ChREBP rather than USF2 Regulates Glucose Stimulation of Endogenous L-Pyruvate Kinase Expression in Insulin Secreting Cells

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Running title:
Elucidation of Glucose Action on L-Pyruvate Kinase

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There is controversy whether or not upstream stimulatory factors (USF) regulate the glucose-responsiveness of L-pyruvate kinase (L-PK) promoter activity in hepatocytes. It has been suggested that USF-2 is required for glucose stimulation of L-PK promoter activity in single islet \( \beta \)-cells and INS-1 cells (J. Biol. Chem., 1997, 272, 20636-20640). In the present study, the tet-on system has been employed to achieve tightly controlled and inducible expression of USF-1 & -2 and their dominant-negative mutants DN-USF-1 (\( \Delta bTDU1 \)) & -2 (TDU2) in INS-1 cells. Quantitative Northern blot analysis shows that neither basal level nor glucose-responsiveness of endogenous L-PK mRNA is affected by overexpression of USF-1 & -2. Likewise, the L-PK expression is unaltered by dominant-negative suppression of USF function. Western blotting demonstrates that USF-1 & -2 and DN-USF-1 & -2 proteins are stably expressed in nuclear fractions of INS-1 cells. Immunofluorescence staining indicates the uniform induction of these transgene-encoded proteins in the nuclei of INS-1 cells. Electrophoretic mobility shift assays (EMSA) using the L-PK promoter segment reveal that induction of USF-1 & -2 dramatically enhances the USF-binding activity, whereas DN-USF-1 and -2 abolish binding. DN-USF-1 and -2 exert their dominant-negative effect by forming non-functional heterodimers with endogenous USF proteins. Carbohydrate response element binding protein (ChREBP) was recently shown to regulate the glucose-responsiveness of the L-PK promoter activity in hepatocytes. We now report the presence of this transcription factor in rat islets and INS-1 cells. Glucose stimulates ChREBP transcription in INS-1 cells, as show by nuclear run-on experiments. Overexpression of ChREBP in INS-1 cells using the tet-on system results in a left-shift of glucose-responsiveness of L-PK expression and an enhanced L-PK promoter activity. Both endogenous and doxycycline-induced ChREBP proteins bind to the L-PK promoter in a glucose-dependent manner. These unprecedented results suggest that ChREBP rather than USF mediates glucose-promoted L-PK expression in insulin secreting cells.
A set of glucose-responsive genes in hepatocytes has evolved to control the conversion of glucose into triglycerides, when food is sufficient, for storing energy to be used during fasting (1). The pancreatic β-cell responds to increased glucose metabolism by releasing insulin, which is essential for maintaining glucose and fatty acid homeostasis (1). L-Pyruvate kinase (L-PK) represents a typical glucose-responsive gene in both hepatocytes and pancreatic β-cells (2-9). However, there is controversy over the identity of the transcription factor controlling the glucose-stimulated L-PK expression. A carbohydrate response element (ChoRE) containing the E-box sequence CACGTG separated by 5 bp has been located in the L-PK promoter (3,4). Accordingly it has been postulated that a member of the basic/helix-loop-helix/leucine zipper (b/HLH/LZ) family of transcription factors may be involved in carbohydrate-mediated regulation (3,4). Upstream stimulatory factor (USF) was the first transcription factor of this family proposed to regulate the glucose response of the L-PK gene promoter in hepatocytes (3). Using similar experimental approaches, Kaytor et al. (4) obtained opposite results and excluded any involvement of USF in glucose regulation of the L-PK gene in hepatocytes. Homozygous deletion of USF2 in mice resulted in a delayed glucose-responsiveness of hepatic L-PK expression, possibly suggesting an indirect role for USF2 (6). In addition, microinjection of USF2 antibodies into the nucleus of single INS-1 cells caused a drastic inhibition of glucose-stimulated L-PK promoter activity (5). Conversely, Kaytor et al. (7) demonstrated that adenovirus-mediated overexpression of a dominant-negative form of USF in INS-1 cells failed to block the glucose-induced L-PK promoter activity and again negated the involvement of USF in glucose-regulation in INS-1 cells. The present study was aimed to clarify these conflicting results. We employed the tet-on system (10) in INS-1 cells to achieve tightly controlled and conditional expression of USF-1 & -2 and their dominant-negative mutants (3) DN-USF-1 (ΔbTDU1) & -2 (TDU2). These stable cell clones allowed evaluation of the involvement of USF in the regulation of endogenous L-PK mRNA levels at various concentrations of glucose.

