REGULATION OF THE L-TYPE PYRUVATE KINASE GENE BY GLUCOSE AND cAMP IN ISLET β CELLS

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Submitted to the Graduate Faculty of

The University of Pittsburgh School of Medicine in partial fulfillment

of the requirements for the degree of Doctor of Philosophy

University of Pittsburgh

2009

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

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Extracellular signals generated during both feeding and fasting coordinately regulate transcription of metabolic enzyme genes that control glucose metabolism in the β cell. A post-prandial rise in extracellular glucose levels promotes expression of various genes including the gene encoding the glycolytic enzyme L-type pyruvate kinase (L-PK). Conversely, under conditions of fasting, a rise in hormones that stimulate increased intracellular levels of cAMP results in suppression of glucose-activated genes such as L-PK. The L-PK gene is coordinately regulated by these two opposing stimuli. Therefore, we explored the mechanism of induction and repression of the L-PK gene by glucose and cAMP, respectively, using the 832/13 rat insulinoma cell line.

Glucose mediates induction of the L-PK gene by stimulating the recruitment of two primary DNA binding transcription factors, the basic helix-loop-helix/leucine zipper protein Carbohydrate Response Element Binding Protein (ChREBP) and the orphan nuclear receptor, Hepatic Nuclear Factor 4α (HNF4α) to their respective response elements in the proximal L-PK promoter. In addition, glucose stimulates the recruitment of the coactivator CREB binding protein (CBP) to the L-PK gene promoter. Assembly of these three factors on the L-PK gene promoter facilitates alterations in the pattern of

acetylation and methylation of histones associated with the promoter and coding region, respectively. These changes in histone modifications correlate with increased occupancy of the RNA Polymerase II (Pol II) holoenzyme on the L-PK promoter. Finally, glucose promotes changes in the phosphorylation state of the carboxyl-terminal domain (CTD) of Pol II at serines 5 and 2, which are necessary for the promoter clearance and elongation phases of transcription.

cAMP represses the glucose-mediated induction of the L-PK gene by inhibiting the assembly of the ChREBP, HNF4α and CBP-containing complex on the L-PK promoter. The cAMP-dependent decrease in complex assembly on the promoter is associated with alterations in the acetylation and methylation status of histones on both the promoter and coding region. Furthermore, cAMP inhibits the glucose-mediated recruitment and phosphorylation of Pol II CTD, ultimately blocking initiation and elongation of the L-PK gene by Pol II.

In summary, these studies provide a detailed insight into the mechanism of regulation of the L-PK gene by glucose and cAMP in islet β cells.

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1.0 INTRODUCTION

1.1 ORGANIZATION OF CHROMATIN WITHIN THE CELL NUCLEUS

1.1.1 Packaging of the genome into nucleosomes

Packaging of the eukaryotic genome into higher-order chromatin fibers allows for compaction of the genome inside the nucleus. The fundamental repeating unit of chromatin is the nucleosome (76). Nucleosomes are composed of an octamer of four highly conserved histone proteins (145), a H3-H4 tetramer (78, 125) and two H2A-H2B dimers (70), around which 146 bp of DNA are wound (77, 114). The current compaction model states that nucleosomes with 'linker DNA' form a 10 nm fiber termed 'beads on a string'. Higher order compaction involves organizing nucleosomes into a 30 nm fiber with the aid of histone H1 (for reviews see (54, 126, 144)). Eukaryotic genomes can be differently compacted with euchromatin being less densely packed than heterochromatin. Euchromatin is enriched with genes that are transcriptionally active. Heterochromatin is more densely packaged than euchromatin and is characterized by transcriptionally silent genes and is further classified as pericentric or constitutive and facultative heterochromatin. Pericentric heterochromatin is found around the chromosome centromeres and telomeres; it remains constitutively silenced throughout cell division.

Facultative heterochromatin however has the ability to become transcriptionally active again (149).

Although nucleosomes allow for tight condensation of DNA, they form a repressive structure that impedes access of regulatory proteins involved in processes such as transcriptional regulation. Post-translational covalent modifications of histones overcome the repressive environment of the nucleosome and thus enhance accessibility of proteins to target DNA sequences.

1.1.2 Covalent modification of histones

Histones serve as targets for a well-documented array of modifications in their N-terminal tails, and also to a lesser extent on the C-terminal tails and globular domains, including acetylation, methylation, phosphorylation, ADP ribosylation, sumoylation and ubiquitination. Since the early nineties, it has been recognized that these histone covalent modifications can work as 'receptor' or 'recognition' signals that function to directly recruit effector proteins. Moreover, there has been growing awareness in the field that specific and distinct patterns of covalent modifications can be attributed to certain histone tails and it was proposed that a histone 'language' or 'code' was encoded in unique combinations of modifications, that are subsequently read by effector modules (138).

Histone acetylation and deacetylation, mediated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively, of specific lysine residues in the N-terminal tails of core histones plays a fundamental role in transcriptional regulation (79). The most common sites for acetylation are lysines 9, 14, 18 and 23 in histone H3 and lysines 5, 8, 12 and 16 in histone H4. The first documented HAT was isolated from

Tetrahymena by Allis and colleagues (12) and since then a myriad of other HATs have been identified in a wide range of species. HATs comprise several families, including the p300/CBP family of global transcriptional coactivators (101). The p300/ CBP family of HATs were first recognized to have intrinsic acetyltransferase activity by Bannister and Kouzarides (4), and are capable of acetylating all four core histone proteins. HATs catalyze the transfer of an acetyl group to the histone thus reducing the affinity between histone and negatively charged DNA, thereby rendering DNA more accessible to transcription factors. In addition, histone modifications present a surface for the recruitment of proteins containing domains that recognize specific modifications.

Acetylated histones are targets for bromodomain-containing proteins. A bromodomain was first documented in the *Drosophila* Brahma protein (142) and since then they have been identified in many HATs e.g. p300/CBP and $TAF_{II}250$ (64) and other chromatin-associating proteins and enzymes including histone methyltransferases (e.g. MLL), ATP-dependent remodeling enzymes (e.g. BRM and BRG1). These domains specifically recognize and bind to acetylated lysines, thereby linking HAT activity to a cascade of events that collectively activate transcription (39).

Histone methylation can occur on either arginine or lysine residues. Methylation of arginine residues on either histone H3 or H4 is catalyzed by a PRMT (protein arginine N-methyltransferases) family of histone methyltransferases (HMTs). Arginines can be mono- or di-methylated either symmetrically or asymmetrically, and activate or repress transcription dependent upon the specific arginine methylated e.g. methylation of H4R3 by PRMT1 is associated with transcriptional activation (158), whereas methylation of H3R8 by PRMT5 is indicative of repression (121). In addition, Coactivator Associated

Arginine Methyltransferase 1 (CARM1/PRMT4) possesses histone H3-specific methyltransferase activity that contributes to transcriptional activation (22).

Similar to arginine methylation, lysine methylation by HMTs is complex; lysines can be mono-, di-, or tri-methylated and again site-specificity determines whether a gene is activated or repressed. Methylation of histone H3-K4 is generally associated with active gene transcription, whereas methylation of histone H3-K9 or H3-K27 modulates gene silencing (for review see (85)). With the exception of methylation of H3-K79 by Dot1L methyltransferase (46), lysine methylation is carried out exclusively by family of proteins containing a SET domain. This domain was originally identified in three *Drosophila* proteins: suppressor of variegation, enhancer of zeste, and trithorax, hence SET (43, 68, 135). SET7/9 has been shown in humans to methylate H3-K4 thus contributing to the activation of a number of genes including the insulin gene (40), whereas Suv39h1 catalyzes the methylation of H3-K9 leading to the biological outcome of transcriptional silencing and/or heterochromatin formation (124).

Methylation states are recognized by proteins containing chromodomains. This domain was first identified in Heterochromatin Protein 1 (HP1) and Polycomb (Pc) of *Drosophila* (122), but has been found in many other chromatin regulators including ATP-dependent remodeling factors, histone acetyltransferases and methyltransferases including the human Suv39h family (for review see (39)).

A recent advancement in the field of histone methylation was the discovery of histone demethylases (HDMs). Shi and coworkers reported that Lysine Specific Demethylase 1 (LSD1) exhibits demethylase active specific for mono- and di-methylated

histone H3-K4 (131). A new family of demethylases have subsequently been discovered, the Jumonji C domain-containing proteins (152).

1.2 CELLULAR GENE TRANSCRIPTION

1.2.1 Factors involved in transcription initiation

Transcription initiation from the promoters of protein-encoding genes is a highly coordinated process requiring the cooperative assembly and concerted functions of a large group of proteins and transcription factors in order to ensure that specific genes and/ or subsets of genes are turned on or off in a temporally and spatially-regulated manner. These factors can be subdivided into three groups: 1) primary DNA binding transcription factors which bind to promoter elements and thus regulate expression of their target genes in response to various stimuli; 2) general (or basal) transcription factors (GTFs) bind TATA or initiator (INR) DNA elements and stimulate the recruitment of RNA Polymerase II (Pol II); and 3) coactivators and corepressors that can interact with primary DNA binding factors in addition to the GTFs and Pol II (10).

1.2.2 Primary DNA binding factors

Primary DNA binding factors are a group of proteins that can interpret and translate the genetic blueprint encoded in the DNA. In response to intercellular or environmental

signals, these factors bind response elements in DNA and in association with other factors including cofactors and GTFs coordinate a program of increased or decreased gene transcription; therefore play a pivotal role in cellular processes including development, adaptation to stress and cell cycle control.

Transcription factors can be subdivided into classes based on regulatory function or sequence homology/tertiary structure. Transcription factors classified according to their regulatory functions are subdivided into two groups, constitutively or conditionally active (8). Nuclear receptor hormones are activated by intracellular ligands, and are thus conditionally active, whereas GTFs are constitutively active. Structural classification is based on sequence similarity in a factor's DNA binding domain. Transcription factors are divided into classes containing e.g. basic domains or zinc-coordinating DNA-binding domains. Members of the former class include the basic helix-loop-helix/leucine zipper (bHLH/LZ) family of transcription factors, with members of this group including SREBP, c-myc and Carbohydrate Response Element Binding Protein (ChREBP) (8). Hepatocyte Nuclear Factor 4α (HNF4α) represents a zinc finger domain-containing protein (61).

