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Involvement of specific proteins (Sp1/Sp3) and nuclear factor Y in basal transcription of the distal promoter of the rat pyruvate carboxylase gene in β-cells☆

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Abstract

Pyruvate carboxylase plays diverse roles in different biosynthetic pathways, including glucose-induced insulin secretion in pancreatic β-cells. We have localized the control region of the P2 promoter by generating a series of 5′-nested deletion constructs, and both 25- and 9-bp internal deletion constructs, as well as by performing site-directed mutagenesis. Transient transfections of these constructs into INS-1 cells identified a CCAAT box and a GC box that are located at −65/−61 and −48/−41, respectively, as the important determinants. Disruption of the GC box resulted in a 4-fold reduction of the reporter activity, while disruption of the proximal CCAAT box (−65/−61) but not the distal CCAAT box (−95/−91) increased the reporter activity by 3-fold. Simultaneous disruptions of both the GC box and the CCAAT box reduced the reporter activity to a level that was close to that of the single GC box mutation. Electrophoretic mobility shift assays (EMSAs) and supershift EMSAs using nuclear extract from INS-1 cells demonstrated that Sp1 and Sp3 bind a GC box while the nuclear factor Y was shown to bind the proximal but not the distal CCAAT box.

Keywords: Pyruvate carboxylase; Biotin enzyme; Glucose-induced insulin release; Gene transcription; Sp1; Sp3; NF-Y

The pancreatic β-cell is the major fuel sensor in our body in that it quickly responds to an altered level of plasma glucose. After meals, an elevated level of absorbed glucose triggers the transportation of glucose via the glucose transporter (GLUT2) into β-cells. This allows glucose to enter glycolysis to produce pyruvate. Pyruvate then enters the mitochondria and is metabolized via two routes, i.e., oxidative decarboxylation by pyruvate dehydrogenase and carboxylation by pyruvate carboxylase (PC) [1,2]. This is strongly indicated by the substantial abundance of PC and pyruvate dehydrogenase mRNAs in pancreatic islets [1]. Short-term exposure to elevated concentrations of glucose also increases the transcription rates of both genes [3]. Inhibition of PC activity with phenylacetic acid results in the reduction of glucose-induced insulin release by both INS-1 cells and pancreatic islets, and further suggests the important role of PC in insulin secretion [4]. A recent study has also shown that the increased pyruvate flux via PC was essential for glucose-stimulated insulin secretion in β-cells [5]. Taken together, it has been proposed that the oxidation of pyruvate by pyruvate

☆ *Abbreviations:* PC, pyruvate carboxylase; Sp, specific protein; NF-Y, nuclear factor Y; P1, proximal promoter; P2, distal promoter; EMSA, electrophoretic mobility shift assay; WT, wild type.

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dehydrogenase and its carboxylation by PC allow an increase of ATP/ADP, NADPH/NADP, and malonyl CoA as the coupling factors that indirectly trigger insulin biosynthesis and secretion [6,7].

The physiological data on PC and insulin secretion were further supported in animal studies. In the genetically obese Zucker fatty rat and the GK rat, it has been shown that PC protein and activity in the pancreatic islets are 50% lower than in normal rats [8,9]. This has been suggested to be an adaptive response of β-cells to prevent self-destruction caused by overproduction of insulin to compensate for insulin resistance [8]. A recent study has shown that the hypoglycemia induced by a partial pancreatectomy causes a progressive hypertrophy of β-cells associated with the loss of insulin secretion machinery and decreased expression of β-cell metabolic enzymes including PC [10]. These data further support the link between PC and insulin secretion in β-cells.

Mammalian PC is located in the mitochondrial matrix [11], with its activity being highest in liver, kidney, adipose tissue, and pancreatic islets [12]. Characterization of the PC gene in rat demonstrated that it possesses two tissue-specific promoters, i.e., the proximal (P1) and the distal (P2) promoter, which are responsible for alternative transcription from a single gene [13]. Transcripts generated from both promoters share the same coding sequence but differ in their 5′-untranslated regions as a result of differential splicing [13,14]. The P1 promoter functions in gluconeogenic (liver and kidney) and lipogenic tissues. Conversely, the P2 promoter is highly active in pancreatic β cells where it serves the anaplerotic role mentioned above [15]. Here we have identified the region of the P2 promoter and the transcription factors that together regulate PC promoter activity. We demonstrate that the binding sites specifically recognized by the Sp-transcription factor family and NFY nuclear factor are important for the expression of a reporter gene in transfected insulin secreting cells (INS-1).

Materials and methods

Generation of reporter constructs. A 1.1 kb fragment of the rat PC distal promoter [P2] [13] was deleted from its 5′-end by using *Sac*I,