Recent studies showed that a ChoRE binding protein (ChREBP), another member of b/HLH/LZ family, bound to the L-PK promoter in a glucose-dependent manner and that transient transfection of primary hepatocytes with ChREBP led to increased L-PK promoter activity (8,9). The present study demonstrates that ChREBP is also expressed in isolated pancreatic islets and in INS-1 cells. We provide the first direct evidence that ChREBP left-shifts the glucose-
responsiveness of endogenous L-PK expression in INS-1 cells engineered for inducible ChREBP action.

EXPERIMENTAL PROCEDURES

Cloning of the Mouse ChREBP cDNA, Construction of Plasmids, and Generation of Stable Cell Lines—Two mouse ChREBP cDNA fragments, 750 bp (1-750, containing Xba I site at 743) and 2335 bp (705-3040, carrying Xba I site at 743 and Bgl II site at 2689), were cloned by reverse transcription-PCR using mouse liver RNA and two pairs of primers, respectively: 5\'atgccgacgaggccatgctcgcgcgtg3', 5'ggctgctagaagccggccccca3'; 5'ttgagccggccagggagag3', 5'gacccagtggcctcagtcag3'. The PCR products were inserted into the pGEM-T Easy Vector (promega/Catalys, Wallisellen, CH) and sequenced. The full length ChREBP cDNA was constructed by subcloning of Sac II-Xba I (1-743) and Xba I-Bgl II (743-2689) fragments into the expression vector PUHD10-3 (a kind gift from Dr. H. Bujard) (10). Four cDNAs encoding respectively USF-1 & -2 (11) (kindly supplied by Dr. M. Sawadogo) and DN-USF-1 (\(\Delta b\)TDU1) & -2 (TDU2) (3) (generously provided by Dr. M. Raymondjean and A. Kahn) were also subcloned into PUHD10-3 for the secondary stable transfection. The first-step INS-1 derived clone INSr\(\beta\), which expresses the reverse tetracycline-dependent transactivator, was reported previously (12). The stable transfection and the clone selection and screening procedures were described by Wang and Iynedjian (13). The USF-1 and -2 antibodies used for screening USF-positive clones were also supplied by Dr. A. Kahn and Dr. M. Sawadogo.

Total RNA Isolation and Northern Blotting—Total RNA was extracted and blotted to nylon membranes according to Wang and Iynedjian (13). The membrane was pre-hybridized and then hybridized to \(^{32}\)P-labeled random primer cDNA probes as previously described (13). To ensure equal RNA loading and even transfer, all membranes were stripped and re-hybridized with a probe encoding the "housekeeping gene" cyclophilin. cDNA fragments used as probes for USF1, USF2, ChREBP mRNA detection were digested from corresponding plasmids.

Cell Fractionation—Cells in 10-cm diameter dishes were cultured with or without 500 ng/ml doxycycline for 24 h. After washing twice with ice-cold PBS, the cells were suspended and allowed to swell for 15 min at 4°C in 400 \(\mu\)l hypotonic buffer composed of 20 mM Tris (pH7.4), 5 mM EDTA, 2 mM DTT and 0.2 mM phenylmethylsulfonyl fluoride. After 3-cyles of freeze-
thaw, the cytosolic proteins (supernatant) were separated from the nuclear fraction (pellet) by centrifugation. The nuclear proteins were further isolated from the pellet according to Schreiber et al. (14).

