c-Myc and ChREBP regulate glucose-mediated expression of the L-type pyruvate kinase gene in INS-1-derived 832/13 cells

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Submitted 19 July 2006; accepted in final form 1 March 2007

Collier JJ, Zhang P, Pedersen KB, Burke SJ, Haycock JW, Scott DK. c-Myc and ChREBP regulate glucose-mediated expression of the L-type pyruvate kinase gene in INS-1-derived 832/13 cells. Am J Physiol Endocrinol Metab 293: E48–E56, 2007. First published March 6, 2007; doi:10.1152/ajpendo.00357.2006.—Increased glucose flux generates metabolic signals that control transcriptional programs through poorly understood mechanisms. Previously, we demonstrated a necessity in hepatocytes for c-Myc in the regulation of a prototypical glucose-responsive gene, L-type pyruvate kinase (L-PK) (Collier JJ, Doan TT, Daniels MC, Schurr JR, Kolls JK, Scott DK. J Biol Chem 278: 6588–6595, 2003). Pancreatic β-cells have many features in common with hepatocytes with respect to glucose-regulated gene expression, and in the present study we determined whether c-Myc was required for the L-PK glucose response in insulin-secreting (INS-1)-derived 832/13 cells. Glucose increased c-Myc abundance and association with its heterodimer partner, Max. Manipulations that prevented the formation of a functional c-Myc/Max heterodimer reduced the expression of the L-PK gene. In addition, glucose augmented the binding of carbohydrate response element binding protein (ChREBP), c-Myc, and Max to the promoter of the L-PK gene in situ. The transactivation of ChREBP, but not of c-Myc, was dependent on high glucose concentrations in the contexts of either the L-PK promoter or a heterologous promoter. The glucose-mediated transactivation of ChREBP was independent of mutations that alter phosphorylation sites thought to regulate the cellular location of ChREBP. We conclude that maximal glucose-induced expression of the L-PK gene in INS-1-derived 832/13 cells involves increased c-Myc abundance, recruitment of c-Myc, Max, and ChREBP to the promoter, and a glucose-stimulated increase in ChREBP transactivation.

Max: carbohydrate response element-binding protein; pancreatic β-cells; transcription

THE LIVER-TYPE PYRUVATE KINASE (L-PK) gene, encoding a key glycolytic enzyme, is induced in vivo in hepatocytes and islet β-cells during the fasting-to-fed transition (16, 35, 45, 48, 50, 53). The predominant signals regulating this gene are not generated by insulin but, rather, by the metabolism of glucose (12, 44). Although many genes are regulated by glucose, the L-PK gene has become a prototypical model system for determining the molecular mechanisms of glucose signaling in both hepatocytes and β-cells (16, 33, 35, 45, 46, 48, 50, 52, 53). Our group recently demonstrated that c-Myc is required for glucose-regulated expression of the L-PK gene in hepatocytes (10), and the present study focuses on the role of c-Myc in the regulation of this gene in β-cells.

c-Myc and its heterodimer partner, Max, are basic helix-loop-helix leucine-zipper (bHLH-LZ) transcription factors that influence cellular growth, proliferation, differentiation, apoptosis, and metabolism (11, 20). The Myc/Max heterodimer recognizes the consensus E-box sequence (5′-CACGTG-3′) as well as other similar noncanonical sequences in the promoters of their target genes (4). Once bound to DNA, Myc/Max recruits coactivators with histone acetyltransferase (HAT) activity, which opens chromatin for the efficient assembly of preinitiation complexes (1, 13, 14, 36). Mad, another bHLH-LZ transcription factor, also binds Max, and Mad/Max heterodimers bind the same elements as Myc/Max. However, Mad/Max heterodimers promote gene repression through the recruitment of corepressor complexes that contain histone deacetylase activity (17).