Table 1
Sequences of the oligonucleotides used for mutagenesis and EMSA

Oligonucleotide	Sequences
Mu1F	5′-CTCGAGGTCGACAGGGGTAGGTGG-3′
Mu1R	5′-CCACCTACCCCTGTCGACCTCGAG-3′
Mu2F	5′-CGCTTAAACGCGACCTGAAAGGGG-3′
Mu2R	5′-CCCCTTTCAGGTCGCGTTTAAGCG-3′
Mu3F	5′-TGCAGGGAGGAGTAGGGTCATTCA-3′
Mu3R	5′-TGAATGACCCTACTCCTCCCTGCA-3′
Mu4F	5′-AGCGGTGAATGAGGGGATGGGCTG-3′
Mu4R	5′-CAGCCCATCCCCTCATTCACCGCT-3′
Mu5F	5′-CCAATCTTTGGAAAAGTCTTACGG-3′
Mu5R	5′-CCGTAAGACTTTTCCAAAGATTGG-3′
Mu6F	5′-CTCAACCAATGGCTGCAGCAAGTT-3′
Mu6R	5′-AACTTGCTGCAGCCATTGGTTGAG-3′
Mu7F	5′-CTCAACCAATGGCGGGCGGAGCCA-3′
Mu7R	5′-TGGCTCCGCCCGCCATTGGTTGAG-3′
Mu8F	5′-AATGGAAAGTCTAGCCAGTGCTGC-3′
Mu8R	5′-GCAGCACTGGCTAGACTTTCCATT-3′
Mu9F	5′-AGTCTTACGGGCCTCCAGCAAGTT-3′
Mu9R	5′-AACTTGCTGCAGGCCCGTAAGACT-3′
Mu10F	5′-TGGAAAGTCTTACCAGTGCTGCAG-3′
Mu10R	5′-CTGCAGCACTGGTAAGACTTTCCA-3′
WT Sp1(−58/−35) [+]	5′-AAAGTCTTACGGGCGGAGCCAGTG-3′
WT Sp1 (−58/+35) [−]	5′-TGCAGCACTGGCTCCGCCCGTAAG-3′
Sp1m (+)	5′-AAAGTCTTACTTTTGGAGCCAGTG-3′
Sp1m (−)	5′-CACTGGCTCCAAAAGTAAGACTTT-3′
Cons Sp1 (+)	5′-TATTCGATCGGGGCGGGGCGAGC-3′
Cons Sp1 (−)	5′-TGCTCGCCCCGCCCCGATCGAAT-3′
WT CCAAT (−65/−61) [+]	5′-GGGCTGTCTCAACCAATGGAAAGT-3′
WT CCAAT (−65/−61) [−]	5′-GTAAGACTTTCCATTGGTTGAGAC-3′
WT CCAAT (−96/−92) [+]	5′-AGGGTCATTCATCCAATCTTTGGA-3′
WT CCAAT (−96/−92) [−]	5′-TCCCCTCCAAAGATTGGATGAATG-3′
MuNF-Y1F	5′-GCTGTCTCAAAACGCGGAAAGTCTT-3′
MuNF-Y1R	5′-AAGACTTTCCGCGTTTTGAGACAGC-3′
MuNF-Y2F	5′-GGTCATTCATAACGCCTTTGGAGG-3′
MuNF-Y2R	5′-CCCTCCAAAGGCGTTATGAATGACC-3′

*Kpn*I, *Xho*I, *Sal*I or *Pst*I which cut at the 5′-end of the fragment. These fragments were gel-purified and cloned into the multiple cloning site of pGL-3 basic vector (Promega) to yield pGL-P2Δ *Sac*I, pGL-P2Δ *Kpn*I, pGL-P2Δ *Xho*I, pGL-P2Δ *Sal*I, and pGL-P2Δ *Pst*I containing different lengths of PC promoter fused to the luciferase reporter gene.

Site-directed mutagenesis. The following pairs of oligonucleotides were used with the QuickChange site-directed mutagenesis (Stratagene) to generate six 25-bp internal deletion mutants; Mu1F/Mu1R (see Table 1) for Mut1 mutant, Mu2F/Mu2R for Mut2, Mu3F/Mu3R for Mut3, Mu4F/Mu4R for Mut4, Mu5F/Mu5R for Mut5, and Mu6F/Mu6R for Mut6 while the following pairs of oligonucleotides were used to generate four 9-bp internal deletion mutants; Mu7F/Mu7R for Mut7, Mu8F/Mu8R for Mut8, Mu9F/Mu9R for Mut9, and Mu10F/Mu10R for Mut10, respectively. The Sp1 substitution mutant (Sp1m) was generated using Sp1(1m)F/Sp1(1m)R. These 10 mutants were generated using pGL-P2Δ *Sal*I as the template. The presence of mutations was verified by automated sequencing using BigDye (ABI) and these mutagenic constructs were prepared using a Qiagen kit and transfected into INS-1 cells.

The NF-Y mutants (NF-Y1m and NF-Y2m) were generated using MuNF-Y1F/MuNF-Y1R and MuNF-Y2F/MuNF-Y2R, respectively. A double mutant, MuNF-Y1,2m, was generated using NF-Y1m mutant as the template and MuNF-Y2F/MuNF-Y2R as mutagenic primers.

