ChREBP-Mlx Is the Principal Mediator of Glucose-induced Gene Expression in the Liver*5

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In mammals, glucose-regulated gene expression has been best characterized in the liver, where increased glucose metabolism induces transcription of genes encoding enzymes involved in de novo lipogenesis. ChREBP and Mlx dimerize and function together as a glucose-responsive transcription factor to regulate target genes, such as liver-type pyruvate kinase, acetyl-CoA carboxylase 1, and fatty acid synthase. To identify additional glucose-responsive genes in the liver, we used microarray analysis to compare gene expression patterns in low and high glucose conditions in hepatocytes. Target genes of ChREBP-Mlx were simultaneously identified by gene profiling in the presence or absence of a dominant negative Mlx. Of 224 genes that are induced by glucose, 139 genes (62%) were also inhibited by the dominant negative Mlx. Lipogenic enzyme genes involved in the entire pathway of de novo lipogenesis were found to be glucose-responsive target genes of ChREBP-Mlx. Genes encoding enzymes in other metabolic pathways and numerous regulators of metabolism were also identified. To determine if any of these genes are direct targets of ChREBP-Mlx, we searched for ChoRE-like sequences in the 5′-flanking regions of several genes that responded rapidly to glucose. ChoRE sequences that bound to ChREBP-Mlx and supported a glucose response were identified in two additional genes. Combining all of the known ChoRE sequences, we generated a modified ChoRE consensus sequence, CAYGNNGN5CNCRTG. In summary, ChREBP-Mlx is the principal transcription factor regulating glucose-responsive genes in the liver and coordinately regulates a family of genes required for glucose utilization and energy storage.

One of the primary organs responsible for controlling energy homeostasis in mammals is the liver. In fasting conditions, the liver produces glucose through glycogenolysis and gluconeogenesis to maintain glucose homeostasis and meet the energy requirements of other tissues. In times of severe energy shortage, liver will supply alternative energy sources in the form of ketone bodies. After food intake, it is a major site for carbohydrate storage in the form of glycogen. In addition, triglycerides, the major energy storage form in mammals, are synthesized in the liver for packaging into very low density lipoprotein particles and export to other tissues. However, in nonalcoholic fatty liver disease, accumulation of triglyceride occurs in the liver. This condition is rising in proportion to obesity in the human population and may contribute to pathologies associated with the metabolic syndrome (1).

Triglycerides are produced from either fatty acids obtained directly from the diet or synthesized de novo when excess carbohydrates are consumed. De novo lipogenesis in the liver is regulated by multiple hormonal and nutritional signals (2). Enzymes involved in glucose uptake, glycolysis, and lipogenesis are induced by consuming a meal high in carbohydrate. One signaling pathway critical for induction of lipogenic enzyme genes involves insulin, acting in part through the transcription factor SREBP-1c3 to induce gene expression (3–5). Increased glucose metabolism has been shown to trigger an independent signaling pathway that transcriptionally activates lipogenic genes, in many cases synergistically with insulin (6, 7). Genes that respond to glucose contain a specific regulatory site, the carbohydrate response element (ChoRE), in their promoter regions. To date, ChoREs have been mapped within the promoter regions of the liver-type pyruvate kinase (PK), S14, fatty acid synthase (FAS), acetyl-CoA carboxylase 1 (ACC), and thioredoxin-interacting protein genes (7, 8). Based on these known sites, the consensus ChoRE has been proposed to consist of two E box motifs with the sequence CACGTG separated by 5 base pairs (7).

ChREBP is a basic helix-loop helix/leucine zipper transcription factor involved in mediating glucose-responsive gene activation (9). ChREBP is most abundantly expressed in tissues where lipogenesis is highly active, such as the liver. It shuttles between the cytoplasm and nucleus in a glucose-responsive manner in hepatocytes (10). Mice with a disruption of the ChREBP gene or hepatocytes treated with siRNA to reduce ChREBP expression cannot induce lipogenic gene expression in response to carbohydrate (11, 12). In hepatocytes prepared from ChREBP null mice, the induction can be restored by the

* This work was supported by National Institutes of Health Grant DK26919 and Minnesota Obesity Center Grant P30 DK50456. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1–4.
2 These two authors contributed equally to this work.
3 The abbreviations used are: SREBP, sterol regulatory element-binding protein; ChoRE, carbohydrate response element; ChREBP, carbohydrate response element-binding protein; PK, liver-type pyruvate kinase; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase 1; Mlx, Max-like factor X; dnMlx, dominant negative Mlx; FGF-21, fibroblast growth factor-21; G0S2, G0/G1 switch gene 2; GKRP, glucokinase regulatory protein; GPDH, glyceraldehyde-3-phosphate dehydrogenase 1; MTTP, microsomal triglyceride transfer protein; TXNIP, thioredoxin-interacting protein; RT, reverse transcription; HA, hemagglutinin; GLUT, glucose transporter.
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addition of a ChREBP expression vector (13). Thus, ChREBP is essential for regulating lipogenic gene expression. However, we have previously reported that ChREBP requires an interaction partner, Mlx, to efficiently bind to ChoRE sequences and exert its functional activity (14). Mlx is a basic helix-loop helix/leucine zipper protein that heterodimerizes with several partners, including ChREBP; MondoA, a paralog of ChREBP expressed predominantly in skeletal muscle; and the repressors Mad1, Mad4, and Mnt (15–17). Expressing a dominant negative form of Mlx in hepatocytes completely inhibits the glucose response of a number of lipogenic enzyme genes, including PK, S₁₄, ACC, and FAS (18). This inhibition is rescued by overexpressing ChREBP but not MondoA. Therefore, Mlx is an obligatory partner of ChREBP in regulating glucose-responsive lipogenic enzyme genes.