1.2.3 Transcription coactivators and corepressors

Transcription cofactors (coactivators or corepressors), many of which exist as large multiprotein complexes, mediate a link between primary sequence-specific transcription factors and the general transcriptional apparatus, thus facilitating the activation or repression of gene transcription. Due to their functional contributions to transcriptional

regulation, cofactors can be generally classified into two groups: 1) cofactors that bind and recruit or are themselves components of the general transcriptional machinery e.g. Mediator; and 2) cofactors with enzymatic activity that (a) modify chromatin e.g. coactivators and corepressors with histone acetylase, deacetylase, methylase and demethylase activity (discussed below); or (b) remodel chromatin e.g. Swi/Snf complex in yeast or its mammalian counterpart, Brg1 and BRM proteins.

Although it was initially believed that the assembly of individual transcriptional coactivator or corepressor complexes, with each separate complex displaying its own specialized role, it has now emerged that these complexes are not distinct entities but display a high degree of functional and structural overlap. These complexes allow for the exchange of enzymatic activities, thus forming heterogeneous complexes that can easily access and modify a vast array of sequence-specific transcription factors in response to various cellular signals (for review see (108)).

1.2.4. Stages of eukaryotic transcription

Transcription is subdivided into sequential steps: 1) formation of the pre-initiation complex (PIC); 2) promoter clearance and 5' capping; 3) pausing; and 4) productive elongation with co-transcriptional processing of the nascent transcript by splicing, polyadenylation and cleavage factors; and 5) termination of transcription.

1.2.5 Basal transcription machinery promotes formation of a pre-initiation complex (PIC)

The basal transcriptional apparatus is composed of six GTFs (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH) in addition to Pol II (14, 120). Pol II alone is unable to recognize core promoter elements; therefore initiation of transcription requires the binding of GTFs to TATA and INR elements and subsequent assembly of GTFs and Pol II into a PIC at the promoter in close proximity to the transcription initiation site. TFIID is composed of if TATA-binding protein (TBP) and 14 TBP-associated factors (TAF_{II}s) (16). In TATA-containing promoters, the first step in PIC formation is the association of TBP with the TATA box and TAF interactions with INR or other elements in the vicinity of the transcription start site (18, 155). TFIIA functions to stabilize TBP-TATA and TAF-DNA interactions (50, 143). TFIIB further stabilizes the TBP-DNA interaction and allows the recruitment of TFIIF and Pol II (48, 112, 150). TFIIE recruits TFIIH and stimulates the kinase activity of TFIIH. TFIIH, in addition to its kinase activity (described below) (92), contains two DNA helicases that promote ATP-dependent promoter melting at the transcription start site, thus forming an open PIC complex that supports transcription initiation (118, 119).

1.2.6 RNA Polymerase II and phosphorylation of its carboxyl-terminal domain (CTD)

Pol II is a large complex composed of 12 subunits, Rpb1-Rpb12, that comprise a mass of approximately 0.5MDa. The largest subunit of Pol II, Rbp1, contains a highly conserved domain known as the carboxyl-terminal domain (CTD). The CTD consists entirely of heptapeptide Tyr^1-Ser^2-Pro^3-Thr^4-Ser^5-Pro^6-Ser^7 repeats. The number of repeats varies among species; yeast contains 26 repeats (2), with human (163) and mouse (31) containing 52 repeats of which 21 exactly match the consensus heptapeptide.

Pol II exists in two forms: Pol II_A, the hypo- or unphosphorylated form found in PIC (83) and paused complexes, whereas Pol II_o is hyperphosphorylated on Serines 2 and 5 of the CTD found in elongating complexes (130). Although Pol II is recruited to the promoter in an unphosphorylated form (91), dynamic and reversible phosphorylation of the CTD controls efficient synthesis of transcripts. Pol II undergoes a cycle of phosphorylation and dephosphorylation during transcription, with serine 5 being phosphorylated during initiation and promoter clearance, and serine 2 phosphorylation occurring predominantly during the elongation phase of transcription (58).

The CTD forms a tail-like extension from the catalytic core of Pol II and is located near the RNA exit channel, and therefore is situated to serve as a docking platform for a wide range of factors that play vital roles in the generation of RNA transcripts. Recruitment of these factors is linked to the phosphorylation state of CTD, particularly serines at positions 2 and 5 of the heptapeptide repeat. Phosphorylation of serine 5 by the kinase activity of the cdk7 subunit of TFIIH (92) supports the recruitment

of enzymes (e.g. guanylyltransferase and (guanine-7)-methyltransferase) that add a methylguanosine cap to the 5' end of the nascent transcript (59, 103).

Following initiation of transcription from a promoter, the default mode of transcription is the production of short, abortive transcripts. Two factors, negative elongation factor (NELF) and DRB Sensitivity Inducing Factor (DSIF), cooperate to block elongation by trapping Pol II near the promoter (156, 166). Switching from abortive transcript production to processive elongation with concomitant generation of long transcripts that eventually become mRNAs requires the action of positive transcription elongation factor b (p-TEFb) complex to overcome the negative effects of NELF and DSIF (102). P-TEFb is composed of cyclin-dependent kinase 9 and its regulatory subunit cyclin T. Phosphorylation of NELF and DSIF by cdk9 promotes the release of NELF; in addition, serine 2 of the Pol II CTD is phosphorylated by p-TEFb (123) and thus provides a platform for the association of CTD-binding proteins involved in splicing, (105, 106) polyadenylation, and cleavage factors (6, 104). CTD phosphatases are required to restore Pol II to the unphosphorylated form, Pol II_A form, and allow for its recycling to promoters to reinitiate the transcriptional cycle (19, 20, 24, 52, 73).

1.2.7 mRNA 3'-end processing

Precursor mRNA (pre-mRNA) 3' end processing, including cleavage and polyadenylation, of the newly synthesized transcript is a vital step in the generation of the mature mRNA. Mammalian RNAs have three defined elements that constitute a 'polyadenylation signal', thus specifying the site of cleavage of the nascent RNA. A

highly conserved AAUAAA motif is generally found 10 – 20 nucleotides upstream of the cleavage site (161). A less conserved U or GU-rich region resides approximately 30 nucleotides downstream of the site of cleavage. The cleavage or Poly (A) site is determined by the distance between the AAUAAA and U/GU-rich motifs. The actual sequence of this Poly (A) site is not conserved; however, it is defined by a CA dinucleotide.

The 3' end processing machinery consists of multiple conserved proteins. Mammalian factors that cleave the synthesized RNA include cleavage and polyadenylation specificity factor (CPSF), cleavage stimulatory factor (CSF), cleavage factors I and II (CF I and CF II), and poly (A) polymerase (PAP). CPSF and PAP have dual function in that they are also required for polyadenylation; poly (A)-binding protein II (PAB) is also required for polyadenylation (97, 107, 127, 141, 162).

The cleavage and polyadenylation complex assembles on the newly synthesized RNA; CPSF binds to the AAUAAA motif and CSF to the U/GU- rich sequence (97, 140). Recruitment of CF I, CF II and PAP facilitate cleavage of the mature mRNA. Following cleavage, addition of approximately 150-250 adenosine residues (37, 45) to the growing poly (A) tail requires the cooperative interactions of CPSF, PAP and PAB. The poly (A) tail provides stability to the mRNA during its export to the cytoplasm where the mRNA undergoes translation (for review see (44, 99)).

1.3 THE L-TYPE PYRUVATE KINASE (L-PK) GENE AS A MODEL TO STUDY TRANSCRIPTION

1.3.1 Extracellular signals regulating expression of the L-PK gene

Blood glucose levels are elevated with dietary increases in carbohydrates; fasting conditions stimulate a rise in hormones that promote increased intracellular cAMP levels (27, 67). Switching between the fasted-to-fed and fed-to-fasted states alters patterns of gene expression to coordinately regulate abundance of metabolic enzymes e.g. enzymes of glucose and fatty acid metabolism (27).

Glucose is taken up into the β cell via the GLUT2 transporter, and is subsequently converted to glucose-6-phosphate by glucokinase. The metabolism of glucose mediates numerous functions in the β cell, including glucose-stimulated insulin secretion (111), and regulation of metabolic enzyme genes, including the glycolytic gene L-type pyruvate kinase (L-PK) and the lipogenic genes Acetyl CoA Carboxylase (ACC) and Fatty Acid Synthase (FAS) (13, 100, 116).

Stimulation of heterotrimeric G-protein coupled receptors by various hormones leads to the activation of adenylate cyclase and an ensuing rise in intracellular cAMP levels. Effects of this second messenger can by mediated by either cAMP-dependent protein kinase (PKA) or exchange protein activated by cAMP (EPACs) (23).

Thus, the L-type pyruvate kinase (L-PK) gene is an excellent model for studying the coordinate changes in gene transcription by glucose and cAMP as its expression in the islet β-cell is solely controlled by these two signaling inputs (100).

1.3.2 Carbohydrate Response Element Binding Protein (ChREBP)

A crucial step in understanding the mechanism of the glucose-mediated induction of the L-PK gene was the discovery by Towle and colleagues that the carbohydrate responsive portion of the L-PK gene is located between -96 and -197 bp with respect to the transcription start site (146). This group later identified a 17 bp DNA sequence, known as the Carbohydrate Response Element (ChoRE), composed of two E-boxes separated by 5 bp, which binds a member of the bHLH/LZ family, and is critical for carbohydrate regulation of the L-PK gene (132).

A decade later Uyeda and coworkers purified a 95 kDa protein they called the Carbohydrate Response Element Binding Protein (ChREBP) (167). ChREBP contains a nuclear localization signal (NLS) and a nuclear export signal (NES), allowing the protein to be trafficked to various compartments of the cell based on various signaling inputs. ChREBP also contains a bHLH/LZ domain and several putative PKA phosphoacceptor sites, making this protein an attractive candidate to mediate not only the glucose-induction, but also the cAMP-repression of the L-PK gene. Indeed, ChREBP was shown to bind the ChoRE as a heterodimer with another member of the bHLH/LZ family, Max-like protein (Mlx), and together these factors mediate the glucose-responsiveness of several metabolic genes including the L-PK gene (95, 96, 136).

