Upstream stimulatory factor regulates *Pdx-1* gene expression in differentiated pancreatic β -cells

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The homeobox gene *Pdx-1* plays a key role in the development of the pancreas. In the adult, however, expression of the *Pdx-1* gene is restricted to pancreatic β -cells and endocrine cells of duodenal epithelium. Recently, the transcription factor, upstream stimulatory factor (USF), has been shown to bind *in vitro* to a mutationally sensitive E-box motif within the 5'-flanking region of the *Pdx-1* gene [Sharma, Leonard, Lee, Chapman, Leiter and Montminy (1996) *J. Biol. Chem.* **271**, 2294–2299]. In the present study, we show that USF not only binds to the *Pdx-1* gene promoter but also functionally regulates the expression of the *Pdx-1* gene in differentiated pancreatic β -cells. Adenovirus-mediated overexpression of a dominant negative form of USF2 decreased binding of endogenous USF to the E-box element by

~ 90%. This reduction in endogenous USF binding led to a greater than 50% decrease in *Pdx-1* gene promoter activity, which, in turn, resulted in marked reductions in *Pdx-1* mRNA and protein levels. Importantly, the lower *Pdx-1* protein levels led to a greater than 50% reduction in *Pdx-1* binding activity to the A3 element on the insulin gene promoter, and a significant reduction in insulin mRNA levels. Overall, our results show that USF functionally regulates *Pdx-1* gene expression in differentiated pancreatic β -cells and provide the first functional data for a role of USF in the regulation of a normal cellular gene.

Key words: E box, INS-1, insulin, transcription factors.

INTRODUCTION

The adult mammalian pancreas is composed of exocrine and endocrine cells which appear to develop from a common precursor cell. Recently, a variety of transcription factors have been identified that function at different stages during pancreas development [1–7]. *Pdx-1* (also termed IPF1, STF-1, IDX-1) is a homeodomain transcription factor that is expressed early (mouse embryonic day 8.5) during the development of the pancreas [8–10]. Mice with a targeted disruption of the *Pdx-1* gene are born apancreatic [1,2], emphasizing the importance of *Pdx-1* in pancreatic development. In the adult pancreas, *Pdx-1* expression is restricted to the pancreatic β -cells residing in the islets of Langerhans [8,11]. *Pdx-1* has been proposed to regulate the expression of a variety of pancreatic endocrine cell genes, including insulin [8,12–14], somatostatin [9,10], the glucose transporter GLUT2 [15], islet amyloid polypeptide [16] and glucokinase [17] through its ability to interact at A-T rich regions contained within the promoter of these genes. A recent study by Ahlgren et al. [18] has shown that selective disruption of *Pdx-1* in pancreatic β -cells markedly alters β -cell phenotype by decreasing insulin, islet amyloid polypeptide and GLUT2 expression while increasing glucagon expression, thus providing evidence that *Pdx-1* plays an essential role in maintenance of the β -cell phenotype.

Although *Pdx-1* plays an important role in the maintenance of β -cell phenotype, the transcriptional mechanisms involved in *Pdx-1* gene expression remain poorly understood. Sharma et al. [19] have shown that a rat *Pdx-1* gene promoter fragment containing 6500 bp of 5'-flanking sequences was sufficient for targeting expression of a β -galactosidase (β -GAL) reporter gene to pancreatic β -cells and endocrine cells in the duodenum of transgenic mice. Similarly, Wu et al. [20] found that mouse *Pdx-*

1 gene promoter sequences, including 4500 bp upstream of the transcription start site, were also sufficient for targeting reporter gene expression to these tissues. Although these promoter regions have not been completely characterized, HNF3 β has been shown to bind and regulate both rat and mouse distal *Pdx-1* promoter elements [20,21].

Within the rat proximal *Pdx-1* promoter, Sharma et al. [19] have also defined a mutationally sensitive E-box element located between –107 and –102. This E-box motif is capable of binding *in vitro* to the upstream stimulatory factor (USF). Based on this evidence, USF has been suggested to regulate *Pdx-1* gene expression. USF proteins are members of the basic/helix-loop-helix/leucine zipper family of transcription factors and were first identified for their role in regulating transcription of the adenovirus major late promoter [22–24]. Two forms of USF, USF1 and USF2, are encoded by separate genes [25–28]. USF proteins primarily bind as USF1/USF2 heterodimers to specific sites with a CACGTG consensus sequence [22,25,27]. USF proteins are ubiquitously expressed and USF binding sites have been identified in a variety of cellular and viral genes (for examples see [29–34]). Nonetheless, other members of basic/helix-loop-helix/leucine zipper family of transcription factors such as Myc, Max, Myn, Mad, Mxi, TFE3, TFEB, MITF and SREBP (ADD-1) can also bind similar DNA elements as USF [35–43], thus making it impossible to determine by mutational analysis which member of the family functionally regulates a particular gene *in vivo*. Due to this major caveat, site-directed mutagenesis of DNA regulatory elements and *in vitro* DNA binding do not constitute sufficient evidence to establish that USF functionally regulates *Pdx-1* gene expression.

To address whether USF plays a functional role in the regulation of *Pdx-1* gene expression, we have used a recombinant adenovirus engineered to overexpress a dominant-negative form

Abbreviations used: USF, upstream stimulatory factor; β -GAL, β -galactosidase; CMV, cytomegalovirus; LUC, luciferase; p.f.u., plaque-forming units; EMSA, electrophoretic mobility-shift assay.