The carbohydrate-responsive portion of the L-PK gene promoter, and the promoters of several other glucose-responsive genes, contain sequences very similar to those recognized by the Myc-Max-Mad network (3, 43). Carbohydrate response elements (ChoREs) contain two noncanonical E-boxes separated by 5 bp (41). A number of transcription factors have been proposed to bind to ChoREs and mediate the glucose response (26–28, 30, 50). However, the identification of carbohydrate response element binding protein (ChREBP) has provided a plausible mechanism for glucose-mediated gene expression (26). ChREBP binds to ChoREs as a heterodimer with Mlx and is a member of the c-Myc superfamily (42, 50, 54). According to a model developed by Kawaguchi et al. (26), ChREBP resides in the cytoplasm and cannot bind DNA during the fasted state because of the phosphorylation of Ser196 and Thr666 by cAMP-dependent protein kinase. During the fed state, increased glucose flux through the hexose monophosphate pathway increases the concentration of xylulose-5-phosphate, which activates protein phosphatase 2A (25). The increased activity of this phosphatase (and perhaps a second nuclear phosphatase) leads to the dephosphorylation of residues 196 and 666, allowing nuclear transport, targeted DNA binding, and, ultimately, glucose-regulated gene expression. However, the details of this model have recently been challenged (33, 46, 52).

In the present study, we demonstrate that reducing functional c-Myc levels or augmenting the abundance of Mad blunts L-PK gene expression. In addition, we present evidence that glucose facilitates formation of Myc/Max heterodimers and increases c-Myc and Max binding to the L-PK promoter. Furthermore, we show that although both c-Myc and ChREBP...
are recruited to glucose-responsive promoters, only ChREBP requires glucose for transactivation.

**MATERIALS AND METHODS**

**Cell culture.** The clonal 832/13 rat insulinoma cell line was isolated from the original INS-1 insulinoma cell population and cultured as previously described (22).

Construction, preparation, and use of recombinant adenoviruses. The adenoviruses containing cDNAs encoding antisense c-myc (AdCMV-ASmyc) (10), Mad1 (AdCMV-Mad1) (7), or β-galactosidase (AdCMV-βGal) (21), were described previously. The adenovirus expressing luciferase (AdCMV-Luc) was obtained from the Louisiana State University Health Sciences Center Vector Core. Transduction efficiency was determined by treating 832/13 cells with twofold serial dilutions of AdCMV-βGal for 1 h. Cells were washed with phosphate-buffered saline (PBS), and the adenovirus was allowed to express for ∼40 h, at which time the cells were fixed and incubated with X-gal substrate according to the protocol provided by Stratagene. This experiment showed that 100 plaque-forming units (pfu) per cell of AdCMV-βGal were required to produce 100% positive (stained blue) cells (data not shown). Thus, for experiments with adenoviruses, incubations of up to 100 pfu/cell for 1 h were used. The medium was refreshed, and cells were incubated overnight, followed by treatment with effectors as described in legends.

siRNA-mediated suppression of gene expression. The expression of c-myc was decreased by transfecting preannealed duplexes from Ambion (catalog no. 189043; Austin, TX; negative control, catalog no. AM4611) into 832/13 cells using Dharmafect reagent 1 (Dharmacon, Lafayette, CO) according to the manufacturer’s suggested protocol.

RNA isolation and measurement of RNAs by RT-PCR. Total RNA was isolated from 832/13 cells using Tri reagent (Molecular Research Designs) according to the manufacturer’s instructions to create pSG424 to contain restriction sites for SpeI and PacI using the QuikChange site-directed mutagenesis kit (Stratagene) and inserting SpeI- and PacI-digested cDNAs encoding wild-type, Ser196Ala, Thr666Ala, and the double mutant ChREBP [cDNAs provided by Dr. Howard Towle (42)] into pSG424 so that Gal4-DDB and the ChREBP proteins were expressed as chimeras. The plPK-183 plasmid (a gift from Dr. Howard Towle) was constructed as described previously (43). The ChRE in the L-PK promoter (from −167 to −150) was replaced with the 17-bp Gal4 DNA binding site (6) by using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions to create plPK*. The plasmid, 5xGal4-E1b, was a gift from Dr. Daryl Ganner.