Uyeda proposed a model for the ChREBP-dependent regulation of the L-PK gene by glucose and cAMP (69). In this model, glucose metabolism activates the phosphatase PP2A via an uncharacterized metabolite. PP2A dephosphorylates ChREBP at serine 196, stimulating entry of ChREBP into the nucleus. PP2A catalyzes a second

dephosphorylation event in the nucleus at threonine 666, promoting binding of ChREBP to the ChoRE and consequently activating transcription of the L-PK gene. Conversely, at non-stimulatory glucose concentrations, agents that increase intracellular cAMP repress expression of the L-PK gene. PKA-mediated phosphorylation of threonine 666 and serine 196 block binding of ChREBP to the ChoRE and promote its expulsion from the nucleus where it is sequestered by 14-3-3 proteins (87, 128).

A number of groups have provided evidence that this model is incomplete and needs revision (86, 151). Putative PKA sites at serine 196 and threonine 666 were believed to promote nuclear localization and DNA binding upon dephosphorylation in response to glucose signaling (69). If this were the case, it would be possible for ChREBP to promote L-PK transactivation at non-stimulatory glucose concentrations when mutated to prevent phosphorylation at these sites. However, Collier *et al.* demonstrated that even in the presence of phosphoacceptor site mutations (serine196alanine and threonine666alanine) in ChREBP, stimulatory glucose concentrations are still required to transactivate ChREBP in the context of the L-PK promoter (28). Tsatsos et al. corroborated these findings, demonstrating that a ChREBP S196A/T666A mutant that blocks PKA-mediated phosphorylation at these sites does not create a glucose-independent transactivator of a ChoRE-containing promoter (151). Furthermore, a study by Chan and colleagues highlights that a S196A/T666A ChREBP mutant requires glucose for transactivation of an L-PK promoter construct. Moreover, a ChREBPΔ1-196 deletion mutant was shown to be active at non-stimulatory glucose concentrations. At low glucose conditions, according to the Uyeda model, Thr666 should

be phosphorylated, thus inhibiting ChREBP from binding DNA. Finally, cantharidic acid, a PP2A inhibitor did not prevent glucose mediated activation of ChREBP (86).

A major advance in understanding the glucose-responsiveness of ChREBP came with the discovery by Chan and colleagues that ChREBP contains a glucose-sensing module, composed of a low-glucose inhibitory domain (LID) and glucose-response activation conserved element (GRACE) domain. Under conditions of low glucose LID inhibits the transactivation activity of GRACE, and elevations in cellular glucose concentration alleviate this inhibition (86). Thus, this factor is able to "sense" changes in glucose and alter gene expression patterns accordingly.

1.3.3 Hepatic Nuclear Factor 4 α (HNF4α)

HNF4α or nuclear receptor subfamily 2, group 1 (NR2A1), a member of the nuclear hormone receptor superfamily, was identified based on amino acid and sequence similarity to other nuclear receptors (133). Endogenous ligands of this new class of receptors were not identified based on structural similarity; therefore HNF4α and other factors identified were referred to as the 'orphan nuclear receptors'. Williams and coworkers discovered by X-ray crystallography that HNF4α is constitutively bound by a fatty acid in its binding pocket (164). Radiolabeled fatty acids could not displace the embedded fatty acid suggesting that it becomes embedded in the pocket during translation. Moreover, fatty acyl-CoA thioesters have been shown to function as ligands of HNF4α (56). HNF4α, a zinc-finger protein, binds DNA as a homodimer (66) to a recognition site known as a direct repeat (DR) of the consensus AGGTCA separated by

one or two nucleotides, known as DR1 and DR2, respectively (113). Upon DNA binding, HNF4α recruits coactivators and other accessory factors necessary to modulate expression of target genes; HNF4α has been shown by GST-pulldown studies to interact with the coactivator CBP (168).

HNF4α is involved in the glucose-stimulated induction of the L-PK gene (1, 7). Kahn demonstrated that the ChoRE requires the neighboring HNF4α binding site (a DR1 element), alternatively known as an L3 element, for maximal efficiency in mediating induction of the L-PK gene (53). Wollheim observed that overexpression of a dominant-negative HNF4α construct in an INS-1-derived clone reduced the expression of the L-PK gene (159).

1.4 GOALS OF THIS STUDY

It has been known for almost two decades that glucose induces expression of the L-PK gene in insulinoma cells, and simultaneous treatment with a cAMP agonist dominantly represses expression of this gene (100). However, signals that regulate the induction by glucose and, in particular, the repression by cAMP are incompletely understood. In the following studies, we used the 832/13 rat insulinoma cell line to:

1) Elucidate the mechanism(s) by which ChREBP and HNF4α regulate the glucose-induction and cAMP-repression of the L-PK gene.

2) Determine the coactivator required for glucose-mediated induction and cAMP-directed repression of the L-PK gene.

3) Identify the histone modifications involved in the glucose-mediated induction and cAMP-directed repression of the L-PK gene.

4) Understand the mechanism(s) by which glucose and cAMP promote modification of histones and recruitment of Pol II to regulate expression of the L-PK gene.

2.0 cAMP OPPOSES THE GLUCOSE-MEDIATED INDUCTION OF THE L-PK GENE BY PREVENTING THE ASSEMBLY OF A FUNCTIONAL COMPLEX CONTAINING CHREBP, HNFα AND CBP*

2.1 ABSTRACT

Glucose-mediated activation of the L-type pyruvate kinase (L-PK) gene is repressed by cAMP, making this an excellent model for studying the mechanism by which these contrary signals regulate gene expression. Using the 832/13 rat insulinoma cell line we demonstrate using RNA interference and chromatin immunoprecipitation that Carbohydrate Response Element Binding Protein (ChREBP), Hepatic Nuclear Factor 4α (HNF4α), and the coactivator CREB Binding Protein (CBP) are required for the glucose response of the L-PK gene, and are recruited to the promoter by glucose. The cAMP agonist forskolin blocked the glucose-mediated induction of the L-PK gene in a PKA-dependent manner, and blocked the recruitment of ChREBP, HNF4α, and CBP to the L-PK promoter, while simultaneously recruiting CBP to the cAMP-inducible gene, nuclear

* This chapter is originally published: Burke, S.J., Collier, J.J. and Scott, D.K. (2009) cAMP opposes the glucose-mediated induction of the L-PK gene by preventing the recruitment of a complex containing ChREBP, HNF4α and CBP. *FASEB Journal*.

receptor subfamily 4, group A, member 2 (NR4A2). Overexpression of CBP, but not ChREBP reversed the cAMP repression of the L-PK gene. In addition, CBP augmented the glucose response of the L-PK promoter. We conclude that cAMP and glucose signaling converge on a complex containing ChREBP, HNF4α and CBP, and that cAMP acts by disrupting this transcriptional complex assembled by glucose-derived signals.

2.2 INTRODUCTION

cAMP and glucose are opposing physiological signals. cAMP is elevated in cells experiencing metabolic fuel deprivation, whereas glucose represents metabolic fuel abundance (147). Cells respond to these contrary signals by altering gene expression patterns to adjust their metabolic phenotype. The L-type pyruvate kinase (L-PK; gene symbol: Pklr) gene encodes a key regulatory enzyme of glycolysis that catalyzes the terminal step in the oxidation of glucose to pyruvate with concomitant generation of ATP; therefore, its expression is tightly regulated. Glucose induces expression of the L-PK gene, whereas cAMP agonists, such as forskolin (an activator of adenylate cyclase), block this expression (100). However, the mechanisms underlying glucose-mediated induction as well as repression by cAMP are not well understood.

The basic Helix-Loop-Helix Leucine Zipper (bHLH-LZ) transcription factor Carbohydrate Response Element Binding Protein (ChREBP) is an important mediator of glucose action on induction of glycolytic (L-PK) and lipogenic enzyme genes (ACC, FAS) (28, 33, 41, 63, 69, 160, 167) . The original model proposed by Uyeda and

colleagues states that under low glucose conditions ChREBP is sequestered in the cytoplasm, presumably through PKA-mediated phosphorylation by a cAMP-generating signal, and that an increase in glucose metabolism promotes dephosphorylation and entry of ChREBP into the nucleus (167). Once in the nucleus, ChREBP mediates expression of target genes by binding to the Carbohydrate Response Element (ChoRE; composed of two E-boxes separated by 5 bp), as a heterodimer with its partner Mlx (96, 136). Signals that increase intracellular cAMP levels have been suggested to provide inhibitory phosphorylation of ChREBP, consequently abolishing its nuclear localization and DNA binding, and subsequently, transactivation of the L-PK gene. Recent studies, however, have shown that this model is most likely incomplete and that mechanisms in addition to the phosphorylation of ChREBP at sites 196 and 666 are necessary for controlling regulation of the L-PK gene. For example, double mutations of these phosphoacceptor sites fail to be dominant positive mutations, and several highly conserved domains in the amino terminus (Mondo Conserved Regions, MCR1-5) have been identified that confer inhibitory and stimulatory functions (28, 38, 86, 151). Furthermore, it has been suggested recently that although glucose promotes a modest increase in the rate of nuclear ChREBP influx, cytoplasmic-nuclear shuttling is not the major mechanism of ChREBP regulation by glucose (38, 87), although this is still unresolved (128).

Several groups have shown that a second transcription factor, Hepatocyte Nuclear Factor 4 α (HNF4α), plays an important complementary role to ChREBP in the induction of L-PK gene expression (1, 7, 42). Both the ChREBP (the ChoRE; located from -165 to -145 bp with respect to the transcription start site) and the HNF4α binding sites (the L3

element; located from -145 to -127 bp) are required for maximal glucose-induced transactivation of the L-PK gene (7, 42).

While ChREBP and HNF4α have been implicated as contributing factors in the regulation of the L-PK gene by glucose, the mechanisms underlying both the glucose activation and cAMP-mediated repression of this gene are largely unknown. Therefore, two key questions regarding regulation of the L-PK gene remain unanswered: 1) how does cAMP mediate repression of the glucose-stimulated induction? and 2) is there a coactivator necessary to provide the maximum response to glucose?

CBP is a versatile transcriptional coactivator that plays a pivotal role in coordinating signaling events with the transcription machinery, thus permitting the appropriate level of gene activity to occur in response to diverse stimuli (21). HNF4α, a member of the nuclear hormone receptor superfamily, interacts with the coactivator CREB binding protein (CBP) (168), leading us to hypothesize that CBP may be the coactivator necessary to communicate multiple signals (e.g., glucose and cAMP) to the L-PK gene promoter.