Transient transfection and reporter assays. Routinely 80% confluent INS-1 cells [16] grown in RPMI 1640 media (Sigma) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, USA), 1 mM sodium pyruvate, 10 mM Hepes, pH 7.5, 50 μM β-mercaptoethanol, and 10% (v/v) fetal bovine serum (Gibco) were trypsinized and plated on 6-well plates with a cell number of 1×10^6 cells/well in antibiotic-free media overnight. One picomole of luciferase reporter plasmid and 2 μg of β-galactosidase expression plasmid, pRSV β-Gal, were complexed with 5 μl (1 μg/μl) Lipofectamine 2000 reagent (Invitrogen) in Opti-MEMI (Gibco) and added to the cells which were incubated at 37 °C for 5 h. Antibiotic-free medium (5 ml) was then added to each well and further incubated at 37 °C for 24 h. The cells were then harvested and suspended in 50 μl of 1× cell culture lysis buffer (Promega). Luciferase and β-galactosidase activities were assayed as previously described [17]. Results from three independent experiments with three different preparations of reporter plasmids, each with triplicate wells, were normalized for β-galactosidase activity, and averaged. Data are expressed as means ± SD.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared as follows: 2×10^7 cells were suspended in 1 ml of 10 mM Hepes, pH 7.9, containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, and 0.2 mM DTT, and incubated on ice for 15 min. The swollen cells were centrifuged and resuspended in 250 μl of 20 mM Hepes, pH7.9, containing 420 mM NaCl, 1.5 mM $MgCl_2$, 10 mM KCl, 25% glycerol, 0.5 mM DTT, and 0.2 mM PMSF, and then incubated on ice for 30 min before being centrifuged at 13,000 rpm at 4 °C for 5 min. For the EMSA, probes were obtained by annealing complementary oligonucleotides and end-labelled with [α-^{32}P]dATP (3000 Ci/mmol) [Amersham–Pharmacia] using Klenow enzyme (New England Biolabs). Double-stranded DNA probes were purified by ethanol precipitation. The DNA–protein binding assay was performed at 4 °C for 20 min in a final volume of 20 μl containing 1× binding buffer (20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 10 mM KCl, and 25% glycerol), 1 μg poly(dI–dC), 10 μg nuclear extract, and 1×10^5 cpm probe. The DNA–protein complexes were separated on 4% polyacrylamide gel electrophoresis

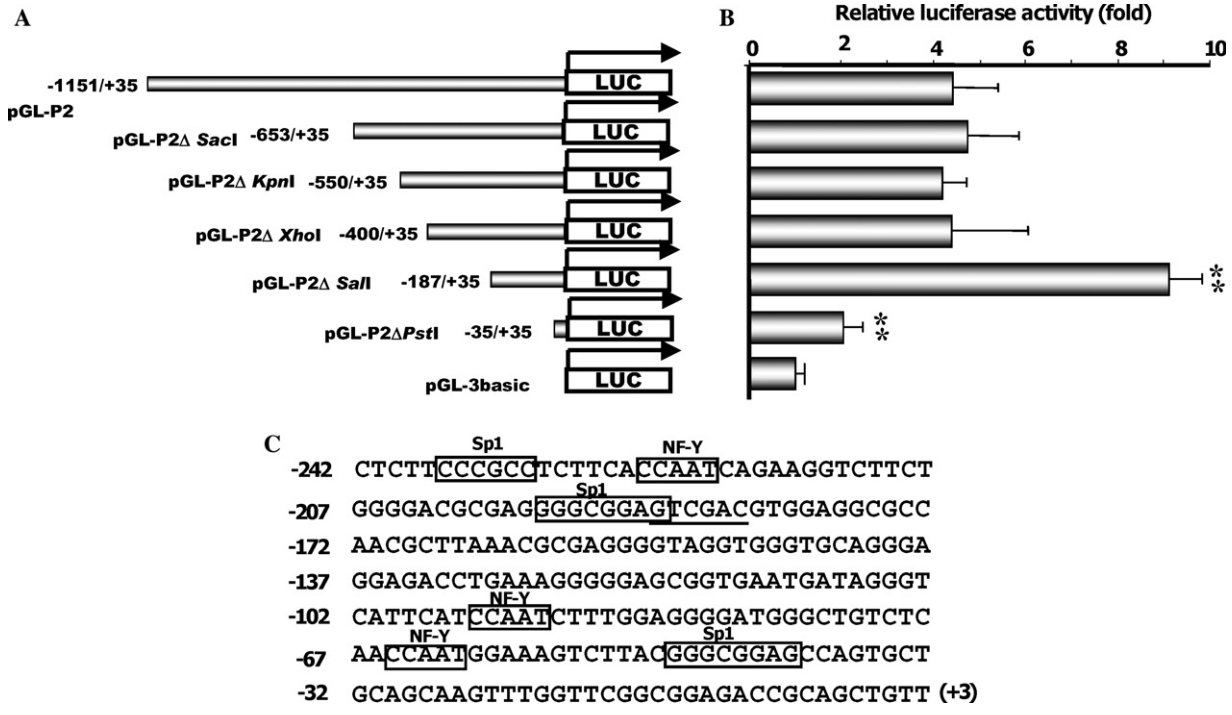

Fig. 1. Localization of minimal elements of the distal promoter in the INS-1 cell line. (A) A schematic diagram showing the positions of 5′-nested deletion mutants generated by restriction enzyme digestions linked with the luciferase reporter gene (LUC). The number represents the nucleotide number relative to the transcription start site(+1). (B) These mutants were transiently transfected into INS-cells. The relative luciferase activity of each construct was compared with the pGL3-basic vector that was arbitrarily set as 1. (C) The nucleotide sequence between +3 and −242. Boxes represent the putative transcription factor binding sites for Sp1 and NF-Y. Underline indicates *Sal*I restriction site. The luciferase activity of each construct was normalized in comparison with co-expressed β-galactosidase activity. The relative luciferase activities shown are means ± SD of three independent experiments, each in triplicate. Relative luciferase activities are also shown as fold of the activities of the pGL-3 basic vector, which was arbitrarily set as 1. $^{**}p \leqslant 0.001$ compared with pGL-P2.

in 0.5× TBE at 250 V at 4 °C for 2 h. The gel was dried and exposed to X-ray film for 48 h. For the competition assay, 5×, 10× or 25× more concentrated double-stranded DNAs were included in the binding reaction. For supershift EMSA, 5 μg of the polyclonal antibody specific for Sp1 (sc59), Sp3 (sc644) or NF-Y (sc7712) [Santa Cruz Biotech, USA] was pre-incubated with the nuclear extract for 30 min before the binding assay was performed.