**Transient transfection.** Transient transfection experiments using Lipofectamine (Invitrogen) were carried out as previously described (10). Luciferase activity was detected using the Dual-Luciferase reporter assay system (Promega) in a TD-20/20 luminometer (Turner Designs).

**ChREB** antibodies. Polyclonal rabbit antibodies were raised against a synthetic peptide (residues 431–442 of rat ChREBP) that was conjugated to keyhole limpet hemocyanin (Sigma) via the sulf-hydryl moiety of a Cys residue added at the end of the ChREBP sequence. Anti-ChREBP antibodies were affinity purified using column chromatography on a peptide-SulfoLink Plus column (Pierce) as previously described (19).

Chromatin immunoprecipitation assays. 832/13 cells, after treatment for 6 h with 2 or 20 mM glucose and reaching ∼90% confluence, were exposed to 1% formaldehyde for 10 min at room temperature. Glycine was added to a final concentration of 0.125 M, and after 5 min, the samples were washed twice and harvested in cold PBS with protease inhibitors (catalog no. 1183617001; Roche Diagnostics). Cells were collected by centrifugation for 4 min at 2,000 g and suspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1). Chromatin immunoprecipitation (ChIP) assays were performed by following the Upstate Biotechnology ChIP assay kit protocol (catalog no. 17-295) with slight modifications. Briefly, the cell lysate was sonicated to yield 100- to 1,000-bp genomic DNA fragments. The lysate (2 ml) was precleared with 75 µl of a 50% slurry of protein A-agarose that contained 32 µg of sonicated salmon sperm DNA, 80 µg of BSA, and 160 µg of recombinant protein A-agarose suspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.05% sodium azide for 30 min at 4°C with agitation. After centrifugation at 1,000 g for 2 min, aliquots of the supernatant were incubated with an antibody directed against ChREBP (see above), c-Myc, or Max or with normal rabbit IgG (catalog nos. SC764, SC222, and SC2027, respectively; Santa Cruz Biotechnology) overnight with agitation at 4°C. Immuno-complexes were recovered by incubation with a 50% slurry of salmon sperm DNA-protein A-agarose, in the buffer described above, for 1 h at 4°C. The beads were washed for 5 min each with low-salt (catalog no. 20-154; Upstate Biotechnology), high-salt (catalog no. 20-155; Upstate Biotechnology), and LiCl immune complex buffer (catalog no. 20-156; Upstate Biotechnology) and twice with TE buffer (10 mM Tris, pH 8.0, 0.5 mM EDTA). The chromatin complexes were eluted by adding freshly prepared elution buffer (1% SDS, 0.1 M NaHCO$_3$) with rotation at room temperature for 15 min, followed by centrifugation and collection of the supernatant. The process was repeated, and the two eluates were combined. The cross-linking was reversed by adding NaCl to a final concentration of 500 mM and heating at 65°C for 4 h. After incubation with 20 mg of proteinase K for 1 h, the DNA was purified using a Qiagen PCR purification column, and target genes were quantified by real-time PCR using the purified DNA as template and the reaction conditions described above. Standard curves were constructed using 2-fold serial dilutions of the DNA extracted from the 2 mM glucose IgG treatment (2.5 µl) as a reference input. The protein content of the different treatment groups was normalized using the Pierce BCA protein assay before immunoprecipitation, and the quantity of the amplicons was expressed as a percentage of the
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The viral genome could be a total reference input. The primer sequences for the PCR reactions were as follows (upstream and downstream, respectively): glucokinase, 5'-CAGTTGTCCTGTCTCTGCTCCATCAG-3' and 5'-ATA CCCTGCTAGTGTCACAAGG-3'; L-PK, 5'-GGATGCCAATA-TAGCCT CA-3' and 5'-CCATGCTGCTAGTGTCCTATGTTGGCA-3'; and rat nucleolin, 5'-CCGCGTCCGAGGCACTG-3' and 5'-TCATCTAC-GTCACCGGTGAGC-3'.