Many questions still remain regarding the physiological role of ChREBP-Mlx. What is the profile of genes induced in hepatocytes by glucose? Is ChREBP-Mlx the common regulator for these glucose-responsive genes, or are additional transcription factors involved? What is the relationship between genes induced by ChREBP-Mlx and the insulin-stimulated transcription factor SREBP-1c? In this study, we used microarray analysis to characterize glucose-responsive genes in primary hepatocytes. Moreover, we determined whether these glucose-responsive genes are target genes of ChREBP-Mlx by comparing differentially expressed genes in the presence or absence of dominant negative Mlx (dnMlx) in hepatocytes cultured in high glucose conditions. These studies demonstrate that ChREBP-Mlx is the principal glucose-responsive transcription factor in the liver regulating a range of metabolic pathways involved in glucose homeostasis and energy storage.

EXPERIMENTAL PROCEDURES

Primary Hepatocyte Culture and Isolation of RNA—Male Sprague-Dawley rats (200–300 g) were fed ad libitum, and primary hepatocytes were isolated by the collagenase perfusion method (19). After a 3-h attachment, cells were transduced with adenovirus expressing either dnMlx (Mlx b/a) or green fluorescent protein (18) (for 3 h in Medium 199 (Invitrogen) containing 5.5 mM (low) glucose, 0.01 μM dexamethasone, and 0.1 units/ml insulin. The amount of virus used was sufficient to ensure >95% transduction and to completely block glucose activation of a ChoRE-containing promoter. Cells were maintained in low glucose medium for 16 h and then cultured in either low or high (27.5 mM) glucose medium for an additional 24 h. Total cellular RNA was isolated using TRIzol reagent (Invitrogen) and further purified for cRNA synthesis using the RNeasy kit (Qiagen). Each RNA aliquot was isolated from a pool of three plates to minimize variability, and RNA samples were prepared in triplicate.

Affymetrix Microarray Analysis—RNA samples were converted into biotin-labeled cRNA using the Qiagen Target Prep Robot by the University of Minnesota Biomedical Genomics Center Affymetrix Microarray core. Labeled samples were hybridized to the rat genome 230 2.0A array. Expressionist (Genedata Inc.) software was used for microarray data analysis. Robust multiarray average (20) probe level analysis was implemented using quantile normalization, robust multiarray average model-based background correction, perfect match only probe selection, and median polish summarization methods. Differentially expressed genes were selected at a threshold of 1.8-fold difference between groups with a significance level of p ≤ 0.01.

Measurement of mRNAs by Quantitative RT-PCR—Selected gene products from the microarray analysis were confirmed by real time RT-PCR performed by a two-step procedure described previously (18). Primers were designed using MacVector (Accelrys Software Inc.) and are shown in supplemental Table 4. For the time course experiment, results of RT-PCR are expressed as the fold induction by normalizing the mean of Ct values from individual time points to the mean of Ct values from low glucose hepatocytes at zero time. All samples were analyzed in triplicate and expressed as mean ± S.D.

Identification of Potential ChoREs—Sequences of 10 glucose-responsive, dnMlx-repressed genes (dermatopontin, FGF-21, fructokinase, G0S2, glucokinase regulatory protein (GKRP), GLUT4, glycogen synthase 2, glyceral-3-phosphate dehydrogenase 1 (GPDH), microsomal triglyceride transfer protein (MTTP), and protein phosphatase 1 G₁ subunit) were searched for potential ChoREs. DNA sequences from −15,000 to +3,000 relative to the transcriptional start site for each gene in rats, mice, and humans were extracted (except GKRP, which is not mapped to the rat genome). These sequences were subsequently entered into the Transcriptional Element Search System Web site (available at www.cbil.upenn.edu/tess) and analyzed using search strings from all known ChoREs as well as a perfect E-box sequence with appropriate spacing (CACGT-GnmmnCCGTG). Sequences that matched one or more of the test sequences at a minimum of 9 of 12 positions and found in all three genomes within a region of sequence homology were selected. An electrophoretic mobility shift assay was conducted as described previously (30). FLAG-tagged ChREBP (S196A/T666A) and HA-tagged Mlx were expressed in 293 cells, and cell lysates were prepared using the whole cell extract protocol (Active Motif, Carlsbad, CA). Double mutant ChREBP was used for electrophoretic mobility shift assay experiments, because it enhances DNA binding to ChoREs (14). A typical reaction contained 100,000 cpm of 32P-labeled oligonucleotide (see supplemental Table 4) and 5 μg of whole cell extract. For reactions with antibodies, proteins were first incubated with HA or FLAG antibodies (Sigma) for 30 min at 4°C.

