mediated P1-promoter activity; the opposite was true for the HNF4α2 site.

It is unclear why mutation of the HNF4α1 site did not affect HNF4α-mediated transcriptional activation of the P1-promoter despite binding purified HNF4α with an almost identical affinity as the HNF4α3 site (Fig. 5). An explanation might lie in the relative specificities of the two binding sites. The HNF4α1 site resembles the DR1 motif that, unlike the H4-SBM, is recognized by multiple NRs. Therefore, while unlikely at the H4-SBM-like HNF4α3 site, it is reasonable to expect competition between HNF4α and other NRs for binding to the HNF4α1 site. While such competition could explain the HNF4α1 site's lack of responsiveness to HNF4α [30], it is perhaps an unlikely explanation in the context of exogenous HNF4α-overexpression (used in the present study). Promoter context, therefore, might better explain the difference between the HNFα1 and HNFα3 site's abilities to mediate HNF4α transactivation. Promoter context is known to influence the specific transcriptional response to different NRs. Nakshatri and Bhat–Nakshatri [31] have shown that the DR1 of the acyl-CoA oxidase (ACO) gene promoter acts as a PPRE, allowing robust transcriptional induction by PPARγ. However, when placed in the enhancer region of cellular retinol-binding protein II (CRBPII) promoter, the same DR1 sequence acts as an HNF4α-responsive element. The distal location of the HNF4α1 (PPRE) site compared to the proximal location of the HNF4α3 site may also restrict HNF4α interplay with the basal transcriptional complex that facilitates activation of the P1-promoter. Whatever the mechanism, our results suggest that the functional importance of the HNF4α1 (PPRE) site is restricted to PPARγ induction in adipocytes.

As suggested above, the proximal location of the HNF4α3 site to the transcription start site may facilitate interaction of HNF4α with

RNA polymerase II to assist initiation of PC gene transcription. It is also noted that the HNF4α3 site is close to GC box 3, a binding site for Sp1 and Sp3 [15]. This site has previously been shown to be an important binding site for basal transcription as well as for Sp1- and Sp3-mediated activation of the P1-promoter [15]. The close proximity of the HNF4α binding site and GC box may allow interplay between Sp1, Sp3 and HNF4α that in turn stimulates transcription from the P1-promoter. In the promoters of the human sterol 27-hydroxylase [32] and haem oxygenase-1 genes [33], Sp1 and Sp3 cooperate with HNF4α to enhance both basal and HNF4α-mediated transcription. Synergistic transactivation of the apolipoprotein CIII gene promoter by Sp1 and HNF4α is also mediated through a direct interaction [34]. It is interesting to note that the H4-SBM found in the HNF4α3 site appears to overlap with the initiator site [11] (see Fig. 2) which resembles the initiator of many housekeeping genes [35]. This element is known to bind to housekeeping initiator protein 1 (HIP1). The 25% reduction in promoter activity observed in the H4-SBM: DR1 mutant under basal conditions is likely due to loss of the HIP-1 binding site resulting from substitution mutation. Several studies have shown that in TATA-less genes, mutation of this initiator sequence reduces the efficiency of transcription, possibly due to improper positioning of RNA polymerase II [35–37]. The ability to respond to HNF4α was retained in the chimeric H4-SBM:DR1 promoter, with the magnitude of HNF4α mediated transactivation reduced compared to wild type by a similar extent as basal activity. This result suggests that DR1 might, to some extent, be functionally substituted with the H4-SBM in this promoter context.

The affinity of purified HNF4α for the HNF4α2 site, which possesses a non-perfect DR1, was relatively poor compared to the HNFα1 and HNFα3 sites. Consistent with this observation, mutation of the HNFα2 site had no effect on HNF4α-mediated transcription of the P1-promoter; it did, however, significantly reduce basal promoter activity. This is at least in part attributed to the presence of two adjacent E-boxes that co-localize with the HNF4α2 site and serve as a binding site for the non-tissue specific factors, USF1 and USF2 [23]. These two transcription factors have been shown to play a role in the regulation of glucose responsive genes such as L-type pyruvate kinase and glucokinase [38]. The reduction in basal P1-promoter activity caused by the HNF4α2 site mutation is likely due to a disruption of the interaction with USF1. This conclusion comes from two pieces of evidence. Firstly, as shown by EMSA using anti-USF1 antibody, USF1 was capable of binding to an HNF4α2 site probe. Secondly, the 10-fold increase in wild type P1-promoter activity caused by overexpression of USF-1 was reduced by 25% by disruption of the HNF4α2 site, and by 50% when the HNF4α2 site was disrupted together with the downstream E-box (E-box 3). Interestingly, overexpression of USF2 caused a 50-fold increase in P1-promoter activity and this activation was mediated solely through E-box 3. These data indicate that USF1 rather than USF2 stimulates P1-promoter activity through the HNF4α2 site. In the rat PC gene, USF1 and USF2 have previously been implicated in the regulation of distal (P2) promoter activity in pancreatic β-cells via interplay with the β-cell specific factor, FoxA2 [39].

Based on the results of the present study and that already known about PC regulation in the adipocyte, we propose the following model (illustrated in Fig. 8) for the tissue-specific regulation of the PC gene. Adipocytes are abundant in PPARγ2 which binds the PPRE (HNF4α1 site) and turns on PC expression [17]. In contrast, the liver expresses high levels of HNF4α to regulate PC expression via binding to the H4-SBM (HNFα3 site). This is supported by Western blot analysis demonstrating that PPARγ2 expression is extremely low while HNF4α is highly abundant in AML12 cells (data not shown). Other

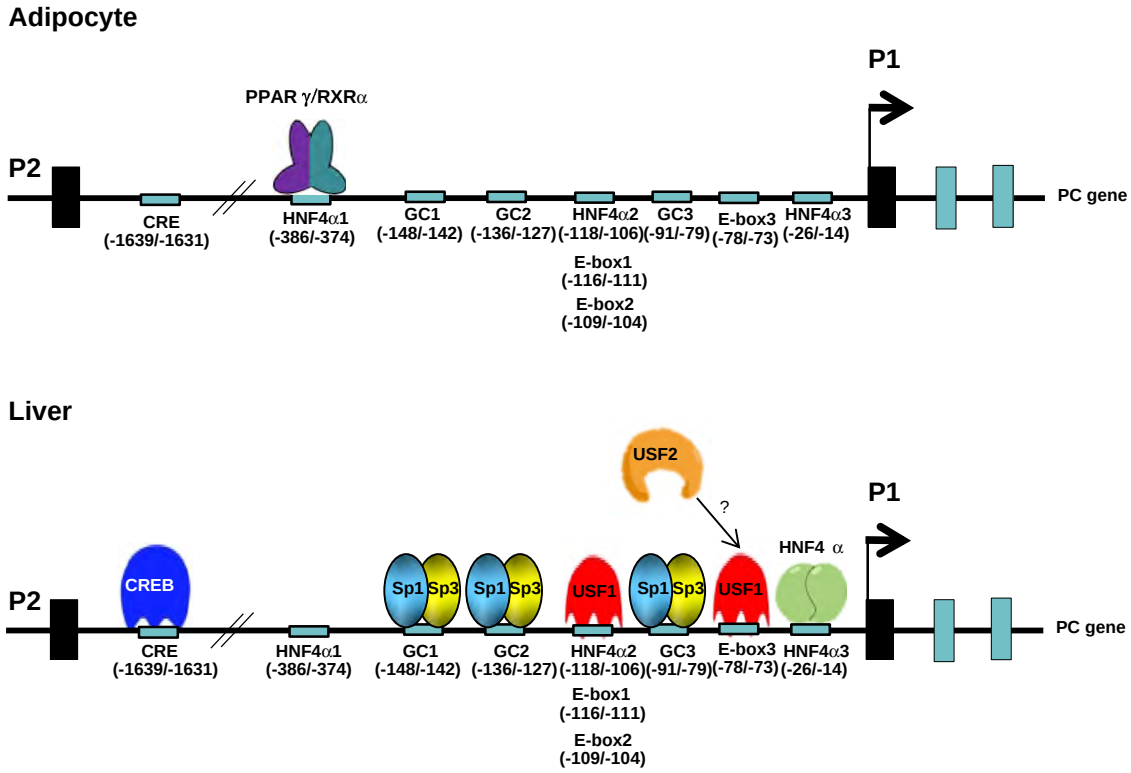


Fig. 8. Schematic diagram showing differential regulation of PC in the adipocyte and liver. The PC gene is regulated by P1 (proximal) and P2 (distal) promoters. P1 is active in the liver and adipocytes while P2 is active in pancreatic β-cells [8]. In adipocytes, P1 is solely regulated by PPARγ via the −386/−374 HNF4α1 site (DR1) [17]. In the liver, PC is regulated by an interplay of transcription factors at the P1-promoter: HNF4α binding to the −26/−14 HNF4α3 site (H4-SBM), Sp1/Sp3 binding to GC box 1 (GC1), GC box 2 (GC2), and GC box 3 (GC3) [15], and USF1 binding to E-box 1 (−116/−111), E-box 2 (−109/−104) and E-box 3 (−78/−73). The −1639/−1631 CRE serves as a cAMP-responsive binding protein (CREB) during cAMP-mediated transcriptional activation [16].

ubiquitously expressed transcription factors, including Sp1 and Sp3 via GC box 3 [15], USF1 via HNF4α2 site, and USF2 via other E-boxes, cooperate with HNF4α to stimulate P1-activity in a liver-specific manner. Furthermore, CREB regulates P1-promoter activity via the distal CRE [16].

HNF4α also regulates the gluconeogenic G6Pase and PEPCK genes, but by a substantially different mechanism than we have shown here for the PC gene. In the promoters of G6Pase and PEPCK genes, HNF4α binding sites are part of larger regulatory units that mediate glucocorticoid and cAMP induction of gene expression. The rat G6Pase gene promoter contains a single HNF4α binding site (located at −455/−431) that acts as an accessory factor binding site (AF1) in a complex glucocorticoid responsive unit (GRU) [40]. Binding of HNF4α to this site facilitates glucocorticoid binding to the glucocorticoid responsive element and, thereby, the induction of PEPCK gene expression; deletion of the site lowers glucocorticoid-mediated induction of the PEPCK gene by approximately 50% [41,42]. In the G6Pase gene, four HNF4α binding sites, located at −672/−667, −516/−511, −76/−64 and +9/+15, form part of a GRU [43] and a protein kinase A/cAMP-responsive unit (CRU) [44].

Finally, the functional importance of HNF4α in the regulation of the PC gene was validated in a cellular context. siRNA mediated suppression of HNF4α expression resulted in a 60% reduction in PC mRNA expression, a 50% reduction in PC protein, and approximately halved the expression of G6Pase mRNA. While total ablation of HNF4α in mice is embryonic lethal [45], support for these findings can be found in the liver-specific HNF4α knockout mice which are viable but display abnormal metabolism of cholesterol, glucose, and fatty and bile acids [46–49]. Holloway et al. [50] examined the effect of liver-specific HNF4α ablation on sex-specific genes by microarray analysis and found that PC mRNA was 85% lower in both male and female knockout mice [50]. Although not reporting on PC expression, Rhee et al. [24] reported reduced expression of both PEPCK and G6Pase genes in these mice. The present report on the transcriptional regulation of PC by HNF4α provides novel molecular insight into how ablation of the HNF4α gene affects PC expression. It appears that HNF4α regulates an overall gluconeogenesis program through the regulation of at least three out of four gluconeogenic enzymes, namely, PC, PEPCK and G6Pase.

Acknowledgements

This work was supported by the Thailand Research Fund (BRG 5480002) and Faculty of Science, Mahidol University to SJ. TC and PR were supported by the DPST, and a PhD scholarship from the Higher Education Commission, respectively. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors thank Professor John Wallace and Clair Alvino, University of Adelaide for editing the manuscript.