**Statistical analysis.** The ChIP experiments were analyzed as simple split-plot designs by ANOVA according to Ref. 32, with whole plots derived from the two groups of formaldehyde-treated, sheared chromatin collected from cells grown at either 2 or 20 mM glucose and with the split plots being the two aliquots of the chromatin preparations that were “precipitated” with either a control IgG antibody or an antibody directed against a particular transcription factor (ChREBP, c-Myc, or Max). Separate ANOVAs were calculated for each target that was amplified by PCR. Since the standard deviations of target concentrations tended to be proportional to the means, the data were logarithm-transformed before the ANOVA was performed. After ANOVA, group means were compared using the least significant difference procedure analysis. Differences of the target concentration at 2 compared with 20 mM glucose for a particular antibody/antiserum were made after calculation of the approximate degrees of freedom by using Satterthwaite’s procedure. Means ± SE were calculated and retransformed back to linear scale.

All results apart from the ChIP assay data are expressed as means ± SE. Data analyses were performed using the statistics module of Microsoft Excel (version 9.0). A two-tailed t-test was used to generate P values, of which those <0.05 were considered statistically significant.

**RESULTS**

Glucose increases the expression of c-Myc in 832/13 cells. Several laboratories have demonstrated that high glucose levels (20–30 mM) increase c-Myc transcript and protein levels in rat islets and insulinoma cells (23, 24, 55). As shown in Fig. 1, A and B, a 16-h treatment with 20 mM glucose resulted in greater than threefold higher c-myc mRNA levels than in cells treated with either 2 mM glucose or 2 mM glucose plus 18 mM mannitol, used as a control for osmotic pressure on the cells (40). These results are consistent with those obtained from other cell lines and from isolated rat islets (23, 55).

Since the 832/13 cells were selected from the INS-1 population based on their robust insulin-secreting properties (22), we tested whether autocrine or paracrine effects of secreted insulin contributed to the increased expression of the c-myc gene. Addition of insulin (from 10^{-10} to 10^{-8} M) in the presence of 2 mM glucose did not augment c-myc mRNA levels (data not shown), demonstrating that secreted insulin is not responsible for the glucose effect. These findings are in agreement with those reported for RINr cells, in which neither insulin nor IGF-1 is able to induce expression of the c-myc gene (55), and those reported for rat islets, in which neither addition of exogenous insulin nor inhibition of insulin secretion affects c-myc mRNA levels (23). Furthermore, as shown in Fig. 1, C and D, 20 mM glucose increased c-Myc protein abundance sevenfold over the controls. We conclude that glucose increases c-Myc mRNA and protein abundance in 832/13 rat insulinoma cells, in agreement with previous observations using similar model systems (23, 24, 55).

L-PK gene expression is increased by glucose. The metabolism of glucose generates potent signals that control expression of various genes, including L-PK, in pancreatic β-cells (23, 31, 51). A 16-h treatment of 832/13 cells with 20 mM glucose produced a 4.6-fold increase in L-PK mRNA levels relative to 2 mM glucose, whereas 2 mM glucose supplemented with 18 mM mannitol had no effect (Fig. 2). These results are in agreement with previous observations (35, 37).

We tested whether the autocrine or paracrine effects of secreted insulin were responsible for the increase in expression of the L-PK gene. At 2 mM glucose, increasing the insulin concentration in the culture medium from 10^{-10} to 10^{-8} M for 16 h did not augment L-PK mRNA levels (Fig. 2). Furthermore, treatment over this same range of insulin concentrations at 20 mM glucose did not have any noticeable effect on L-PK mRNA levels (data not shown). This result confirms an earlier
cells and HIT cells) than the 832/13 cells. This experiment allowed us to determine whether or not there was a correlation between proliferation and the glucose-mediated induction of the c-Myc and L-PK genes. We found that both c-Myc and study in which insulin did not induce expression of the L-PK gene in INS-1 cells (35). Furthermore, the L-PK gene promoter is not expected to respond to insulin because it does not contain a sterol response element (30). We therefore conclude that insulin does not increase L-PK mRNA levels.