Functional Analysis of Putative ChoREs—PCR of rat genomic DNA was used to generate promoter sequences from the rat GPDH (−2014 to +11), rat GLUT4 (−1010 to +26), mouse GKRP (−529 to +33), and rat G0S2 (−499 to +24) genes. In each case, the cloned genomic segment, containing the putative ChoRE sequence, was cloned into the pGL3 reporter vector. The putative ChoRE sequences in G0S2 (−213/−197) and GLUT4 (−838/−822) reporter vectors were deleted by site-directed mutagenesis (Stratagene). Oligonucleotides containing two copies of putative ChoRE sequences (17-bp ChoRE sequence plus 5 bp of flanking DNA on both sides) were cloned in a head-to-tail fashion upstream of the PK(−40/−12) basal promoter in the reporter plasmid pGL3. Each construct was transfected with an internal control that expresses Renilla lucif-
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RESULTS AND DISCUSSION

Identification of Glucose-responsive Genes and ChREBP-Mlx Target Genes by Microarray Analysis—Primary hepatocytes were cultured in three different conditions: low glucose, transduced with a control green fluorescence protein adenovirus; high glucose, transduced with a control green fluorescence protein adenovirus; and high glucose, transduced with a dnMlx adenovirus. Insulin was present to allow effective glucose metabolism but was held constant between samples. The dnMlx construct was prepared by mutating two conserved basic residues in the basic region of Mlx (18). The resulting mutant Mlx heterodimerizes with ChREBP, since its HLH/LZ domain is intact, but this heterodimer is not able to bind to DNA. Therefore, the dnMlx prevents the binding and function of ChREBP-Mlx. To minimize interanimal variability, all cells originated from a single animal. Furthermore, each RNA sample was isolated from a pool of three plates to minimize intersample variability. Subsequently, RNA samples were processed to produce cRNA and were hybridized to the Affymetrix rat genome 230 2.0 gene chip containing transcripts from over 28,000 well substantiated rat genes. The algorithm used for microarray data analysis was robust multiaarray average, which is a statistically robust averaging method that yields background-adjusted, normalized, and log-transformed perfect match values (20).

The expression of 283 genes was significantly changed at a threshold of 1.8-fold by switching from low glucose to high glucose conditions (supplemental Table 1). Of these, 224 genes were induced in high glucose conditions, whereas 59 were repressed (Fig. 1). Comparing genes influenced by dnMlx in high glucose conditions, the expression of 204 genes was affected at least 1.8-fold (supplemental Table 2). Of these, 178 were repressed by dnMlx, and 26 were increased. Of the 224 genes induced by glucose, 139 were also repressed by dnMlx (Fig. 1, supplemental Table 3). Previously characterized glucose-responsive target genes of ChREBP-Mlx (PK, S14, ACC, and FAS) were identified in the group of overlapping gene products. Furthermore, the results of the microarray analysis were confirmed by RT-PCR for 20 selected gene products with no false positive found. Hence, the experimental strategy was effective in detecting known targets of ChREBP-Mlx. The extent of overlap between glucose-induced and dnMlx-repressed gene products was 62%. However, this number is probably a serious underestimate of the true degree of overlap. Of the 85 genes in the group that were induced by glucose but not repressed by dnMlx at least 1.8-fold, about 50% of these genes were repressed by dnMlx between 1.5- and 1.7-fold. Similarly, of the 39 genes in the group repressed by dnMlx but not induced more than 1.8-fold by glucose, about 80% of these genes were induced by glucose between 1.5- and 1.7-fold. These genes are probably glucose-responsive genes regulated by ChREBP-Mlx as well. If we include these genes, the extent of overlap between glucose-induced and dnMlx-repressed genes increases to 83%. Since ChREBP is the only known Mlx-interacting activator in the liver, we conclude that ChREBP-Mlx is the major transcriptional complex responsible for activating hepatic glucose-responsive genes.

Additionally, 59 genes are repressed by glucose, and 26 genes are increased in the presence of dnMlx. Between these two groups, only eight genes are shared (Fig. 1). The minimal extent of overlap in these two groups suggests that ChREBP is not a major glucose-responsive repressor in the liver. We have not further analyzed genes in these groups. It is worth noting, however, that genes expressed more highly in the presence of dnMlx but not affected by glucose could represent targets of Mlx interacting with one of its repressive partners, Mad1, Mad4, or Mnt. Since no target genes are known for Mlx interacting with any of its repressive partners, this group is worthy of further investigation.

ChREBP-Mlx Influences the Entire Pathway of de Novo Lipogenesis—The synthesis of triglycerides from simple carbohydrate requires multiple metabolic pathways: glycolysis and pyruvate oxidation to provide acetyl-CoA, fatty acid synthesis from acetyl-CoA, NADPH generation to supply the reductive power, packaging of fatty acids into the glycerophosphate backbone, and lipoprotein packaging to transport triglycerides. Interestingly, glucose-responsive genes that are inhibited by dnMlx were found in all of these pathways, indicating that ChREBP-Mlx activates the entire program of de novo lipogenesis (Fig. 2). Three different hexose transporters were identified as glucose-responsive target genes of ChREBP-Mlx: GLUT2, GLUT4, and GLUT5. GLUT2 is the major glucose transporter in the liver and has previously been shown to be induced by glucose in both hepatocytes and β cells (21, 22). GLUT4 is not expressed at significant levels in the liver (23). Surprisingly, GLUT4 is the most highly affected gene product in response to both glucose