Results and discussion

Core promoter of P2 resides within the first 187-bp upstream of the transcription initiation site

To determine the regions of the P2 promoter that contributed to its maximal activity, we progressively deleted the 1.1 kb P2 promoter fragment from the 5′-end using restriction enzyme sites that are naturally present in this fragment. As shown in Fig. 1A, all six constructs containing 35 bp (+35) of the 5′-end of exon 1D and different lengths of the P2 promoter region that lie immediately upstream of exon 1D were transiently transfected into INS-1 cells. Fig. 1B shows the luciferase activities of six different constructs relative to the promoterless construct, pGL-3 basic in INS-1 cells. The longest construct (pGL-P2) shows a relative luciferase activity approximately 4.5-fold higher than the promoterless construct. Progressive deletions to regions −653/+35 (pGL-P2Δ *Sac*I), −550/+35 (pGL-P2Δ *Kpn*I), and −400/+35 (pGL-P2Δ *Xho*I) slightly affect the luciferase activity whilst a further deletion to region −187/+35 (pGL-P2Δ *Sal*I) increased the luciferase activity 2-fold suggesting the presence of a negative element(s) between regions −400 and −187. However, deletion from regions −187/+35 to −35/+35 (pGL-P2Δ *Pst*I) results in a dramatic decrease in luciferase activity by 80% to only 2-fold higher than the promoterless construct, suggesting that the core promoter resides within the first 187 bp upstream of transcription initiation site. We previously used the same reporter constructs for identifying minimal promoter elements in COS-1 cells [13] and found differences in the reporter activities obtained from some constructs in these two different cell lines. These could well be due to (i) different tissue-specific controls of the P2 promoter in COS1 cell (kidney) and INS-1

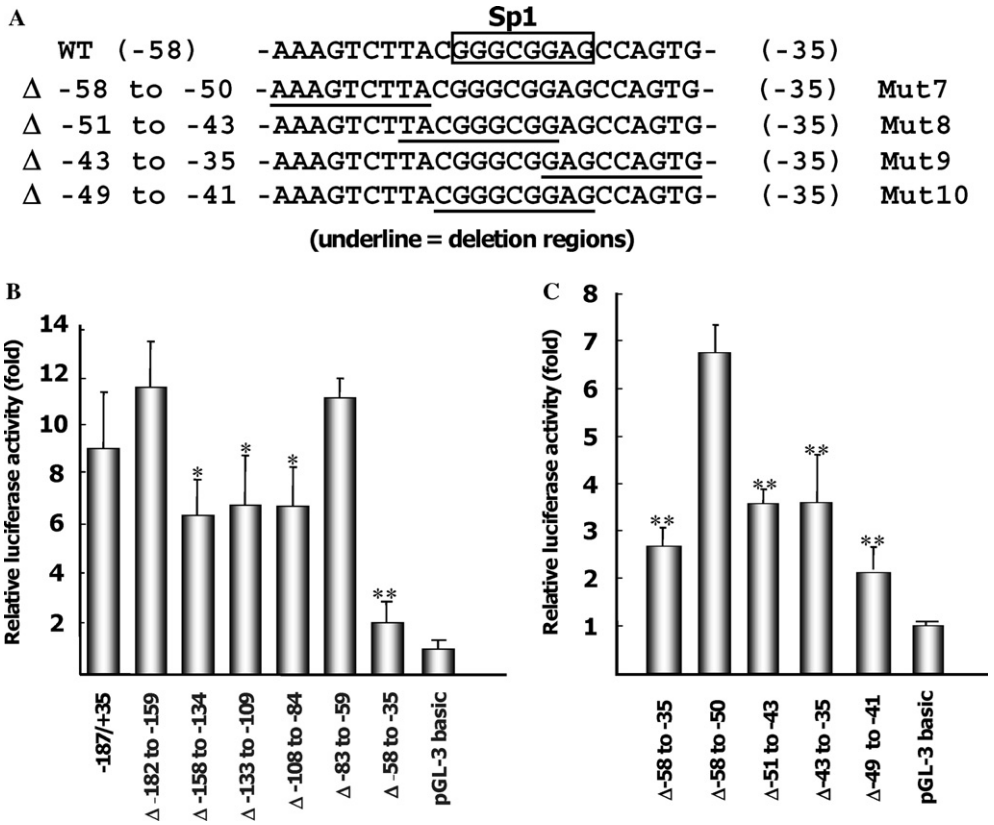

Fig. 2. Transient transfection of 25- and 9-bp internal deletion mutants in INS-1 cells. (A) The positions of deletions in each mutant relative to the putative Sp1 binding site are shown. Underlines indicate deleted nucleotide sequence. (B) A series of mutants containing 25 nucleotide internal deletions spanning −187 to −35 were prepared from the pGL-P2Δ *Sal*I construct and transfected into INS-1 cells. (C) A series of mutants containing 9 nucleotide deletions spanning −58 to −35 were prepared from the pGL-P2Δ *Sal*I construct and transfected into INS-1 cells. The relative luciferase activities shown are means ± SD of three independent experiments, each in triplicate. The relative luciferase activity of each construct was compared with the pGL3-basic vector that was arbitrarily set as 1. $*p \leqslant 0.05$, $**p \leqslant 0.001$ compared with pGL-P2Δ *Sal*I (−187/+35).

cells, and (ii) the luciferase activity and transfection efficiency of COS1 cells are much higher than those of INS-1 cells. Nevertheless, both studies clearly show that the core promoter appears to be located within the DNA sequence 187 bp upstream of the transcription start site.