AdCMV-ASmyc blocks the glucose-mediated accumulation of c-Myc. AdCMV-ASmyc, an adenovirus expressing antisense c-myc mRNA, effectively reduces c-Myc protein levels in rat hepatoma cells, pancreatic β-cells, and developing mouse lung tissue (8, 10, 47). c-Myc abundance was reduced by ~80% (P < 0.01, n = 3) in glucose-stimulated 832/13 cells that expressed AdCMV-ASmyc relative to AdCMV-βGal-treated cells (Fig. 3, A and B).

Preventing the formation of functional c-Myc/Max heterodimers interferes with L-PK gene expression. We tested whether manipulations that either decreased c-Myc or increased Mad levels would interfere with the glucose-mediated induction of the L-PK gene. The addition of 20 mM glucose to the 832/13 cells increased L-PK mRNA levels more than fivefold compared with treatment with 2 mM glucose (Fig. 3C). However, treatment with AdCMV-ASmyc reduced the glucose-mediated induction by ~60%, whereas AdCMV-βGal had no significant effect. To ensure that the effects seen were not due to a nonspecific adenoviral effect, we decreased the abundance of c-Myc using a siRNA approach. The transfection of siRNA duplexes targeting the c-Myc transcript significantly decreased mRNA levels of this gene (data not shown). This maneuver lowered basal levels of L-PK mRNA and decreased the glucose response of the L-PK gene to basal levels, whereas a control siRNA had no effect (Fig. 3D).

Rapidly dividing cells express very little Mad protein (2). Not surprisingly, Mad is undetectable in nuclear fractions of 832/13 insulinoma cells. However, transducing cells with AdCMV-Mad1 generated immunodetectable quantities of this transcription factor (Fig. 4A). We found that overexpression of Mad1 reduced the glucose-mediated induction of L-PK mRNA by ~40% compared with cells transduced with the control adenovirus (Fig. 4B).

Because c-Myc plays a direct role in controlling cellular proliferation (1), we examined pancreatic β-cell lines that are less sensitive to glucose-induced proliferation (e.g., βTC-1

Fig. 2. Glucose increases liver-type pyruvate kinase (L-PK) mRNA levels in 832/13 cells. Total RNA was isolated from 832/13 cells treated for 16 h with media containing 2 mM glucose, 20 mM glucose, or 2 mM glucose + 18 mM mannitol. In addition, some cells were treated for the same length of time with 2 mM glucose + 10^{-10}, 10^{-9}, or 10^{-8} M insulin. The graph represents relative RNA levels, as measured by real-time RT-PCR, normalized to 18S RNA levels. Values represent means ± SE from 3 independent experiments. *P < 0.05 vs. 2 mM glucose.