erase into hepatocytes in low glucose medium and switched to either low or high glucose after 16 h. Twenty-four h later, cells were harvested, and luciferase activity was measured and normalized to Renilla luciferase expression.
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FIGURE 2. Genes of de novo lipogenesis are induced by glucose and repressed by the dnMlx. A schematic of the de novo lipogenesis pathway is shown. Genes induced by glucose and repressed by dnMlx are indicated in boldface type. PK, fructokinase; AldB, aldolase B; GKR, glucokinase regulatory protein; GPI, glucose phosphate isomerase; G6PDH, glucose-6-phosphate dehydrogenase; TKT, transketolase; PDH, pyruvate dehydrogenase subunit B; MDH, malate dehydrogenase 1; ME, malic enzyme; ACL, ATP citrate lyase; ACS, acetyl-CoA synthase; FAE, fatty acid elongase 2; GPDH, glycerol-3-phosphate dehydrogenase 1; MTTP, microsomal triglyceride transfer protein.

and dnMlx. The high response of GLUT4 mRNA reflects a very low basal level in low glucose conditions. The finding that the GLUT4 gene is capable of responding to glucose suggests that it may be subject to glucose regulation at the level of gene expression under certain not yet identified conditions in liver or in muscle and adipose, where it is highly expressed. GLUT5 functions primarily as a fructose transporter, and its expression in muscle and adipose, where it is highly expressed. GLUT5 function in dietary fructose to the glycolytic pathway. With the dramatic increase in dietary fructose due to the consumption of high fructose corn syrup and the ability of fructose to bypass the major regulatory steps of glucose metabolism (glucokinase, phosphofructokinase, and pyruvate dehydrogenase complex), ChREBP-Mlx could be a contributory factor in promoting increased obesity in the population.

The expression of glucokinase, the major hexokinase expressed in the liver, is regulated by insulin but not glucose (25). However, glucokinase activity is controlled acutely via its interaction with an inhibitory protein, GKR (26). The identification of GKR as a glucose-responsive gene that is inhibited by dnMlx demonstrates the overlapping and coordinate regulation that occurs between insulin and glucose signaling pathways in the liver.

The NADPH required for fatty acid synthesis is furnished by the pentose phosphate shunt and malic enzyme. Glucose-6-phosphate dehydrogenase was induced in high glucose, and the induction was inhibited by dnMlx, suggesting that it is a target gene of ChREBP-Mlx. In the nonoxidative phase of the pentose phosphate shunt, ribulose 5-phosphate is converted to ribose 5-phosphate and intermediates of the glycolytic pathway. Transketolase, which is required for these interconversions, was also identified as a novel glucose-responsive gene regulated by ChREBP-Mlx. Alternatively, NADPH can be generated by cytosolic malic enzyme. Malic enzyme can participate in both the pyruvate/malate and pyruvate/citrate shuttles (27). The result of these shuttles is to transfer reducing equivalents from NADH to NADPH in the process known as pyruvate cycling (28). Malic enzyme and several genes encoding enzymes involved in this shuttle were found to be glucose-responsive and regulated by ChREBP-Mlx. The coordinated regulation of this set of genes emphasizes the importance of NADPH for the reductive reactions of fatty acid synthesis.

In the fatty acid biosynthetic pathway in which FAS and ACC were previously known targets of ChREBP-Mlx, several other gene products were identified in the group of overlapping genes. These included acetyl-CoA synthetase, ATP citrate lyase, and fatty acid elongase 2. In addition, mRNA levels of GPDH were significantly induced by high glucose, and the induction was abolished by dnMlx. The up-regulation of GPDH fits well with its role in forming glycerol 3-phosphate, the glycerol backbone, for triglyceride synthesis. A previous report showed that GPDH mRNA was induced by glucose in adipose tissue (29). Several lines of evidence have connected GPDH with obesity. For instance, mice lacking both GPDH1 (cytosolic) and GPDH2 (mitochondrial) display a decreased adiposity and reduced body weight (30). In addition, enhanced GPDH activity was observed in adipose tissue of obese humans (31). Together, these observations suggest an important role for GPDH in the physiological regulation of triglyceride synthesis.

MTTP catalyzes the rate-limiting step in the production of apoB-containing VLDL and was found as a novel target of ChREBP-Mlx. The main role of MTTP is to transfer lipids onto the apoB polypeptide in the endoplasmic reticulum of lipoprotein-secreting cells (32). Inhibition of the MTTP activity in animals lowers lipoprotein levels (33, 34), whereas overexpression of MTTP in mouse liver increases the secretion of apoB-containing lipoproteins (35, 36). Interestingly, hepatic expression of MTTP and in vivo secretion of triglyceride-rich lipoproteins are increased in obese diabetic mice (37). The identification of GPDH and MTTP as ChREBP-Mlx targets indicates that this complex regulates genes encoding enzymes in the final stages of lipogenesis as well as the earliest steps.

The results of the microarray analysis are consistent with the phenotype of mice globally deleted for the ChREBP gene (11). ChREBP knock-out mice displayed lower mRNA levels of several lipogenic enzymes, such as PK, ATP citrate lyase, FAS, and malic enzyme, and reduced rates of fatty acid synthesis com-
pared with wild type mice. ChREBP deficiency also caused intolerance of fructose in the diet, leading to death after several days. The intolerance to fructose is probably due to the reduced expression of enzymes that are required for fructose to enter into the glycolytic pathway. Together, our results and those of the ChREBP null mouse indicate a critical role of ChREBP-Mlx in supporting the pathways of carbohydrate metabolism and de novo lipogenesis.