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of the USF2 protein. We demonstrate that overexpression of the dominant-negative USF, termed USF2ΔB, reduced *Pdx-1* gene promoter activity, *Pdx-1* mRNA and protein levels. The reduction in *Pdx-1* protein levels led to a commensurate reduction in *Pdx-1* binding activity to the A3 element on the insulin promoter and a significant reduction in insulin mRNA levels. Overall, these results indicate that *Pdx-1* gene transcription is functionally regulated by USF.

EXPERIMENTAL

Materials

Cell culture materials, lipofectamine, random primer labelling kits, and enzymes were from Gibco-BRL (Gaithersburg, MD, U.S.A.); luciferase (LUC) assay kits were from Promega (Madison, WI, U.S.A.); anti-USF1 antibodies (C-20) and anti-USF2 antibodies (N-18) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.); Nytran membranes were from Schleicher & Schuell (Keene, NH, U.S.A.); chemiluminescence detection kits were from Pierce (Rockford, IL, U.S.A.); anti-N-terminal XIHbox8 antibodies were provided by Dr Christopher Wright (Vanderbilt University, Nashville, TN, U.S.A.); USF2ΔB cDNA was provided by Dr Michele Sawadogo (University of Texas M. D. Anderson Cancer Center, Houston, TX, U.S.A.); the recombinant adenovirus, cytomegalovirus (CMV)-βGAL (AdCMV-βGAL), was provided by Dr Chris Newgard (University of Texas, Southwestern, Dallas, TX, U.S.A.); *Pdx-1* reporter genes were provided by Dr Marc Montminy (Joslin Diabetes Center, Harvard Medical School, Boston, MA, U.S.A.).

Cell culture

INS-1 cells were routinely cultured in CO₂/air (1:19) at 37 °C in RPMI-1640 media containing 11.1 mM glucose and supplemented with 10 % fetal-bovine serum, 1 mM pyruvate, 10 mM Hepes, 50 μM 2-mercaptoethanol, 100 units penicillin/ml and 100 μg streptomycin/ml. In all experiments the RPMI-1640 medium contained the supplements described above. Cells were passaged weekly by trypsin/EDTA detachment. All studies were performed on INS-1 cells between passages 70 and 80.

Preparation of recombinant adenovirus encoding dominant negative USF2

A recombinant adenovirus containing the cDNA sequences encoding for USF2ΔB was prepared according to the method of Becker et al. [44]. The recombinant adenovirus, AdCMV-USF2ΔB, was prepared by insertion of the murine USF2ΔB cDNA sequences [29] into the plasmid pACCMV.pL.pA adjacent to the CMV promoter. pACCMV.pL.pA containing the USF2ΔB cDNA was then co-transfected with pJM17 plasmid into HEK-293 cells. The resultant recombinant virus was amplified in HEK-293 cells and titred by plaque assay [44]. INS-1 cells were untreated or were transduced with AdCMV-USF2ΔB or with a control virus containing the β-GAL gene [each at 50 plaque-forming units (p.f.u.) per cell] for 1 h. The viral incubation media was then replaced with RPMI-1640 media.

Nuclear extracts and electrophoretic mobility-shift assays (EMSA)

INS-1 cells were plated at a density of 10 × 10⁶ cells in a 100-mm dish 2 days before viral treatment. Cells were then mock transduced or transduced with AdCMV-βGAL or AdCMV-USF2ΔB (50 p.f.u. per cell) and, 2 to 3 days after treatment, nuclear extracts were isolated as described previously [45]. Protein

concentrations were determined by method of Lowry et al. [46]. Double-stranded oligodeoxynucleotide probes to the rat *Pdx-1* E-box element (−118 GGCCCCAGGCTGAACACGTGGGGTGCC −95), human insulin gene A3 element (previously termed the CT2 motif; −230 CCCCTGGTTAAGACTCTAATGACCCGCTGG −201) and rat pyruvate kinase HNF-4 binding site (−146 TCCTGGACTCTGGCCCCCAGTGT −124) were labelled with [α ³²P]dCTP by filling in overhanging 5' ends with the large fragment of DNA polymerase 1. Binding reactions (5–10 μg of protein per lane) and electrophoresis were performed as described previously by Olson et al. [45]. Supershift analysis for *Pdx-1* binding activity was performed by the addition of 2 μl of anti-N-terminal XIHbox8 antibodies. This anti-N-terminal XIHbox8 antiserum was raised against amino acid residues 1–75 of the XIHbox8 protein, a region highly conserved within the *Pdx-1* protein [12]. Previous studies have shown that the anti-N-terminal XIHbox8 antiserum specifically supershifts the *Pdx-1* protein–DNA complex when HIT cell and INS-1 cell nuclear extracts are used [12,45,47]. Supershift analysis for USF was performed by the addition of 2 μl of anti-USF1 antibodies.

Western-blot analysis of nuclear proteins

Nuclear extracts (30 μg) were resolved by SDS/PAGE (10 % gel). Resolved proteins were then transferred to nitrocellulose membranes and *Pdx-1*, USF1 or USF2 immunoreactivity was detected using anti-N-terminal XIHbox8, anti-USF1 antibodies and anti-USF2 antibodies respectively. Immunoreactivity was visualized by chemiluminescence and bands were quantified by scanning densitometry and NIH image software.