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Multiple E-Boxes in the Distal Promoter of the Rat Pyruvate Carboxylase Gene Function as a Glucose-Responsive Element

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Abstract

Pyruvate carboxylase (PC) is an anaplerotic enzyme that regulates glucose-induced insulin secretion in pancreatic islets. Dysregulation of its expression is associated with type 2 diabetes. Herein we describe the molecular mechanism underlying the glucose-mediated transcriptional regulation of the PC gene. Incubation of the rat insulin cell line INS-1 832/13 with glucose resulted in a 2-fold increase in PC mRNA expression. Transient transfections of the rat PC promoter-luciferase reporter construct in the above cell line combined with mutational analysis indicated that the rat PC gene promoter contains the glucose-responsive element (GRE), comprising three canonical E-boxes (E1, E3 and E4) and one E-box-like element (E2) clustering between nucleotides −546 and −399, upstream of the transcription start site. Mutation of any of these E-boxes resulted in a marked reduction of glucose-mediated transcriptional induction of the reporter gene. Electrophoretic mobility shift assays revealed that the upstream stimulatory factors 1 and 2 (USF1 and USF2) bind to E1, the Specificity Protein-1 (Sp1) binds to E2, USF2 and the carbohydrate responsive element binding protein (ChREBP) binds to E4, while unknown factors binds to E3. High glucose promotes the recruitment of Sp1 to E2 and, USF2 and ChREBP to E4. Silencing the expression of Sp1, USF2 and ChREBP by their respective siRNAs in INS-1 832/13 cells blunted glucose-induced expression of endogenous PC. We conclude that the glucose-mediated transcriptional activation of the rat PC gene is regulated by at least these three transcription factors.

Citation: Wutthisathapornchai A, Vongpipatana T, Muangsawat S, Boonsaen T, MacDonald MJ, et al. (2014) Multiple E-Boxes in the Distal Promoter of the Rat Pyruvate Carboxylase Gene Function as a Glucose-Responsive Element. PLoS ONE 9(7): e102730. doi:10.1371/journal.pone.0102730

Editor: Yoshiaki Tsuji, North Carolina State University, United States of America

Received February 20, 2014; **Accepted** June 21, 2014; **Published** July 23, 2014

Funding: This work was supported by grant no. BRG5480002 from the Thailand Research Fund and Faculty of Science, Mahidol University to SJ and by NIH grant DK28348 to MJM. AW and TB were supported by the MD-PhD scholarship, Mahidol University. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Glucose homeostasis is tightly regulated by glucagon and insulin which counteract each other to maintain the concentration of plasma glucose within a narrow range. Glucagon secreted during prolonged starvation raises the blood glucose level by stimulating glycogen breakdown and gluconeogenesis [1] while insulin secreted during fed conditions [glucose-induced insulin secretion (GSIS)] lowers blood glucose levels by stimulating glucose uptake, glycolysis and glycogen synthesis in liver and skeletal muscle [2]. This process occurs efficiently in the pancreatic β-cell due to the presence of GLUT2 and glucokinase [3], which together acts as a sensor allowing high concentrations of glucose to enter to the cell for aerobic glycolysis and oxidative phosphorylation [4]. These biochemical pathways raise the concentrations of cellular ATP which drive insulin secretion known as K_{ATP}-dependent GSIS [5]. Although pyruvate formed after glycolysis appears to enter beta cell mitochondria through pyruvate decarboxylation catalyzed by pyruvate dehydrogenase complex (PDH) and pyruvate carboxylation by pyruvate carboxylase (PC) with similar proportions, flux toward the latter reaction is tightly associated with the glucose concentration the cells is exposed to and thus correlates with the magnitude of insulin secretion [6,7]. In further support of this observation, PC protein levels in rat pancreatic islets were found to be rapidly induced by exogenous glucose [8]. Subsequent studies have clearly revealed that indeed pyruvate carboxylation by PC constitutes an important component of the "pyruvate cycling" which provides NADPH, one of coupling factors required for GSIS in pancreatic β-cells [9–11]. Suppression of PC expression in rat insulinoma cells impairs anaplerosis, concomitant with the lowered GSIS [12–13] while overexpression of PC enhances GSIS [13], indicating the critical role of PC in supporting insulin secretion. Several studies performed in models of diabetic rats and human subjects with type 2 diabetes clearly show that down-regulation of PC expression in pancreatic islets is associated with type 2 diabetic phenotypes further supporting the role of PC in GSIS [14–16].

In the rat, mouse, and human, PC expression is regulated by two alternative promoters, the proximal promoter is active in liver and adipose tissue where PC is involved in gluconeogenesis and

lipogenesis, respectively while the distal promoter is active in β-cells where PC is required to support GSIS [17–20]. Characterization of the distal promoter of rat PC gene revealed that a member of the forkhead box protein 3β (FoxA2) regulates PC expression in a β-cell specific manner [21]. Although previous work has shown that PC expression is inducible by exogenous glucose, it remains largely unknown how glucose increases PC expression [8,17].

Specificity protein 1 (Sp1) is a zinc-finger and ubiquitously expressed transcription factor which regulates expression of a variety of genes. Sp1 can regulate transcription of several metabolic genes in response to physiological changes including alterations in glucose levels. High glucose stimulates activity of protein phosphatase 1 which in turn dephosphorylates Sp1, enhancing its DNA binding activity [22]. The upstream stimulatory factors (USFs), comprising USF1 and USF2, and the carbohydrate response element binding protein (ChREBP) are the basic helix-loop-helix leucine zipper transcription factor. While USFs as a homo- or heterodimer recognize the canonical E-box (CANNTG), ChREBP is heterodimerized with Mlx, and bind to two adjacent E-boxes, termed the carbohydrate responsive element (ChoRE) in their target genes. Elevated glucose concentrations increase the abundance of ChREBP mRNA [23] and the activity via xylulose-5-phosphate, an intermediate of the pentose phosphate pathway by stimulating the activity of protein phosphatase 1A which in turn dephosphorylates ChREBP, enhancing its entry into nucleus [24]. Furthermore, a conformational change within the glucose-sensing module of ChREBP induced by high glucose also contributes to its ability to transactivate its target genes [25].

Although Pedersen *et al*. [26] have previously reported that ChREBP regulates glucose-induced PC expression via the −408/−392 ChoRE, locating in the distal promoter of rat PC gene, a weak binding of ChREBP to this ChoRE has been observed. Moreover most of this study was performed using a heterologous promoter-reporter and the functional importance of this transcription factor with respect to transcriptional regulation of endogenous PC expression was not demonstrated. Here we identify the glucose-responsive element (GRE) within the 1 kb enhancer region of rat PC gene that mediates transcriptional induction of PC by glucose. Our results reveal that the ChREBP, USF2 and Sp1 transcription factors act in concert via binding to the tandem E-boxes located within the first 600 nucleotides upstream of the transcription start site. We also confirmed the functional importance of these three transcription factors with respect to the regulation of glucose-induced expression of the endogenous PC gene with gene knockdown experiments.

Materials and Methods

Plasmids construction

The construction of the 1.2 kb 5′-flanking region representing the distal promoter of rat PC gene-luciferase reporter construct (pGL-P2) and its 5′-truncated mutant constructs were described previously [27]. The pGL-P2 reporter construct with various E-boxes mutated, namely, MuE1, MuE2, MuE3, MuE4 were constructed using pGL-P2 as a template while the double mutant MuE2&E4 was constructed using MuE4 as the template. The pGL-P2ΔKpnI mutants containing 25 bp or 23 bp internal deletions (Δ1, Δ2, Δ3, Δ4, Δ5 and Δ6) were constructed using pGL-P2ΔKpnI as template. The mutagenic reaction was performed using Quik-change XL site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions. The mutagenic primers used to generate the above constructs are shown in Table1. The nucleotide sequences of the mutagenic clones were verified by automated DNA sequencing (Macrogen Inc, USA).

The plasmids encoding USF1 and USF2 were cloned from cDNA prepared from INS-1 832/13 cells by RT-PCR using the primers (USF1CDS F/R and USF2CDS F/R) designed from published cDNA sequences [28]. The USF1 and USF2 cDNAs were cloned into the *Hind*III and *Kpn*I sites of pcDNA3 expression vector (Invitrogen). Plasmids encoding Sp1 and Sp3 were constructed as described previously [29].

Cell culture and transient transfection

INS-1 and INS-1 832/13 cell lines [30] were kindly provided by C.B. Newgard, Duke University. INS-1 and INS-1 832/13 cells were maintained in RPMI 1640 medium (Gibco) supplemented with 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 10 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) heat-inactivated fetal bovine serum (Gibco), at 37°C with 5% CO_2. Cultures were maintained to 70–80% confluence before being used in the transfection experiments.

INS-1 832/13 cells were seeded at a density of 1×10^6 cells/well in antibiotic-free medium containing 5.5 mM glucose in 6-well plates for 4 days prior to transfection. Cells were transfected with mixtures containing 5 μg of Lipofectamine 2000 transfection reagent (Invitrogen), 1 pmole of a firefly luciferase reporter construct and 2 μg of pRSV-βgal plasmid encoding *E. coli* β-galactosidase in Optimem I-reduced serum medium. The transfected cells were maintained in this medium for 24 h before it was replaced with RPMI medium containing either 5.5 mM or 25 mM glucose for the next 24 h.

For transactivation assays, 1.5 μg of the luciferase reporter construct, 1.5 μg of plasmid overexpressing USF1, USF2, Sp1 (pSp1), Sp3 (pSp3) or pcDNA3 (empty vector) and 2 μg of pRSV-β-gal were mixed with 5 μg of Lipofectamine 2000 (Invitrogen) in Optimem I-reduced serum medium and transfected to INS-1 832/13 as described above. The luciferase and β-galactosidase assays were performed as described previously [21]. The luciferase activity was normalized with the β-galactosidase activity and presented as the relative luciferase activity.

siRNA transfection

INS-1 832/13 cells were transfected with Sp1, USF1, USF2 and ChREBP siRNAs. In brief, 5×10^5 INS-1 832/13 cells were plated in 6-well plates 24 h before transfection in Optimem I-reduced serum medium before transfected with with 25 ng of siRNAs targeted to rat Sp1, USF1, USF2, ChREBP or scrambled siRNA (Ambion) and 2 μg of lipofectamine 2000 in the presence of 2 ml growth medium for 5 min. The transfected cells were maintained at 37°C with 5% CO_2 for 48 h before being harvested for RNA extraction and real time PCR analysis.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from INS-1 832/13 cell were prepared as described previously [21]. EMSA was performed using a non-radioactive EMSA. The two complementary oligonucleotides with their 3′-end labeled with biotin (BioBasic, Canada) were annealed and subjected to binding assays as described previously [31]. Supershift assays were performed by pre-incubating 2 μg of polyclonal antibodies against USF1 (sc-22), USF2 (sc-862), ChREBP (sc-21189), Sp1 (sc-59) or Sp3 (sc-644-G) [Santa Cruz Biotechnology] with nuclear extracts for 10 min before binding reactions were performed. The DNA-protein complexes were separated by 5% native polyacrylamide gel electrophoresis using 0.5% TBE. The DNA-protein complexes were transferred onto Biodyne membrane (PALL) and the bands were detected using the

Table 1. Oligonucleotides used in this study. Underline indicates mutated nucleotides.