**Immunoblot**-Immunoblotting procedures were performed as described previously using enhanced chemiluminescence (Pierce, Rockford, IL) for detection (12). The dilution for antibodies against, respectively, human USF-1 or -2 C-terminus (Santa Cruz Biotech./LabForce, Nunningen, CH) was 1:2,000.

**Immunofluorescence**-For immunofluorescence cells grown on polyornithine treated glass coverslips were treated with or without 500 ng/ml doxycycline for 24 h. The cells were then washed, fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline containing 1% BSA (PBS-BSA). The preparation was then blocked with PBS-BSA before incubating with rabbit polyclonal antibodies against, respectively, the C-terminus of human USF-1 and -2 (1:200 dilution), followed by the second antibody labeling.

**Isolation of Cell Nuclei and Transcriptional Run-On Assay**-INS-1E cells cultured in 15-cm dishes were equilibrated in 2.5 mM glucose medium for 24 h, and then incubated for further 6 h at 2.5 and 24 mM glucose, respectively. The cells were rinsed twice with phosphate-buffered saline at 4 °C, scraped in the same buffer, and harvested by centrifugation at 500 g for 5 min at 4 °C. The cell pellet was resuspended in 4 ml lysis buffer containing 10 mM Tris-Cl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, and 0.5% (v/v) NP-40 and incubated on ice for 5 min (15). Cell nuclear pellet were obtained by centrifugation for 5 min at 500 g, 4 °C, and then washed once with the same lysis buffer. The cell nuclei were resuspended in 200 µl storage buffer containing 50 mm Tris-Cl, pH 8.3, 40% glycerol, 5 mM MgCl₂, and 0.1 mM EDTA, and immediately snap-frozen in liquid nitrogen (15).

For the run-on assay, cell nuclei were washed by centrifugation in labeling buffer (20 mM Tris-Cl, pH 8.0, 140 mM KCl, 10 mM MgCl₂, 1 mM MnCl₂, 20% (v/v) glycerol, and 14 mM β-mercaptoethanol), and then incubated for 30 min at 30 °C in 80 µl labeling buffer containing 1 mM each of ATP, GTP, and CTP, 10 mM phosphocreatine, 100 µg/ml phosphocreatine kinase, and 10 µCi ³²P-UTP (16). The radio-labeled nascent RNA transcripts were purified using QIAshredder and RNase columns (Qiagen) following manufacture’s protocol. Hybridization of the transcripts to filter-bound cDNA plasmids was carried out as described (15).
Transient Transfection and Luciferase Assay-Cells were transfected with the L-PK gene promoter luciferase plasmid, PK(-183)Luc (kindly provided by Dr. H. Towle) (4), using the calcium-phosphate-DNA precipitation method as described previously. Cells were then cultured for 24 h with or without 500 ng/ml doxycycline at indicated concentrations of glucose. Luciferase reporter enzyme assays were performed as previously reported (17).

Nuclear Extract Preparation and Electrophoretic Mobility-Shift Assay (EMSA)-Nuclear extracts from cells cultured at indicated conditions were prepared according to Schreiber et al. (14). The double-stranded oligonucleotides probe corresponding to the L-PK promoter ChoRE element was described previously: 5'gggcgcacgaggccactccctgggtcc3'; 5'ggaaccacgggagtggcgcgtgcgccc3' (9). EMSA procedures including conditions for probe labeling, binding reactions, and antibody supershift were performed as in Wang et al. (12). Antibodies against, respectively, human USF-1 or -2 C-terminus were used.

RESULTS

Establishment of INS-1 Stable Cell Lines Expressing USF-1 & -2, DN-USF-1 & -2, and ChREBP Using tet-on System-After screening 60-80 hygromycine-resistant clones for each transgene with Western blotting, we obtained 5-10 clones expressing USF-1 &-2, and DN-USF-1 & -2 proteins under induced conditions. The clones designated as USF-1#63, DN-USF-1#2, USF-2#15, and DN-USF-2#21, which displayed the highest inducibility and no "leakage" of extrinsic genes in the absence of doxycycline, were chosen for the present study. The mouse ChREBP cDNA was cloned as described in the Experimental Procedures. Four ChREBP-positive clones were detected out of 40 hygromycine-resistant lines using Northern blotting analysis. The clone termed ChREBP#16 was chosen for the following experiments.