RNA isolation and Northern-blot analysis

Cells were plated at a density of 5 × 10⁶ cells per 60-mm dish in RPMI-1640 medium containing 11.1 mM glucose, and 2 days later the cells were mock transduced or transduced with 50 p.f.u. per cell AdCMV-βGAL or AdCMV-USF2ΔB. Total RNA was extracted 2 to 3 days after infection and Northern blots were performed as described previously [45]. Briefly, 10–20 μg of total RNA was separated on a 1.5 % (w/v) agarose gel containing 6.7 % formaldehyde and transferred on to a Nytran nylon membrane. Blots were probed with cDNAs from human β-actin, Syrian hamster preproinsulin and rat *Pdx-1*, as described previously [45]. Northern blots were quantified using a phosphor-imager.

Reporter genes studies

The plasmids *Pdx-1*[−6.5kb]LUC and *Pdx-1*[−190]LUC contain the *LUC* gene under transcriptional regulation by the rat *Pdx-1* gene sequences −6500 to +68 and −190 to +78 respectively [19]. The plasmids *Pdx-1*[−6.5kbEm]LUC and *Pdx-1*[−190Em]LUC contain a mutated E-box motif (−118/−95) in the context of the 6500 or 190 bp *Pdx-1* promoter respectively [19]. Construction of CMV4-USF2 and CMV4-USF2ΔB expression vectors has been described previously by Kaytor et al. [34].

For transfection of adenovirus-infected cells, INS-1 cells were subcultured at a density of 1.5 × 10⁶ cells/well (diam. 3.5 cm) in RPMI-1640 medium 2 days before transduction. Cells were then mock transduced or transduced with 50 p.f.u. per cell AdCMV-βGAL or AdCMV-USF2ΔB. Twenty-four hours later cells were transfected with 1 μg of reporter gene DNA for 4 h at 37 °C using 4 μl of Lipofectamine. The cells were harvested 24 h after transfection and LUC activity was assayed.

For all co-transfection experiments, INS-1 cells were subcultured at a density of 1.5×10^6 cells/well in RPMI-1640 medium 2 days before transfection. In experiments designed to test the ability of wild-type USF2 to reverse USF2 Δ B suppression of *Pdx-1* gene promoter activity (see Figure 3), cells were transfected with 1 μ g of Pdx-1[–190]LUC, 2 μ g of CMV4–USF2 Δ B or 1 or 2 μ g of CMV4–USF2 and 20 μ l Lipofectin. All cells were transfected with equal amounts of DNA by use of a CMV4-expression plasmid containing no insert. For co-transfection experiments designed to examine the role of the *Pdx-1* E box (see Table 2), INS-1 cells were transfected with 1 μ g of reporter DNA, 2 μ g of CMV4–USF2 Δ B and 16 μ l of Lipofectamine. For these experiments, control cells were transfected with 1 μ g of reporter DNA and 2 μ g of CMV4-expression plasmid containing no insert. Cells were harvested 48 h after co-transfection and LUC activity assayed.

RESULTS

Dominant negative form of USF2 decreases USF binding activity in INS-1 cells

Recently, Sharma et al. [19] identified an E box located within the *Pdx-1* gene (–107/–102) that, when mutated, strongly reduced promoter activity. Although this E box serves as a binding site for USF *in vitro*, it has yet to be determined whether USF functionally regulates activity of the *Pdx-1*-gene promoter

Figure 1 Expression of USF and USF2 Δ B in AdCMV–USF2 Δ B treated INS-1 cells

Upper panel: schematic comparison of the structures of USF2 and USF2 Δ B. USF2 Δ B was generated from mouse USF2 [66] and lacks the basic region (B) contained within the DNA-binding domain. All other regions of the molecules, including helix-loop-helix (HLH), leucine zipper (LZ) and USF specific region (USR) are identical. Lower left panel: Western blot of USF2 protein expression in INS-1 cells 48 h after (lane 1) mock transduction (Control), (lane 2) AdCMV– β GAL transduction or (lanes 3 and 4) AdCMV–USF2 Δ B transduction. To allow detection of a slight mobility shift between endogenous USF2 and USF2 Δ B, 30 μ g of nuclear extract was applied to lanes 1, 2 and 4, and only 7.5 μ g was applied to lane 3. Lower right panel: Western blot of USF1 protein expression in INS-1 cells 48 h after (lane 1) mock transduction (Control), (lane 2) AdCMV– β GAL transduction and (lane 3) AdCMV–USF2 Δ B transduction. The blots shown are representative Western blots of three independent experiments, which yielded similar results.

Figure 2 Expression of USF2 Δ B specifically decreases USF binding activity in INS-1 cells

EMSA were performed using INS-1-cell nuclear extracts and a radiolabelled oligodeoxynucleotide probe containing the *Pdx-1* gene E box (left panel) or HNF-4 binding element (right panel). Left panel: lane 1, free probe; lane 2, mock transduced cells (Control); lane 3, AdCMV– β GAL transduced cells; lane 4, AdCMV–USF2 Δ B transduced cells; lane 5, AdCMV– β GAL transduced cells plus 2 μ l anti-USF1 antibodies (ab); lane 6, AdCMV–USF2 Δ B transduced cells plus 2 μ l anti-USF1 antibodies (ab). Right panel: lane 1, free probe; lane 2, mock transduced cells (Control); lane 3, AdCMV– β GAL transduced cells; lane 4, AdCMV–USF2 Δ B transduced cells. The results are representative EMSAs from four independent experiments, which yielded similar results.

