Transcriptional regulation of the distal promoter of the rat pyruvate carboxylase gene by hepatocyte nuclear factor 3β/Foxa2 and upstream stimulatory factors in insulinoma cells

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PC (pyruvate carboxylase) plays a crucial role in intermediary metabolism including glucose-induced insulin secretion in pancreatic islets. In the present study, we identified two regions of the 1.2 kb distal promoter, the −803/−795 site and the −408/−403 E-box upstream of the transcription start site, as the important *cis*-acting elements for transcriptional activation of the luciferase reporter gene. Site-directed mutagenesis of either one of these sites in the context of this 1.2 kb promoter fragment, followed by transient transfections in the insulinoma cell line, INS-1, abolished reporter activity by approx. 50 %. However, disruption of either the −803/−795 or the −408/−403 site did not affect reporter gene activity in NIH 3T3 cells, suggesting that this promoter fragment is subjected to cell-specific regulation. The nuclear proteins that bound to these −803/−795 and −408/−403 sites were identified by gel retardation assays as HNF3β (hepatocyte nuclear factor 3β)/Foxa2 (forkhead/winged helix transcription factor box2) and USFs (upstream stimulatory factors), USF1 and USF2, respectively. Chromatin immunoprecipitation assays using antisera against HNF3β/Foxa2, USF1 and USF2 demonstrated that endogenous HNF3β/Foxa2 binds to the −803/−795 Foxa2 site, and USF1 and USF2 bind to the −408/−403 E-box respectively *in vivo*, consistent with the gel retardation assay results. Although there are weak binding sites located at regions −904 and −572 for PDX1 (pancreatic duodenal homeobox-1), a transcription factor that controls expression of β-cell-specific genes, it did not appear to regulate PC expression in INS-1 cells in the context of the 1.2 kb promoter fragment. The results presented here show that Foxa2 and USFs regulate the distal promoter of the rat PC gene in a cell-specific manner.

Key words: forkhead/winged helix transcription factor box2 (Foxa2), glucose-induced insulin secretion, pancreatic β-cell, pyruvate carboxylase, transcription, upstream stimulatory factor (USF).

INTRODUCTION

The pancreatic β-cell plays a central role in the regulation of glucose homoeostasis through the secretion of insulin in response to an elevated level of blood glucose. Rapid uptake of the glucose followed by glycolysis and oxidative phosphorylation are essential biochemical pathways, providing the metabolic coupling factor, i.e. ATP, required for insulin secretion. In addition to ATP, NADPH is believed to be another important coupling factor required for glucose-induced insulin secretion. The generation of a high level of NADPH is achieved through pyruvate cycling between the mitochondria and cytoplasm, known as the pyruvate–malate or pyruvate–citrate shuttle [1,2]. Both of these shuttles are initiated by the carboxylation of pyruvate to oxaloacetate by the mitochondrial enzyme PC (pyruvate carboxylase). The pyruvate then exits the mitochondria as citrate or malate. Pharmacological inhibition of PC activity in cultured islets or in animal studies has been shown to impair glucose-induced insulin secretion, indicating the important role of PC in this process [2,3] apart from its gluconeogenic role in liver and kidney, and its lipogenic role in adipocytes [4,5].

As PC is involved in different metabolic pathways, its regulation is highly complex. In mammals, transcription of the PC gene is regulated by two alternative promoters, namely the proximal and the distal promoters [6]. These two promoters mediate the production of five distinct mRNAs with different 5′-UTRs (5′-untranslated regions) but identical in their coding sequences [6,7]. These PC mRNA isoforms are also subjected to post-transcriptional regulation [8]. The proximal promoter is highly active in gluconeogenic and lipogenic tissues, while the distal promoter is highly active in pancreatic β-cells and is inducible by glucose [8]. These two promoters lack a canonical TATA-box but contain multiple GC-boxes [6]. We have previously identified Sp1/Sp3 together with NF-Y (nuclear factor-Y) as the general transcription factors that regulate the distal promoter in insulinoma cells, INS-1 [9]. Since the distal promoter is highly active in β-cells, we sought to identify sequences within this promoter that confer this cell-type-specific expression. We identified HNF3β (hepatocyte nuclear factor 3β)/Foxa2 (forkhead/winged helix transcription factor box2) as a novel transactivator of the PC gene distal promoter, acting via its cognate sequence located 803 nt upstream of the transcription start site. Furthermore, we found that USFs (upstream stimulatory factors), USF1 and USF2, acting through the classical E-box consensus sequence located 408 nt upstream of the transcription site, contribute to positive regulation of the distal promoter in the insulinoma cell line, INS-1. The present study provides an explanation of the alternative usage of the two promoters in different cell types.

Abbreviations used: ChIP, chromatin immunoprecipitation; ChRE, carbohydrate responsive element; ChREBP, ChRE-binding protein; CRE, cAMP-response element; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility-shift assay; Foxa2, forkhead/winged helix transcription factor box2; HNF3β, hepatocyte nuclear factor 3β; NF-Y, nuclear factor-Y; PC, pyruvate carboxylase; PDX1, pancreatic duodenal homeobox-1; PPAR, peroxisome-proliferator-activated receptor; USF, upstream stimulatory factor; 5′-UTR, 5′-untranslated region.

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Table 1 Oligonucleotides used for mutagenesis and EMSA

Putative binding sites of transcription factors are underlined. Mutated bases are indicated as lower-case letters in boldface.