In this report we demonstrate that glucose and cAMP converge on a complex containing ChREBP, HNF4α and CBP to regulate L-PK gene transcription. We present multiple lines of evidence that ChREBP, HNF4α, and CBP are co-resident on the L-PK gene promoter and are equally important for full activation of the L-PK gene by glucose. Additionally, we demonstrate that the cAMP-mediated repression of the L-PK gene involves disassembly of a complex of proteins containing these factors, and can be overcome by increasing CBP abundance.

2.3 MATERIALS AND METHODS

2.3.1 Cell Culture, RNA isolation and quantification by RT-PCR

832/13 cell culture conditions were as described previously (60). Total RNA was isolated from 832/13 cells using TRI-reagent (Molecular Research Center; Cincinnati, OH, USA) according to the manufacturer's protocol. iScript (Bio-Rad; Hercules, CA, USA) was used for first-strand synthesis of cDNA using 0.5 µg of RNA. 2.5 µl cDNA was combined with 12.5 µl 2x iTaq SYBR Green Supermix with ROX (BioRad), 0.5 µl each of forward and reverse primers, in a total reaction volume of 25 µl. Real-time RT-PCR was performed using the Applied Biosystems Prism 7300 sequence detection system and software. Relative mRNA levels for individual genes were reported after normalization to cyclophilin mRNA levels. The forward and reverse primer sequences used for L-PK, NR4A2, and cyclophilin were as follows: L-PK (F) 5'- AACCTCCCCACTCAGCTACA, (R) 5'- TGCTCCACTTCTGTCACCAG; NR4A2 (F) 5'- GGCTGGTCAAGATGGGTAGA, (R) 5'- AAATGCCCTTTCACGTTCTG; cyclophilin (F) 5'-TGGTGGCAAGTCCATCTACG, (R) 5'- AAAATGCCCGCAAGTCAAAG.

2.3.2 Isolation of nuclear and cytosolic proteins, and immunoblotting

Nuclear and cytosolic fractions were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce; Rockford, IL, USA). The protein concentration was determined using the BCA assay (Pierce) with bovine serum albumin as the standard.

Immunoblotting was performed as previously described (26). Antibodies used for quantification of CBP (Cat: #: sc-369) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), ChREBP (Cat #: NB400-135) from Novus Biologicals (Littleton, CO, USA), HNF4α (Cat #: MAB10090) from Millipore (Temecula, CA, USA), GFP (Cat #: DB064) from Delta Biolabs (Gilroy,CA, USA), β Actin (Cat #: 4967) and Tubulin (Cat #: 2144) from Cell Signaling Technology (Danvers, MA, USA). Mlx antibody was a gift from Dr. Donald Ayer (University of Utah) (9).

2.3.3 Plasmids

The L-PK wild-type plasmid (pLPK-183), pL-PK* (constructed with the ChoRE replaced by a Gal4 DNA binding site), Gal4-DNA Binding Domain (DBD), Gal4-ChREBP, Gal4-S196A, Gal4-T666A or Gal4-S196A/T666A were described previously (28). The pG5-luc (5x Gal4) plasmid was from Promega (Madison, WI, USA). The pRc/RSV-CBP plasmid was a gift from Dr. Richard Goodman (Oregon Health & Science University) (80). RSV-PKA Cα plasmid was a gift from Dr. William Walker (University of Pittsburgh) (154). The pLPK- CAT plasmids (wild-type and linker scanning mutants LS-2, LS-3 and LS-4) were kindly provided by Dr. Howard Towle (University of Minnesota) (89). Sequencing was conducted by the Genomics and Proteomics Core Laboratories of the University of Pittsburgh.

2.3.4 Transient transfection and luciferase assay

Transfection of 832/13 cells was performed using LipofectAMINE (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were transfected with DNA plasmids in serum-free media. Following an 18 h incubation, cells were treated for 6 h with 2 mM or 20 mM glucose in the presence or absence of 10 μM forskolin. Cells were lysed in Passive Lysis Buffer (Promega) and luciferase assays were performed with lysates using the Dual-luciferase Reporter Assay System (Promega) in a Luminoskan Ascent luminometer (Thermo Scientific; Waltham, MA, USA). Relative light units were normalized to protein content using a BCA protein assay (Pierce).

2.3.5 siRNA-mediated Suppression of Gene Expression

The expression of ChREBP, HNF4α and CBP was decreased by transfecting pre-annealed duplexes (ChREBP: siRNA ID# 57311 and 190928, HNF4α: siRNA ID # 51661 and 199193, CBP: siRNA ID # 199670 and 57051) from Ambion (Austin, TX, USA) into 832/13 cells using Dharmafect reagent 1 (Dharmacon; Lafayette, CO, USA) according to the manufacturer's suggested protocol. Suppression of the targeted genes was analyzed by immunoblotting.

2.3.6 CAT ELISA assay

Transfection of 832/13 cells was performed using LipofectAMINE (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were transfected with 1 μg of wild-type or mutant L-PK plasmids in addition to either 1 μg of CBP or control plasmid in serum-free media. Following an 18 h incubation, cells were treated for 6 h with 2 mM or 20 mM glucose in the presence or absence of 10 μM forskolin. CAT ELISA assay was performed using a kit (Cat # 11363727001) from Roche Diagnostics (Indianapolis, IN, USA) according to the manufacturer's instructed protocol.

2.3.7 Chromatin Immunoprecipitation assay

832/13 cells were cultured in 2 mM or 20 mM glucose in the presence or absence of 10 μM forskolin. Following a 6 h incubation, chromatin immunoprecipitation assays were performed by following the Upstate (Temecula, CA, USA) Biotechnology ChIP assay kit protocol, as described previously (28). The ChoRE containing portion of the L-PK promoter and a fragment of the coding region 5 kb downstream of the L-PK transcriptional start site were targeted for amplification. Relative binding of each factor is reported after normalization to IgG control conditions. Forward and reverse primers used to amplify the ChoRE- containing region of the L-PK gene promoter were as follows: 5'-GGATGCCCAATATAGCCTCA-3' and 5'-CCATGCTGCTACGTTGCTTA-3' (upstream and downstream, respectively). Primers used to amplify the CRE of the NR4A2 promoter (located from -116 to -109 with respect to the transcriptional start site)

were as follows: 5'- TTGCTTGTACCAAATGCCC -3' and 5'- TTGTAGTAAACCGACCCGC-3' (upstream and downstream, respectively). Antibodies used for immunoprecipitation of HNF4α (Cat # sc-8987), CBP and IgG (Cat #: sc-2027) were from Santa Cruz Biotechnology and ChREBP from Novus Biologicals.

2.3.8 Sequential Chromatin Immunoprecipitation Assay

GFP-ChREBP was transduced into 832/13 cells, incubated for 24 h then cultured in 2 mM or 20 mM glucose in the presence or absence of 10 μM forskolin for 6 h. Sequential chromatin immunoprecipitation was performed as described previously (165). GFP-ChREBP was immunoprecipitated with anti-GFP antibody, followed by elution with GFP peptide and then the eluate was subjected to second immunoprecipitation with either CBP or HNF4α antibodies. GFP antibody and GFP blocking peptide (Cat #: DB064P) were from Delta Biolabs.

2.3.9 Statistical analyses

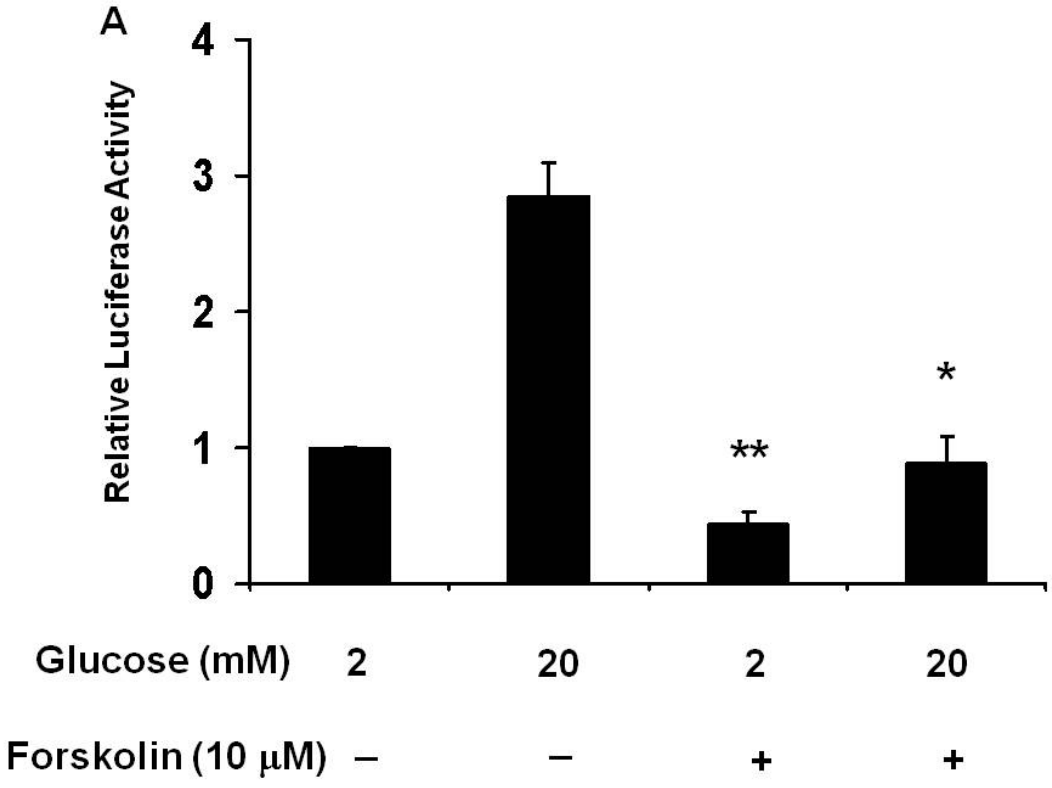
A one-way ANOVA was performed to detect statistical differences (p values < 0.05). A Tukey post hoc test was used to determine statistical differences within the ANOVA. All data are reported as means ± SEM.