in cells. To determine whether USF may regulate the expression of the *Pdx-1* gene, a dominant-negative form of USF2 (USF2 Δ B) was overexpressed in INS-1 cells using a recombinant adenovirus (AdCMV–USF2 Δ B). In the USF2 Δ B protein, the basic region of the USF DNA-binding domain was deleted and the nuclear localization, helix-loop-helix and leucine zipper domains remained intact (Figure 1, upper panel). The USF2 Δ B protein retained the ability to dimerize with endogenous USF1 and USF2, but the heterodimer was unable to bind DNA [29]. The expression of USF2 Δ B was determined by Western-blot analysis of nuclear extracts derived from untreated, AdCMV– β GAL- and AdCMV–USF2 Δ B-treated INS-1 cells. Treatment of INS-1 cells with AdCMV–USF2 Δ B led to a high level of expression of USF2 Δ B within 48 h (Figure 1, lower left panel; lane 4). The mobility difference between endogenous USF2 and USF2 Δ B can be seen when 75% less extract from AdCMV–USF2 Δ B transduced cells was loaded on to the gel (Figure 1, lower left panel; compare lanes 1 and 2 with lane 3). Infection of INS-1 cells with AdCMV–USF2 Δ B had no effect on endogenous expression of USF1 compared with untreated cells or cells treated with AdCMV– β GAL (Figure 1, lower right panel).

The effectiveness of USF2 Δ B for disrupting binding of USF to the *Pdx-1* gene E box (–107/–102) was determined by EMSA using a double-stranded oligodeoxynucleotide probe corresponding to the rat *Pdx-1* gene sequences –118 to –95. Nuclear extracts derived from untreated and AdCMV– β GAL-treated INS-1 cells formed a DNA–protein complex with the *Pdx-1* gene E box (Figure 2, left panel; lanes 2 and 3). This E-box-binding complex could be supershifted by the addition of anti-USF1 antibodies (Figure 2, left panel; lane 5). In nuclear extracts derived from AdCMV–USF2 Δ B-treated INS-1 cells, the E-box

Table 1 Expression of USF2ΔB diminishes *Pdx-1* gene promoter activity

INS-1 cells were mock transduced (control) or transduced with either AdCMV-USF2ΔB or AdCMV-βGAL. Cells were transiently transfected with either Pdx-1[−6.5kb]LUC or Pdx-1[−190]LUC 24 h after treatment. The cells were incubated for an additional 24 h in RPMI-1640, harvested and LUC activity was assayed. Values are the means ± S.E.M. of three individual experiments.

Reporter gene	Cell treatment	Luminescence (arbitrary units)
Pdx-1[−6.5kb]LUC	Control	96.3 ± 8.5
	AdCMV-βGAL	86.4 ± 4.4
	AdCMV-USF2ΔB	40.2 ± 3.4
Pdx-1[−190]LUC	Control	22.8 ± 1.8
	AdCMV-βGAL	32.1 ± 5.8
	AdCMV-USF2ΔB	12.7 ± 3.3

binding activity was reduced to $11 \pm 1.6\%$ ($n = 4$, $P < 0.005$) when compared with control cells (Figure 2, left panel; lane 4). Most of the remaining binding activity could be attributed to residual USF binding because it was disrupted by anti-USF1 antibodies (Figure 2, left panel; lane 6). Adenovirus-mediated overexpression of USF2ΔB in INS-1 cells and rat hepatocytes also led to reduced USF binding to the E-box sequences (−165/−149) contained within the carbohydrate-response element [48,49] of the L-pyruvate kinase gene promoter (J. Qian, E. N. Kaytor, H. C. Towle and L. K. Olson, unpublished work). In contrast to USF, the DNA binding activity to the HNF-4-binding site located within the L-pyruvate kinase gene (−146/−124) remained unchanged in AdCMV-USF2ΔB treated INS-1 cells (Figure 2, right panel). These data demonstrate that overexpression of USF2ΔB can specifically displace USF binding activity and thus provide a means for determining the functional role of USF in *Pdx-1* gene expression.

Dominant-negative USF diminishes *Pdx-1* gene promoter activity

To determine whether USF plays a functional role in *Pdx-1* gene promoter activity, INS-1 cells were transiently transfected with *Pdx-1* promoter/reporter constructs 24 h after cells were infected with AdCMV-USF2ΔB or AdCMV-βGAL. *Pdx-1* promoter activity was assessed by using two LUC reporter genes regulated by *Pdx-1* gene sequences −6500 to +68 (Pdx-1[−6.5kb]LUC) or −190 to +78 (Pdx-1[−190]LUC). Pdx-1[−6.5kb]LUC contains sufficient *Pdx-1* gene promoter sequences for β-cell specific expression, whereas Pdx-1[−190]LUC contains the *Pdx-1* minimal promoter and other putative regulatory sites including the *Pdx-1* gene E box [19]. The expression of Pdx-1[−6.5kb]LUC was not significantly affected by treatment of INS-1 cells with AdCMV-βGAL compared with uninfected cells (Table 1), indicating that viral transduction does not non-specifically reduce *Pdx-1* promoter activity. In contrast, Pdx-1[−6.5kb]LUC expression was reduced to $46 \pm 3.1\%$ ($n = 3$, $P < 0.001$) in AdCMV-USF2ΔB treated cells compared with AdCMV-βGAL treated cells. The expression of Pdx-1[−190]LUC was increased by approx. 40% in AdCMV-βGAL treated cells compared with control cells, indicating that viral transduction does have some non-specific effects on the −190 *Pdx-1* promoter. The mechanism whereby adenoviral infection increased −190 *Pdx-1* promoter activity remains unknown. As observed with the Pdx-1[−6.5kb]LUC reporter gene, the expression of Pdx-1[−190]LUC was also decreased to $39 \pm 3.3\%$ ($n = 3$, $P < 0.005$) in AdCMV-USF2ΔB treated cells compared with AdCMV-βGAL treated cells (Table 1). These data suggest that