Fig. 3. Interfering with the induction of c-Myc inhibits the glucose-mediated expression of L-PK. A: 832/13 cells were treated with 100 pfu/cell of adenovirus expressing either β-galactosidase (AdCMV-βGal) or antisense c-Myc (AdCMV-ASmyc) for 1 h. The cells were then incubated for 18 h in medium containing 5 mM glucose, followed by an additional 16-h treatment at 20 mM glucose. Nuclear extracts were harvested, and the abundance of c-Myc was determined by immunoblotting. Equal protein loading was confirmed by immunoblotting for the amount of tubulin. B: the graph represents the means ± SE of relative protein levels normalized to tubulin levels and determined by densitometry. *P < 0.05 vs. βGal. C: 832/13 cells were treated for 18 h with 25, 50, or 100 pfu of either AdCMV-βGal or AdCMV-ASmyc or were left untreated. The cells were then cultured in 2 or 20 mM glucose for 16 h, and total RNA was extracted. The graph represents relative L-PK mRNA levels analyzed by RT-PCR and densitometry of reverse-imaged ethidium bromide-stained agarose gels of the PCR products, normalized to the abundance of cyclophilin mRNA. The experiment was performed 3 times, each in duplicate to triplicate. *P < 0.05 vs. 20 mM βGal. D: 832/13 cells were transfected with a control siRNA duplex (siCon) or with siRNA duplexes targeted to a 21-nt region of the rat c-myc gene (siMyc). Following 48 h of culture at 11 mM glucose, the cells were treated with media containing either 2 or 20 mM glucose for an additional 6 h. The levels of L-PK mRNA were measured by quantitative RT-PCR. Data are expressed as means ± SE from 4 independent experiments. **P < 0.01 vs. siCon.
Glucose augments ChREBP, c-Myc, and Max occupancy on the L-PK gene promoter. ChREBP appears to mediate the glucose response of the L-PK gene in both hepatocytes and pancreatic β-cells (25, 33, 50, 52). To assess the role of c-Myc in this process, we tested the ability of glucose to promote the binding of ChREBP, c-Myc, and Max to the L-PK gene promoter using a quantitative ChIP assay with antibodies raised specifically against each of these transcription factors (Fig. 6). Using primers specific for the promoter region containing the ChoRE of the L-PK gene, we found that precipitations with antibodies against ChREBP, c-Myc, and Max yielded a higher concentration of PCR amplicons than a control IgG at both low and high glucose concentrations. By contrast, the ChIP experiments revealed no (ChREBP and c-Myc) or only weak (Max) binding of the transcription factors to the GK gene promoter, which provided a negative control. As a positive control for c-Myc binding, we used a primer set specific for the regulatory region of the nucleolin gene, a known target of c-Myc that contains several E-boxes (18). We found that c-Myc and Max bound abundantly to the nucleolin regulatory region compared with negative controls. Indeed, binding of c-Myc and Max to the L-PK gene was modest by comparison. Furthermore, there was more ChREBP, c-Myc, and Max bound to the L-PK gene promoter after treatment with 20 mM glucose compared with 2 mM glucose and more c-Myc bound to the nucleolin promoter after treatment with 20 mM glucose. Thus glucose promotes the recruitment of the transcriptional regulators ChREBP, c-Myc, and Max to the L-PK gene promoter.

Glucose transactivates ChREBP but not c-Myc. c-Myc, Max, and ChREBP are recruited to the L-PK gene promoter upon glucose stimulation (Fig. 6) and thus may play direct roles in glucose-mediated gene expression in pancreatic β-cells. To determine whether glucose affects the activity of these regulatory molecules after binding to DNA, we used plasmids expressing chimeras of the yeast transcription factor Gal4 DNA binding domain (pGal4-DBD) and either ChREBP or c-Myc (pGal4-ChREBP and pGal4-Myc, respectively).

Glucose promotes Myc-Max association. Our working hypothesis is that glucose regulates gene expression by initiating the recruitment of active regulatory complexes to their target gene promoters. We first tested whether glucose promotes a functional Myc/Max heterodimer complex using a coimmunoprecipitation assay. Nuclear fractions incubated with antisera directed against Max coimmunoprecipitated amounts of c-Myc that directly correlated with the glucose concentration (Fig. 5). This is consistent with the rise in c-Myc abundance generated by glucose (Fig. 1) and confirms that glucose flux generates concentration-dependent increases in heterodimer complexes.

L-PK genes were induced by glucose in βTC-1 cells (in our hands, βTC-1 cells grew slower than 832/13 cells but faster than HIT cells) after a 16-h treatment of 20 mM glucose (~2-fold each, n = 3, P < 0.05; data not shown) but that only the L-PK gene was induced by glucose in HIT cells with the same treatment (~2-fold, n = 3, P < 0.05; data not shown). Taking these data together, we conclude that a functional c-Myc/Max complex is necessary for the maximal induction of the L-PK gene in INS-1-derived 832/13 cells cultured in either low or high concentrations of glucose. Furthermore, these observations may be reflective of their proliferative capacity.