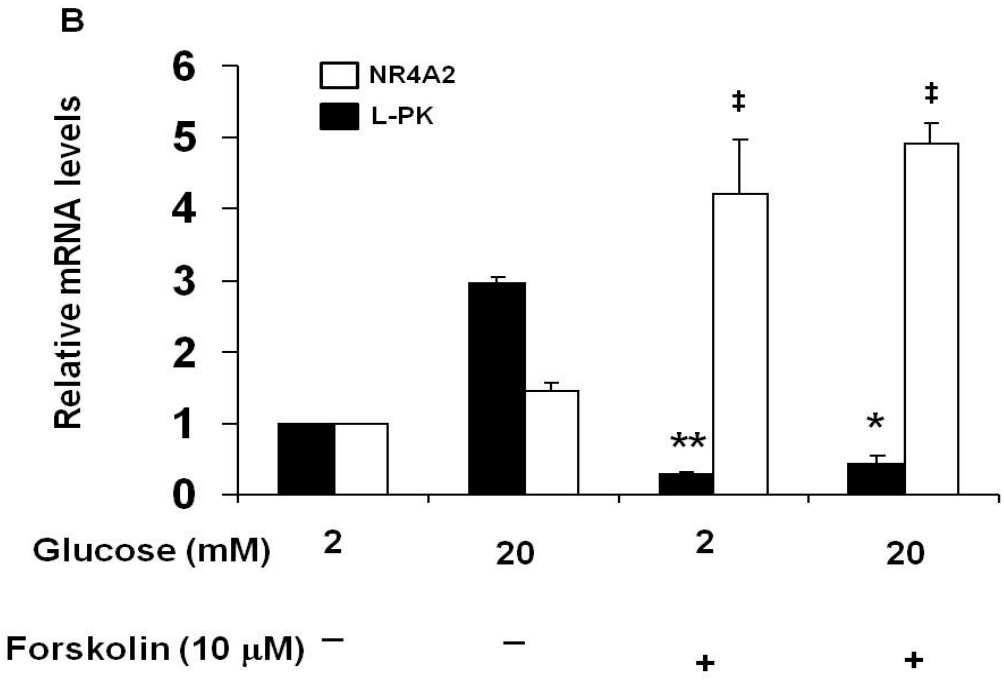
2.4 RESULTS

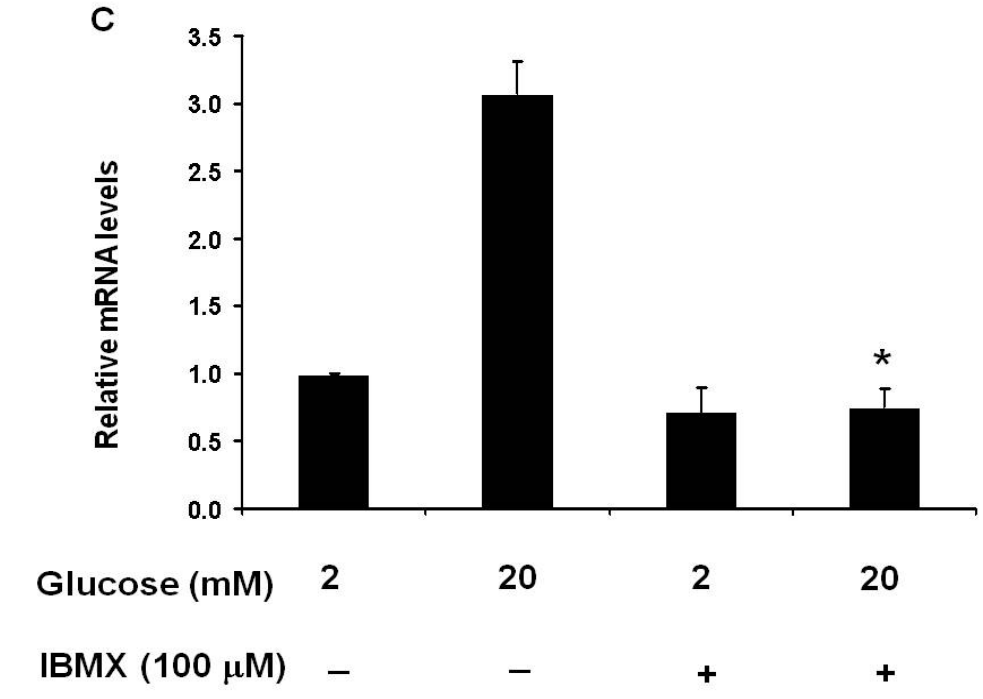
2.4.1 cAMP agonists block the glucose-mediated induction of the L-PK gene.

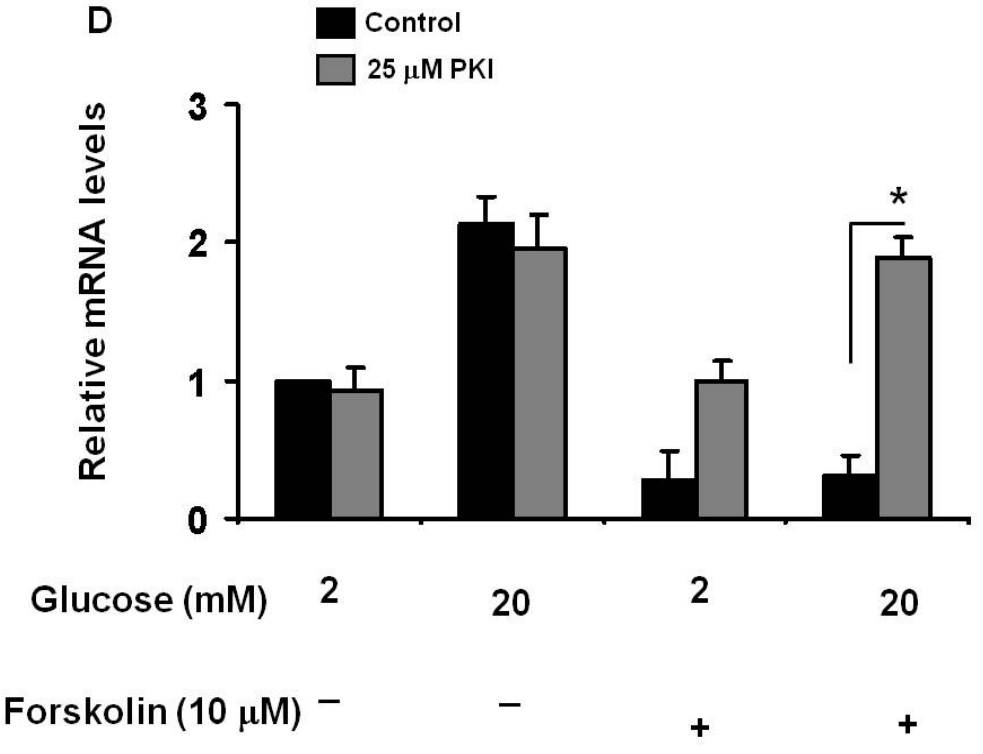
Glucose increases the expression of the L-PK gene while cAMP agonists block this induction (100). To further investigate a mechanism for the glucose-stimulated induction and cAMP-mediated repression of the L-PK gene, we used the INS-1-derived 832/13 β-cell line, which has been selected for its fine-tuned sensitivity to glucose (60). 832/13 cells treated with 20 mM glucose for 6 h had an approximate 3-fold induction of L-PK promoter activity, which has been previously reported (28). Here we now show that this induction was completely blocked by co-treatment with 10 μM forskolin, an activator of adenylate cyclase (Figure 1A). Similar to the effects seen with promoter activity, the 3-fold increase in L-PK mRNA levels induced by glucose was also blunted by cAMP agonists forskolin (Figure 1B) and IBMX (Figure 1C); whereas the NR4A2 (Nurr1) control gene was induced approximately 5-fold under the same conditions, demonstrating specific and coordinate regulation of gene expression by cAMP (Figure 1B). Forskolin-mediated repression of L-PK expression is completely blocked in the presence of 25 μM myristoylated PKI, a specific PKA inhibitor, demonstrating a cAMP-PKA pathway requirement for the repression of this gene (Figure 1D). In addition, transfection of a constitutively-active PKA mimicked the effect of forskolin. Importantly, addition of 10 μM forskolin in the presence of constitutively-active PKA showed no further repressive effects on L-PK promoter activity, as compared to either of these treatments alone (Figure 1E). Taken together, it is clear that the repressive effects of cAMP are mediated

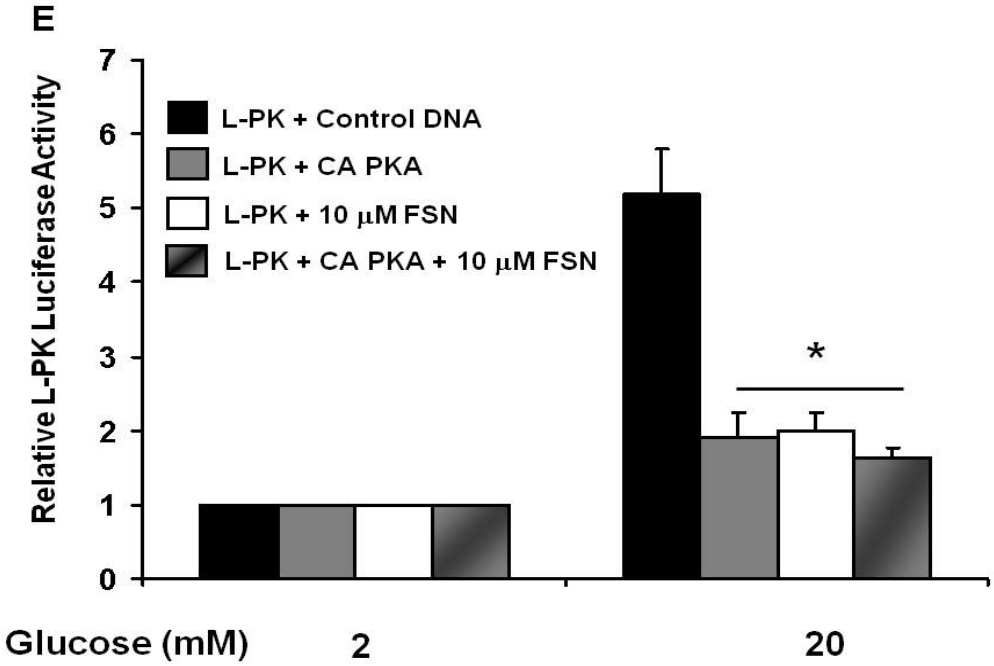
predominately via a PKA-dependent pathway. In addition, because forskolin completely inhibited L-PK gene promoter activity (Figure 1A) but did not appreciably affect mRNA stability in either the unstimulated (data not shown) or glucose-induced state (Figure 1F); we conclude that the cAMP-mediated repression of the L-PK gene occurs largely at the transcriptional level.