Figure 3 Regulation of *Pdx-1* gene promoter activity by wild-type USF2 and USF2ΔB

INS-1 cells were transiently transfected with 1 μg Pdx-1[−190]LUC and CMV4-USF2ΔB and/or CMV4-USF2 expression vectors, as indicated. In all cases, equal amounts of DNA (5 μg) were transfected into cells by adding control CMV4 expression vector containing no insert. Cells were harvested 48 h after transfection and LUC activity was assayed. The activities are shown as arbitrary units of luminescence and are the means ± S.E. of three independent experiments.

Table 2 USF does not exclusively regulate *Pdx-1* promoter activity through the E box located at sequences −107/−102

INS-1 cells were transiently transfected with either 1 μg Pdx-1[−6.5 kb]LUC, Pdx-1[−6.5 kbEm]LUC, Pdx-1[−190]LUC or Pdx-1[−190Em]LUC and 2 μg of control CMV4 expression vector or CMV4-USF2ΔB. Cells were harvested 48 h after transfection, and LUC activity was assayed. Values are the means ± S.E.M. of three individual experiments.

Reporter gene	Expression vector	Luminescence (arbitrary units)
Pdx-1[−6.5kb]LUC	CMV4-control	80.90 ± 4.83
	CMV4-USF2ΔB	31.51 ± 4.77
Pdx-1[−6.5kbEm]LUC	CMV4-control	6.59 ± 0.40
	CMV4-USF2ΔB	3.43 ± 0.81
Pdx-1[−190]LUC	CMV4-control	16.33 ± 0.84
	CMV4-USF2ΔB	5.29 ± 0.17
Pdx-1[−190Em]LUC	CMV4-control	2.47 ± 0.23
	CMV4-USF2ΔB	1.35 ± 0.14

disruption of USF binding by overexpression of USF2ΔB diminishes *Pdx-1* gene promoter activity.

To confirm that USF2ΔB suppressed promoter activity by directly interacting with USF, we examined the ability of wild-type USF2 to reverse USF2ΔB inhibition of *Pdx-1* promoter activity. For these studies, INS-1 cells were transiently transfected with a CMV4-USF2ΔB expression vector. Transient transfection of INS-1 cells with 2 μg of CMV4-USF2ΔB reduced the expression of Pdx-1[−190]LUC to $65 \pm 6.6\%$ ($n = 5$, $P < 0.004$) of that observed for control cells (Figure 3). The reduction in Pdx-1[−190]LUC expression in this experiment was not as large as that shown in Table 2, most likely because of the less effective expression of USF2ΔB resulting from the addition of CMV4 carrier DNA. Transfection of 2 μg of CMV4-USF2 completely reversed the dominant negative action of USF2ΔB overexpression on the Pdx-1[−190]LUC reporter gene (Figure 3).

Figure 4 Expression of USF2 Δ B diminishes *Pdx-1* mRNA and protein levels

INS-1 cells were mock transduced or transduced with either AdCMV-USF2 Δ B or AdCMV- β GAL. Left panel: Northern blot of *Pdx-1* mRNA levels. Lane 1, cells harvested 48 h after mock transduction (Control); lane 2, cells harvested 48 h after AdCMV- β GAL transduction; lane 3, cells harvested 48 h after AdCMV-USF2 Δ B transduction; and lane 4, cells harvested 72 h after AdCMV-USF2 Δ B transduction. This is a representative Northern blot of three identical experiments, which yielded similar results. Northern blots were then stripped and reprobed for β -actin mRNA to ensure even loading. The reprobed blot of the left panel is shown in Figure 6. Right panel: Western blot of *Pdx-1* protein. Lane 1, cells harvested 48 h after mock transduction (Control); lane 2, cells harvested 48 h after AdCMV- β GAL transduction; and lane 3, cells harvested 48 h after AdCMV-USF2 Δ B transduction. This is a representative Western blot of two identical experiments, which yielded similar results.

These results indicated that USF2 Δ B acts directly by disruption of USF *in vivo* and that USF functions as a transcriptional activator on the *Pdx-1* promoter. To further verify the transactivation ability of USF on the *Pdx-1* gene promoter, INS-1 cells were transfected with either 1 μ g or 2 μ g of a CMV4-USF2 expression vector. Transient transfection of INS-1 cells with 2 μ g of CMV4-USF2 induced a 1.7 ± 0.2 -fold ($n = 3$, $P < 0.027$) increase in *Pdx-1* promoter activity compared with control cells (Figure 3), consistent with USF having a role as a transcriptional activator of the *Pdx-1* gene promoter.