**Identification of Potential ChREBP-Mlx Targets Involved in Other Metabolic Processes**—Although the pathways of de novo lipogenesis are most widely affected by glucose and ChREBP-Mlx in hepatocytes, genes in other metabolic processes were also influenced. Genes in the glycogen synthesis pathway, glycolgen synthase 2 and protein phosphatase 1 regulatory subunit 3B (G_subunit), were identified as glucose-responsive genes inhibited by dnMlx. The G_subunit of protein phosphatase 1 is the hepatic glycolgen-targeting subunit that enhances the dephosphorylation and activation of glycogen synthase (38). The promotion of glycogen formation represents an alternative pathway in the hepatocyte for storing excess glucose following a high carbohydrate meal.

Paradoxically, several genes encoding gluconeogenic enzymes (glucose-6-phosphatase catalytic subunit, glucose 6-phosphate transporter, and fructose-1,6-bisphosphatase) were found to be induced by glucose in an Mlx-dependent manner. Glucose-6-phosphatase has been previously reported to be induced by glucose in hepatocytes and liver (39, 40). The physiological basis for the induction of these enzymes in hyperglycemic conditions is not clear but certainly could contribute to dysregulated hepatic glucose production in diabetic states.

In addition to genes encoding metabolic enzymes, several genes that play roles in regulating metabolic and energy homeostasis were identified as potential targets of ChREBP-Mlx (Table 1). For example, the adiponectin receptor, glucagon receptor, adiponutrin, and FGF-21 were found in the group of overlapping genes. FGF-21 is a recently discovered novel member of the FGF family (41). FGF-21 is preferentially expressed in the liver and can function as a potent activator of glucose uptake in adipocytes (42). The administration of FGF-21 to diabetic ob/ob or db/db mice or obese ZDF rats resulted in dramatically lower circulating glucose and triglyceride levels as well as improved glucose and insulin tolerance. Most strikingly, FGF-21 transgenic mice are resistant to diet-induced weight gain and fat accumulation, although they consume more food relative to their weight than controls. FGF-21 functions by inducing the insulin-independent glucose transporter GLUT1, but not the insulin-dependent glucose transporter GLUT4, in adipocytes. Therefore, FGF-21 may represent a “hepatokine,” a metabolic regulator produced in the liver that functions in another tissue to coordinate metabolic activities.

**Comparison of Target Genes of ChREBP-Mlx and SREBP**—Both glucose and insulin are required for the full induction of most lipogenic enzyme genes. The actions of insulin in regard to lipid metabolism are mediated through the transcription factor SREBP-1c. SREBP target genes in the liver have been identified by comparing the gene expression patterns in the livers of three lines of mice: transgenic mice overexpressing SREBP-1a, transgenic mice overexpressing SREBP-2, and mice null for the SREBP cleavage-activating protein SCAP, a protein required for the nuclear localization of SREBPs (43). Target genes of SREBP identified in this manner were compared with target genes of ChREBP-Mlx. Shared target genes of ChREBP-Mlx and SREBP are indicated with Footnote b in Table 1. Most metabolic genes in the fatty acid synthesis pathway are regulated by both SREBP and ChREBP-Mlx. Interestingly, ChREBP-Mlx regulates genes encoding enzymes in the glycolytic pathway, whereas few of the SREBP target genes are found in this pathway. Moreover, most genes involved in NADPH generation are targets of ChREBP-Mlx, but only one of them, malic enzyme, is controlled by SREBP. In contrast, SREBP mediates activation of the entire pathway for cholesterol synthesis in the liver, whereas ChREBP has only two targets in this pathway. Thus, ChREBP and SREBP regulate genes with overlapping but distinct patterns in major metabolic pathways in the liver.

**Glucose Regulation in β Cells**—Pancreatic β cells are also a primary organ in controlling glucose homeostasis. β cells can sense changes in blood glucose levels and respond by increasing the secretion of insulin. In addition, the production of insulin is regulated by glucose as well. Two transcription factors, PDX-1 and MafA, have been shown to transcriptionally regulate the insulin gene in a synergistic manner (44). However, the intracellular mechanism by which glucose metabolism is coupled with transcription factor activity is unknown. The L-type pyruvate kinase gene is expressed in the insulinoma β cell line INS-1 and is transcriptionally induced by glucose (45). ChREBP is expressed in islets, and its transcription is regulated by glucose as well (46). Overexpression of ChREBP in INS-1 cells induced the mRNA level of endogenous PK (46). This response was dependent on the ChoRE sequence of the PK gene (46). These observations suggest that, in addition to PDX-1 and MafA, ChREBP-Mlx functions as a glucose-responsive transcription factor in the β cells.