Oligonucleotide	Sequence	Used for
pGL-P2 ΔKpnI Mu1F	5′ TCTCTATCGATA-TGCCCTTAGCTA 3′	Δ1 Mutant
pGL-P2 ΔKpnI Mu1R	5′ TAGCTAAGGGCA-TATCGATAGAGA 3′	
pGL-P2 ΔKpnI Mu2F	5′ TCTAATTCCTCG-GACCTCTTCTGT 3′	Δ2 Mutant
pGL-P2 ΔKpnI Mu2R	5′ ACAGAAGAGGTC-CGAGGAATTAGA 3′	
pGL-P2 ΔKpnI Mu3F	5′ CGTTTCTCCTGC-TACGTGCATCTG 3′	Δ3 Mutant
pGL-P2 ΔKpnI Mu3R	5′ CAGATGCACGTA-GCAGGAGAAACG 3′	
pGL-P2 ΔKpnI Mu4F	5′ TCTGCTAAAGAG-AACCCCCGTGCA 3′	Δ4 Mutant
pGL-P2 ΔKpnI Mu4R	5′ TGCACGGGGGTT-CTCTTTAGCAGA 3′	
pGL-P2 ΔKpnI Mu5F	5′ CAGCACCGCTCC-CGGTTTGAAGAG 3′	Δ5 Mutant
pGL-P2 ΔKpnI Mu5R	5′ CTCTTCAAACCG-GGAGCGGTGCTG 3′	
pGL-P2 ΔKpnI Mu6F	5′ CTGTTATGGTTG-CTCGAGTGAATG 3′	Δ6 Mutant
pGL-P2 ΔKpnI Mu6R	5′ CATTCACTCGAG-CAACCATAACAG 3′	
(−465/−460)mut_F	5′ TAAAGAGTACGTG**g**A**at**T**c**GCAGCACCGCTCC 3′	MuE1 mutant
(−465/−460)mut_R	5′ GGAGCGGTGCTGC**g**A**at**T**c**CACGTACTCTTTA 3′	
(−442/−437)mut_F	5′ CACCGCTCCAACC**gaat**T**c**CATCTGTTATGG 3′	MuE2 mutant
(−442/−437)mut_R	5′ CCATAACAGATG**g**A**attc**GGTTGGAGCGGTG 3′	
(−436/−431)mut_F	5′ TCCAACCCCCGTG**g**A**at**T**c**TTATGGTTGCGGT 3′	MuE3 mutant
(−436/−431)mut_R	5′ ACCGCAACCATAA**g**A**at**T**c**CACGGGGGTTGGA 3′	
(−408/−403)mut_F	5′ GGTTTGAAGAGA**g**A**at**T**c**CTACTCGA**t**T**c**AATGAATTGC 3′	MuE4 mutant
(−408/−403)mut_R	5′ CTGCAATTCATT**g**A**a**TCGAGTAG**g**A**at**T**c**TCTCTTCA 3′	
USF2CDS-F	5′ AAGCTTATGGACATGCTGGACCCGGGTCTG 3′	USF2 cloning
USF2CDS-R	5′ GGTACCTCACTGCCGGGTGCTCTCGCCC 3′	
USF1CDS-F	5′ AAGCTTATGAAGGGGCAGCAGAAAACAGC 3′	USF1 cloning
USF1CDS-R	5′ GTACCTTAGTTGCTGTCATTCTTGATGAC 3′	
M4 (+)	5′ GCTAAAGAGTACGTGCATCTGGCAGCACCG 3′	EMSA
M4 (−)	5′ CGGTGCTGCCAGATGCACGTACTCTTTAGC 3′	
M5 (+)	5′ CCAACCCCCGTGCATCTGTTATGGTTGCGG	EMSA
M5 (−)	5′ CCGCAACCATAACAGATGCACGGGGGTTGG 3′	
M5pmut E-box2 (+)	5′ CCAACCGAATTCCATCTGTTATGGTTGCGG 3′	EMSA
M5pmut E-box2 (−)	5′ CCGCAACCATAACAGATGGAATTCGGTTGG 3′	
M4+M5 (+)	5′ CATCTGGCAGCACCGCTCCAACCCCCGTGCATCTG 3′	EMSA
M4+M5 (−)	5′ CAGATGCACGGGGGTTGGAGCGGTGCTGCCAGATG 3′	
M4/5pmut (+)	5′ CATCTGGCAGCAGAATTCCAACC**gaat**T**c**CATCTG	EMSA
M4/5pmut (−)	5′ CAGATG**g**A**attc**GGTTGGAGCGGTGCTGCCAGATG 3′	

doi:10.1371/journal.pone.0102730.t001

Lightshift Chemiluminescent EMSA kit (Pierce). The sequences of oligonucleotides used in these experiments are shown in Table 1.

Chromatin immunoprecipitation assay (ChIP)

INS-1 832/13 cells were seeded at a density of 6×10^6 cells in a 100 mm dish and maintained in RPMI medium containing 5.5 mM glucose for 4 days. The cells were cultured in RPMI medium containing 5.5 mM or 25 mM glucose for 12 h. DNA and proteins were cross-linked by adding formaldehyde to the culture medium to the final concentration of 1% (v/v) at 37°C for 5 min. ChIP was performed as described previously [21] except that the pre-cleared chromatin was precipitated with 1 μg of anti-USF1, anti-USF2, anti-Sp1, anti-Sp3 or anti-ChREBP as described above. The transcription factor-bound DNA was amplified using Q-PCR with FastStart Universal SYBR Green master mix (Roche). Input (unbound DNA) of each group (5.5 or 25 mM glucose) was used to normalize the target DNA (set as 100%) and non-IgG condition was used as the reference in real time PCR. The primers used for amplifying the target site are −408 F/R (5′-GCGACCTCTTCTGTATCTGCTAA-3′ and 5′-AGACCTTCTGATTGGTGAAGAGG-3′) which flanked the Sp1 and USF sites of rat PC promoter [19], and Ex2 F/R primers (5′-GCCCATCAAGAAAGTAATGGTA-3′ and 5′-CTTGGCCACCTTAATGATGTCT-3′) that are located within exon 2 of the rat PC gene [27].

Quantitative RT-PCR (Q-PCR)

Total RNA was isolated from cells using TRI Reagent (Molecular Research Center) and its concentration was determined by spectrophotometry. cDNA synthesis was carried out in a 20 μl-reaction mixture containing 500 ng of total RNA, 200 ng of random hexamers (Promega), 1x first-strand buffer (50 mM Tris-

HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 0.1 mM DTT, 1 mM each of dNTP and 200 units of SuperscriptIII reverse transcriptase (Invitrogen). Quantitation of PC expression was performed by real time PCR (Q-PCR) using *Taq*man probe. The Q-PCR was performed in a 12 µl-reaction mixture containing 1x *Taq*man PCR master mix (Roche), 2 µl of 1:10 diluted cDNA, 1 µM of each primer and 0.5 µM of *Taq*man probe. The thermal cycling consisted of an initial incubation at 50°C for 2 min and 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min using MxPro 3005 (Agilent Technologies). Expression of PC was normalized with that of 18 s rRNA and the results are shown as "relative gene expression". The sequences of primers and probes for PC mRNA and 18 s rRNA gene are the same as previously described [19]. Q-PCR for the detection of USF1, USF2, Sp1 and ChREBP expression in INS-1 832/13 was performed using Sybergreen. The primers used for detection of rat USF1 (PPR45083A), USF2 (PPR49799A), Sp1 (PPR49794A) and ChREBP (PPR49636B) were purchased from Qiagen.

Western blot analysis

Forty micrograms of nuclear and cytosolic extracts from INS-1 832/13 cells were subjected to 10% discontinuous SDS-PAGE before transferring onto PVDF membrane (PALL). The blots were incubated with appropriate dilution of anti-Sp1 (sc-59), anti-USF1 (sc-22), anti-USF2 (sc-862), anti-ChREBP (sc-21189) [Santa Cruz Biotechnology], anti-phospho Sp1 (ab59257), anti-tubulin (ab6046) or anti-laminB (ab16048) [Abcam], in 5% BSA in TBS-T overnight. The blots were washed with TBS-T before incubating with appropriate secondary antibodies conjugated with HRP for 3 h. The immunoreactive bands were visualized by an enhanced chemiluminescence using ECL Western substrate kit (Pierce).

Statistical Analysis

All data are presented as the means ± SD from three independent experiments. Statistical analyses were performed using the ANOVA test. A P value$<$0.05 was considered to be statistically significant.

Results

Glucose induces PC mRNA expression in a time-dependent manner

Previous work has shown that incubation of rat islets with elevated concentrations of glucose resulted in the up-regulation of PC protein [9]. To examine whether the increased PC protein was due to up-regulation of PC mRNA, INS-1 cells were exposed to glucose at normal (5.5 mM) and high (25 mM) concentrations, followed by quantitative real time PCR analysis of PC mRNA expression. As shown in Figure 1A, incubation of the INS-1 cells with a high concentration of glucose resulted in a significant increase in PC mRNA expression at 2 h, 6 h, 12 h, 24 h, 48 h and 72 h ($P<0.01$).

Multiple E-boxes in the distal promoter of rat PC gene function as the glucose-responsive element

Because the distal promoter of the rat PC gene is the only promoter that is transcriptionally active in rat pancreatic β-cells [17] and mostly active in the insulinoma cell line, INS-1 832/13 [26] we examined whether the distal promoter of the PC gene contains a glucose-responsive element (GRE) by transfecting the 1.2 kb distal promoter-luciferase chimeric reporter constructs into the INS-1 832/13, a cell line that responds to glucose more robust than the INS-1 cell line [30]. This promoter fragment has previously been characterized to contain full basal and tissue-specific *cis*-acting elements [21]. The transfected cells were maintained in the medium containing 5.5 mM or 25 mM glucose for 24 h. As shown in Figure 2, the transfected cells maintained in medium containing 25 mM glucose possessed luciferase activity approximately 2-fold higher than those maintained at 5.5 mM glucose, suggesting that glucose exerts its stimulatory effect on PC expression via GRE located within the 1.2 kb distal promoter of PC gene. To define the GRE, we transfected a series of 5′-truncated distal promoter-luciferase reporter constructs into INS-1 832/13 cells. Truncations from nucleotides −1146 to −653 (pGL-P2ΔSacI construct) or to nucleotide −546 (pGL-P2ΔKpnI) did not affect glucose-mediated transcriptional activation of the luciferase reporter gene (Figure 2). However, truncation to nucleotide −399 (pGL-P2ΔXhoI) not only completely eliminated the glucose induction effect but this also unexpectedly increased the luciferase reporter gene under low glucose conditions. Further truncation of the P2 promoter to the −34 region (pGL-P2ΔPstI), yielded a similar result with that of the pGL-P2ΔXhoI construct, suggesting that the GRE was located between nucleotides −546 and −399.

To precisely localize the GRE, we generated a series of internal deletions across the −546 to −399 region and the resulting constructs [Δ1(−546/−522), Δ2(−521/−497), Δ3−496/−472), Δ4(−471/−447), Δ5(−446/−422) and Δ6(−421/−399)] were transfected into INS-1 832/13 cells. As shown in Figure 3A, deletion of the nucleotides −546/−522 (Δ1), −521/−497 (Δ2) and −496/−472 (Δ3) did not affect the glucose response, however deletion of the nucleotides −471/−447 (Δ4) resulted in a complete loss of the glucose response. Similar results were observed when the nucleotides −446/−422 (Δ5) and −421/−399 (Δ6) were deleted. Of particular interest, the deleted nucleotides in Δ4, Δ5 and Δ6 mutant constructs corresponded to the three copies of canonical E-boxes (CANNTG), designated E1 (−465/−460), E3 (−436/−431) and E4 (−408/−403), and one E-box-like element [E2 (−442/−437), respectively (See Figure 3A). E-boxes have previously been reported to be involved in many glucose-responsive genes [32–37]. To examine whether these four E-boxes confer glucose-mediated transcription induction of the PC gene, each of them was mutated. As shown in Figure 3B, mutations of E1, E2 or E3, resulted in a complete loss of glucose-mediated transcription induction of the reporter gene but had no effect on transcriptional induction under normal glucose (5.5 mM). In contrast, mutation of a whole ChoRE consisting of E4 and E-box-like (see ChoRE in Figure 4A) not only eliminated high glucose induction effect but also resulted in a 2-fold increase in the reporter activity under low glucose condition. This result was in agreement with those of the pGL-P2ΔXhoI (Figure 2) and Δ6 mutants (Figure 3A), suggesting ChoRE functions as an activator under high glucose induction condition and as a repressor under low glucose condition.