Oligonucleotide	Sequence
−408/−403 E-box mutF	5′-GGTTTGAAGAGA**g**A**at**T**C**CTACTCGA**t**T**c**AATGAATTGC-3′
−408/−403 E-box mutR	5′-CTGCAATTCATT**g**A**a**TCGAGTAG**g**A**at**T**c**TCTCTTCA-3′
−436/−431 E-box mutF	5′-TCCAACCCCCGTG**g**A**at**T**c**TTATGGTTGCGGTTT-3′
−436/−431 E-box mutR	5′-TTCAAACCGCAACCATAA**g**A**at**T**c**CACGGGGGTTG-3′
−539P/−530 PDX1 mutF	5′-ACCGGCGCTCTC**gcc**TTCCTCGTGCCCT-3′
−539/−530 PDX1 mutR	5′-TAAGGGCACGAGGAA**ggc**GAGAGCGCCG-3′
−902/−893 PDX1 mutF	5′-GGTTAGTAAGCAA**ggc**TTGTTTAACGCC-3′
−902/−893 PDX1 mutR	5′-TGGCGTTAAACAA**gcc**TTGCTTACTA-3′
−803/−795 Foxa2 mutF	5′-TGATGATGGAGGACTT**gga**T**cc**TTACTGTCTGGTCT-3′
−803/−795 Foxa2 mutR	5′-AGACCAGACAGTAA**gg**A**tcc**AAGTCCTCCATCATCA-3′
−903/−895 Foxa2 mutF	5′-TTGGAGGTTAGTAA**tgg**AT**cc**TTGTTTAACGCCA-3′
−903/−895 Foxa2 mutR	5′-TGGCGTTAAACAA**gg**AT**cca**TTACTAACCTCCAA-3′
−408/−403 E-box WT (+)	5′-GTTTGAAGAGACACGTGCTACTCGAGTGAATG-3′
−408/−403 E-box WT (−)	5′-TGCAATTCATTCACTCGAGTAGCACGTGTCTCTTC-3′
−436/−431 E-box WT (+)	5′-TCCAACCCCCGTGCATCTGTTATGGTTGCGGTT-3′
436/−431 E-box WT (−)	5′-TTCAAACCGCAACCATAACAGATGCACGGGGGTTGG-3′
Cons ChRE (+)	5′-ATGGGCGCACGGGGCACTCCCGTGGTTCCTGG-3′
Cons ChRE (−)	5′-CCAGGAACCACGGGAGTGCCCCGTGCGCC-3′
Cons USF (+)	5′-CACCCGGTCACGTGGCCT-3′
Cons USF (−)	5′-GGTGTAGGCCACGTGACCGGGTG-3′
−803/−795 Foxa2 (+)	5′-TGATGATGGAGGACTTATTTGTTTACTGTCTGGTCT-3′
−803/−795 Foxa2 (−)	5′-GATAGGAGACCAGACAGTAAACAAATAAGTCCTCCA-3′
−903/−895 Foxa2 (+)	5′-GGAGGTTAGTAAGCAATTATTGTTTAACGCC-3′
−903/−895 Foxa2 (−)	5′-TGGCGTTAAACAATAATTGCTTACTA-3′
Cons HNF3β (+)	5′-TGACTAAGTCAATAATCAGAATCAG-3′
Cons HNF3β (−)	5′-CTGATTCTGATTATTGACTTAGTCA-3′

MATERIALS AND METHODS

Generation of reporter constructs and site-directed mutagenesis

A 1.2 kb fragment of the PC distal promoter (pGL-P2) linked to the luciferase reporter gene plasmid [6] was used as the template for site-directed mutagenesis. The single mutants, (−408/−403) E-box mut, (−436/−431) E-box mut, (−539/−530) PDX1 (pancreatic duodenal homeobox-1) mut, (−902/−893) PDX1 mut, (−803/−795) Foxa2 mut and (−903/−895) Foxa2 mut, were performed with an XL site-directed mutagenesis kit (Stratagene) using the pGL-P2 plasmid as the template. The mutagenic oligonucleotide primers used to generate the above constructs are shown in Table 1. The double mutants, (−408/−403)/(−436/−431) E-box, (−902/−893)/(−539/−530) PDX1 and (−903/−895)/(−803/−795) Foxa2, were generated using single mutants, (−408/−403) E-box mut, (−539/−530) PDX1 mut and (−803/−795) Foxa2 mut, as the templates respectively. The (−48/−41) GC-box mutant was generated using the same primers described previously [9] but using pGL-P2 [6] as the template. The mutagenic reactions were performed following the manufacturer's instructions and the correct mutagenic sequences were verified by automated sequencing using BigDye (ABI). All mutant constructs were prepared using a Qiagen Miniprep kit (Qiagen) and used for the transient transfection experiments.

Cell culture and transient transfection

The insulinoma cell line INS-1 was routinely cultured to 70–80 % confluence in RPMI 1640 medium (Gibco) supplemented with 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 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. Cells were trypsinized and seeded at a density of 2×10^6 cells/well in 6-well plates and cultured in antibiotic-free medium for 24 h before transfection. The transfection was carried out on the next day using Lipofectamine™ 2000 reagent (Invitrogen). Briefly, 1 pmol of the luciferase reporter construct and 2 μg of pRSV-βgal plasmid encoding *Escherichia coli* β-galactosidase were complexed with 5 μg of Lipofectamine™ reagent in 500 μl of Optimem I reduced serum-free medium. After 5 h of incubation, 2 ml of complete medium was added to the transfected cells and further incubated at 37 °C for 24 h. For transfection of the NIH 3T3 cell line, cells were routinely maintained in DMEM (Dulbecco's modified Eagle's medium; Gibco), supplemented with 10 % heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin, at 37 °C with 5 % CO_2. Cells (2×10^5) were plated on a 24-well plate in antibiotic-free DMEM. Transfections were carried out as described for the INS-1 cells, except that 0.25 μg of reporter plasmid and pRSV β-gal plasmid and 2 μg of transfection reagent were used per well. The transfected cells were maintained in the complete medium for 48 h before being harvested.

For the transactivation assay of the distal promoter-linked luciferase construct with Foxa2, 5 μg of both plasmids was co-transfected into HEK-293 cells (human embryonic kidney cells) as described previously [4]. The plasmid overexpressing Foxa2 was generated as follows. Briefly, the cDNA encoding Foxa2 was isolated from 5 μg of total RNA of INS-1 cells using the one-step RT (reverse transcriptase)–PCR system (Invitrogen) with Foxa2-forward (5′-GAAGCTTATGCTGGGAGCCGTGAAGATG-3′) and Foxa2-reverse (5′-CCTCGAGCTTAGGACGAGTTCATAATGGGCC-3′) primers designed from the rat Foxa2 cDNA sequence [10]. The cDNA was cloned into the pcDNA3 expression vector (Invitrogen) and sequenced.

Luciferase and β-galactosidase assays

The transfected cells were scraped from the culture dishes and centrifuged at 160 ***g*** for 5 min. The cell pellet was resuspended in 100 μl of 1× reporter lysis buffer (Promega) and freeze-thawed for three cycles before the supernatant was collected by centrifugation at 14 000 ***g*** for 5 min at 4 °C. Total protein (20 μg) was assayed using the luciferase assay system (Promega) in a Berthold luminometer. β-Galactosidase was assayed using *o*-nitrophenyl β-D-galactopyranoside as the substrate as previously described [9]. The luciferase activity was normalized with the β-galactosidase activity, and expressed as 'relative luciferase' activity. Results from at least two independent experiments using different preparations of plasmids, each with duplicate wells, were averaged and presented as means ± S.D.