A
Relative Luciferase Activity
4
3
2
1
0
**
*
Glucose (mM) 2 20 2 20
Forskolin (10 μM) – – + +


B
Relative mRNA levels
NR4A2
L-PK
6
5
4
3
2
1
0
‡
‡
**
*
Glucose (mM) 2 20 2 20
Forskolin (10 μM) – – + +


C
Relative mRNA levels
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Glucose (mM) 2 20 2 20
IBMX (100 μM) – – + +


D
Control
25 μM PKI
Relative mRNA levels
3
2
1
0
*
Glucose (mM) 2 20 2 20
Forskolin (10 μM) – – + +


Relative L-PK Luciferase Activity
7
6
5
4
3
2
1
0
L-PK + Control DNA
L-PK + CA PKA
L-PK + 10 μM FSN
L-PK + CA PKA + 10 μM FSN
*
Glucose (mM)
2
20

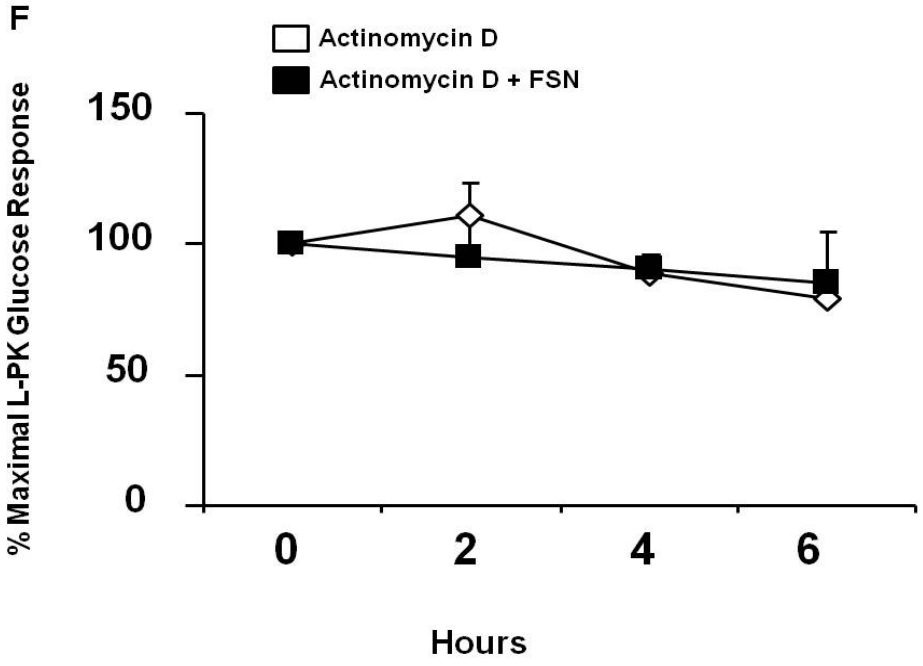
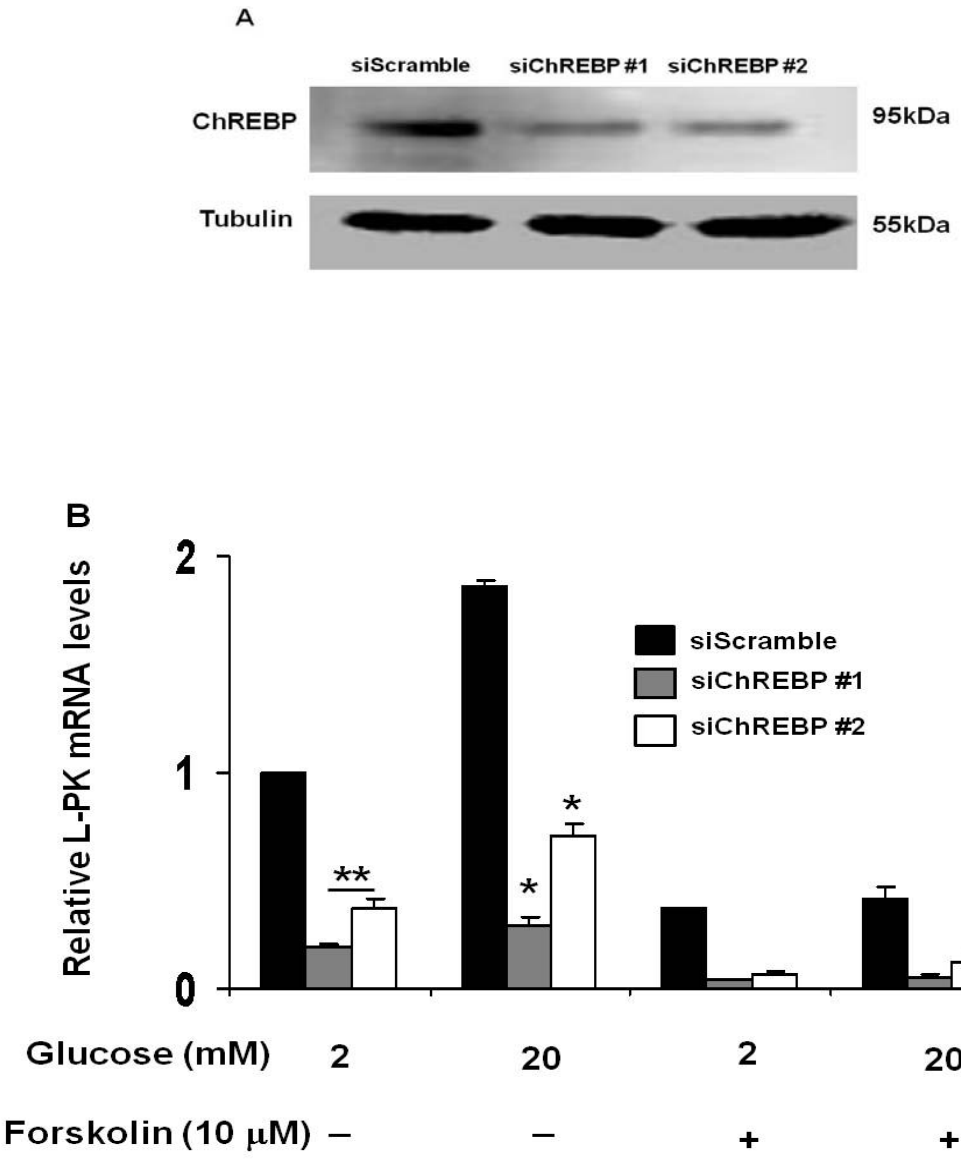
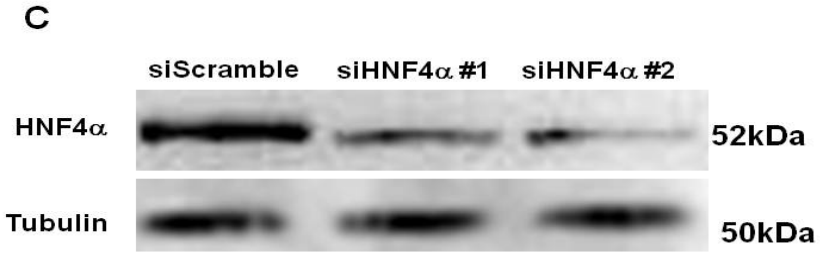

% Maximal L-PK Glucose Response
Actinomycin D
Actinomycin D + FSN
150
100
50
0
0
2
4
6
Hours

Figure 1. cAMP represses L-PK promoter activity and mRNA levels, but does not decrease mRNA stability in 832/13 cells. *A*. 832/13 cells were transiently transfected with 2 μg L-PK promoter driven-luciferase construct (pLPK-183) for 18 h and luciferase activity was measured following an additional 6 h incubation with 2 mM or 20 mM glucose in the presence or absence of 10 μM forskolin. ***P* < 0.05 vs. 2 mM glucose, **P* < 0.01 vs. 20 mM glucose. 832/13 cells were treated for 6 h with 2 mM or 20 mM glucose in the presence or absence of 10 μM forskolin (B) or 100 μM IBMX (C) . *D*. Cells were pretreated for 1 h with 25 μM PKI, followed by a 6 h incubation with 2 mM or 20 mM glucose in the presence or absence of 10 μM forskolin. Following extraction of total RNA (B-D), relative levels of L-PK and NR4A2 mRNA were analyzed by real-time RT-PCR. ***P* < 0.05 vs. 2 mM glucose, **P* < 0.001 vs. 20 mM glucose, ‡ *P* < 0.005 vs. 20 mM glucose. *E.* 832/13 cells were co-transfected with 1 μg pLPK-183 and either control pUC19 (1 μg) or constitutively active RSV-PKA Cα (1 μg). Cells were treated with 2 mM or 20 mM glucose in the presence or absence of 10 μM forskolin. Following a 6 h incubation, luciferase activity was measured. Data are presented relative to 2 mM glucose (note that forskolin treatments decreased basal promoter activity as in panel 1A when expressed as absolute values) **P* < 0.05 vs. 20 mM glucose. *F.* 832/13 cells were treated with 20 mM glucose for 6 h, followed by treatment with or without 10 μM forskolin in the presence of the transcriptional inhibitor Actinomycin D. The decay of mRNA was determined by quantifying the amount of specific mRNA for the L-PK gene at 2, 4 and 6 h time points. Data shown are means ± SEM from three independent experiments.

2.4.2 Depletion of either ChREBP or HNF4α blunts expression of the L-PK gene.

Whether or not ChREBP and its neighboring factor HNF4α, are equally required for glucose-mediated expression of the L-PK gene in β cells is undetermined. To address this issue, we suppressed endogenous ChREBP levels using two different siRNA duplexes. This resulted in a 74% and 80% decrease of ChREBP abundance (Figure 2A), which was sufficient to decrease the expression of the L-PK gene by 84% and 62%, for duplex 1 and 2, respectively (Figure 2B). Additionally, siRNA-directed suppression of HNF4α abundance by 73% and 51%, for duplex 1 and 2, respectively (Figure 2C), revealed that glucose was unable to induce the L-PK gene without this factor (Figure 2D), similar to that seen in the data above with siChREBP. Importantly, silencing either ChREBP or HNF4α further decreased expression of the L-PK gene at 2 mM glucose (Figures 2B and D), similar to results obtained with forskolin treated cells (see Figure 1).We therefore conclude that an siRNA-mediated decrease in the abundance of HNF4α or ChREBP is equally sufficient to blunt the expression of the glucose-responsive L-PK gene.