Gene profiling of glucose-responsive genes has also been performed in the murine β cell-derived cell line Min6 (47) as well as in human (48) and porcine islets (49). The set of genes induced by glucose in these cells is largely distinct from that observed in hepatocytes. In β cells, the largest group of genes induced by glucose are involved in the secretory pathway. No genes involved in fatty acid or triglyceride synthesis were observed. One possible explanation for the distinct populations of glucose-responsive genes in hepatocytes and β cells is that PDX-1 and MafA are the primary transcription factors regulating glucose-responsive genes in β cells (44). However, the most dramatically glucose-induced gene in both human and porcine islets is thioredoxin-interacting protein (TXNIP). TXNIP mRNA levels are significantly reduced in the HcB-19 mouse strain, which displays hypertriglyceridemia (50). Moreover, mice deleted for the TXNIP gene displayed increased lipogenesis as well as increased hepatic triglyceride and cholesterol levels (51). Most interestingly, a ChoRE similar to sequence in the ChoREs in lipogenic enzyme genes has been identified in the promoter region of TXNIP (8). TXNIP appeared in the group of both glucose-responsive genes and genes affected by dnMlx in hepatocytes. Therefore, TXNIP is likely to be a
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Table 1

Selected genes induced by glucose and repressed by dnMlx

| Pathway and gene                      | Accession number | RT-PCR | H/L ratio | H/DN Ratio |
|---------------------------------------|------------------|--------|-----------|------------|
| Glycolysis pathway                    |                  | ×      | 2.1       | 2.3        |
| GLUT2                                 | NM_012879        |        |           |            |
| GLUT4                                 | NM_012751        | ×      | 11.7      | 17.8       |
| GLUT5                                 | NM_013741        |        | 3.9       | 3.7        |
| Glucose phosphate isomerase           | BE283882         |        | 3.7       | 2.5        |
| Fructosekinase                        | NM_031855        | 2.8    | 2.5       |            |
| Aldolase B                            | M10149           | 2.0    | 1.8       |            |
| Liver-type pyruvate kinase            | NM_012624        | ×      | 8.4       | 7.9        |
| NADH generation                       |                  |        |           |            |
| Glucose-6-phosphate dehydrogenase     | NM_017006        | ×      | 2.2       | 3.0        |
| Transketolase                         | NM_022592        | ×      | 2.2       | 2.0        |
| Malic enzyme                          | NM_012600        | ×      | 2.3       | 1.9        |
| Malate dehydrogenase 1               | BG671530         | ×      | 1.8       | 2.2        |
| Dicarboxylate transporter             | NM_133418        | 2.2    | 2.2       |            |
| Pyruvate dehydrogenase β             | BM389223         | 2.6    | 2.7       |            |
| Fatty acid synthesis                  |                  |        |           |            |
| ATP citrate lyase                     | NM_016987        |        | 2.6       | 3.2        |
| Acetyl-CoA synthetase b               | AA849497         | 2.6    | 2.6       |            |
| Acetyl-CoA carboxylase 1 b            | BI296153         | 2.7    | 4.1       |            |
| Fatty acid synthase b                 | AI179334         | ×      | 2.7       | 3.4        |
| S-2 chain                              | NM_012703        | ×      | 3.9       | 11.5       |
| Fatty acid elongase 2 b               | BE116152         | 1.9    | 3.5       |            |
| Triglyceride formation and transport  |                  |        |           |            |
| Glycol-3-phosphate dehydrogenase 1    | NM_022215        | ×      | 3.2       | 4.6        |
| Microsomal triglyceride transfer protein | BM385230    |        | 3.2       | 3.5        |
| Glycogen synthesis                    |                  |        |           |            |
| Glycogen synthase 2                   | NM_013089        | ×      | 4.3       | 3.3        |
| Protein phosphatase 1 G6 subunit      | BI274037         | ×      | 3.1       | 2.7        |
| Gluconeogenesis                       |                  |        |           |            |
| Glucose-6-phosphatase, catalytic      | NM_013098        |        | 2.6       | 2.9        |
| Glucose-6-phosphate transporter       | NM_013589        | 2.6    | 2.3        |            |
| Fructose-1,6-bisphosphatase 1         | NM_012558        | 2.2    | 1.8        |            |
| Cholesterol metabolism                |                  |        |           |            |
| HMG-CoA synthase 2 b                  | M33684           | 1.9    | 2.0        |            |
| 7-Dehydrocholesterol reductase b      | NM_022389        | 2.9    | 3.0        |            |
| Metabolic regulators                  |                  |        |           |            |
| Fibroblast growth factor 21           | NM_130752        | ×      | 6.0       | 5.9        |
| Thio-redoxin-interacting protein      | U30289           |        | 4.8       | 5.9        |
| G0S2                                  | AI406939         | ×      | 4.4       | 4.1        |
| RGS16                                 | BF391820         | ×      | 4.0       | 4.4        |
| Adiponectin receptor 2                | BI290608         | 3.0    | 2.7       |            |
| Glucokinase regulatory protein        | NM_013120        | ×      | 2.8       | 2.7        |
| Interleukin 6 receptor                | NM_017020        | 2.8    | 3.1        |            |
| ChREBP 29                             | NM_133552        | ×      | 2.5       | 1.9        |
| INSIG2                                | AAI818627        | 2.3    | 1.9        |            |
| PDH kinase, isoenzyme 2               | NM_030872        | ×      | 2.2       | 2.1        |
| Adiponutrin                           | BI277600         | 2.0    | 2.2        |            |
| Glucagon receptor                     | L04796           | 1.9    | 2.0        |            |

* a Induction by glucose and repression by dnMlx was confirmed by real time RT-PCR.
  b Genes characterized as direct target genes of SREBP.

Target glucose-responsive gene of ChREBP-Mlx in islets and hepatocytes.