Basal or Glucose-Stimulated Endogenous L-PK mRNA Levels Are Not Altered by USF-The consequences of induction of USF-1 & -2 and DN-USF-1 & -2 on the endogenous L-PK expression was evaluated quantitatively by Northern analysis in USF-1#63 (Fig. 1A), DN-USF-1#2 (Fig. 1B), USF-2#15 (Fig. 1C), and DN-USF-2#21 (Fig. 1D) cells. As shown in Fig. 1, glucose increased L-PK mRNA levels in these four cell lines. USF-1 and -2 bind to the ChoRE L-PK promoter by forming homo- and hetero-dimers (3,4,11). We demonstrate that over 10-fold
induction of USF-1 (Fig. 1A) and USF-2 (Fig. 1C) failed to raise the level of L-PK mRNA. Titration of the induction levels of USF-1 and -2 by reducing the dose of doxycycline gave similar results (data not shown).

DN-USF-1 and DN-USF-2 exert their dominant-negative function by forming non-functional heterodimers with endogenous USF proteins (3). Three days have been suggested to be required for the effects of dominant-negative USF on L-PK expression (18). We analyzed total RNA samples isolated from DN-USF-1#2 (Fig. 1B), and DN-USF-2#21 (Fig. 1D) cells treated with or without 500 ng/ml doxycycline for 80 h. We found that neither basal nor glucose-stimulated L-PK mRNA levels were altered by dominant-negative suppression of USF function (Fig. 1B & D).

Transgene Encoded Proteins are Expressed in Nuclear Fractions of INS-1 Cells—Regarding the involvement of USF in the regulation of glucose-stimulated L-PK promoter activity, opposite conclusions were drawn by two different groups using similar approaches (3,4). Because protein levels of USF and DN-USF in transiently transfected hepatocytes were not monitored in these studies (3,4), it is difficult to judge the molecular bases underlying the discrepancy. As shown in Fig. 2, USF-1 (Fig. 2A), DN-USF-1 (Fig. 2B), USF-2 (Fig. 2C), and DN-USF-2 (Fig. 2D) proteins were detected predominantly in the nuclear fractions of our INS-1-derived stable clones. Induction of DN-USF-1 (Fig. 2B) and -2 (Fig. 2D) did not affect protein levels of endogenous USF-1 and -2.

Transgene Encoded Proteins are Uniformly Induced—To confirm that these INS-1-derived cells behave as a homogenous population in response to doxycycline-induction, we performed immunofluorescence experiments. As shown in Fig. 3, nuclear localized USF-1 & -2, and DN-USF-1 & -2 proteins were induced homogeneously.

Induction of USF Enhances Its Binding to the L-PK Promoter and DN-USF Abolishes Such Binding—USF-1 and -2 bind to the ChoRE of L-PK as both homo- and heterodimers (3,4,11). As demonstrated in Fig. 4A and B, induction of USF-1 and -2 resulted in, respectively, 10- and 20-fold increases in the USF-binding activities. The specificity of both endogenous and induced USF binding to the L-PK ChoRE was proved by supershift assays with antibodies directed against the C-terminus of either USF-1 (Fig. 4A) or USF-2 (Fig. 4B). DN-USF-1 (3), which contains the intact dimerization motif but lacks transactivation and DNA-binding domains, exerts its dominant-negative action by forming non-functional heterodimers with endogenous USF proteins. As expected, induction of DN-USF1 almost completely eradicated the endogenous USF
binding (Fig. 4C). DN-USF-2 (3), which lacks the transactivation domain, exerts its dominant-negative function by forming USF/DN-USF-2 heterodimers and DN-USF-2 homodimers to compete with endogenous USF for the cognate DNA binding. As shown in Fig. 4D, induction of DN-USF-2 eliminated endogenous USF binding. Due to the large excess of DN-USF-2, its homodimer binding was predominant over the USF/DN-USF-2 heterodimer (Fig. 4D). Antibody supershift assay indicated the identity of these retarded binding complexes (Fig. 4D).