Sp1 functions as a glucose-responsive transcription factor binding to E-box like element

To identify which transcription factors might bind to these four E-boxes, we performed EMSA using two double stranded oligonucleotide probes corresponding to various E-boxes. Incubation of M4+M5 probe which covers E1, E2 and E3 (Figure 4A) with nuclear extract of INS-1 832/13 cells produced two prominent species of DNA-protein complexes (C1 and C2, respectively) (Figure 4B, lane 2) compared to control with no nuclear extract (lane 1). Since the transcription factors, USF1,

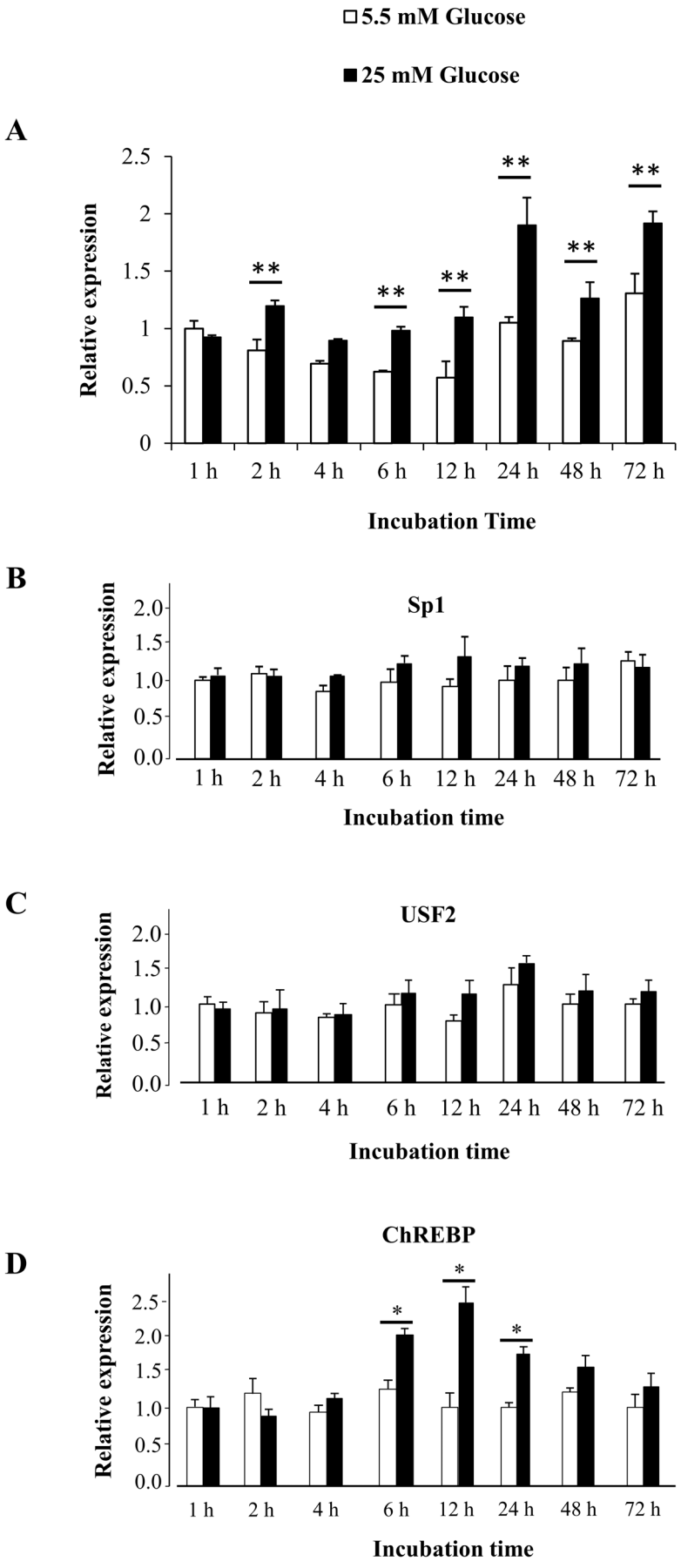
5.5 mM Glucose
25 mM Glucose
A
Relative expression
Incubation Time
1 h
2 h
4 h
6 h
12 h
24 h
48 h
72 h
B
Sp1
Relative expression
Incubation time
C
USF2
Relative expression
Incubation time
D
ChREBP
Relative expression
Incubation time

Figure 1. Glucose increases expression of PC and ChREBP mRNAs in INS-1 832/13 cells. INS-1 832/13 cells were cultured in RPMI medium containing normal (5.5 mM) or high (25 mM) glucose for 1, 2, 4, 6, 12, 24, 48 and 72 h. Total RNA was prepared from cells harvested at the indicated time points, converted to cDNA and analyzed by real time PCR. The expression of PC (A), Sp1 (B), USF2 (C) and ChREBP (D) was normalized with that of 18 s rRNA and shown as relative PC expression. Relative expression values of each gene obtained from cells maintained in RPMI containing 5.5 mM and 25 mM glucose at various time points were compared with those obtained from cells cultured in RPMI containing 5.5 mM at 1 h, which was arbitrarily set as 1. The statistical analysis was conducted using ANNOVA test where *P<0.05; **P<0.01.
doi:10.1371/journal.pone.0102730.g001

USF2 and ChREBP have been reported to regulate several glucose responsive genes via binding to E-boxes in their enhancer regions, we performed a supershift assay using antibodies against these three transcription factors. As shown in Figure 4B, neither of these antibodies affected the formation of both C1 and C2 complexes, indicating that these two complexes could not be attributed to the above three transcription factors (lanes 3–5, respectively). Re-examination of the nucleotide sequence surrounding these E-boxes by the PROMO transcription factor binding site database [38] revealed the presence of CCCCCG (positions −444/−439), within E2. This sequence is similar to the GC-rich, a putative binding site of Sp1 and Sp3 transcription factors [39]. We examined if this is the case by incubating the binding reaction in the presence of anti-Sp1 or anti-Sp3 antibody. Addition of anti-Sp1, anti-Sp3 or both antibodies reduced the formation of C1 complex (lanes 7–9, respectively). Incubation of the probe with nuclear extract of INS-1 832/13 overexpressing Sp1 or Sp3 produced a predominant strong band of C1 (lanes 13 and 15, respectively) which was much stronger than that observed from nuclear extract of cells transfected with an empty vector (lane 12). Incubation of anti-Sp1 or anti-Sp3 antibody to the nuclear extract of the Sp1- or Sp3-overexpressing cells prior to the reaction markedly eliminated this strong band (lanes 14 and 16, respectively), further confirming that Sp1 and Sp3 bind to this CCCCCG within E2. To examine whether the CCCCCG motif indeed mediates C1 and C2 formation, we performed a competition EMSA in which the competitor sequence lacking this sequence (M4+5 pmut competitor) was used in the assay. In contrast to the use of WT sequence as a competitor which can eliminate the complex formation (lane 11), the competitor lacking CCCCCG sequence failed to prevent the complex formation (lane 10), suggesting that Sp1 and Sp3 bind to this CCCCCG within E2.

To examine whether over-expression of Sp1 or Sp3 in INS-1 832/13 could influence PC promoter activity, we performed a reporter assay in which we co-transfected the WT PC promoter-reporter construct or promoter-reporter construct containing mutated CCCCCG with plasmids over-expressing Sp1 or Sp3 in the INS-1 832/13 and cultured the transfected cells in the medium containing normal (5.5 mM) or high (25 mM) of glucose. As shown in Figure 5A, in the presence of 5.5 mM glucose, over-expression of Sp1 or Sp3 increased the reporter activity of the WT promoter construct approximately 2-fold while in the presence of 25 mM glucose, over-expression of Sp1 or Sp3 further increased the reporter activity of the WT promoter to 5-fold. In the mutant construct, in the presence of 5.5 mM, over-expression of Sp1 or

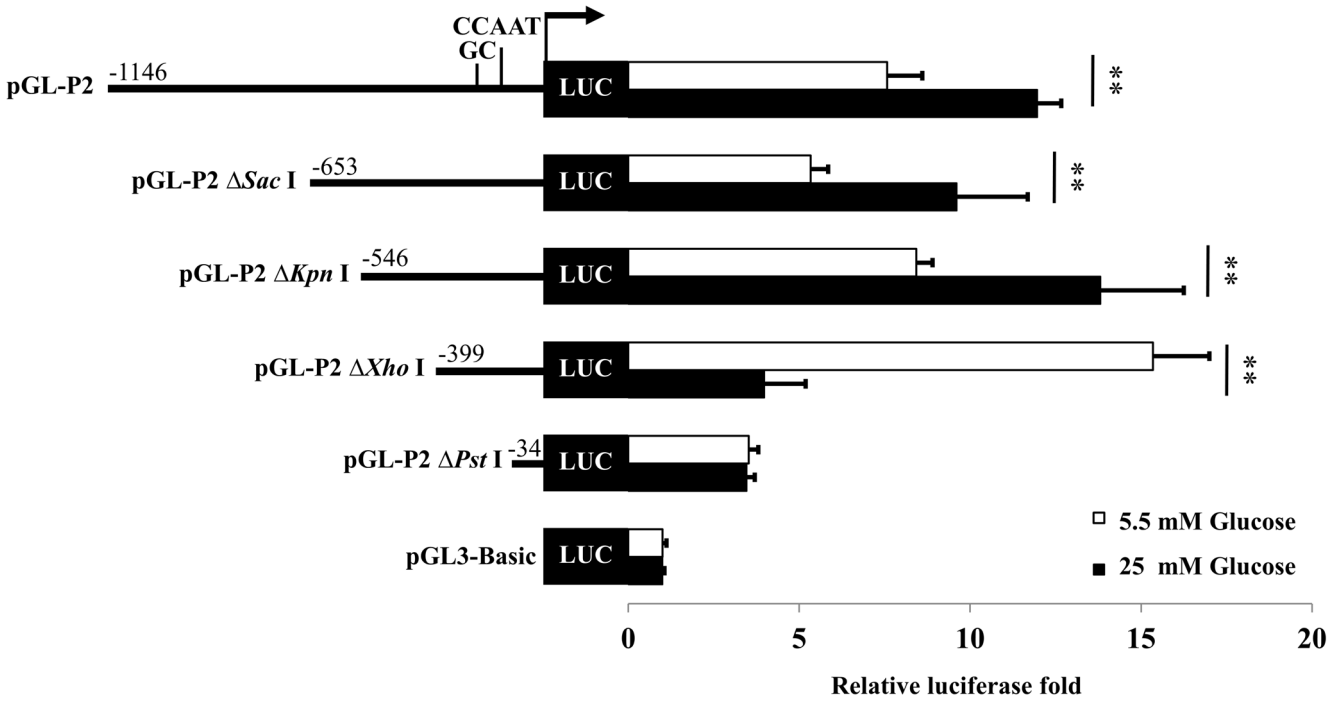

Figure 2. The P2 promoter of rat PC gene contains glucose-responsive element(s) (GRE). A series of 5′-truncated P2-luciferase reporter constructs were transfected into INS-1 832/13 cells. The transfected cells were maintained in RPMI containing normal (5.5 mM) or high (25 mM) glucose for 24 h. The luciferase activity of each construct was normalized to the β-galactosidase activity and expressed as relative luciferase activity. Relative luciferase values obtained from transfected cells maintaining in high glucose medium were presented as fold change relative to those maintaining in normal concentration of glucose, each of which was arbitrarily set as 1. The statistical analysis was conducted using ANOVA test where **P<0.01.
doi:10.1371/journal.pone.0102730.g002