EMSA (electrophoretic mobility-shift assay)

Nuclear extracts of INS-1 cells were prepared as described previously [9]. Oligonucleotide sequences used to prepare probes in the present study are shown in Table 1. Briefly, probes were obtained by annealing two complementary oligonucleotides with 3–6 nt overhanging at their 5′-ends, followed by end-filling reaction with 50 μCi of [α-^{32}P]dATP (3000 Ci/mmol; GE-Health Sciences) using Klenow enzyme (New England Biolabs). Double-stranded probes were purified by phenol/chloroform extraction followed by ethanol precipitation. The DNA–protein binding reactions were performed at 4 °C for 20 min in a final volume of 20 μl consisting of 1× binding buffer [20 mM Hepes/KOH, pH 7.9, 25 % (v/v) glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol and 0.2 mM PMSF], 10 μg of nuclear extract, 2 μg of poly(dI-dC) · (dI-dC)

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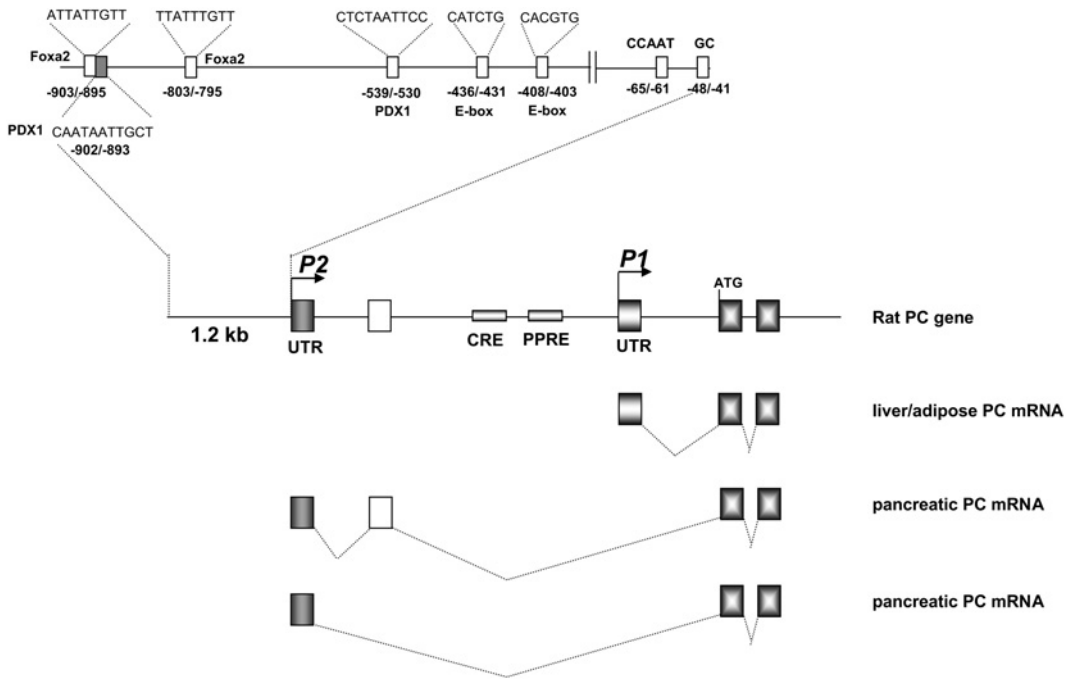

Figure 1 Structural organization of the 5′-end of the rat PC gene and the generation of various mRNA isoforms by alternative splicing

The rat PC gene possesses two alternative promoters, i.e. the proximal (P1) and the distal (P2) promoters. The P1 promoter produces a PC mRNA that is exclusively found in liver and adipose tissues, while the P2 promoter generates PC mRNAs that are abundant in pancreatic β-cells. These PC mRNA isoforms contain identical coding regions but different 5′-UTRs [7]. The P1 promoter activity is regulated by CRE [11] and PPRE (PPAR-responsive element) [4]. Several *cis*-acting elements including −48/−41 GC-box and −65/−61 CCAAT-box are shown. Also shown are two E-boxes located at −408/−403 and −436/−431, two PDX1-binding sites at −539/−530 and −902/−893, and two Foxa2 sites at −803/−795 and −903/−885 located upstream of the GC- and CCAAT-boxes identified by TRANSFAC and COMPELS databases [12,13].

(Sigma) and 100000 c.p.m. of ^{32}P-labelled probe. The DNA–protein complexes were separated by 5% native PAGE in 0.5× TBE (1 × TBE = 45 mM Tris/borate and 1 mM EDTA) at 250 V at 4 °C for 2.5 h. For the competition assay, a 10, 50 or 100 times excess of unlabelled double-stranded probes were included in the binding reactions. For the supershift assay, 5 μg of the specific antibodies, Foxa2/HNF3β (sc-6554), USF1 (sc-8983) or USF2 (sc-861) (Santa Cruz Biotechnology), was pre-incubated with nuclear extract for 30 min prior to the binding reactions.

ChIP (chromatin immunoprecipitation) assay

The ChIP assay was performed using a ChIP assay kit (Upstate Biotechnology). Briefly, 2 × 10^6 INS-1 cells grown in a six-well plate were cross-linked with 0.5% (v/v) formaldehyde at 37 °C for 5 min. The cells were sonicated for 3 × 20 s before centrifugation at 16000 ***g*** for 15 min at 4 °C. The specific DNA-bound transcription factor complexes were precipitated with either 20 μl of anti-USF1, 20 μl of anti-USF2 or 20 μl of anti-HNF3β/Foxa2 at 4 °C overnight before the addition of Protein A–agarose beads. The proteins were removed from DNA by digestion with 10 μg/ml proteinase K at 65 °C for 30 min. The DNA was recovered from the solution by Qiaquick PCR purification kit (Qiagen), and eluted in 50 μl of sterile water. Eluted DNA (20 μl) was subjected to PCR with −803 F/R (5′-CTCCAGCTTCAGTCTTCCAAGT-3′ and 5′-TCTTTCTAGAGGGTTCGGTTCA-3′) or −408 F/R (5′-GCGACCTCTTCTGTATCTGCTAA-3′ and 5′-AGACCTTC-TGATTGGTGAAGAGG-3′) primers, which flank the binding sites of HNF3β/Foxa2 or USF respectively. The eluted DNA was also amplified using Ex2 F/R primers (5′-GCCCATCAAGA-AAGTAATGGTA-3′ and 5′-CTTGGCCACCTTAATGATGTCT-3′) that are located within exon 2 of the rat PC gene [7]. This exon is > 10 kb downstream of the distal promoter of the PC gene.