**ChREBP Is Expressed in Islets and in INS-1 Cells; Glucose Induces Its Transcription**

ChREBP was identified in hepatocytes (8,9). We found that ChREBP mRNA was also expressed in rat islets and in INS-1E cells but not in brain or spleen (Fig. 5A). Glucose is also inducing the ChREBP mRNA in INS-1E cells (Fig. 5B). The expression pattern of ChREBP and its glucose-responsiveness correlate to that of L-PK (Fig. 5AB). To characterize whether the increased ChREBP mRNA levels in response to rising glucose concentrations is regulated at the rate of transcription, we performed the nuclear run-on assay. A representative experiment is shown in Fig. 5C. Glucose stimulated the transcriptional rate of ChREBP gene by an average of 3.2 ± 0.4 (four independent experiments).

**Overexpression of ChREBP in INS-1 Cells Affects Glucose-Responsiveness of L-PK mRNA**

As shown in Fig. 6, L-PK mRNA levels in ChREBP#16 cells were elevated dose-dependently in response to extracellular glucose concentrations. Induction of ChREBP led to a typical leftward shift of glucose-dependent expression of endogenous L-PK mRNA (Fig. 6).

**Glucose Regulates Binding of Endogenous and Induced ChREBP to the L-PK Promoter**

EMSA with the L-PK ChoRE probe was performed to examine the effect of glucose on ChREBP binding activities in both INS-1E cells (Fig. 7A) and ChREBP#16 clone (Fig. 7B). In INS-1E cells, the endogenous ChREBP binding activity was enhanced in response to glucose concentrations (Fig. 7A). Similarly, in ChREBP#16 cells, both endogenous and induced ChREBPs bound to the L-PK promoter in a glucose-concentration dependent manner (Fig. 7B). Induction of ChREBP led to an 8-fold increase in the signal density of retarded ChREBP complexes (Fig. 7C). The binding activity of ChREBP correlated well with the expression of L-PK mRNA (Fig. 6).

**ChREBP rather than USF2 regulates the glucose-stimulated L-PK promoter activity**

To define whether ChREBP indeed activates L-PK gene transcription by directly acting on its promoter, we examined the effects of overexpression of ChREBP on the L-PK gene promoter luciferase activity. As demonstrated in Fig. 8A, induction of ChREBP increased the L-PK promoter activity
by 3- and 4-fold, respectively, at 2.5 mM and 24 mm glucose. In contrast, similar induction of USF2 (Fig. 8B) and DN-USF2 (Fig. 8C) did not alter the L-PK promoter activity.

**DISCUSSION**

There is controversy regarding the role of USF in the regulation of glucose-stimulated L-PK expression in hepatocytes, pancreatic β-cells and INS-1 cells (3-7). Two groups showed contradictory results of the L-PK reporter enzyme activity, using transient transfection of hepatocytes with USF and DN-USF (3,4). However, the expression levels of USF and DN-USF were not examined in either study. Vallet et al. (6) have reported a delayed response of glucose-induced L-PK expression in the livers of USF-2-deficient mice. This could be secondary to alterations in expression of USF-2-target genes such as the liver glucokinase. USF positively regulates the expression of this gene (19). Microinjection of USF antibodies into the nuclei of INS-1 cells could have pleotropic effects on the L-PK promoter luciferase activity (5) and data showing diminished luciferase activity should therefore be interpreted with caution. Indeed, this work (5) is strongly challenged by Kaytor et al. (7) who suggest that adenovirus-mediated expression of DN-USF does not interfere with glucose-induced L-PK promoter activity. Unfortunately, the expression level and cellular localization of DN-USF in the latter study was not monitored (7).