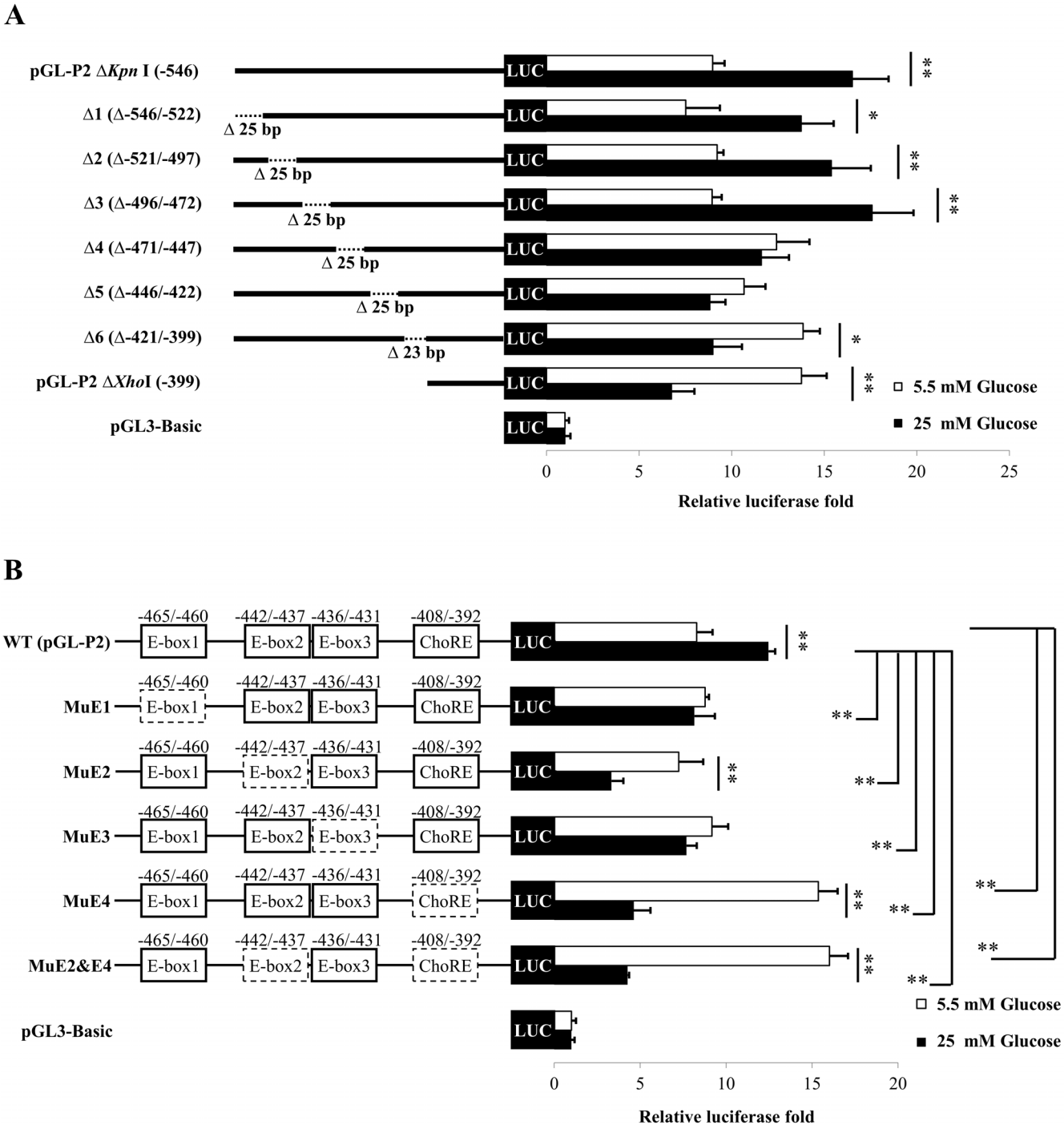

Figure 3. Multiple E-boxes in the P2 promoter of the PC gene function as GREs. A, A series of 25 or 23-nucleotide internal deletions were generated across the −546 to −399 of P2 promoter (Δ1, Δ2, Δ3, Δ4, Δ5 and Δ6). **B**, The WT P2 promoter construct containing mutation at E1, E2, E3 and whole ChoRE (MuE1, MuE2, MuE3, MuE4 and MuE2&E4) were generated and transiently transfected into INS-1 832/13 cells. The transfected cells were maintained in RPMI containing normal (5.5 mM) or high (25 mM) glucose for 24 h. The luciferase activity of each construct was normalized to the β-galactosidase activity and expressed as relative luciferase activity. Relative luciferase values obtained from transfected cells maintaining in high glucose medium were presented as fold change relative to those obtained from those maintaining in normal concentration of glucose, each of which was arbitrarily set as 1. The statistical analysis was conducted using ANOVA test where *P<0.05; **P<0.01.
doi:10.1371/journal.pone.0102730.g003

Sp3 was capable of increasing the reporter activity to the similar levels as that of the WT construct. However, further increase of reporter activity of this mutant construct mediated by Sp1 or Sp3 was lost, indicating that mutation of CCCCCG sequence blunts Sp1- and Sp3-mediated transactivation under high glucose concentration. We next confirmed the above result by performing a ChIP assay that showed *in situ* binding of Sp1 and Sp3 to CCCCCG in the P2 promoter of the rat PC gene in INS-1 832/13

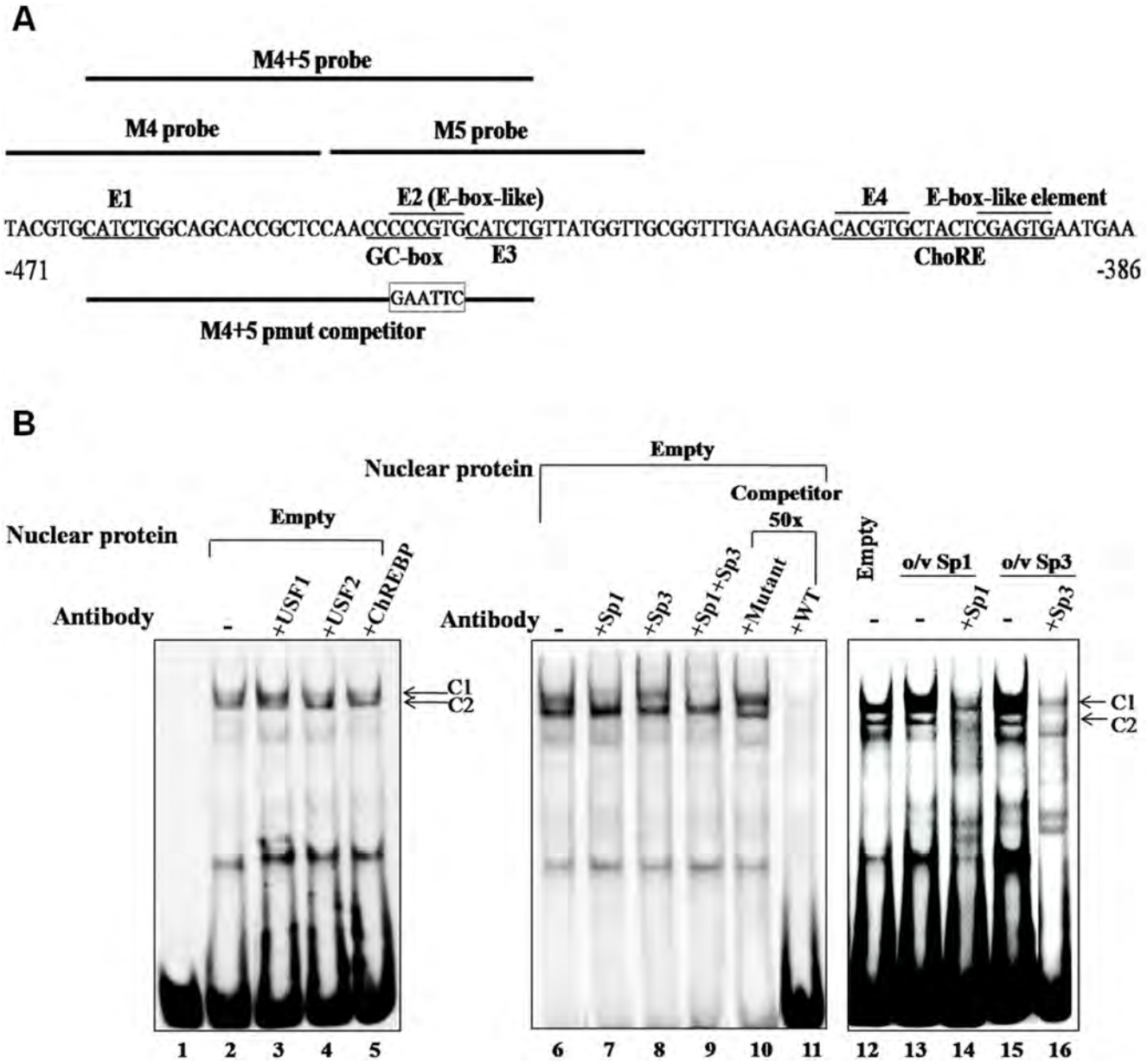

Figure 4. Sp1 and Sp3 bind E-box-like element (E2) *in vitro*. A, Nucleotide sequence of M4+M5 and location of various E-boxes [E1, E2 (E-box like), E3 and E4]. **B**, M4+M5 probe was incubated with nuclear extract of INS-1 832/13 cells and subjected to EMSA. Lanes 1, M4+5 probe alone; lanes 2 and 6, probe incubated with nuclear extract; lanes 3–5, nuclear extracts were pre-incubated with anti-USF1, anti-USF2, anti-ChREBP, respectively. Lanes 7–9, nuclear extracts incubated with anti-Sp1, anti-Sp3 antibodies or both before the probes were added into the reaction, respectively. Lanes 10–11, nuclear extracts were pre-incubated with 50-fold excess mutant or wild type unlabeled oligonucleotide before the probes were added into the reaction, respectively. Lane 12, probe incubated in nuclear extract of INS-1 832/13 cells transfected with an empty vector (Empty) or nuclear extract of INS-1 832/13 cells transfected with plasmid over-expressing Sp1 (o/v Sp1) (lane 13) or Sp3 (o/v Sp5) (lane 15). Lanes 14 and 16, nuclear extracts of INS-1 832/13 cells over-expressing Sp1 or Sp3 were pre-incubated with anti-Sp1 or anti-Sp3 antibody, respectively before probe was added into the reaction. Arrows (C1, C2) represent DNA-protein complexes.
doi:10.1371/journal.pone.0102730.g004

cells maintained in medium containing either 5.5 mM or 25 mM glucose. As shown in Figure 5B, in the presence of 25 mM glucose, Sp1 appeared to bind to the CCCCCG sequence approximately 2-fold greater than in the presence of 5.5 mM glucose. Although we have shown that Sp3 also bound to E2 by EMSA, we were not able to show that Sp3 bound to E2 in the presence of either 5.5 mM or 25 mM glucose using the ChIP assay. This may suggest that Sp1 rather than Sp3 regulates PC expression via CCCCCG sequence *in vivo*. This enhanced binding of Sp1 to CCCCCG sequence in the presence of high glucose cannot be attributed to an increased level of Sp1 mRNA as its mRNA expression did not change after cells were exposed to high glucose from 1 h to72 h. (Figure 1B). Likewise, the increased binding of Sp1 was not due to the increased entry rate of Sp1 into nucleus because the amounts of Sp1 detected by Western blot analysis in the nucleus and cytoplasm of cells maintained in the presence of 5.5 mM and 25 mM glucose at the time when ChIP was performed (12 h) were no difference from one another