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long with an L-PK reporter construct wherein the ChoRE was replaced with a Gal4 DNA binding site (pLPK*). Transfection of the wild-type L-PK promoter-luciferase reporter plasmid [pLPK-183, (43)] resulted in an 18-fold (± 0.8SE, n = 3) glucose response when cells grown in 20 mM glucose were compared with cells cultured in 2 mM glucose (Fig. 7A). Cotransfection of pLPK* and pGal4-Myc led to modest transcriptional activity when cells were cultured in either 2 or 20 mM glucose compared with cotransfection with a vector that expressed the Gal4 DNA binding domain alone (6.1-fold ± 0.7SE, n = 3, and 5.3-fold ± 0.8SE, n = 3, respectively). A key finding is that the transcriptional activity provided by pGal4-Myc was not significantly different in cells treated with 2 or 20 mM glucose, which is distinct from that observed for ChREBP (see below). Indeed, a similar result was observed for ChREBP, since they agree with the model proposed by Kawaguchi et al. (26) for ChREBP transcriptional activity is independent of phosphorylation on known regulatory residues.

Under physiological conditions, the abundance of c-myc RNA and protein is increased in β-cells treated acutely with high glucose (Refs. 23, 55; Fig. 1). This same treatment also stimulates transcription of the β-cell L-PK gene (35, 37). Manipulations that decreased c-Myc levels or increased levels of the antagonistic transcription factor Mad reduced expression of the L-PK gene (Figs. 3 and 4). In addition, we found that glucose increases c-Myc/Max interaction (Fig. 5). These observations are consistent with the idea that the relative abundance of Mad, c-Myc, Max, ChREBP, and Mlx may be important control points in the regulation of glucose-responsive genes (46, 52).

The current study is consistent with both ChREBP and c-Myc having important but functionally distinct roles in glucose-regulated gene expression. Glucose promotes the recruitment of ChREBP and, to a lesser extent, c-Myc and its heterodimer partner Max to the L-PK promoter (Fig. 6). These observations were expected for ChREBP, since they agree with the model proposed by Kawaguchi et al. (26) for ChREBP activity when cells were exposed to 20 mM glucose (8.8-fold ± 0.8SE, n = 3; Fig. 7A). Kawaguchi et al. (26) have shown that phosphorylation of Ser196 results in the retention of the transcription factor in the cytoplasm and that phosphorylation of Thr666 prevents DNA binding. We obtained constructs wherein these two amino acids were changed to alanine, either separately or together, to determine whether the phosphorylation state of these residues had any effect on the transcriptional activity of Gal4-ChREBP. As shown in Fig. 7, A and B, none of the mutations of Gal4-ChREBP had transcriptional activity when cultured in 2 mM glucose, whereas all of the Gal4-ChREBP constructs displayed similar transcriptional activity when exposed to 20 mM glucose. This was true in the context of the pLPK* reporter construct (~9-fold activation compared with 2 mM glucose) and in the context of the 5xGal4-E1b reporter gene (~1,700-fold). We therefore conclude that 1) glucose is required for the transcriptional activity of ChREBP but not c-Myc, 2) this effect is independent of the phosphorylation state of residues 196 and 666, and 3) the glucose-dependent transactivation of ChREBP is independent of promoter context.

**DISCUSSION**

The L-PK gene responds to changes in glucose metabolism in both hepatocytes and pancreatic β-cells (16, 33, 35, 45, 46, 48, 50, 52, 53). Thus the L-PK gene is an excellent model for investigating the mechanism of glucose-regulated gene expression in both cell types. In previous studies using hepatocytes, we demonstrated that c-Myc, a bHLH-LZ transcription factor, regulates expression of the L-PK gene (10). In the present study, we used the INS-1-derived β-cell line, 832/13 (22), to investigate the role of c-Myc in the glucose-mediated induction of L-PK gene expression. Several key findings emerged: 1) maneuvers that diminish c-Myc levels or enhance Mad abundance blunt L-PK gene expression, in both low and high glucose; 2) glucose facilitates the recruitment of c-Myc, Max, and ChREBP to the promoters of glucose-responsive genes; 3) ChREBP, but not c-Myc, requires glucose for its transcriptional activity; and 4) the glucose-dependent increase in ChREBP transcriptional activity is independent of phosphorylation on known regulatory residues.