The present study was designed to assess the role of USF in the regulation of glucose-induced L-PK expression in a more controlled manner. We established four stable INS-1-derived clones, permitting inducible expression of USF-1 & -2, and DN-USF-1 & -2, respectively. The expression level and cellular localization of these transgene-encoded proteins are well documented in our study. We also show that these nuclear localized USF and DN-USF proteins are induced homogeneously in a doxycycline-dependent manner. Furthermore, we demonstrate that induction of USF-1 and -2 led to over-10 fold increase in the USF binding to the L-PK ChoRE, whereas induction of DN-USF-1 and -2 abolished endogenous USF binding activity. Moreover, we illustrate with EMSA that DN-USF-1 exerts its dominant-negative action by forming non-functional heterodimers with endogenous USF, while DN-USF-2 forms predominantly homodimers to compete for the cognate DNA-binding. Finally, using quantitative Northern blot analysis we evaluate the consequences of up- and down-regulation of USF function.
on glucose-responsiveness of endogenous L-PK mRNA. We conclude that USF has no effect on either basal or glucose-stimulated L-PK expression in INS-1 cells. Consistently, induction of USF2 or DN-USF2 does not affect the L-PK promoter activity in INS-1 cells, which supports the study of Kaytor et al. (7) but contradicts the report of Kennedy et al. (5).

It has been suggested that a novel b/HLH/LZ transcription factor distinct from USF regulates the glucose response of the L-PK promoter (20). Yamashita et al. (9) purified this protein based on its ability to bind to the L-PK ChoRE and identified it as ChREBP by sequencing the digested peptides. In addition, ChREBP binds to the L-PK promoter and translocates to the nucleus of hepatocytes in a glucose-dependent manner (8,9). Furthermore, overexpression of ChREBP in primary hepatocytes by transient transfection causes enhanced glucose-induced L-PK promoter activity (8,9). The present study includes the following novel findings. We show that ChREBP is also expressed in rat islets and INS-1 cells. More importantly, we find that glucose stimulates the expression of ChREBP at the transcriptional level in INS-1 cells. Our results provide the first direct evidence that ChREBP induces the expression of endogenous L-PK mRNA in insulin-secreting cells by activating the L-PK promoter activity. Furthermore, in INS-1 cells both endogenous and doxycycline-induced ChREBP proteins bind to the L-PK promoter in response to glucose. It has been well documented in hepatocytes that glucose activates the nuclear translocation of ChREBP protein by dephosphorylation of Ser\(^{196}\) in the cytoplasm and also stimulates the DNA-binding activity by dephosphorylation of Thr\(^{666}\) in the nucleus (8,9). It remains to be established whether similar mechanisms also apply to INS-1 cells.

We therefore conclude that ChREBP rather than USF regulates the glucose-responsiveness of L-PK expression in INS-1 cells. It is possible that a similar mechanism participates in the regulation of other glucose-sensitive genes in hepatocytes and \(\beta\)-cells.

Acknowledgments—We are grateful to D. Cornut-Harry, Y. Dupre, and V. Calvo for expert technical assistance. We are indebted to Drs A. Kahn (DN-USF-1 and -2 cDNAs and USF antibodies), M. Sawadogo (USF-1 and -2 cDNAs and USF-antibody), H. Towle (PK(-183)Luc construct), P. B. Iynedjian (INS-r9 cells), H. Bujard (PUHD 10-3 vector), and N. Quintrell (pTKhygro plasmid). This work was supported by the Swiss National Science Foundation (grant no. 32-49755.96 and 32-66907.01).
The abbreviations used are: L-PK, liver-type pyruvate kinase; ChoRE, carbohydrate response element; ChREBP, ChoRE binding protein; USF, upstream stimulatory factor; DN-USF, dominant-negative mutant of USF; b/HLH/LZ, basic/helix-loop-helix/leucine zipper; PCR, polymerase chain reaction.