Direct Target Genes of ChREBP-Mlx—Since the effects of glucose and dnMlx were assessed following 24 h of treatment, genes that are affected could either be direct or indirect targets of ChREBP-Mlx. To identify direct targets of ChREBP-Mlx, promotor sequences from 10 genes were computationally analyzed for putative ChoREs (see “Experimental Procedures”). These genes were selected for their relatively high response to glucose and their potential physiological importance. Sequences from −15,000 to +3,000 relative to the transcriptional start site for each gene in rats, mice, and humans were analyzed with previously characterized ChoREs as input sequences. Multiple ChoRE-like sequences were found in each promoter. To predict which ChoRE-like sequences might be functional, the ChoRE-like matches for each gene were analyzed between species for conservation. ChoRE-like matches near the transcriptional start site that also had high similarity between species were found in GPDH, GLUT4, GKR, and G0S2 genes (Fig. 3A). The failure to find ChoREs in the other selected genes may be due to the constraints of the search or the possibility that some or all of these could be indirect targets of ChREBP-Mlx.

Genes that are direct targets of ChREBP-Mlx should respond rapidly to glucose treatment. To test this supposition for the candidate genes, time course studies were conducted in primary hepatocytes. Since PK is a well characterized direct target gene of ChREBP-Mlx, its induction by glucose was used for comparison. Accumulation of PK mRNA after switching hepatocytes from low to high glucose could be observed within 2 h of treatment (Fig. 4). The mRNA levels of each of the candidate direct target genes were induced by glucose within the same time frame. These data are consistent with the possibility that
GLUT4, GPDH, GKRP, and G0S2 are direct target genes of ChREBP/H18528Mlx. Interestingly, FGF-21 mRNA responded to glucose as rapidly as any of the above gene products (Fig. 4). This result suggested that FGF-21 might also be a direct target of ChREBP/H18528Mlx. However, no ChoRE-like sequence that was shared between rat, mouse, and human genes was identified in the promoter region of FGF-21. The FGF-21 gene is transcribed divergently from the fucosyl transferase 1 gene with only 2000 bp separating them. Interestingly, fucosyl transferase 1 was also found in the group of glucose-induced, dnMlx-repressed genes, suggesting that a shared, but unrecognized, ChoRE element may coordinately control the expression of these two genes.

To further characterize the putative ChoREs of GPDH, GLUT4, GKRP, and G0S2, we tested for binding of ChREBP/H18528Mlx by electrophoretic mobility shift assay. Cell extracts from 293 cells expressing FLAG-tagged ChREBP and HA-tagged Mlx were used with oligonucleotides containing the PK ChoRE or the newly identified, putative ChoREs. As observed previously (14), extracts from 293 cells expressing ChREBP and Mlx formed a slowly migrating complex with the PK ChoRE (Fig. 3B). A complex migrating at the same mobility was observed with the candidate GPDH, GLUT4, GKRP, and G0S2 ChoRE-containing oligonucleotides. Furthermore, the migration of these complexes was retarded with either FLAG or HA antibodies, indicating that the complexes contain ChREBP and Mlx. Thus, all four of the putative ChoRE sequences are able to bind *in vitro* to the ChREBP-Mlx complex.

To assess whether these sites behave as functional ChoREs, we first tested their activity when linked in two copies to a minimal TATA-containing promoter (PK/H11002 S14/H11001). This context provides a highly sensitive measure for potential ChoRE function, since no other transcription factors that might interfere with its activity should bind to this reporter construct (52). Each construct was transfected into primary hepatocytes under low glucose condition and induced by high glucose for 24 h. As expected, we found that the putative ChoREs from the GOS2, GLUT4, and GKRP genes all supported a functional response to glucose at a level equal to or better than the well established PK and S14 ChoRE sequences (Fig. 5A). However, the ChoRE from the GPDH gene did not support any increased activity in the presence of high glucose. Hence, although this sequence supported ChREBP-Mlx binding, it was not capable of...
Role of ChREBP-Mlx in Glucose-induced Gene Expression

A functional analysis of putative ChoREs in the context of a minimal promoter. Two copies of putative ChoRE sequences were cloned in a head-to-tail fashion upstream of the PK(-40/+12) basal promoter in the reporter plasmid pGL3. Each construct was cotransfected with a Renilla luciferase plasmid into hepatocytes in low glucose medium and switched to either low or high glucose after 16 h. Twenty-four h later, cells were harvested, and luciferase activity was measured. Data are expressed as the fold induction by comparing the relative luciferase activity from high glucose samples divided by low glucose samples. Values represent means ± S.D. of triplicate samples.

FIGURE 5. Functional activity of putative ChoRE sequences. A, functional analysis of putative ChoREs in the context of a minimal promoter. Two copies of putative ChoRE sequences were cloned in a head-to-tail fashion upstream of the PK(-40/+12) basal promoter in the reporter plasmid pGL3. Each construct was cotransfected with a Renilla luciferase plasmid into hepatocytes in low glucose medium and switched to either low or high glucose after 16 h. Twenty-four h later, cells were harvested, and luciferase activity was measured. Data are expressed as the fold induction by comparing the relative luciferase activity from high glucose samples divided by low glucose samples. Values represent means ± S.D. of triplicate samples. B, functional analysis of putative ChoREs in the context of a minimal promoter. G052, GPDH, GLUT4, and GKR promoter sequences containing the putative ChoRE sequences were cloned into pGL3 firefly luciferase vector. The putative ChoRE sequences in G052 and GLUT4 promoter were deleted by site-directed mutagenesis (ΔE). These constructs were tested as described in A.

directing activated gene transcription. This discrepancy between binding and functional activity has been observed previously for other transcription factors (53, 54). The fact that binding does not lead to activation suggests that the DNA does not act solely to tether ChREBP-Mlx to the promoter but rather is an active component in allowing ChREBP-Mlx to adopt an active conformation. We surmise that the context of the ChoRE-like sequence in GPDH is not capable of allowing this conformation of ChREBP-Mlx to form. A possible basis for this difference is discussed below.