RESULTS

Cis-acting elements at −803/−795 and −408/−403 confer positive regulation of the distal promoter of the rat PC gene in INS-1 cells

Previously we have identified a GC-box located at −48/−41 as the binding site for Sp1 and Sp3 transcription together with a CCAAT-box located at −65/−61 as the binding site for NF-Y, which maintain the basal transcription activity within the 187 nt upstream of the transcription start site of the rat PC distal promoter in INS-1 cells [9]. Figure 1 shows the organization of the proximal (P1) and the distal (P2) promoters of the rat PC gene that mediate the production of three major transcripts bearing different 5′-UTRs [6]. Computer-assisted analysis of the putative transcription factor-binding site of the longest 1.2 kb rat PC distal promoter fragment [6] with the TESS (transcription element search system), and TFSEARCH with TRANSFAC and COMPELS databases [12,13], identified two copies of a putative PDX1-binding site [14], located at −539/−530 and −902/−893, and two copies of a putative winged-helix transcription factor Foxa2 (formerly HNF3β) located at −803/−795 and −903/−895 with the latter overlapping with the −902/−893 PDX1 site. Furthermore, two copies of an E-box, a potential binding site for basic helix–loop–helix leucine zipper proteins [15], were found at −408/−403 and −436/−431.

To identify the functional importance of these transcription factor-binding sites, we generated single or multiple mutations of these sites within this 1.2 kb P2 promoter-linked luciferase

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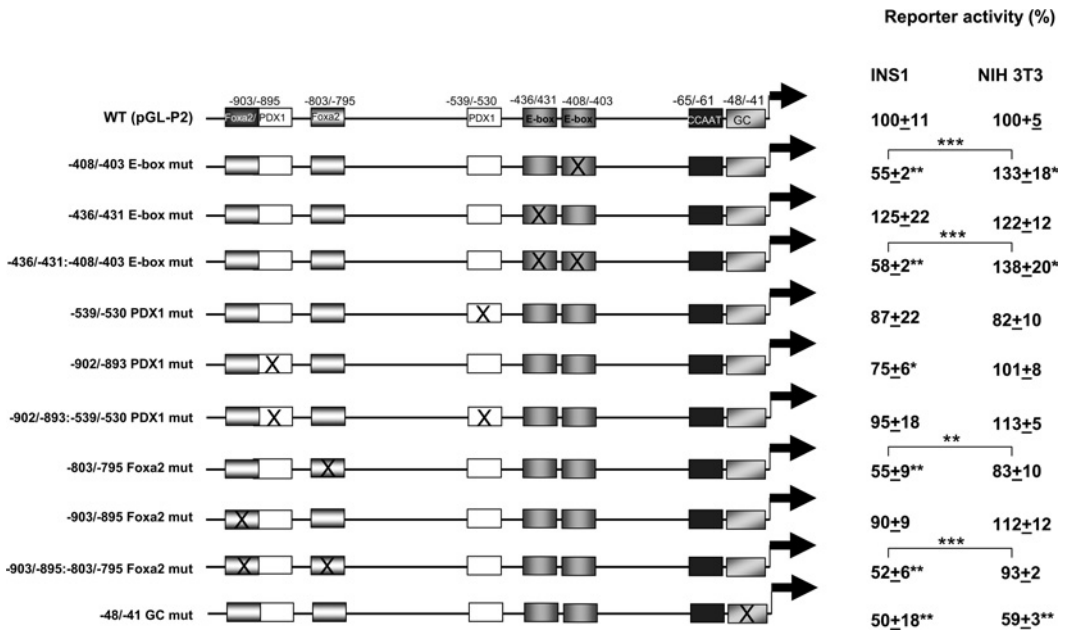

Figure 2 Site-directed mutagenesis of the putative −408/−403 and −436/−431 E-boxes, −539/−530 and −902/−893 PDX1 sites and −803/−795 and −903/−895 Foxa2 sites on P2 promoter expression in INS-1 cells

These mutant promoter constructs harbouring different point mutations introduced at various putative transcription factor-binding sites (X) were generated and transiently transfected into INS-1 and NIH 3T3 cells. The relative luciferase activities of each construct are shown as means ± standard deviations for three independent experiments, each in duplicate. The relative luciferase activity of each construct was compared with the 1.2 kb distal promoter wild-type construct (WT) which was arbitrarily set as 100 %. $*P \leqslant 0.05$, $**P \leqslant 0.01$, $***P \leqslant 0.005$. Arrow indicates the luciferase reporter gene.

reporter gene (Figure 2) and examined these in transient transfection assays in INS-1 cells. Mutations of the −408/−403 E-box but not the −436/−431 E-box reduced the reporter activity to 55 %. Mutation of both of these two E-boxes together did not reduce the reporter activity further than that of the −408/−403 E-box single mutation, suggesting that the −436/−431 E-box is not functional. Single mutation or double mutation of the PDX1 site located at −902/−895 and −539/−530 did not markedly affect the luciferase reporter activity. Mutation of the −803/−795 Foxa2 decreased the reporter activity to 55 %, whereas mutation of the −903/−895 Foxa2 site reduced the reporter activity by only 10 %. Double mutation of the two Foxa2 sites abrogated the reporter activity to a similar level to that of the −803/−795 single mutation, suggesting that the −903/−895 Foxa2 is not functionally active in INS-1 cells. However, neither of the above mutations markedly affects the reporter activities in the NIH 3T3 cell line, suggesting that the −803/−795 Foxa2 site and the −408/−403 E-box are important determinants mediating transcription of the 1.2 kb distal promoter of the rat PC gene in INS-1 cells. However, mutation of the −48/−41 GC-box, a binding site for Sp1/Sp3 [8], reduced the reporter activity by 40–50 % in both INS-1 and NIH 3T3 cells, indicating that this sequence does not work in a cell-specific manner.

Foxa2 and USFs bind −803/−795 and −408/−403 E-box respectively

To identify whether Foxa2 does indeed bind to its cognate sequence at the −803/−795 site, an EMSA was performed. As shown in Figure 3, a double-stranded oligonucleotide spanning −817/−776 [(−803 /−795) Foxa2 site], when incubated with nuclear extract from INS-1 cells, formed a single DNA–protein complex ('C'). The binding specificity of the probe to the nuclear protein was determined by a competition assay using various concentrations of unlabelled double-stranded wild-type sequence, a well-characterized Foxa2 consensus sequence from the transthyretin gene [16] or a mutated (−803/−795) sequence (Figure 3A). As shown in Figure 3(B), the DNA–protein complex ('C') was outcompeted by 10, 50 and 100 times excess wild-type sequence. Similarly, the DNA–protein complex can also be effectively outcompeted by 10 times excess of the unlabelled consensus (Cons) Foxa2 sequence. By contrast, a 100-fold concentration of an oligonucleotide, designated Mut Foxa2, that contains a mutation identical with that described in the −803/−795 Foxa2 mutant construct, failed to compete with the labelled probe for formation of the complex C.