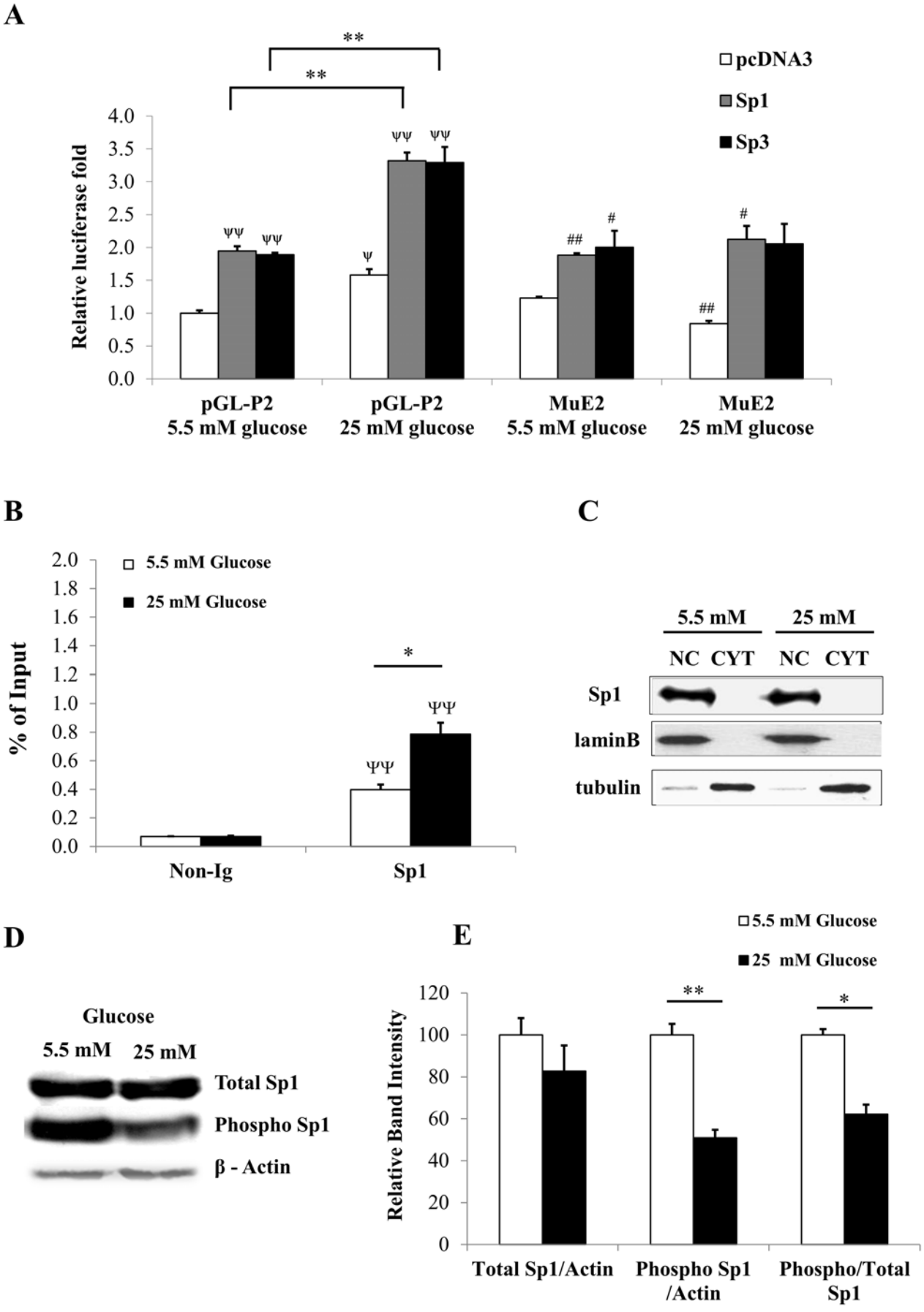
A
pcDNA3
Sp1
Sp3
Relative luciferase fold
pGL-P2 5.5 mM glucose
pGL-P2 25 mM glucose
MuE2 5.5 mM glucose
MuE2 25 mM glucose
B
5.5 mM Glucose
25 mM Glucose
% of Input
Non-Ig
Sp1
C
5.5 mM
25 mM
NC CYT NC CYT
Sp1
laminB
tubulin
D
Glucose
5.5 mM 25 mM
Total Sp1
Phospho Sp1
β - Actin
E
5.5 mM Glucose
25 mM Glucose
Relative Band Intensity
Total Sp1/Actin
Phospho Sp1 /Actin
Phospho/Total Sp1

Figure 5. Sp1 regulates glucose-induced PC expression through E-box-like element (E2) in P2 promoter of the PC gene. A, The WT-P2(pGL-P2) or its mutation at E2-luciferase reporter constructs (MuE2) with plasmid overexpressing Sp1, Sp3 or empty vector (pcDNA) were transfected into INS-1 832/13 cells before they were maintained in medium containing low (5.5 mM) or high (25 mM) glucose before their luciferase activities were assayed. Relative luciferase activity of cells co-transfected with pGL-P2 (WT) or mutant promoter construct with empty vector (pcDNA), pSp1 or pSp3 maintained at 5.5 mM or 25 mM were normalized with that of cells co-transfected with pGL-P2 and empty vector maintained at 5.5 mM, which was arbitrarily set as 1. The statistical analysis was conducted using ANOVA test. Ψ ($P<0.05$) and ΨΨ ($P<0.01$), compared with cells transfected with pGL-P2 and pcDNA maintained at 5.5 mM glucose. # ($P<0.05$) and ## ($P<0.01$), compared with cells transfected with MuE2 maintained at 5.5 mM glucose. **$P<0.01$ (transactivation of WT promoter by Sp1 or Sp3 under 5.5 mM and 25 mM glucose). **B**, The Sp1-bound chromatin was prepared from INS-1 832/13 cells grown in low (5.5 mM) or high (25 mM) glucose, fragmented and immunoprecipitated with anti-Sp1 antibody and subjected to real time PCR. The fluorescence signals obtained from quantitation of immunoprecipitated fraction was normalized to the input levels. The input fraction was the sonicated Sp1-bound DNA before immunopreripitating with anti-Sp1 antibody. The statistical analysis was conducted by ANOVA test where *,ΨΨ $P<0.01$. **C**, Western blot analysis of nuclear (NC) and cysolic (CYT) extracts of INS-1 832/13 cells maintained under 5.5 or 25 mM glucose with anti-Sp1 antibody. Loading controls of the cytosolic and nuclear proteins were assessed by stripping the blot and re-probed with anti-tubulin and anti-lamin B antibody, respectively. **D**, A representative of Western blot analysis of nuclear extracts of INS-1 832/13 cells maintained in the presence of 5.5 or 25 m glucose with anti-Sp1, anti-phospho Sp1. Control loading was assessed by stripping the blot and re-probed with anti-actin antibody. E, The intensity of total Sp1, phospho-Sp1 bands was quantitated and normalized with that of actin band and shown as the ratios of total Sp1/actin, phospho-Sp1/actin and total Sp1/phospho-Sp1 from three independent experiments. The statistical analysis was conducted using ANOVA test where *$P<0.05$ **$P<0.01$.
doi:10.1371/journal.pone.0102730.g005

(Figure 5C). Furthermore, most of the Sp1 was also found in the nucleus. Several reports have demonstrated that phosphorylation/dephosphorylation of Sp1 in response to elevated glucose levels contributes to its transcriptional activity [40]. We detected total and phosphorylated Sp1 in INS-1 832/13 cells cultured in the medium containing low and high glucose. As shown in Figure 5D, INS-1 832/13 cells grown in the presence of 5.5 mM and 25 mM glucose expressed similar amounts of total Sp1. However, most Sp1 presence in the cells maintained under low glucose was detected in the phosphorylated form while only 50% of total Sp1 presence in cells maintained under high glucose was detected in the phosphorylated form (Figure 5E). It is very likely that the enhanced recruitment of Sp1 to CCCCCG sequence detected by the ChIP assay can be attributed to the dephosphorylation of this protein. The dephosphorylation form of Sp1 has been shown to be a transcriptionally active form which contributes to the transcriptional induction of the acetyl-CoA carboxylase 1 (ACC1) gene [41].

USF2 preferentially binds to E1 while an unknown factor(s) binds to E3

Although the EMSA shown in Figure 4B suggested that USF1, USF2 and ChREBP did not bind to the probe, this may not be the only conclusion because E1 and E3 are located close to the extreme 5′- and 3′-ends of M4+M5 probe. This may restrict the efficiency of binding of (an) additional transcription factor(s), if any, apart from Sp1 and Sp3. To rule out this possibility, we synthesized the M4 probe which contains only E1 in the middle for EMSA analysis (Figure. 6A). Incubation of the M4 probe with a nuclear extract of INS-1 832/13 revealed weak DNA-protein complexes (data not shown), suggesting the poor binding of the candidate transcription factor(s) to this probe. However, a prolonged exposure (10 min) of the blot produced two weak DNA-protein complexes (C1 and C2) (Figure 6B, lane 2). Incubation of anti-USF1 or anti-USF2 antibody in the reaction marginally affected both complexes (lanes 3 and 4). However, incubation of M4 probe with the nuclear extract prepared from INS-1 832/13 cell overexpressing USF1 produced an additional weak band corresponding to the C2 complex (lane 5, asterisk) while incubation of the same probe with a nuclear extract of INS-1 832/13 cells overexpressing USF2 also produced an additional band corresponding to the C2 complex but was much stronger (lane 6). Furthermore incubation of anti-USF1 or anti-USF2 antibodies prevented C2 formation (lanes 7 and 8), suggesting that USF2 preferentially binds to E1. Although both anti-USF1 and USF2 antibodies prevented at least one of the two complexes, anti-ChREBP antibody did not affect formation any of these complexes (lane 9), suggesting that ChREBP did not bind to E1.

We also conducted EMSA using the new probe, M5 which contains mutated CCCCCG in E2 (M5 pmut E2 probe in Figure 6A), leaving only E1 intact in the middle (M5 pmut E-box2, Figure 6A). Any binding observed in this assay would result from binding of the nuclear factor to E3 only. Incubation of this probe with a nuclear extract of non-transfected INS-1 832/13 produced one prominent band, M5–1 (Figure 6C, lane 2). Incubation of the binding reaction in the presence of anti-USF1, anti-USF2 or anti-ChREBP (lanes 3–5, respectively) did not affect this complex, suggesting other nuclear factors rather than these three proteins bind to E3.