Given the synthetic nature of the two copy reporter constructs, it was also important to test ChoRE activity in the natural context of the promoter regions. Genomic fragments from each of the four candidate genes containing the putative ChoREs were cloned into the pGL3 reporter vector. The PK promoter served as a positive control and was induced by glucose ~4-fold (Fig. 5B). G052 and GLUT4 promoters were also induced by glucose about 2.5- and 4-fold, respectively. Deletion of the putative ChoRE sequence in these promoter regions resulted in the loss of glucose response, confirming the critical nature of the identified sequence. Hence, the putative ChoRE sequences from G052 and GLUT4 represent bona fide binding sites for ChREBP-Mlx and functional ChoREs. Despite its ability to function when linked in two copies to a minimal promoter, the GKR promoter fragment containing the putative ChoRE sequence surprisingly failed to respond to glucose. One possible explanation may be that the promoter cloned from GKR lacks additional enhancer sequences required to support a glucose response in the context of the natural promoter. For example, previous work has shown that the PK and S14 ChoREs only support a glucose response in conjunction with additional nuclear factors (e.g. HNF-4) binding to distinct sites when in the context of their natural promoter regions (55). Further experiments are needed to test this possibility.

Based on functional ChoRE sequences from seven different genes, we generated a refined consensus sequence (CAYGNNGNCNCRGTG) to better represent critical elements of a functional ChoRE sequence. The sequence logo showing the relative conservation of bases at each position is shown in Fig. 6. This consensus sequence is palindromic, and each of the motifs contains a (5’-)CAGY sequence. Positions 5 in the first E box and 2 in the second E box are not critical for glucose regulation. Consistent with this consensus, we have previously shown that mutations in any of the first 4 bp of a synthetic ChoRE containing two perfect CACGTG motifs disrupt glucose regulation, whereas mutations in position 5 do not (19). The importance of the base in position 6 is not as clear, since it is conserved in the first E box but only weakly in the second. Previous mutational studies have suggested that his base is not critical. To test the validity of this ChoRE consensus sequence, we compared it to the nonfunctional ChoRE-like sequence from the GPDH gene. This sequence contains a G at position 3, whereas all functional ChoRE sequences have a pyrimidine at this position. This difference may explain the lack of activity for the GPDH sequence noted above. We are using the refined ChoRE consensus sequence to further analyze genes from the microarray to identify functional ChoREs and to better understand the mechanism of ChREBP-Mlx in transcriptional activation.

FIGURE 6. Sequence logo representing consensus ChoRE sequence. Rat PK, S14, ACC, FAS, G052, GLUT4, and human TXNIP ChoRE sequences were used to generate a consensus ChoRE sequence using WebLogo (available on the World Wide Web at weblogo.berkeley.edu) (56). The height of each letter within a stack indicates the relative frequency of each nucleotide at that position, whereas the overall stack height of all letters represent the sequence conservation at that position.

Sequence logo representing consensus ChoRE sequence. Rat PK, S14, ACC, FAS, G052, GLUT4, and human TXNIP ChoRE sequences were used to generate a consensus ChoRE sequence using WebLogo (available on the World Wide Web at weblogo.berkeley.edu) (56). The height of each letter within a stack indicates the relative frequency of each nucleotide at that position, whereas the overall stack height of all letters represent the sequence conservation at that position.
Conclusions—The liver plays a central and essential role in sensing nutrients and regulating metabolic pathways related to glucose and energy homeostasis. In this study, we sought to investigate glucose-regulated gene expression in the liver using microarray analysis. The results suggest that glucose-upregulates genes encoding enzymes and proteins involved in the entire pathway of de novo lipogenesis. Additionally, genes encoding enzymes in other related metabolic pathways and numerous metabolic regulators are also activated by glucose. More than 60% of glucose-responsive genes are inhibited by dnMlx in high glucose. Thus, ChREBP is the principal mediator that transcriptionally regulates glucose-responsive genes. We have identified additional direct target genes for ChREBP-Mlx and refined the consensus sequence for supporting a functional glucose response. The central role of ChREBP-Mlx in regulating expression of genes involved in glucose metabolism and homeostasis highlights the importance of its further evaluation with respect to the pathologies associated with the metabolic syndrome.

Acknowledgments—We thank Dr. Wayne Xu and Dr. Zheng Jin Tu (Supercomputing Institute, University of Minnesota) for assistance in analyzing the microarray data. Also, we acknowledge the University of Minnesota Biomedical Genomics Center Affymetrix Microarray Core for cRNA sample preparation and hybridization. We thank Nikolai Tsatsos for critical reading of the manuscript.

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