To confirm whether the protein binding to −803/−795 is indeed a Foxa2, a supershift assay was performed using an anti-Foxa2 polyclonal antibody. As shown in Figure 3(C), incubation of INS-1 nuclear extract with this antibody resulted in the disruption of complex 'C', concomitant with the formation of an apparent supershifted band (SS). Furthermore, when the −803/−795 Foxa2 probe was incubated with the nuclear extract from HEK-293 cells transfected with the plasmid encoding rat Foxa2 cDNA, a similar DNA–protein complex was obtained. Incubation of this nuclear extract with anti-Foxa2 antibody also produced supershifted band, similar to that observed in the INS-1 cells. In contrast, a double-stranded oligonucleotide corresponding to the −903/−895 Foxa2 site probe, when incubated with INS-1 cells or HEK-293 overexpressing Foxa2, failed to produce any prominent DNA–protein complex (Figure 3C). These results suggest that the C complex represents Foxa2 binding to the −803/−795 Foxa2.

To determine the identity of the nuclear protein binding to the −408/−403 E-box, we performed similar experiments to those described for the Foxa2 site. A double-stranded oligonucleotide corresponding to −419/−381 (−408/−403 E-box) (Figure 4A), when incubated with the INS-1 nuclear extract, produced two major DNA–protein complexes, designated CI and CII

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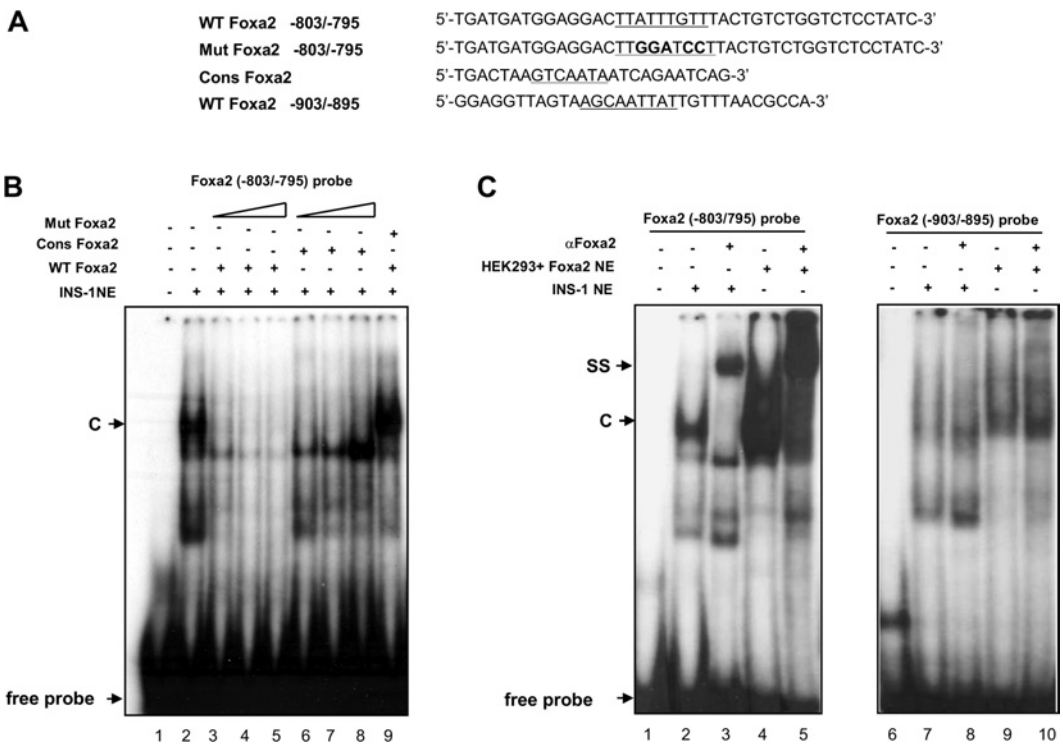

Figure 3 EMSA of the putative −803/−795 and −903/−895 Foxa2 sites of the rat PC distal promoter

(**A**) Nucleotide sequences of −817/−776 (−803/−795) and −907/−876 Foxa2 site (−903/−895) of the distal promoter compared with −803/−795 Foxa2 mutant sequence (Mut) and consensus Foxa2 sequence [16]. (**B**) The ^{32}P-labelled double-stranded oligonucleotide probe corresponding to the −817/−776 region of the distal promoter (WT) was incubated with nuclear extract of INS-1 cells in the absence or presence of unlabelled competitors. Lane 1, probe alone; lane 2, probe with INS-1 nuclear extract. The WT Foxa2 −803/−795, consensus Foxa2 (Cons Foxa2) and mutant Foxa2 (Mut Foxa2) double-stranded oligonucleotides were included as the competitor with nuclear extract in the assay (lanes 3–9 respectively). The triangles refer to the use of increasing amounts of the unlabelled competitor (WT sequence or consensus Foxa2 sequence) (10:1, 50:1 and 100:1 excess respectively) and 100:1 excess for the Foxa2 mutant. (**C**) Supershift assays of the −803/−795 and −903/−895 Foxa2 sites. These two probes were radiolabelled and incubated with nuclear extracts of INS-1 or HEK-293 cells overexpressing Foxa2, in the absence or presence of Foxa2 antibody. Lanes 1 and 6, probes alone. Probes were incubated with nuclear extracts from INS-1 (lanes 2 and 7), or HEK-293 cells overexpressing Foxa2 (lanes 4 and 9). Anti-Foxa2 antibody was pre-incubated in the nuclear extracts of INS-1 (lanes 3 and 8) and HEK-293 cells overexpressing Foxa2 (lanes 5 and 10) before probes were added. 'C' indicates specific DNA–nuclear protein complex, while 'SS' represents the supershifted complex; NE, nuclear extract.