A
siScramble
siChREBP #1
siChREBP #2
ChREBP
95kDa
Tubulin
55kDa
B
Relative L-PK mRNA levels
2
1
0
siScramble
siChREBP #1
siChREBP #2
**
*
*
Glucose (mM) 2 20 2 20
Forskolin (10 μM) – – + +


C
siScramble
siHNF4α #1
siHNF4α #2
HNF4α
52kDa
Tubulin
50kDa

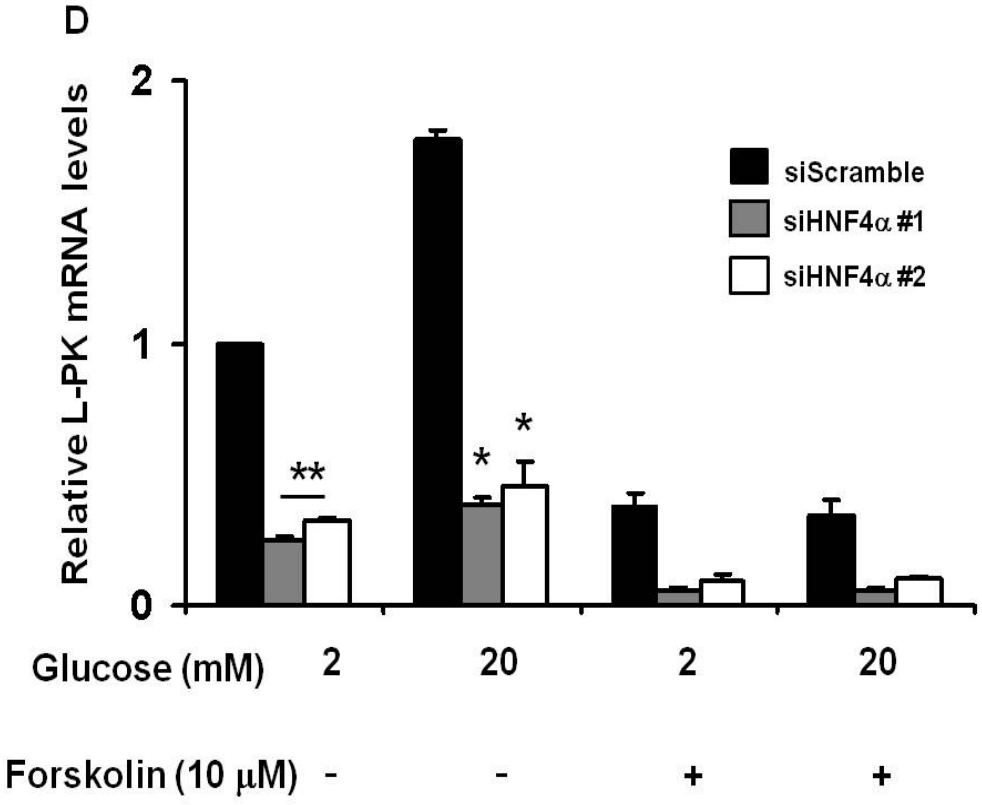
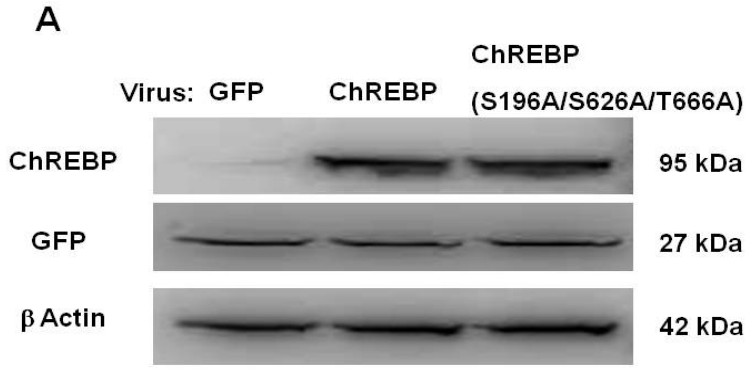

D
Relative L-PK mRNA levels
2
1
0
siScramble
siHNF4α #1
siHNF4α #2
**
*
*
Glucose (mM) 2 20 2 20
Forskolin (10 μM) - - + +

Figure 2. Decreasing the abundance of ChREBP or HNF4α inhibits expression of the L-PK gene. 832/13 cells were transfected with either a scrambled control siRNA duplex (siScramble) or siRNA duplexes targeted to a 21 base pair region of the rat ChREBP or HNF4α genes. After 48 h of duplex exposure, nuclear extracts were harvested for immunoblotting using antibodies directed against either (A) ChREBP or (C) HNF4α, with the loading control, tubulin. The immunoblots are a representative of 2 independent experiments. 832/13 cells were transfected with above duplexes for 48 h, followed by a 6 h culture with 2 mM or 20 mM glucose, in the presence or absence of 10 μM forskolin. L-PK mRNA levels were analyzed by RT-PCR (B and D). Values represent the means ± SEM from three independent experiments. $^{**}P < 0.05$ vs. siScramble at 2 mM glucose, $^{*}P < 0.05$ vs. siScramble at 20 mM glucose for mRNA experiments.

2.4.3 Overexpression of ChREBP is not sufficient to prevent cAMP-mediated repression of the L-PK gene.

Because a decrease in ChREBP nuclear abundance clearly impaired the expression of the L-PK gene (Figures 2A and B), we hypothesized that simply enhancing ChREBP abundance may be sufficient to rescue the forskolin-mediated repression of this gene. To test this hypothesis, we overexpressed either wild-type ChREBP or a mutant ChREBP (S196A / S626A / T666A), wherein these three previously defined PKA phosphoacceptor sites have been removed (69, 87). Adenovirus-mediated overexpression of wild-type and the triple phospho-mutant ChREBP in the 832/13 rat insulinoma cell line led to a five-

and eight-fold increase in ChREBP mRNA levels, respectively (data not shown). This translated to quantitative increases in the nuclear abundance of wild-type and phosphoacceptor mutant ChREBP proteins, as compared to barely detectable endogenous nuclear ChREBP protein levels (Figure 3A). Despite these large increases in the nuclear abundance of wild-type or phosphoacceptor mutant ChREBP, this maneuver was unable to overcome the repression induced by forskolin (Figure 3B). Because restoring the abundance of ChREBP was insufficient to overcome cAMP-mediated repression, even when the ostensibly repressive PKA sites have been removed, we conclude that mechanisms other than absolute protein quantity or shuttling exist to communicate the induction by glucose and repression by cAMP to the L-PK gene. In addition, from these data and siChREBP data (Figure 2B) we conclude that ChREBP is necessary, but not sufficient, for the glucose-stimulated expression of the L-PK gene.


A
Virus: GFP
ChREBP
ChREBP
(S196A/S626A/T666A)
ChREBP
95 kDa
GFP
27 kDa
β Actin
42 kDa

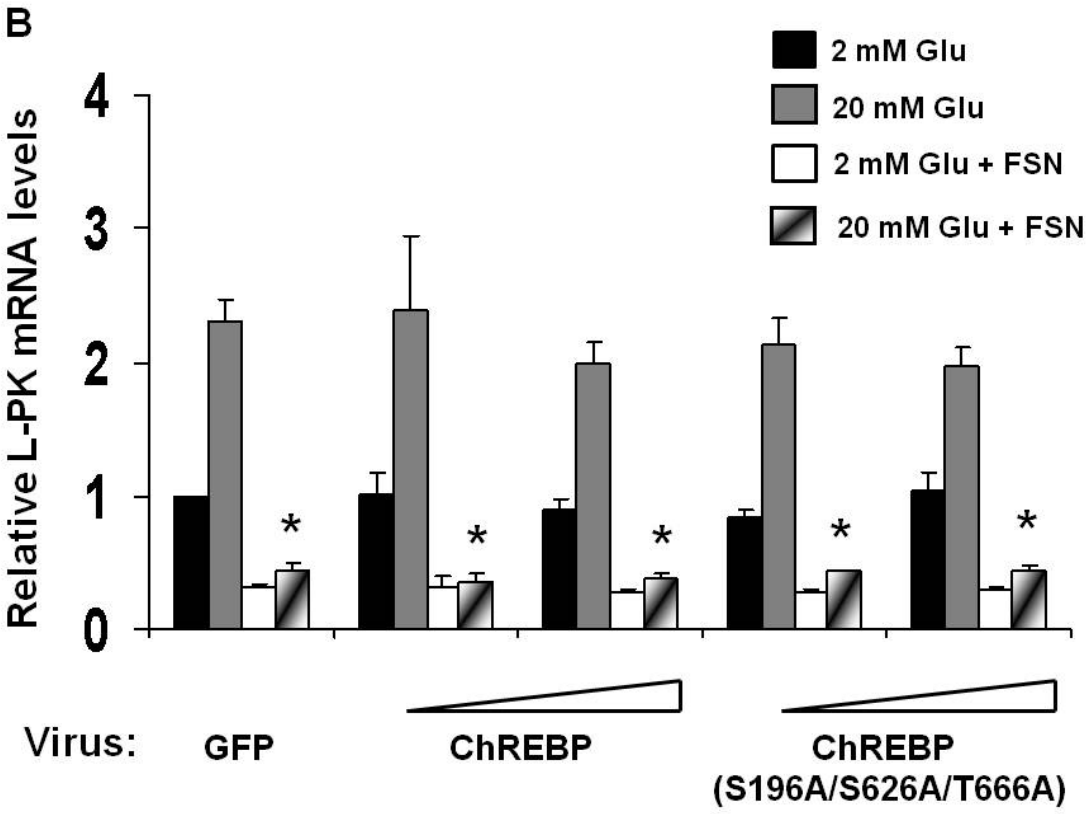

B
2 mM Glu
20 mM Glu
2 mM Glu + FSN
20 mM Glu + FSN
Relative L-PK mRNA levels
0
1
2
3
4
*
Virus:
GFP
ChREBP
ChREBP
(S196A/S626A/T666A)

Figure 3. Overexpression of either wild-type or phospho-mutant ChREBP is not sufficient to prevent cAMP-mediated repression of the L-PK gene. *A.* 832/13 cells were transduced with GFP-tagged wild-type and phospho-mutant ChREBP (S196A/ S626A/ T666A) adenoviruses. Following a 24 h incubation, nuclear extracts were prepared for immunoblotting with antibodies against ChREBP, GFP or β Actin as a loading control. *B.* After a 24 h exposure to adenovirus, cells were additionally treated with 2 mM or 20 mM glucose for 6 h in the presence or absence of 10 μM forskolin. Relative mRNA levels of L-PK were analyzed via RT-PCR. Data are means ± SEM. ***P* < 0.05 vs. 20 mM glucose for each respective group.