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FIG. 1. Effects of up- or down-regulation of USF function on endogenous L-PK mRNA levels. L-PK mRNA was quantified by Northern blotting. 20 µg of total RNA samples were analyzed by hybridizing with a L-PK cDNA probe. The same membrane was stripped and rehybridized with cyclophilin and USF cDNA probes. A, USF-1#63 cells were cultured with or without 500 ng/ml doxycycline in standard glucose medium (11.2 mM) for 24 h and continued for a further 24 h in 2.5 mM glucose medium before incubation at indicated glucose concentrations for an additional 8 h. B, DN-USF-1#2 cells were cultured in the presence or absence of 500 ng/ml doxycycline in standard glucose medium for 48 h. The culture was continued for a further 24 h in 2.5 mM glucose medium and incubated for an additional 8 h at indicated glucose concentrations. C, USF-2#15 cells were treated as described in (A). D, DN-USF-2#21 cells were cultured as indicated in (B). The experiment was repeated three to four times with similar results.

FIG. 2. USF and DN-USF proteins were induced in the nuclear fraction of INS-1 cells. Nuclear extracts and cytosolic proteins were prepared from USF-1#63 (A), DN-USF-1#2 (B), USF-2#15 (C), and DN-USF-2#21 (D) cells cultured with or without 500 ng/ml doxycycline for 24 h in standard (11.2 mM) glucose medium. 10 µg protein from nuclear extracts and 50 µg
protein from the cytosolic fraction were resolved by 11% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with antibodies against, respectively, the USF-1 or USF-2 C-termini. kDa, kilodaltons. Data show a representative Western blotting from two independent experiments.

FIG. 3. Uniform induction of Nuclear located USF and DN-USF proteins. Immunofluorescence staining with antibodies against the USF-1 or USF-2 C-termini is shown. Cells were cultured in the presence or absence of 500 ng/ml doxycycline for 24 h in standard (11.2 mM) glucose medium. The experiment was repeated twice with similar results.

FIG. 4. Effects of USF and DN-USF induction on USF-binding to the L-PK Promoter. Gel-shift assay was performed with oligonucleotide duplex corresponding to the ChoRE of an L-PK promoter. The binding reaction contained 8 µg nuclear extracts from USF-1#63 (A), USF-2#15 (B), DN-USF-1#2 (C), and DN-USF-2#21 (D) cells cultured with or without 500 ng/ml doxycycline for 24 h in standard (11.2 mM) glucose medium. The identity of retarded USF binding complexes was confirmed by supershifting with antibodies against the C-terminus of USF-1 and -2. The experiment was repeated four times with similar results.

FIG. 5. ChREBP is expressed in islets and its transcription is regulated by glucose. A, 20-µg RNA samples were analyzed by Northern hybridization with a 32P-labeled ChREBP cDNA probe. The same membranes were then stripped and re-hybridized with L-PK and 18 S probes. Total RNAs extracted from rat liver, brain and spleen or islets cultured in 24 mM glucose for 4 h. Data represents a typical Northern blotting from three separate experiments. B, Northern blot analysis of total RNA isolated from INS-1E cells cultured 24 h in 2.5 mM glucose medium, and incubated for further 8 h at indicated glucose concentrations. The experiment was repeated twice with similar results. C, Transcriptional run-on assay in nuclei isolated from INS-1E cells cultured 24 h in 2.5 mM glucose medium, and incubated for further 6 h at indicated glucose concentrations. Radio-labeled nascent transcripts were hybridized with indicated filter-bound plasmids. Data show a representative run-on assay from four independent experiments.
FIG. 6. *Induction of ChREBP in INS-1 cells causes a left-shift of glucose-responsiveness of endogenous L-PK expression.* The mRNA levels of L-PK were quantified by Northern blotting. ChREBP#16 cells were cultured with or without 500 ng/ml doxycycline in standard glucose medium (11.2 mM) 24 h, and then a further 24 h in 2.5 mM glucose medium, before incubation at indicated glucose concentrations for an additional 8 h. A, A representative Northern blotting experiment. 20 µg of total RNA samples were analyzed by hybridizing with an L-PK cDNA probe. The same membrane was stripped and rehybridized with ChREBP and cyclophilin cDNA probes. B, Quantification of L-PK mRNA levels from three independent Northern blot analyses using densitometer (Mean ± SEM). The densitometer value from cells cultured without doxycycline at 2.5 mM glucose was assigned as 1.