(Figure 4B). To determine the specificity of the binding, a competition assay was conducted using a 10, 50 and 100 times excess amount of unlabelled double-stranded wild-type −408/−403 E-box-containing sequence and outcompeted by a 10 times excess. In contrast, a 100 times excess amount of an oligonucleotide, designated −408/−403 mut E-box, which contains a mutation identical with that described in the −408/−403 E-box sequence, failed to compete with the wild-type labelled probe for CI and CII formation (Figure 4B). This indicates that this complex represents a specific DNA–protein interaction and that its formation correlates with the basal gene expression conferred by this E-box as illustrated in Figure 2.

Since PC mRNA, both in cultured pancreatic islets and INS-1 cells, was induced by exogenous glucose [8,17] we asked whether the recently discovered ChREBP [ChRE (carbohydrate responsive element)-binding protein] [18], which binds to the E-box of several glucose-responsive genes, can also bind to the −408/−403 E-box of the rat PC gene. We performed a competition assay using an unlabelled double-stranded ChRE sequence of the L-type pyruvate kinase promoter. Incubation of 10, 50 or 100 times excess amount of this ChRE sequence only partially outcompeted the CI and CII complexes at 100 times excess (Figure 4B, lanes 6–8). Indeed when 10, 50 and 100 times excess of unlabelled double-stranded oligonucleotides bearing a consensus E-box-binding site for USFs were included as the competitor, both CI and CII complexes were completely outcompeted by 10 times excess (Figure 4C, lanes 3–5). Furthermore, this USF consensus sequence when used as a probe can form two DNA–protein complexes which migrated with the same mobility as that seen with the −408/−403 E-box probe (Figure 4C, lane 7). These results suggest that USFs rather than ChREBP bind the −408/−403 E-box of the distal promoter of the rat PC gene.

To confirm the binding of USF proteins to this −408/−403 E-box, we performed supershift assays using antibodies to both USF1 and USF2 isoforms. As shown in Figure 5, pre-incubation of INS-1 nuclear extract with an anti-USF1 antibody resulted in the disruption of the CI complex and moderately reduced the CII complex, concomitant with the formation of a supershift SSI band. In comparison, incubation of INS-1 nuclear extract with an anti-USF2 antibody and the −408/403 E-box resulted in a marked reduction of CI and CII complexes, concomitant with the formation of a supershift SS2 complex. Incubation with both anti-USF1 and -USF2 antibodies resulted in a marked reduction of both complexes. These results suggest that both USF1 and USF2 isoforms interact with the −408/−403 E-box, forming both CI and CII complexes. In contrast, incubation of a double-stranded oligonucleotide probe corresponding to −466/−428 (−436/−431 E-box) did not produce a DNA–protein complex identical with that obtained with the −419/−381 (−408/−403 E-box) probe. Furthermore, pre-incubation of the nuclear extract with either anti-USF1 or -USF2 antibody did not produce a supershifted band (results not shown).

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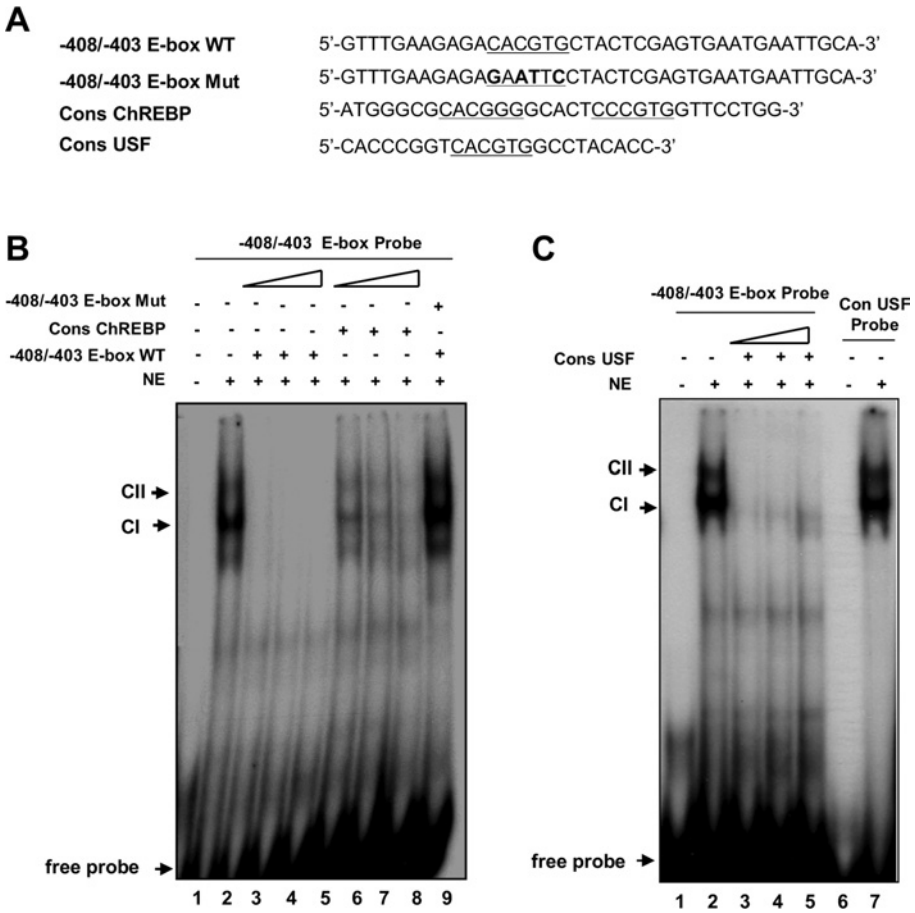

Figure 4 EMSA of putative −408/−403 E-box of rat PC distal promoter

(**A**) Nucleotide sequences of −419 to −381 (−408/−403 E-box) wild-type (WT) of the distal promoter compared with mutant −408/−403 E-box mutant (Mut) element, consensus sequence of ChREBP-binding site (Cons ChREBP) of liver pyruvate kinase (L-PK) promoter and the consensus USF binding site (Cons USF). The putative sequences are underlined and the mutated bases are indicated as boldface, lower-case letters. (**B**) Radiolabelled double-stranded oligonucleotides containing the −419/−381 (−408/−403 E-box WT) region of the distal promoter were incubated with nuclear extracts of INS-1 cells in the absence or presence of unlabelled competitors. Lane 1, probe alone; lane 2, probe with nuclear extract. The −408/−403 E-box WT, Cons ChREBP or −408/−403 E-box unlabelled double-stranded oligonucleotides were included as competitors with nuclear extract in the assay (lanes 3–9 respectively). The triangles refer to the use of increasing amounts of the unlabelled competitor (WT sequence or Cons ChREB) (10:1, 50:1 and 100:1 excess respectively) and 100:1 excess for the −408/−403 E-box mutant sequence. (**C**) Radiolabelled −419/−381 (−408/−403 E-box) probe was incubated with the nuclear extract in the absence (lane 2) or presence of unlabelled consensus USF competitor (lanes 3–5 respectively). The triangles refer to the use of increasing amounts of unlabelled consensus USF sequence (10:1, 50:1 and 100:1 excess respectively). Lanes 6 and 7 were EMSA of consensus USF (Cons USF) probe, respectively with or without nuclear extract. 'CI' and 'CII' indicate specific DNA–nuclear protein complexes; NE, nuclear extract.