To determine whether overexpression of USF2 Δ B decreased *Pdx-1* gene promoter activity solely by interfering with USF binding to the E box located at $-107/-102$, constructs with a mutation of this E-box sequence were tested. Since previous studies by Sharma et al. [19] had demonstrated a role for the $-107/-102$ E box in *Pdx-1* promoter activity in HIT-T15 cells, it was first necessary to test the effect of E-box mutations in INS-1 cells. As predicted, expression of *Pdx-1*[-6.5kbEm]LUC was reduced to $8.2 \pm 0.5\%$ ($n = 3$, $P < 0.002$) of that observed for wild-type *Pdx-1*[-6.5kb]LUC (Table 2). Similarly, expression of *Pdx-1*[-190Em]LUC was $15.0 \pm 1.4\%$ ($n = 3$, $P < 0.002$) of that observed for wild-type *Pdx-1*[-190]LUC (Table 2). These results indicate that the E-box motif located at $-107/-102$ of the *Pdx-1* gene promoter plays an important role in determining promoter activity in INS-1 cells, as previously reported for HIT-T15 cells [19]. These same constructs, containing E-box mutations, were tested in INS-1 cells co-transfected with a CMV4-USF2 Δ B expression vector. Co-transfection of wild-type *Pdx-1* reporter genes, *Pdx-1*[-6.5kb]LUC or *Pdx-1*[-190]LUC with 2 μ g of CMV4-USF2 Δ B reduced LUC expression to $39 \pm 5.9\%$ ($n = 3$, $P < 0.001$) and $32 \pm 1.0\%$ ($n = 3$, $P < 0.003$) of that observed in control cells respectively (Table 2). Surprisingly, co-transfection of CMV4-USF2 Δ B also reduced expression of *Pdx-1*[-6.5kbEm]LUC and *Pdx-1*[-190Em]LUC to $52 \pm 3.1\%$ ($n = 3$, $P < 0.005$) and $55 \pm 5.6\%$ ($n = 3$, $P < 0.01$) of that observed in control cells respectively (Table 2). These results indicate that the ability of USF2 Δ B to reduce *Pdx-1* gene promoter activity is, in part, mediated through the E-box sequence located at $-107/-102$, but that additional USF

regulatory sites present within the *Pdx-1* -190 bp promoter region may also contribute to its action.

Expression of USF2 Δ B diminishes *Pdx-1* mRNA levels and protein levels

A significant reduction in *Pdx-1* promoter activity by USF2 Δ B overexpression should reduce the endogenous levels of both *Pdx-1* mRNA and protein. The effect of USF2 Δ B overexpression on *Pdx-1* mRNA levels was measured by Northern-blot analysis. *Pdx-1* mRNA levels were not significantly affected by treatment of INS-1 cells with AdCMV- β GAL compared with control cells (Figure 4, left panel; compare lane 1 with lane 2), indicating that viral transduction does not non-specifically alter *Pdx-1* mRNA levels. In contrast, 48 h after AdCMV-USF2 Δ B infection *Pdx-1* mRNA levels were reduced to $24 \pm 5.6\%$ ($n = 3$, $P < 0.005$) of control cells (Figure 4, left panel; lane 3), but 72 h after AdCMV-USF2 Δ B treatment, *Pdx-1* mRNA levels were reduced to $45 \pm 2.6\%$ ($n = 3$, $P < 0.005$) of control cells (Figure 4, left panel; lane 4), suggesting some recovery of *Pdx-1* gene expression. Importantly, overexpression of USF2 Δ B had no effect on β -actin mRNA levels (see Figure 6) indicating that the changes in *Pdx-1* mRNA levels were not due to global changes in gene expression.

The effect of USF2 Δ B overexpression on *Pdx-1* protein levels was measured by Western-blot analysis. As observed for *Pdx-1* mRNA levels, there were no significant changes in *Pdx-1* protein levels 48 h after INS-1 cells were treated with AdCMV- β GAL when compared with control cells (Figure 4, right panel; compare lane 1 with lane 2). AdCMV-USF2 Δ B treatment, however, reduced *Pdx-1* protein levels to $14 \pm 0.5\%$ ($n = 2$) of control cells (Figure 4, right panel; lane 3). These data demonstrate that effects of USF2 Δ B on *Pdx-1* promoter activity translate into marked reductions in *Pdx-1* mRNA and protein levels.

Effect of USF2 Δ B on *Pdx-1* binding to the insulin promoter and on insulin mRNA levels

Two A-T rich elements, termed the A1 and A3 elements, are highly conserved among all characterized mammalian insulin gene promoters [12,50–52]. The predominant β -cell binding activity to the A1 and A3 elements is *Pdx-1* [8–10]. Since overexpression of USF2 Δ B markedly reduced *Pdx-1* mRNA and protein levels, one would predict a commensurate reduction in *Pdx-1* binding activity to the insulin promoter A1 and A3 elements. To test this prediction, EMSAs were performed using a double-stranded oligodeoxynucleotide probe containing the human insulin gene A3 element ($-230/-201$). As observed for both *Pdx-1* mRNA and protein levels, no significant differences in A3 element binding was observed in extracts from INS-1 cells treated with AdCMV- β GAL compared with control cells (Figure 5, lane 2 versus lane 3). However, extracts from AdCMV-USF2 Δ B treated cells reduced A3 binding activities to $44 \pm 4.5\%$ ($n = 3$, $P < 0.001$) compared with control cells (Figure 5, lane 4). To confirm that the A3-binding complex corresponded to *Pdx-1*, the ability of an antibody that recognizes *Pdx-1* to supershift the A3 binding complex was tested. Addition of XIHbox8 antibodies to nuclear extracts isolated from AdCMV- β GAL-infected INS-1 cells supershifted the A3 binding complex (Figure 5, lane 5). These results clearly indicated that the reduction in *Pdx-1* protein levels caused by USF2 Δ B overexpression led to a reduction in *Pdx-1* binding to the A3 element contained in the insulin promoter.