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function in hepatocytes wherein dephosphorylation of Ser\textsuperscript{196} and Thr\textsuperscript{666} allows nuclear translocation and DNA binding, respectively (26). This model predicts that alanine-substituted mutations of these residues in ChREBP, expressed as a chimera with a Gal4 DNA binding domain and cotransfected with promoter-reporter constructs with Gal4 DNA binding sites, would be constitutively active in low glucose. By contrast, we found that glucose was absolutely necessary for ChREBP transactivation, regardless of the phosphorylation state of ChREBP or the promoter context of the reporter vector (Fig. 7). This observation suggests that in addition to dephosphorylation, other molecular events, such as the recruitment of a coactivator, perhaps triggered by increased glucose flux and metabolism, are required for ChREBP function. These results are in concert with recent observations in both hepatocytes and β-cells, which act together to relieve repressed transactivation in the presence of glucose (33).

By contrast, we found that Gal4-Myc was not dependent on glucose for transactivation in the context of the L-PK promoter (Fig. 7). Furthermore, we performed transient transfection experiments to test whether the expression of a dominant negative Max interfered with L-PK promoter activity or with transcriptional activation provided by multimerized ChoREs and found no effect (data not shown). This observation suggests that c-Myc/Max heterodimers do not bind as a heterotetramer with ChREBP/Mlx on ChoREs, in concert with the recent observations of Ma et al. (34) showing that two sets of ChREBP/Mlx heterodimers bind to ChoREs. How do c-Myc and ChREBP work together to communicate a glucose signal to the L-PK gene? One possibility is that c-Myc/Max heterodimers bind transiently, recruiting histone acetyltransferase (HAT) activity to open the chromatin of glucose-responsive genes, allowing ChREBP and Mlx to bind and transactivate efficiently. This model is consistent with the relatively modest recruitment of c-Myc and Max after glucose treatment (Fig. 6).
and with a number of recent studies showing that c-Myc recruits HAT activity to target genes and may act primarily to remodel chromatin so that “secondary” transcription factors are able to more effectively mediate transcription of specific genes (1, 9, 14, 15, 29, 36, 49). Another possibility, one that is not mutually exclusive, is that c-Myc/Max heterodimers exert their influence from sites distinct from the ChREB but near enough (200–1,000 bp) to be detected in the ChIP assays. Experiments are underway to differentiate between these and other possibilities, as well as to determine the temporal relationships of transcription factors as they are recruited to the L-PK promoter. It should be noted that our observations are consistent with those in transformed cells in which c-Myc is necessary for increased expression of glycolytic enzyme genes and glycolysis (11). Thus the necessity of c-Myc for a maximal glucose response may be, in part, reflective of the glucose-mediated increase in proliferation seen in insulinoma cells.

In conclusion, glucose induces Myc/Max association and recruits c-Myc, Max, and ChREBP to the L-PK gene promoter in 832/13 rat insulinoma cells. c-Myc depletion or Mad overexpression blunts the expression of the L-PK gene in these cells. Furthermore, ChREBP, but not c-Myc, requires glucose for transactivation. Together, these results demonstrate important roles for both c-Myc and ChREBP in the expression of the L-PK gene.

ACKNOWLEDGMENTS

We thank Dr. Christopher Newgard for the 832/13 cell line, Dr. Sanjeev Gupta for the AdCMV-Mad adenovirus, Dr. Daryl Granner for the 5×E1b plasmid, Dr. Howard Towle for the pLPK-183 plasmid and ChREBP cDNAs, Dr. Peggy Farnham for the Gal4-Myc and pSG424 vectors, Dr. Michael S. Lan for the βTC-1 and HIT cells, and the Louisiana State University Gene Therapy Vector Core, Dr. Jay Kolls, and Dr. Jacob Reiser for assistance with adenoviral production. We also thank members of the Scott and Claycomb laboratories for critical reading of the manuscript.

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GRANTS

This work was supported by American Diabetes Association Career Development and Research Awards and by National Institute of Diabetes and Digestive and Kidney Diseases Grant R01 DK065149 (to D. K. Scott).

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