Analysis of the nucleotide sequence surrounding this region using the TRANSFAC database [18] reveals the presence of three copies of a GC box, providing putative binding sites for Sp1 transcription factors at positions −237/−232 (inverted, distal), −196/−189 (central), and −48/−41 (proximal), respectively, and three copies of a CCAAT box, providing putative binding sites for NF-Y transcription factors at positions −225/−221, −95/−91, and −65/−61, respectively (Fig. 1C).

Localization of control regions of the 187-bp fragment of the P2 promoter in β-cells

Since the construct containing the first 187-bp of P2 promoter plus the first 35-bp of exon 1D possesses the highest luciferase activity in β-cells, a series of six 25-nucleotide internal deletion constructs spanning −187 to −35 were generated in an attempt to identify the important regulatory regions. These constructs including Δ −182 to −159 (Mut1), Δ −158 to −134 (Mut2), Δ −133 to −109 (Mut3), Δ −108 to −84 (Mut4), Δ −83 to −59 (Mut5), and Δ −58 to −35 (Mut6) were transiently transfected into INS-1. As shown in Fig. 2B, deletion of −182 to −159 resulted in a slight increase of luciferase activity while deletions of regions −158 to −134, −133 to −109, and −108 to −84 reduced the luciferase activity to approximately 65%. Further deletion of region −83 to −59 brought the luciferase activity up to approximately the same level as that of the Mut1 construct. However, further deletion of the −58 to −35 region (Mut6) resulted in the reduction of promoter activity by 85% to be close to that of the promoterless construct. This result suggests that a strong positive regulatory element is located between −58 and −35 regions.

As shown in Fig. 2A, the nucleotide sequence in this region contains a GC box, which is a binding site for the Sp1 transcription factor, located at −48/−41. To test whether this putative Sp1 binding site or other sequences may have contributed to the loss of promoter activity when the region −58 to −35 was deleted, we generated four 9-bp internal deletion constructs spanning this region including Δ −58 to −50 (Mut7), Δ −51 to −43 (Mut8), Δ −43 to −35 (Mut9), and Δ −49 to −41 (Mut10). The 9-bp deleted nucleotides relative to the Sp1 binding site are shown in Fig. 2A. Deletion of region −58 to −50 resulted in a 2-fold increase in the promoter activity while further deletions of region −51 to −43 (5′ Sp1 binding site) and −43 to −35 (3′ Sp1 binding site) resulted in a marked decrease of promoter activity similar to that by the deletion of region −58 to −35. Total deletion of the putative Sp1 binding site (Mut10) reduced promoter activity to be not significantly different from that of the Δ −58 to −35 deletion (Fig. 2C). These data suggest that this GC box is a strong positive element that controls P2 promoter activity in β-cells.

Nuclear factors Sp1 and Sp3 from INS-1 bind at −58/−35 of P2 promoter

To determine if Sp1 or any other protein is capable of binding −58/−35 region, we performed an electrophoretic mobility shift assay (EMSA) using the wild type [WT (−58/−35)] and mutated oligonucleotides containing partial (Mut8 and Mut9) or complete (Mut10) deletion of Sp1 binding site as shown in Fig. 2A. As shown in Fig. 3, the wild type −58/−35 double-stranded probe, when incubated with nuclear extract from INS-1, forms three major DNA–protein complexes (C1, C2, and C3). The binding specificity of complexes was determined in competition with excess unlabelled double-stranded wild type oligonucleotide. As shown in Fig. 3, all three complexes could be competed off with 50× and 100× excess amount of unlabelled wild type sequence (WT). In contrast, when double-stranded oligonucleotides devoid of the Sp1 binding site were included as the competitor in excess amounts of 10×, 50×, and 100×, they failed to

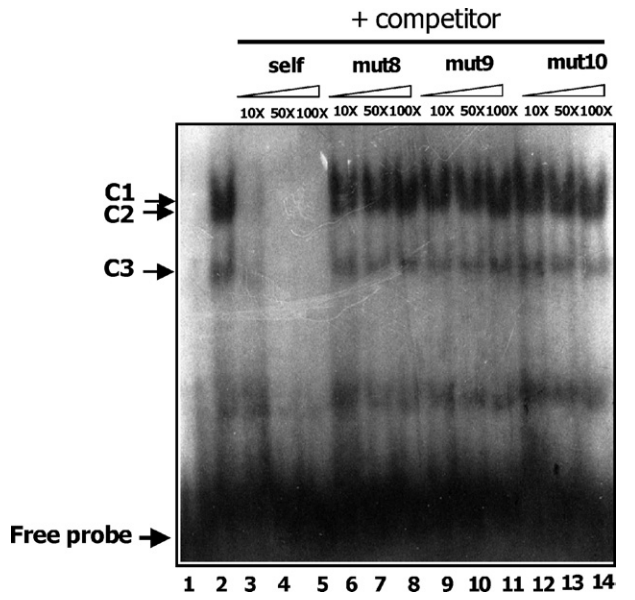

Fig. 3. Electrophoretic mobility shift assay of region −58 to −35 of rat PC distal promoter to nuclear protein. Autoradiogram of EMSA of WT sequence bound with nuclear protein. Lane 1, probe with no nuclear extract; lane 2, probe with nuclear extract. The WT (self), mut8, mut9, and mut10 double-stranded oligonucleotides (sequences shown in Fig. 2A) were included as the competitor with nuclear extract in the assay (lanes 3–14, respectively). The triangles refer to the use of increasing amounts of the unlabelled competitors (10:1, 50:1, and 100:1 excess, respectively). C1, C2, and C3 indicate DNA–nuclear protein complex bands.

abolish the formation of DNA–protein complexes, suggesting that the formation of these complexes requires an intact GC box.