A reduction in *Pdx-1* binding activity to the insulin A3 element would be predicted to cause a reduction in insulin gene expression.

Figure 5 Expression of USF2ΔB decreased Pdx-1 binding activity for the insulin gene promoter

INS-1 cells were mock transduced or transduced with either AdCMV-USF2ΔB or AdCMV-βGAL. Nuclear extracts were isolated 48 h after transduction and analysed by EMSA for binding to the A3 element of the human insulin gene promoter. Lane 1, free probe; lane 2, untransduced cells (Control); lane 3, AdCMV-βGAL transduced cells; lane 4, AdCMV-USF2ΔB transduced cells; and lane 5, AdCMV-βGAL transduced cells plus 2 μl anti-N-terminal XIHbox8 antibodies (ab). The supershifted complex containing Pdx-1 is indicated on the left by an asterisk. This is a representative EMSA of three identical experiments yielding similar results.

Figure 6 Effect of USF2ΔB on insulin mRNA levels

INS-1 cells were mock transduced (Control) or transduced with either AdCMV-βGAL or AdCMV-USF2ΔB. Total RNA was isolated 48 h or 72 h after transduction and insulin and β-actin mRNA levels were determined by Northern-blot analysis. Lane 1, cells harvested 48 h after mock transduction; lane 2, cells harvested 48 hrs after AdCMV-βGAL transduction; lane 3, cells harvested 48 hrs after AdCMV-USF2ΔB transduction; and lane 4, cells harvested 72 hrs after AdCMV-USF2ΔB transduction. The Northern blot shown is the same blot shown in Figure 4 (left panel) after it had been stripped and reprobed for insulin and β-actin. This is a representative Northern blot of four identical experiments.

This prediction was tested by measuring insulin mRNA levels by Northern-blot analysis. As shown in Figure 6, AdCMV-USF2ΔB-treated cells displayed a transient reduction in insulin mRNA levels. Thus 48 h after infection with AdCMV-USF2ΔB, insulin mRNA levels were reduced to $46 \pm 9.9\%$ ($n = 4$, $P <$

0.005) of that observed for control cells (Figure 6, lane 1 versus lane 3). No reductions in insulin mRNA levels were observed 72 h after treatment of INS-1 cells with AdCMV-USF2ΔB. From these data we conclude that the $\sim 55\%$ reduction in Pdx-1 binding activity to the A3 element of the insulin promoter was sufficient to transiently reduce insulin gene transcription. These data are also consistent with the hypothesis that USF functionally regulates *Pdx-1* gene transcription.

DISCUSSION

The critical observation of this study is that overexpression of the dominant-negative USF2 protein, USF2ΔB, reduces *Pdx-1* gene promoter activity. A commensurate reduction in both Pdx-1 mRNA and Pdx-1 protein levels indicates that this change in reporter gene activity detected in a transient transfection assay actually reflected a change in expression of the endogenous *Pdx-1* gene. Thus we conclude that USF plays a functional role in supporting the transcription of the *Pdx-1* gene in the pancreatic β-cell. Two lines of evidence had previously suggested a role of USF in supporting *Pdx-1* promoter activity [19]. First, mutation of a consensus binding site for USF at $-107/-102$ was shown to markedly decrease *Pdx-1* promoter activity in transient transfection experiments. Secondly, USF present in nuclear extracts was shown to bind *in vitro* to the *Pdx-1* E box at $-107/-102$. Similar lines of evidence have been used as an argument for a role for USF in transcriptional activation of a large number of cellular genes. However, given the numerous basic/helix-loop-helix/leucine zipper factors capable of binding to the CACGTG motif recognized by USF, the nature of the actual factor recognizing the E box of the *Pdx-1* (or other cellular genes) *in vivo* has remained unclear. The USF2ΔB form has previously been used to demonstrate a role for USF in supporting the transcription of a bi-directional herpes viral promoter [29] and fatty acid synthase reporter genes [53]. To our knowledge, the present study is the first to demonstrate directly that USF functionally regulates the expression of an endogenous gene encoding a normal cellular protein.

Although it was one of the earliest promoter-specific factors identified, the role of USF as a transcriptional activator has not been well established. Studies using a crude, reconstituted, cell-free system demonstrated that recombinant USF1 could stimulate transcriptional activity from the adenoviral major late promoter and HIV promoters in an E-box dependent manner [54,55]. In co-transfection experiments, ectopic expression of USF1 generally has been found to provide only modest stimulation of promoters containing USF binding E-box sites [55–57]. In other cases, ectopic expression of USF led to significant reductions in promoter activities, perhaps due to displacement of other E-box-binding proteins with stronger activation domains [58]. The most dramatic activities of USF have been detected in situations in which USF acts synergistically with transcription factors from distinct families to stimulate transcription [29,59]. In the case of the *Pdx-1* gene, it is unclear, as yet, whether the actions of USF are mediated in synergy with another factor interacting with the *Pdx-1* promoter sequences.