Foxa2 and USFs bind their cognate sequences *in situ*

To complement the results of the *in vitro* gel-retardation assay, we investigated whether Foxa2, and USFs 1 and 2 bind their cognate sequences at −803/−795 Foxa2 and −408/−403 E-box of the distal promoter of the rat PC gene (Figure 6A) within intact cells, using a ChIP assay [19]. The transcription factor-bound chromatin was cross-linked, sheared and immunoprecipitated with either anti-Foxa2, anti-USF1 or anti-USF2 antibodies. The immunoprecipitated DNA fragments were PCR-amplified with the −408 F/R primers which are located between nt −503 and −210 containing the −408/−403 E-box, or with the −803 F/R primers which amplified nucleotides between −993 and −705 containing the −803/−795 Foxa2 site. With the −803 F/R primers, a 289 bp amplicon was detected from the 'input DNA' and from DNA fractions that were immunoprecipitated with the anti-Foxa2 antibody but not from other fractions (Figure 6B). By using −408 F/R primers, a 293 bp amplicon was detected from the 'input DNA' and from DNA fractions that were immunoprecipitated with either the anti-USF1 or -USF2 antibodies but not from the other fractions (Figure 6B). To test the specificity of the antibody–protein interactions, these immunoprecipitates were also analysed for the presence of exon 2 of the rat PC gene [7] using Ex2 F/R primers that recognize the PC coding sequence between +181 and +387. More than 10 kb of genomic DNA separate exon 2 and the distal promoter of the rat PC gene, and neither Foxa2 nor USF would be expected to associate with exon 2. As expected, only the 217 bp amplicon corresponding to +171 and +387 of PC exon 2 was detected from the input DNA and not from other immunoprecipitated DNA fractions (Figure 6B). These results indicate that endogenous Foxa2 and USF1 and USF2 of INS-1 cells bind the −803/−795 Foxa2 site and −408/−403 E-box respectively of the distal promoter of the rat PC gene *in vivo*.

DISCUSSION

The present study provides insight into the mechanism directing PC gene transcription in pancreatic islet β-cells. We have demonstrated that the distal promoter of the rat PC gene is regulated by Foxa2 and USFs. Foxa2 encodes a transcription factor that has

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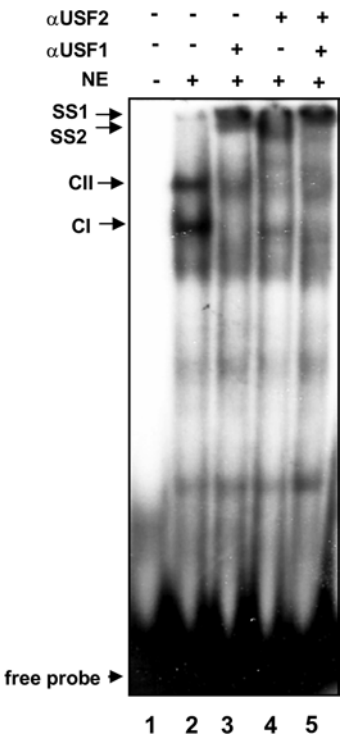

Figure 5 Supershift assays of the putative −408/−403 E-box

Radiolabelled WT −408/−403 E-box probe was incubated with the INS-1 nuclear extracts (lanes 2–5), in the absence or presence of anti-USF1 (αUSF1) and/or anti-USF2 (αUSF2) antibody. 'CI' and 'CII' represent the DNA–nuclear protein complexes, while 'SS' indicates the supershifted band. NE, nuclear extract.

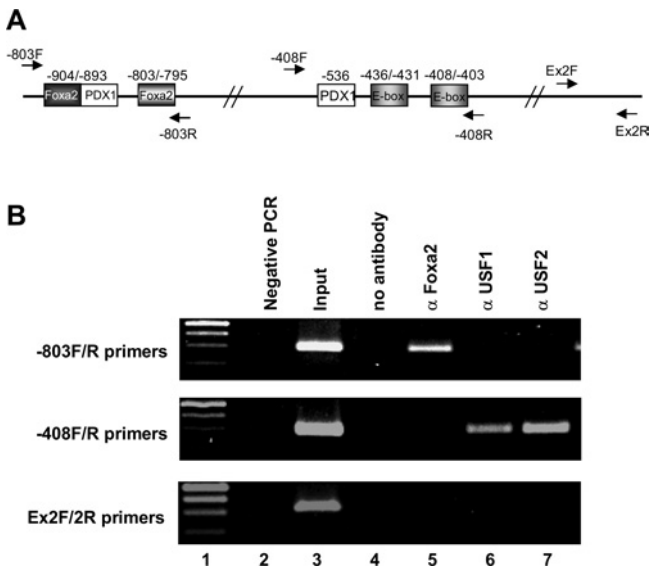

Figure 6 ChIP assay of the −803/−795 Foxa2 and the −408/−403 USF-bound sites of the distal promoter of the rat PC gene

Soluble chromatin was prepared from INS-1 cells. The Foxa2-, USF1- or USF2-associated DNA fragments were immunoprecipitated (IP) with either anti-Foxa2, anti-USF1 or anti-USF2 antibody respectively. (**A**) The location of the primers (arrows) relative to the −803/−795 Foxa2 site or −408/−403 E-box are shown. (**B**) The Foxa2-associated −803/−795 site was PCR-amplified with −803F/R primers, the USF1- and USF2-associated −408/−403 E-box was PCR-amplified with −408F/R primers, and a PCR was also performed with negative control primers, Ex2F/2R primers, which bind and amplify the +171 to +387 region of exon 2 of the rat PC gene [6]. Lane 1, DNA marker; lane 2, negative control of PCR reaction; lane 3, PCR of input DNA (1:250 dilution); lane 4, PCR of IP without antibody; lane 5, PCR of Foxa2-bound −803/−795 site (1:25 dilution); lane 6, PCR of USF1-bound −408/−403 E-box (1:25 dilution); lane 7, PCR of USF2-bound −408/−403 E-box (1:25 dilution).