To confirm whether the formation of DNA–protein complexes observed is due to the binding of Sp1 to a GC box, a competition assay was performed using the conserved Sp1 binding oligonucleotide of the SV40 promoter (Fig. 4A) as the competitor. As shown in Fig. 4B, the DNA–protein complexes could be partially competed off by a 5- and 10-fold excess of unlabelled Sp1 binding site of SV40 and completely competed off by 25-fold excess of the same competitor but not by the mutant oligonucleotide. As Sp1 and Sp3 are the major isoforms of Sp-transcription factor that are ubiquitously expressed in most tissues [19–21], we have used antibodies for these two isoforms to identify which of these isoforms contributed to the formation of C1, C2, and C3 complexes. Incubation of the INS-1 nuclear extract with polyclonal antibody against Sp1 reduced the formation of C1 complex by more than 70% (lane 13) and also supershifted C1 (Fig. 4B, asterisk). Likewise, incubation of INS-1 nuclear extract with Sp3 polyclonal antibody (lane 14) reduced the formation of C2 and C3 complexes by more than 90%. Addition of both Sp1 and Sp3 antibodies completely blocked the formation of C1, C2, and C3 complexes. These data suggested that Sp1 interacts with GC box and forms C1 complex while Sp3 interacts with GC box and forms C2 and C3 complexes, respectively.

Functional interaction of Sp1 and NF-Y in 187-bp P2 promoter

The presence of two nearby CCAAT boxes located at positions −95/−91 (distal) and −65/−61 (proximal), in the distal promoter of the rat PC gene (see Fig. 1C), raises the question of a functional role for these elements in regulating PC expression. To investigate this, we generated substitution mutations of each CCAAT box or both,

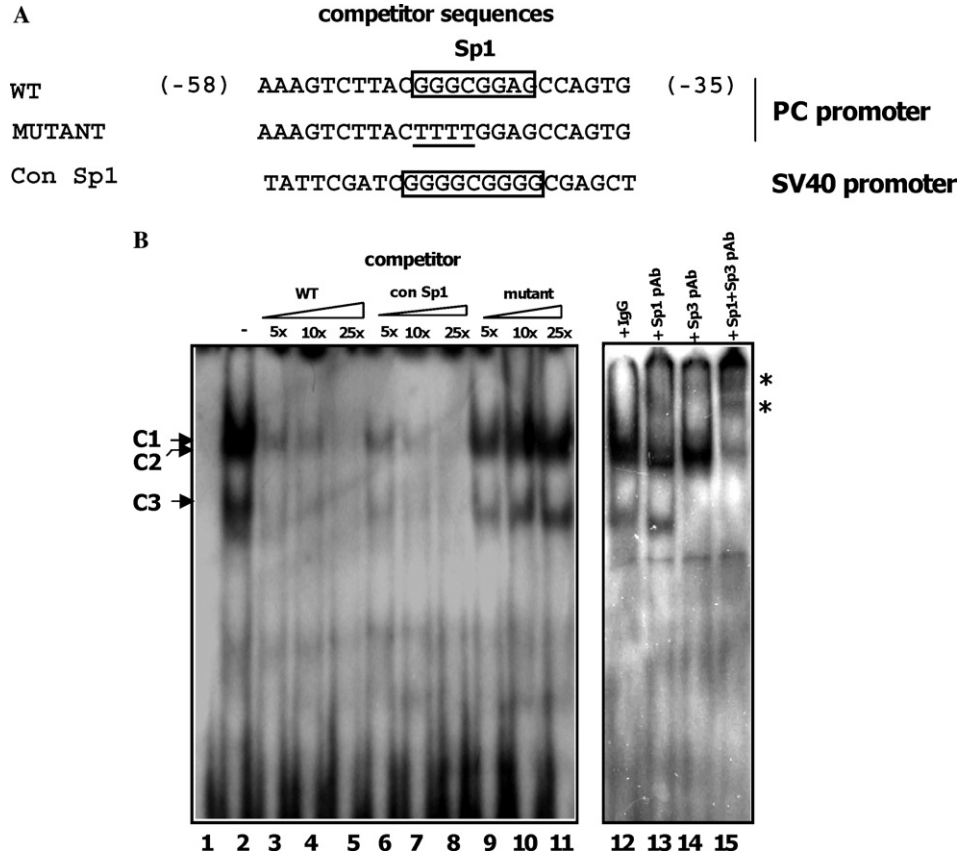

Fig. 4. Electrophoretic mobility shift and supershift assays of −58 to −35 region of distal promoter in the rat PC gene. (A) Nucleotide sequence of the −58 to −35 region of wild type rat PC promoter (WT) compared with mutant sequence and consensus Sp1 binding site of SV40 virus. (B) The ^{32}P labelled double-stranded oligonucleotide probes corresponding to −58 to −35 region of the rat PC promoter were incubated with nuclear extract of INS-1 cells in the absence or presence of anti-Sp1 and anti-Sp3 polyclonal antibodies or both. Lane 1, probe alone; lane 2, probe with nuclear extract. The WT, consensus Sp1 (con Sp1) and WT-mutant double-stranded oligonucleotides were included as the competitor with nuclear extract in the assay (lanes 3–11, respectively). The triangles refer to the use of increasing amounts of the unlabelled competitor (wild type sequence, mutant sequence or consensus SV40 Sp1 sequence [5:1, 10:1, and 25:1 excess, respectively]. Non-specific IgG (lane 12), anti-Sp1 (lane 13), antiSp3 (lane 14) or anti Sp1 + Sp3 (lane 15) was included in the supershifted assay. Arrows represent the nuclear protein–DNA complexes (C1, C2, and C3) indicated as the shifted bands whereas the asterisks represent the antibody/Sp1/Sp3/DNA complexes indicated as the supershifted bands.