FIG. 7. *Endogenous and induced ChREBPs bind to the L-PK promoter in a glucose-dependent manner.* EMSA was performed with an oligonucleotide probe corresponding to the ChoRE of the L-PK promoter. The binding reaction was carried out with 8 µg nuclear extracts. A, INS-1E cells were cultured for 24 h in 2.5 mM glucose medium, and incubated for a further 8 h at indicated glucose concentrations. B, ChREBP#16 cells were cultured with or without 500 ng/ml doxycycline 24 h in 2.5 mM glucose medium, and incubated for a further 8 h at indicated glucose concentrations. C, ChREBP#16 cells were cultured in the presence or absence of 500 ng/ml doxycycline 24 h in standard (11.2 mM) glucose medium.

FIG. 8. *ChREBP rather than USF2 transactivates the L-PK promoter.* ChREBP#16 (A), USF-2#15 (B) DN-USF-2#21 (C) cells were transfected with plasmid PK(-183)Luc. After 24 h culture with or without 500 ng/ml doxycycline at 2.5 and 24 mM glucose, respectively, cells were collected and 20 µg cytosolic protein was assayed for luciferase activity. Luciferase activity measured in non-induced cells cultured at 2.5 mM glucose was defined as 1. Data represent Mean ± SEM from five to six independent experiments. Induction of ChREBP significantly enhanced the L-PK promoter activity (p<0.0001), whereas USF2 and DN-USF2 had no effects.
Glucose  

| 2.5 | 6  | 12 | 24 | 2.5 | 6 | 12 | 24 | (mM) |

Doxycycline  

| - | + | - | + | - | + | - | + | 500 ng/ml |

Cyclophilin

USF1

L-PK

Cyclophilin

USF2

L-PK

FIG. 1
**FIG. 2**

- **A** and **B** show the effects of Doxycycline on the localization of USF1 and DNUSF1 in the nuclear and cytosolic compartments.

- **C** and **D** demonstrate the anti-USF1 and anti-USF2 effects of 500 ng/ml.

- USF1#63 and DNUSF1#2 are indicated with arrows and corresponding bands at 66 kDa, 45 kDa, and 29 kDa.
FIG. 3
|        | A: USF1#63 | B: USF2#15 | C: DNUSF1#2 | D: DNUSF2#21 |
|--------|-----------|------------|-------------|--------------|
| USF antibody | - + - +  | - + - +   | - - +      | - - +       |
| Doxycycline | - - + +  | - - + +   | - + +      | - + +       |

500 ng/ml

**FIG. 4**

USF1/2

USF/DNUSF2

DNUSF2
FIG. 5

A

Liver
Brain
Islets
Spleen

B

INS-1E

Glucose
2.5
6
24 mM

ChREBP →
L-PK →

18 s →

C

Glucose
2.5
24 mM

ChREBP →
Vector →
β-Actin →

Downloaded from www.jbc.org/ by guest on March 23, 2020
A

Cyclophilin
ChREBP
L-PK

Doxycycline -+ -+ -+ -+ 500 ng/ml

Glucose 2.5 6 12 24 (mM)

B

Optical Density (Fold of Basal)

FIG. 6
| Glucose | Doxycycline |
|---------|-------------|
| 2.5     | -           |
| 6       | +           |
| 12      | -           |
| 24      | +           |

**FIG. 7**

A | B | C
---|---|---
INS-1E | ChREBP#16 | ChREBP#16
2.5 | 6 | 12 | 24
- | + | - | + | +
11.2 | - | + | - | +

mM | 500 ng/ml
---|------------
- | ChREBP
- | USF
FIG. 8
ChREBP rather than USF2 regulates glucose stimulation of endogenous L-pyruvate kinase expression in insulin secreting cells
Haiyan Wang and Claes B. Wollheim

J. Biol. Chem. published online June 26, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M201635200

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