been postulated to play a crucial role in β-cell development due to its ability to bind and regulate a β-cell-specific transcription factor, PDX1. Although Foxa2 is expressed in some endodermal-derived tissues, its expression is most abundant in islets and several pancreatic β-cell lines [20]. The importance of Foxa2 in maintaining islet development and function has previously been demonstrated in the pancreatic β-cell-specific Foxa2 knockout mice [21,22]. Mice with Foxa2 disrupted in β-cells showed severely hypoglycaemic and dysregulated insulin secretion in response to both glucose and amino acids [21]. Furthermore, Foxa2 also regulates several β-cell-specific expressed genes, e.g. mafA [23], the Sur1 (sulfonylurea receptor 1), the Kir6.2 (inward rectifier potassium channel membrane 6.2) [21,24], and islet GLUT2 [25]. Previously, Foxa2 has been shown to be a signalling molecule, linking insulin-sensitivity and glucose and lipid homoeostasis [26]. Modulation of Foxa2 activity could be a potential target for the treatment of Type 2 diabetes [27].

We have shown by mutagenesis, *in vitro* DNA binding and ChIP assays that Foxa2 binds to the −803/−795 region of the distal promoter of the rat PC gene, and that it regulates gene transcription. With the luciferase reporter gene-based assay, mutation of this −803/−795 site reduced transcriptional activity by approx. 50 %, indicating the positive role of Foxa2 in the regulation of PC in INS-1 cells. In contrast, the −903/−895 site did not bind Foxa2, which was consistent with the mutational analysis showing that disruption of this site had no impact on the luciferase reporter gene activity. This may perhaps be due to the −903/−895 putative Foxa2 (5′-AATTATTGTTTA-3′) containing two core nucleotides that deviate from the consensus Foxa2 site (5′-VAWTRTTKRYTY-3′) [28] and thus affect protein binding. In contrast, the −803/−795 Foxa2 (5′-ACTTATTTGTTT-3′) site contains only two flanking nucleotides that deviate from the consensus sequence, and hence its binding is still preserved.

PDX1 is clearly a central regulator of pancreas development and subsequent β-cell function. Targeted disruption of the *Pdx1* gene in mice caused pancreas agenesis [29], while islet-specific knockout of *Pdx1* gene caused an impaired glucose tolerance [30]. Gauthier et al. [31] have expressed a dominant-negative PDX1 mutant in rat islets followed by microarray analysis to identify PDX1 target genes. Specifically, PC and other mitochondrial proteins that are essential for the generation of ATP and other coupling factors in the β-cells were affected by this PDX1 mutant overexpression. From our mutagenesis and transient transfection analyses in INS-1 cells, PDX1 apparently did not regulate PC expression through the 1.2 kb distal promoter of the rat PC gene. Furthermore, gel retardation assays performed with either of the putative PDX1 sites (located at −902/−893 and −539/−530) did not produce any evidence of DNA–protein complexes, and overexpression of PDX1 in INS-1 cells did not transactivate the distal promoter of the rat PC gene (results not shown). Alternatively, PDX1 may regulate PC through its cognate sequence located upstream of the existing 1.2 kb distal promoter fragment or in the intron of the gene.

In addition to the Foxa2 tissue-specific transcription factor, we also found that the ubiquitously expressed transcription factor, USF [32], plays a role in regulation of the distal promoter of the rat PC gene. The USF is composed of two isoforms, the 43 kDa USF1 and the 44 kDa USF2. These two isoforms exist as a homodimer or heterodimer [33]. The relative role of USF1 and USF2 in the context of the regulation of the distal promoter of the rat PC gene is unclear. It appears that both USF1 and USF2 bind to the

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−408/−403 E-box since antibodies against both isoforms can prevent the formation of two distinct DNA–protein complexes. Although the −436/−431 E-box resembles the consensus E-box (CANNTG), it was not recognized by USF1 or USF2. The only differences between the −436/−431 and the −408/−403 E-boxes are the two central nucleotides (CATCTG and CACGTG respectively), but this difference could affect USF binding. The two central palindrome nucleotides have been shown to have higher affinity than the non-palindrome nucleotides. It is interesting to note that mutation of the −48/−41 GC-box, a binding site of the Sp transcription factor family, also reduced the reporter activity by 50 %, which is similar to that of the −803/−795 Foxa2 site or the −408/−403 E-box mutation alone. This suggests a positive role for both the general transcription factors, Sp1/Sp3, USF1 and USF2, and the tissue-specific transcription factor, Foxa2, in the transcriptional regulation of the rat PC gene.

Our finding that the distal promoter of the rat PC gene is regulated by USF, which is a non-tissue-specific transcription factor, is not entirely unexpected because several lines of evidence indicate that this family of general transcription factors can control several glucose-regulated genes in pancreas, including insulin [34], PDX1 [35], glucokinase [36], and the islet-specific glucose-6-phosphatase catalytic subunit-related gene [37]. Although another recently discovered transcription factor, ChREBP, can regulate transcription through the classical E-box of energy-related genes such as the L-type pyruvate kinase [18,38], acetyl-CoA carboxylase and fatty acid synthase genes [39], this factor does not appear to bind the −408/−403 E-box, suggesting PC may not be regulated by ChREBP.

Although USF is a ubiquitous transcription factor, it can act in concert with tissue-specific transcription factors to regulate expression of particular genes [40]. For example, USF and the islet-specific transcription factor, NeuroD (neurogenic differentiation)/BETA2 (β-cell E box transactivator 2), can function together to direct transcription of the islet-specific glucose-6-phosphatase catalytic subunit-related protein [37]. Identification of USF and Foxa2 as the regulators of the distal promoter of the rat PC gene is a novel finding of the present study. To our knowledge, this is the first gene that is regulated by these two transcription factors in pancreatic β-cells.

The finding that the distal promoter is regulated by Foxa2 and USFs provides an explanation of the alternative usage of the proximal and the distal promoters of the rat PC gene in different tissues. In liver and adipose tissues where the proximal PC promoter is active, its transcription is solely regulated by PPARγ (peroxisome-proliferator-activated receptor γ) [4] and CREB [CRE (cAMP-response-element)-binding protein] [11]. In contrast, in pancreatic β-cells where the distal PC promoter is active, its transcription is regulated by the interplay of both general transcription factors, i.e. Sp1/Sp3, NF-Y [4] and USFs, and a tissue-specific transcription factor, Foxa2.

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