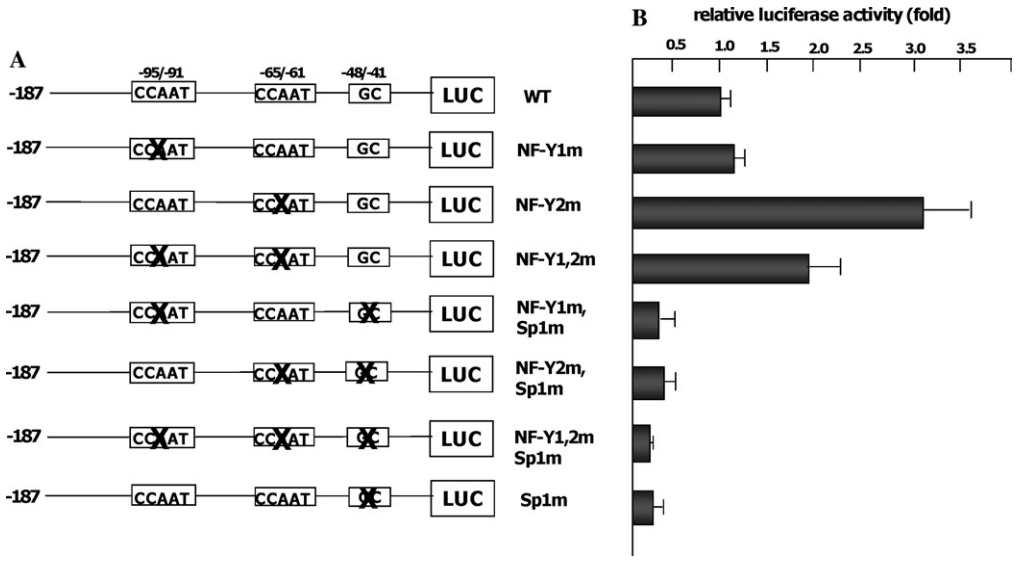

Fig. 5. Site-directed mutagenesis of the GC box and the proximal and the distal CCAAT boxes. Single or double mutations of the distal (−95/−91) and proximal (−65/−61) CCAAT boxes were generated with and without the GC box intact in the WT construct (pGL-P2Δ *Sal*I), and these constructs were transiently transfected into INS-1 cells. (A) The schematic diagram represents the mutations in each construct. (B) The relative luciferase activities of each construct upon transfecting into INS-1 cells are shown as means ± SD of three independent experiments, each in triplicate. The relative luciferase activity of each construct was compared with pGL-P2Δ *Sal*I (−187/+35) that was arbitrarily set as 1.

together with an intact or mutated GC box (see Fig. 5A). As shown in Fig. 5B, mutation of the distal CCAAT box (NF-Y1m) [−95/−91] to AAGGC had a minimal effect on the luciferase activity while the same mutation of the proximal CCAAT box (NF-Y2m) [−65/−61] caused a 3-fold increase in the luciferase activity. Double mutations of the proximal and distal CCAAT boxes (NF-Y1,2m) increased the luciferase activity 2-fold above WT. With the GC box mutated, mutations of either distal (NF-Y1m, Sp1m) or proximal CCAAT box (NF-Y2m, Sp1m) or both (NF-Y1,2m, Sp1m) resulted in the reduction of luciferase activity to be close to that of the single GC box mutant (Sp1m). These data indicate that the proximal CCAAT box acts as a negative repressor in regulating Sp1/Sp3 activity. Deletion of this box relieves its repressive effect resulting in an increase in promoter activity.

As the mutation of proximal but not the distal CCAAT box had affected transcription of the reporter gene, we performed an EMSA on the proximal CCAAT box to examine what nuclear proteins bind to this CCAAT box. As shown in Fig. 6B, a strong protein–DNA complex (C1) was observed when the labelled double-stranded oligonucleotide containing the proximal CCAAT box (WT, −72/−54) was incubated with nuclear extracts of INS-1. To confirm whether the DNA–protein complexes observed are due to binding of NF-Y to the proximal CCAAT box, a competition assay was performed using the conserved NF-Y binding oligonucleotide of the SV40 promoter (Fig. 6A) as the competitor. As shown in Fig. 6B, the C1 complex could be partially displaced by a 5-fold excess of unlabelled NF-Y binding site from SV40 and unlabelled WT sequence but not by the mutant oligonucleotide. Incubation of the INS-1 nuclear extract with polyclonal antibody against NF-Y (b-subunit) reduced the formation of C1 complex by more than 80% and also supershifted C1 (Fig. 6B, asterisk). However, there was no such effect with the anti-Sp1 antibody. These data together with the mutational analysis suggest that NF-Y binds the proximal CCAAT box and modulates Sp1/Sp3 activities. Similar EMSA experiments performed with the distal CCAAT box identified no DNA–protein complex (data not shown), suggesting that this site is not functional or is a relatively poor binding site for NF-Y, and is therefore in agreement with the transfection result (Fig. 5).