Glucose enhances binding of USF2 and ChREBP to E4

We showed that E4 serves as a binding site for USF1 and USF2 [19], however the functional importance of this binding site with respect to the glucose-induction was unknown. Pedersen *et al*. [26] have previously shown that ChREBP binds relatively weakly to this site. To examine whether the recruitment of USF1, USF2 and ChREBP to this E-box is enhanced during high glucose induction, we performed a ChIP assay of this E4 with anti-USF1, anti-USF2 and anti-ChREBP antibodies in INS-1 832/13 cells maintained in the medium containing 5.5 mM and 25 mM glucose. As shown in Figure 7A, cells grown in high glucose medium did not affect the recruitment of USF1, instead recruitment of USF2 and ChREBP to E4 was increased (Figure 7A). The enhanced binding of USF2 to E4 in the presence of high glucose could not result from an increased level of USF2 mRNA expression as its abundance was unchanged after the cells were exposed to 25 mM glucose from 1 h to 72 h (Figure 1C). The increased binding of USF2 to E4 was not due to the increased entry rate of this transcription factor to nucleus because the amounts of USF2 in the nucleus of cells maintained under low and high glucose concentrations were similar (Figure 7B). Also most USF2 were found in the nucleus. We failed to detect expression of the ChREBP protein in INS-1 832/13 cells grown under 5.5 mM and 25 mM glucose by Western blot analysis, suggesting that the expression of ChREBP in INS-1 832/13 may be rather low compared to other types of cells, such as liver. This rather weak expression of endogenous ChREBP in INS-1 832/13 or rat islets was also seen in the previous report [26]. Nevertheless, the increased binding of ChREBP to E4 is partly contributed by increased ChREBP mRNA abundance. As shown in Figure 1D, expression of ChREBP mRNA was increased to 2–2.5-fold after INS-1 832/13 cells were exposed to 25 mM glucose for 6 h, 12 h and 24 h.

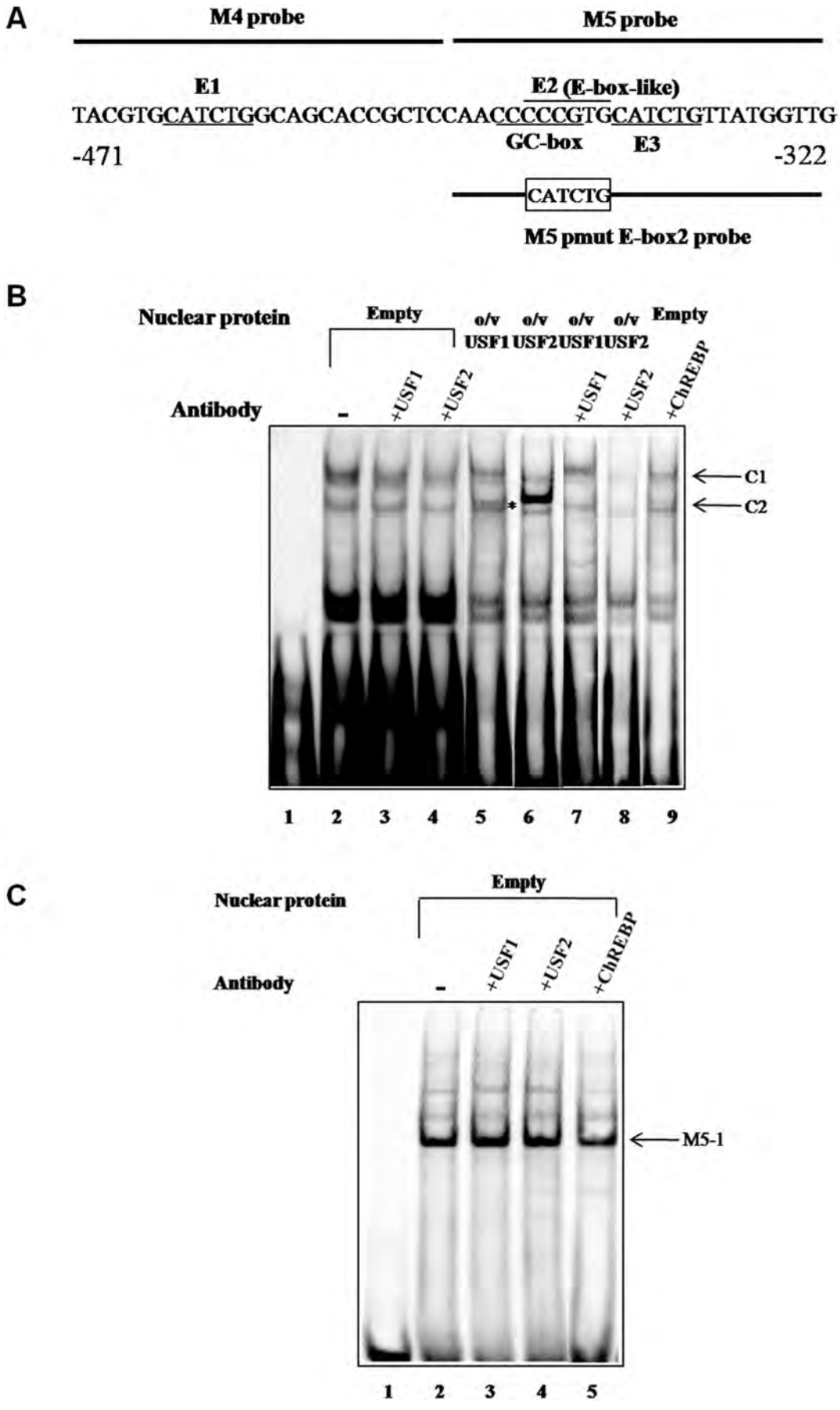
A
M4 probe
M5 probe
E1
E2 (E-box-like)
TACGTGCATCTGGCAGCACCGCTCCAACCCCCGTGCATCTGTTATGGTTG
GC-box
E3
-471
-322
CATCTG
M5 pmut E-box2 probe
B
Nuclear protein
Empty
o/v USF1
o/v USF2
o/v USF1
o/v USF2
Empty
Antibody
-
+USF1
+USF2
+USF1
+USF2
+ChREBP
C1
C2
1 2 3 4 5 6 7 8 9
C
Nuclear protein
Empty
Antibody
-
+USF1
+USF2
+ChREBP
M5-1
1 2 3 4 5

Figure 6. USF1 and USF2 bind to the E1 in the P2 promoter of the PC gene. A. Nucleotide sequences of M4, M5 and M5 pmut E2 probes. E-box and GC-box are underline. **B,** EMSA of M4 probe with INS-1 832/13 nuclear extract. Lane 1, M4 probe alone; lane 2, probe incubated with nuclear extract; lanes 3–4, nuclear extracts pre-incubated with anti-USF1 or anti-USF2 antibody before the probes were added into the reaction, respectively. Lanes 5–6, probe incubated with nuclear extract of INS-1 832/13 overexpressing USF1 or USF2, respectively. Lanes 7 and 8, nuclear extracts of INS-1 832/13 overexpressing USF1 pre-incubated with anti-USF1 antibody or overexpressing USF2 pre-incubated with anti-USF2 antibody before the probe was added into the reaction, respectively. Lane 9, INS-1 832/13 nuclear extract pre-incubated with anti-ChREBP antibody before the probe was added into the reaction. **C.** EMSA of M5 pmut E2 probe with an INS-1 832/13 nuclear extract. Lane 1, M5 pmut E2 probe alone; lane 2, probe incubated with nuclear extract; lanes 3–5, nuclear extracts pre-incubated with anti-USF1, anti-USF2 or anti-ChREBP antibody before the probes were added into the reaction, respectively. Arrows represent DNA-protein complexes.
doi:10.1371/journal.pone.0102730.g006

Finally we confirmed the functional role of Sp1, USF1, USF2 and ChREBP in regulating glucose-mediated transcriptional induction of PC expression. We suppressed expression of these four transcription factors by their respective siRNAs in INS-1 832/13 and maintained the transfected cells in the medium containing normal or high glucose before analyzing the expression of PC mRNA by real time PCR. As shown in Figure 7C, upon transfection of siRNAs targeted to Sp1 (Sp1 KD), USF1 (USF1 KD), USF2 (USF2 KD) or ChREBP (ChREBP KD) resulted in 80%, 90%, 75% and 80% reduction in expression of their respective mRNAs. As shown in Figure 7D, suppression of USF1 did not appear to affect glucose-induced PC mRNA expression while suppression of Sp1, USF2 or ChREBP expression resulted in 50%, 40% and 30% reduction of glucose-induced PC mRNA expression. Combined knockdown of USF2, Sp1 and ChREBP expression completely prevented glucose-induced PC mRNA expression (Figure 7D). These data suggest that the glucose-mediated transcriptional activation of PC in the P2 promoter is regulated by these three transcription factors, albeit their absolute degrees of the control are varied.

Discussion

Pyruvate carboxylation has been shown to be a crucial anaplerotic reaction which regulates glucose-induced insulin secretion in pancreatic islets. PC catalyzes the above reaction and is highly abundant in this tissue where it participates in the pyruvate cycling, a process by which a coupling factor, NADPH is formed and required for glucose-induced insulin secretion (for review see [42,43]). Suppression of PC expression impairs anaplerosis concomitant with the reduced glucose-induced insulin secretion [12,13]. Previous work has shown that expression of PC in isolated rat islets is inducible by exogenous glucose and its expression is highly correlated with the flux toward pyruvate carboxylation [44]. Here we showed that induction of PC expression by glucose in the rat insulinoma cell line, INS-1 832/13 is rather slow, the levels of mRNA expression is increased to only 1.5-fold within the early hours before reaching maximum at 24 h. The slow induction of PC mRNA expression was also observed in the isolated rat islets maintained under high glucose containing medium [8]. It is possible that the allosteric regulation of PC by acetyl-CoA may be important mechanism to increase activity of PC during this early period before transcription of this gene is activated. It is well known that equal amounts of pyruvate enter pyruvate carboxylation by PC and pyruvate decarboxylation by PDH [6,7,9,45]. The acetyl-CoA formed by PDH-catalyzed reaction can in turn allosterically activate PC activity during this early period.

In this report we identified a complex GRE within the P2 promoter of the rat PC gene. This GRE consists of four tandem E-boxes, locating at −465/−460 (E1), −442/−437 (E2), −436/−431(E3) and −408/−403 (E4) and as the glucose sensor. It is interesting to note that although these four E-boxes are well defined in the distal promoter of the rat PC gene, only E3 and E4 are also conserved in the distal promoter of the human PC gene, while the nucleotides corresponding to E1 and E2 contains one nucleotide difference in the distal promoter of the human PC gene [20]. This raises the possibility of subtle difference of transcriptional activation of PC in response to glucose between the rat and the human. Nevertheless, mutational analysis of these sequences of distal promoter of rat PC gene showed that mutating one of these E-boxes is sufficient to eliminate glucose-mediated transcriptional activation of P2 promoter activity, suggesting that these four E-boxes are equally important. It is noted that E1 and E3 resemble the canonical E-box (CANNTG) while E4 is part of the previously reported ChoRE [26]. While both endogenous USF1 and USF2 can bind E1, overexpression of USF1 or USF2 in INS-1 832/13 cells revealed that USF2 preferentially binds E1 much stronger than USF1. However, ChREBP did not bind this E-box, possibly because binding of ChREBP requires two adjacent E-boxes separated by 5 nucleotides [46]. Although the E3 sequence is similar to the E1 sequence, neither USF1 nor USF2 binds to E1, it appears to bind strongly to an unknown nuclear factor whose identity remains unidentified. We have previously shown that E4 serves as a binding site of USF1 and USF2 [21]. Although mutation of E4 moderately reduced P2 activity under basal conditions, its role in glucose-mediated transcriptional induction at that time was unknown [21]. Pedersen *et al* [26] previously reported that this E-box also serves as a binding site of ChREBP which mediates glucose-induced transcriptional induction of the P2 promoter. They have also shown that high glucose promotes the recruitment of ChREBP, but not USF1 or USF2, to this E-box. However, we have found that high glucose not only enhances binding of ChREBP, but also USF2, to this E-box. It is well known that USF2 can form a homodimer or a heterodimer with USF1, but not with ChREBP, to regulate transcription [47]. Likewise, ChREBP/Mlx heterodimer is required to activate glucose-dependent transcription. Although initial reports have clearly demonstrated that ChREBP transcriptional activity is closely associated with elevated concentrations of glucose [23,48–49] via complicated post-translational modifications [24,25,50], growing evidence has now indicated that both USF1 and USF2 are capable of regulating transcription of several glucose-dependent genes [34,51–53]. Although the expression levels of USF2 can be induced by elevated concentrations of glucose in rat mesangial cells [53], we did not observe a significantly increase of USF2 in INS-1 832/13 cells grown under high glucose concentration suggesting the regulation of USF2 expression may be different between cell types. Despite the lack of increased USF2 mRNA level and USF2 protein accumulated in the nucleus in INS-1 832/13 cells maintained in the presence of high glucose, we observed the enhanced binding of USF2 to E4 in the ChIP assay, suggesting that posttranslational modification of this transcription factor may be responsible for the enhanced DNA binding activity. Nowak et al. [52] have reported that elevated concentrations of glucose stimulate expression of the apolipoprotein A5 gene through increased binding of USF1 and USF2 to their cognate E-box without changing protein abundance. Interestingly, this enhanced