USF2ΔB overexpression resulted in similar levels of reduction in both the -6500 bp and -190 bp promoter constructs, suggesting that USF2ΔB lessens *Pdx-1* gene promoter activity through a USF regulatory site(s) located within the -190 bp promoter. Attempts were made to determine whether USF2ΔB was inhibiting *Pdx-1* gene promoter activity through interference with the $-107/-102$ E-box motif. Mutation of the $-107/-102$ E-box motif reduced the ability of USF2ΔB overexpression to inhibit *Pdx-1* gene promoter activity suggesting that USF2ΔB, in

part, is decreasing *Pdx-1* gene promoter activity by interfering with binding at the $-107/-102$ E box. However, a decrease in *Pdx-1* gene promoter activity still occurred in constructs containing mutation of the $-107/-102$ E box. Thus USF appears to act at sites other than just the $-107/-102$ E box. The effects of USF have also been found to occur through initiator elements rather than through classic E boxes in the HIV genome [55]. Although the presence of initiator elements in the *Pdx-1* gene have not been demonstrated, the lack of a TATA box motif would be consistent with a role for initiator element(s) in promoting transcriptional initiation. Whatever the site(s) of USF action, the ability of USF2 expression to reverse the inhibitory effect of USF2 Δ B on *Pdx-1* gene promoter activity supports the conclusion that USF2 Δ B overexpression is reducing *Pdx-1* gene promoter activity by directly interfering with USF.

During the course of these studies, the effect of overexpression of USF2 Δ B on the regulation of the L-pyruvate kinase promoter by glucose was also investigated. USF has been shown to bind to the carbohydrate-responsive element of the L-pyruvate kinase promoter *in vitro* [49,60], but whether USF plays a role in mediating glucose-induced changes in pyruvate kinase promoter activity remains controversial [31,34]. As was observed for the *Pdx-1* promoter E box, overexpression of USF2 Δ B led to a decrease in USF binding to the pyruvate kinase E-box sequences ($-165/-149$) contained within the carbohydrate-responsive element (J. Qian, E. N. Kaytor, H. C. Towle and L. K. Olson, unpublished work). Interestingly, overexpression of USF2 Δ B had no effect on basal or carbohydrate-induced expression of a carbohydrate-responsive L-pyruvate kinase reporter gene in primary hepatocytes [34] or in INS-1 cells (J. Qian, E. N. Kaytor, H. C. Towle and L. K. Olson, unpublished work). The contrasting effect of overexpression of USF2 Δ B on the *Pdx-1* gene promoter versus the L-pyruvate kinase promoter supports the concept that not all E-box elements that serve as USF binding sites are functionally regulated by USF.

The decrease in *Pdx-1* gene expression by overexpression of USF2 Δ B coincided with a transient reduction in insulin mRNA levels. The transient nature for the reduction in insulin gene expression is most likely due to the recovery of *Pdx-1* expression that is observed 72 h after treatment with AdCMV-USF2 Δ B. In a recent study, Ahlgren et al. [18] reported that the loss of Pdx-1 protein in conditional knockout mice resulted in a marked reduction in both insulin and GLUT2 protein levels. In a similar manner, we also observed that a decrease in Pdx-1 protein by overexpression of USF2 Δ B reduced GLUT2 levels (results not shown). The decrease in insulin gene expression by overexpression of USF2 Δ B most likely results from the $\sim 55\%$ reduction in Pdx-1 binding activity. The possibility that overexpression of USF2 Δ B is having a direct effect on insulin promoter activity, however, cannot be excluded. Indeed, Read et al. [61] and Cordle et al. [62] have reported that USF binds both the human insulin gene E2 and the rat insulin II E1 elements respectively. It is unlikely, however, that the decrease in insulin gene expression is due to displacement of USF from the insulin E-box elements, because only minimal amounts of USF bind to the rat insulin E-box elements [62].

In the light of our results, disruption of the USF genes within mice would be predicted to cause marked alterations in pancreatic development and β -cell phenotype. Recent studies by Vallet et al. [63,64] and Sirito et al. [65] characterized both USF2 $-/-$ and USF1 $-/-$ mutant mice. No alterations in development or reproduction and no metabolic disorders were observed in USF1 $-/-$ mutant mice [64,65]. In contrast, USF2 $-/-$ mutant mice displayed a growth defect at birth that was maintained throughout postnatal development [65]. In addition, the USF2 $-/-$

mutant mice had decreased viability, lifespan and fertility, and had brain alterations that led to epileptic seizures [65]. As with the USF1 $-/-$ mutant mice, USF2 $-/-$ mutant mice displayed no metabolic disorders [63]. Importantly, embryonic lethality was observed in double USF2 $-/-$ and USF1 $-/-$ mutant mice [64,65], implying that USF is necessary for embryonic development. This also indicates that USF1 or USF2 homodimers are sufficient for the development and maintenance of viable animals. It is likely that overexpression of USF2 Δ B in INS-1 cells displaces both USF2/USF1 heterodimers and USF1/USF1 and USF2/USF2 homodimers. Thus overexpression of USF2 Δ B most likely models the double USF2 $-/-$ and USF1 $-/-$ mutant mice. Because double USF2 $-/-$ and USF1 $-/-$ mutant mice display embryonic lethality, no direct comparison with our studies is possible. In conclusion, our experimental results strongly support a functional role for USF in the regulation of *Pdx-1* gene expression in differentiated pancreatic β -cells.

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