NF-Y and Sp1 have often been seen to enhance their DNA binding cooperatively and thereby activate transcription synergistically [22]. While mutations of NF-Y binding sites have usually been found to reduce basal transcription of many genes [23,24], mutation of the NF-Y binding site increased basal transcription of the distal promoter of the rat PC gene. It would appear that NF-Y can act as a repressor of basal transcription of PC. It has been shown that NF-Y can exert dual functions either as an activator or a repressor depending on the promoter context [25,26]. The distal promoter of the rat PC gene does not contain a TATA-motif in the vicinity of the transcription start site [13]. Whereas NF-Y binding to the CCAAT box is frequently essential for gene transcription, particularly for a TATA-less promoter, NF-Y by itself is largely unable to activate transcription. In the case of the distal promoter of the rat PC gene, the fact that mutation of the Sp1/Sp3

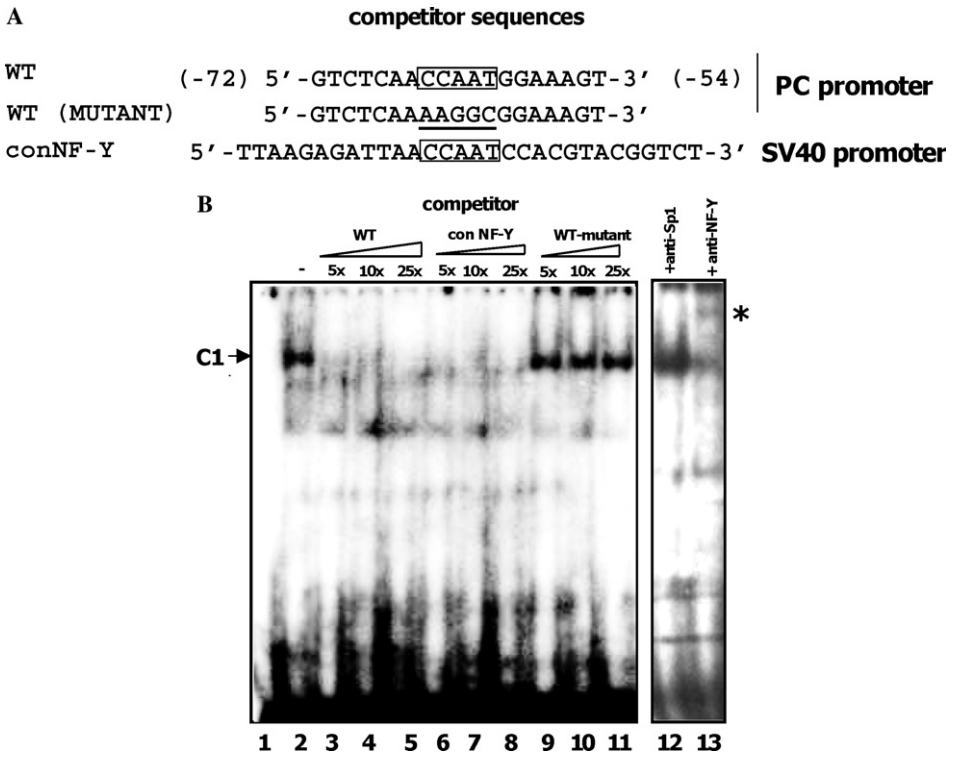

Fig. 6. Electrophoretic mobility shift and supershift assays of the proximal CCAAT box of the distal promoter of the rat PC gene. (A) Nucleotide sequence of −72/−54 containing the proximal CCAAT box (−65/−61) region of the wild type rat PC promoter [WT] compared with the consensus CCAAT box of the SV40 virus. (B) The ^{32}P-labelled double-stranded oligonucleotide probes corresponding to −72/−54 of the rat PC promoter were incubated with nuclear extract of INS-1 cells in the absence or presence of antiNFY polyclonal antibody. Lane 1, probe alone; lane 2, probe with nuclear extract. The WT, consensus NF-Y (con NF-Y), and mutant double-stranded oligonucleotides were included as the competitor with nuclear extract in the assay (lanes 3–11, respectively). The triangles refer to the use of increasing amounts of the unlabelled competitor (wild type sequence, mutant sequence or consensus NF-Y sequence) [5:1, 10:1, and 25:1 excess, respectively]. Anti-Sp1 (lane 12) or anti-NF-Y (lane 13) included in the supershifted assay. Arrow represents the nuclear protein–DNA complexes (C1) indicated as the shifted band whereas an asterisk represents the antibody/NF-Y/DNA complex indicated as the supershifted band.

binding site with the proximal CCAAT box intact also failed to alter reporter gene activity (Fig. 5) strengthens the notion that binding of the NF-Y factor alone is not able to exert its effect on transcription unless it interacts with other relevant nuclear factors. In contrast, the binding of NFY to the proximal CCAAT box tended to repress Sp1/Sp3 mediated transcription activation of the distal promoter of the rat PC gene.

It will be interesting to see if the Sp1/Sp3 general transcription factors can modulate PC transcription during glucose-induced insulin secretion. Both mitochondrial glycerol phosphate dehydrogenase (mGPD) and the cytosolic NAD-linked glycerol phosphate dehydrogenase are thought to be involved in glucose-induced insulin secretion [27]. The promoter of mGPD also contains multiple sites for Sp/Krüppel-like transcription factor [28]. The level of mGPD in cells grown in elevated level concentrations of glucose was down-regulated through the reduction in the amount of Sp1 present in these INS-1 cells. We are currently determining whether Sp1/Sp3 or other transcription factors play dual roles in modulating PC transcription in β-cells.

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