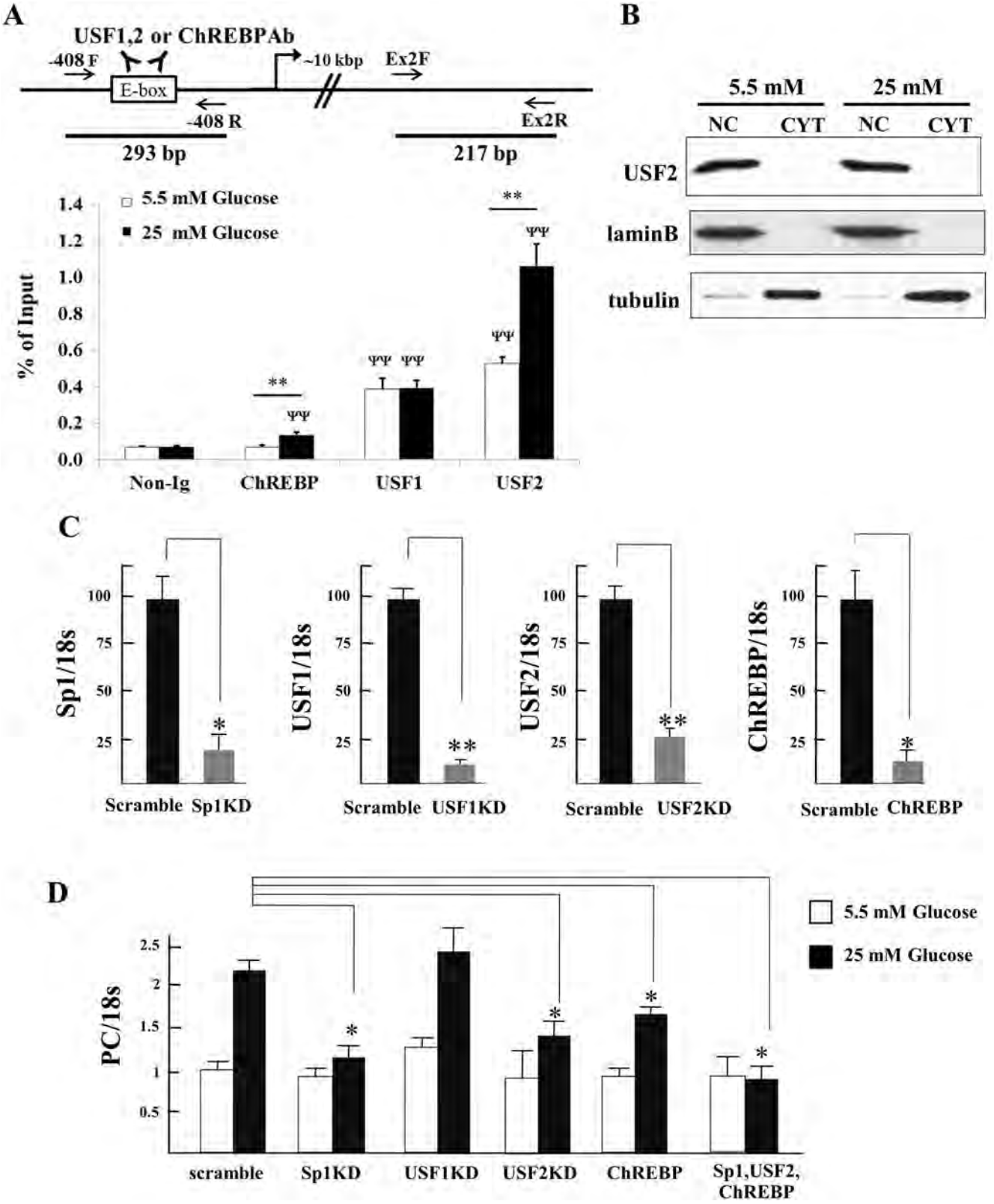

Figure 7. High glucose enhances binding of USF2 and ChREBP to E4 in the P2 promoter of the PC gene and suppression of Sp1, USF2 and ChREBP expression blunts glucose-induced expression of endogenous PC expression in INS-1 8322/13. A, *Top panel*, a schematic representation of the P2 promoter region with E-box4 and primer binding sites for quantitative real time PCR indicated. *Bottom panel*, the USF1-, USF2- or ChREBP-bound chromatin was prepared from INS-1 832/13 cells grown in low (5.5 mM) or high (25 mM) glucose was prepared, immunoprecipitated with their corresponding antibodies and subjected to real time PCR using the primers indicated above. The fluorescence signals obtained from the immunoprecipitated fractions were normalized to those obtained from the input fraction which was the sonicated transcription

factor-bound DNA before immunoprecipitating with the antibodies. The statistical analysis was conducted by ANOVA test where **P<0.01 compared between low and high glucose concentrations. $^{\Psi\Psi}$P<0.001 compared with the fraction that was immunoprecipiated with no antibody at both low or high glucose concentration. **B**, Western blot analysis of nuclear (NC) and cysolic (CYT) extracts of INS-1 832/13 cells maintained under 5.5 or 25 mM glucose with anti- USF2 antibody. Loading controls of the cytosolic and nuclear proteins were assessed by stripping the blot and re-probed with anti-tubulin and anti-lamin B antibodies, respectively. **C**, INS-1 832/13 cells were mock- or transfected with siRNAs targeted to Sp1, USF1, USF2 and ChREBP. The transfected cells were cultured in the medium containing low (5.5 mM) or high (25 mM) glucose for the next 24 h before the expression of Sp1, USF1, USF2, ChREBP and PC mRNAs was measured by quantitative real time PCR and their expression levels were normalized with that of 18 s rRNA. The values obtained from scramble and each knocked down cells are expressed relative to that obtained from the mocked transfection, which was arbitrarily set as 100%. The values shown are means ± standard deviations of the three independent experiments (n = 3). The statistical analysis was conducted using ANOVA test where *P<0.05; **P<0.01.
doi:10.1371/journal.pone.0102730.g007

DNA-binding is at least in part caused by dephosphorylation of both USF1 and USF2. Therefore, it remains unclear whether USF2 and ChREBP co-regulate glucose-dependent transcriptional activation of the P2 promoter simply through competition of the same binding site.

It is also interesting to note that while all E-boxes function as glucose-responsive elements that mediate transcriptional activation of the P2 promoter under high glucose induction, E4 functions as a repressor under a normal glucose concentration as mutation of this E-box resulted in an increased reporter gene expression under a normal concentration of glucose. Jeong et al. [54] have recently reported that ChREBP can also function as a transcriptional repressor as well as an activator, however, a molecular explanation regarding this dual role of ChREBP was not investigated. Growing evidence has also indicated that in the promoters of glucose-responsive genes, a complex ChoRE may consist of the classical core ChoRE and the nearby accessory site which allows maximal glucose-induction. The accessory sites thus far have been reported to be the binding sites of the nuclear factor-Y (NF-Y) (i.e. CCAAT-box), HNF4α or c-myc [55,56]. Jeong et al. [54] have recently employed integrated expression profiling and genome-wide analysis of ChREBP target genes and identified a guanine-rich sequence similar to a Sp1-binding site associated with ChREBP even though this sequence does not bind ChREBP. The authors suggest that this guanine-rich sequence may form part of an accessory site allowing Sp1-family transcription factors to bind and co-regulate with ChREBP. As mentioned above, we found that the CCCCCG sequence (i.e. GGGGGC on the complementary strand) coincided within the E2 and the ChREBP binding site (E4) and are in the close vicinity, which may be the case as reported by Jeong et al. [54].

Although, Sp1 has previously been known to be a ubiquitously expressed transcription factor which controls basal transcription of a variety of "housekeeping" genes [57–58], growing evidence now indicates that the abundance and its transcriptional activity of this transcription factor can be modulated by nutrients and cellular metabolites [59–63]. We have found that the CCCCCG sequence within E2 serves as a glucose-sensor because a high concentration of glucose increases the recruitment of Sp1 to this sequence. Also mutation of this sequence eliminated glucose-induced transcriptional induction of P2 promoter activity. We found that an elevated concentration of glucose did not affect the abundance of Sp1 mRNA or Sp1 protein in the nucleus but rather affects the phosphorylation status of the protein. We found that a high concentration of glucose increases dephosphorylation of threonine 453 of Sp1 and this might in part contribute to the enhanced transcriptional activity under this condition. Phosphorylation of this residue of Sp1 has been reported to decrease its ability to activate transcription of the cystathionine γ-lyase gene in pancreatic beta cells [63]. The regulation of PC expression by glucose appears to be similar to another biotin containing enzyme, namely acetyl-CoA carboxylase (ACC). In mouse preadipocytes, Sp1 also mediates glucose activation of the ACC1 gene via the two GC-rich sequences which form part of the glucose-responsive element [64]. Exposure of preadipocytes to high glucose stimulates dephosphorylation of Sp1 concomitant with an enhanced binding to its cognate binding site in the ACC1 gene promoter [41].

Finally the functional roles of USF1, USF2, ChREBP and Sp1 in transcriptional induction of endogenous PC expression were validated by an siRNA experiment. This clearly demonstrated that silencing expression of USF2, ChREBP and Sp1, but not USF1 mRNA, individually resulted in modest reductions of glucose-induced transcriptional induction of PC mRNA expression, suggesting that these three transcription factors may act in concert allowing maximal induction of PC in response to an elevated concentration of glucose.

The findings that glucose-induced transcriptional activation of PC in pancreatic β-cells is regulated by multiple transcription factors provide a complex paradigm in terms of the disease development because deregulation of some of these transcription factors in part underlies the impaired insulin secretion or hyperglycemia. For example, ChREBP was first known to link glycolysis and *de novo* fatty acid synthesis in liver. There is also a direct and strong association between ChREBP expression and insulin sensitivity in adipose tissue of humans with insulin resistance [65]. Growing evidence now indicates that this transcription factor also regulates transcription of β-cell specific genes [49]. Suppression of ChREBP expression impairs glucose-stimulated pancreatic β-cell proliferation [66]. Recently, glucose-induced ChREBP overexpression has been shown to play a pivotal role in mediating glucotoxicity of pancreatic β-cells, causing impaired insulin secretion [67]. Likewise deregulation of Sp1 via the abundance or its posttranslational modification is well known to at least in part cause diabetes [68].

In summary we identified a complex glucose-responsive unit which mediates glucose-induced transcription of the P2 promoter of the rat PC gene. Although this GRU appears to provide a platform for binding of at least four transcription factors, namely USF1, USF2, ChREBP and Sp1, only the latter three are functionally relevant to glucose-induced transcription of P2 promoter of rat PC gene.

Author Contributions

Conceived and designed the experiments: AW TV SJ. Performed the experiments: AW TV SM TB SJ. Analyzed the data: AW TV SJ. Contributed reagents/materials/analysis tools: MJM SJ. Wrote the paper: AW TV SJ.

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