2.4.4 Glucose-dependent recruitment of ChREBP and HNF4α to the L-PK gene promoter is decreased by cAMP.

We next sought to determine whether ChREBP occupancy on the L-PK gene promoter was impacted by glucose and forskolin treatment. A chromatin immunoprecipitation assay using ChREBP antisera revealed that raising the glucose concentration from 2 to 20 mM induced a 9.5-fold increased recovery of the portion of the L-PK gene promoter containing the ChoRE (Figure 4), consistent with previous reports (28). However, when 832/13 cells are treated concurrently with 20 mM glucose and 10 μM forskolin, there was an 80% decrease in recovery of the same promoter fragments. Similarly, HNF4α recruitment to a region of the L-PK gene promoter containing the L3 element was increased 2.4-fold in the presence of glucose (Figure 4); exposure to forskolin decreased

recovery of this promoter region by 72%. A region that does not contain a ChoRE served as a negative control for transcription factor binding (data not shown). We conclude that cAMP agonists decrease the occupancy of ChREBP and HNF4α on their respective elements in the L-PK promoter despite the presence of a stimulatory concentration of glucose.

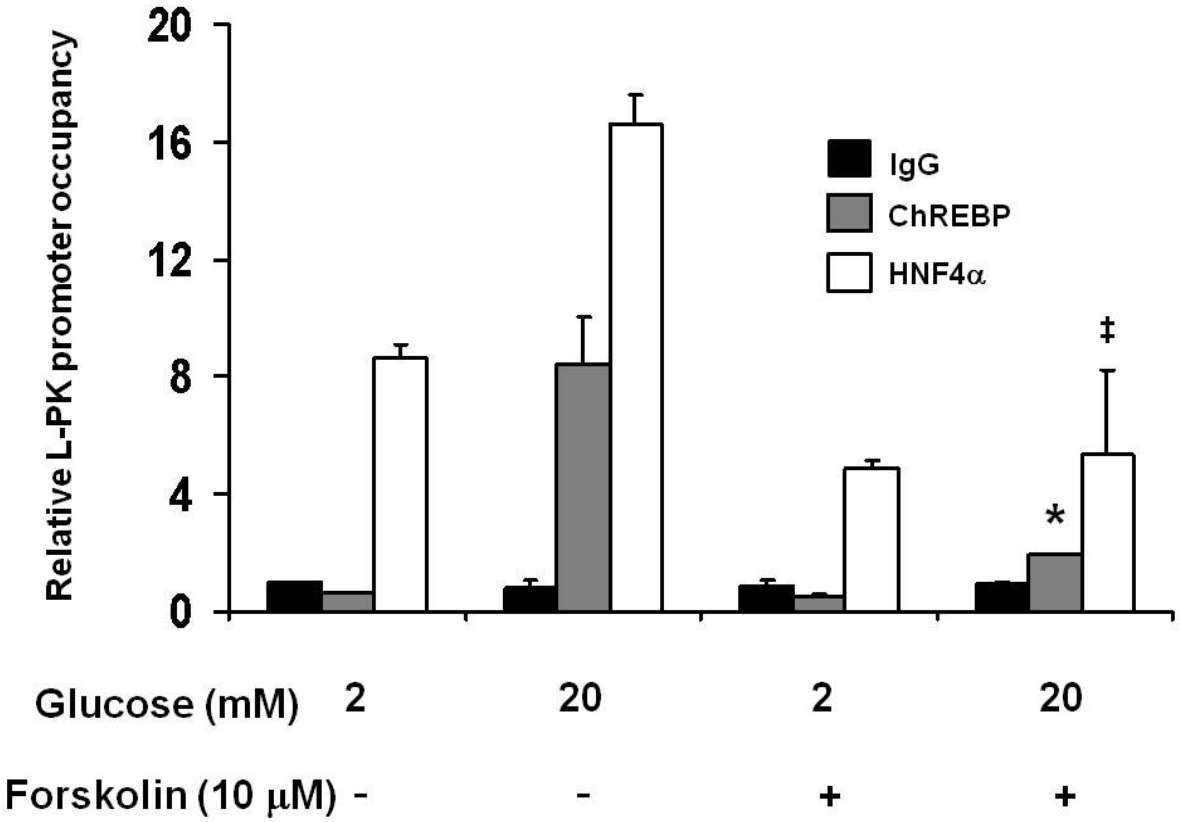


Figure 4. Recruitment of ChREBP and HNF4α to the L-PK gene promoter is decreased in the presence of cAMP. 832/13 cells were cultured in 2 mM or 20 mM glucose for 6 h in the presence or absence of 10 μM forskolin. Chromatin immunoprecipitation experiments were conducted with an antibody against ChREBP, HNF4α or control IgG. The gene promoter for L-PK was targeted for amplification. Data are expressed as means ± SEM from three independent experiments. **P* < 0.05 vs. 20 mM glucose, ‡ *P* < 0.05 vs. 20 mM glucose.

2.4.5 siRNA-mediated Suppression of CBP Prevents Glucose-mediated Induction of the L-PK gene.

It has been shown that CBP associates with HNF4α (53); however, whether CBP contributes to the glucose-mediated induction, or cAMP-directed repression, of the L-PK gene has never been tested directly. Therefore, we used siRNA-directed suppression of CBP to examine its potential role in the glucose-induction of the L-PK gene. Indeed, when CBP abundance is decreased by 63% and 70% using siRNA duplex transfection (Figure 5A), the ability of glucose to induce the expression of the L-PK gene was lost (Figure 5B). Similar to results seen with siChREBP and siHNF4α (Figures 2B and D), siRNA-mediated suppression of CBP abundance further decreased L-PK gene expression levels at the non-stimulatory glucose concentration (Figure 5B). To our knowledge, this is the first report demonstrating an absolute requirement for CBP to mediate the glucose response to this or any other glucose-responsive gene.

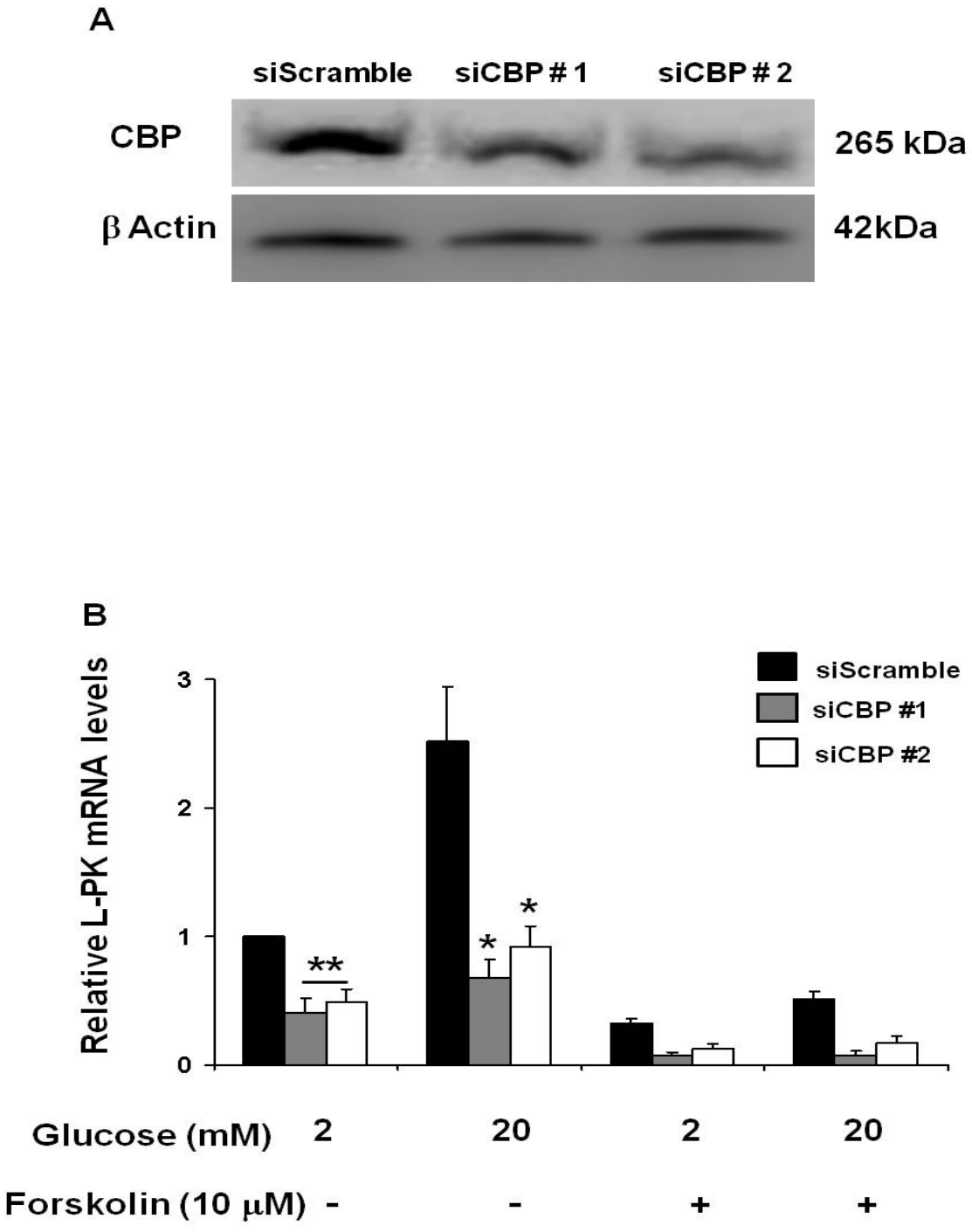
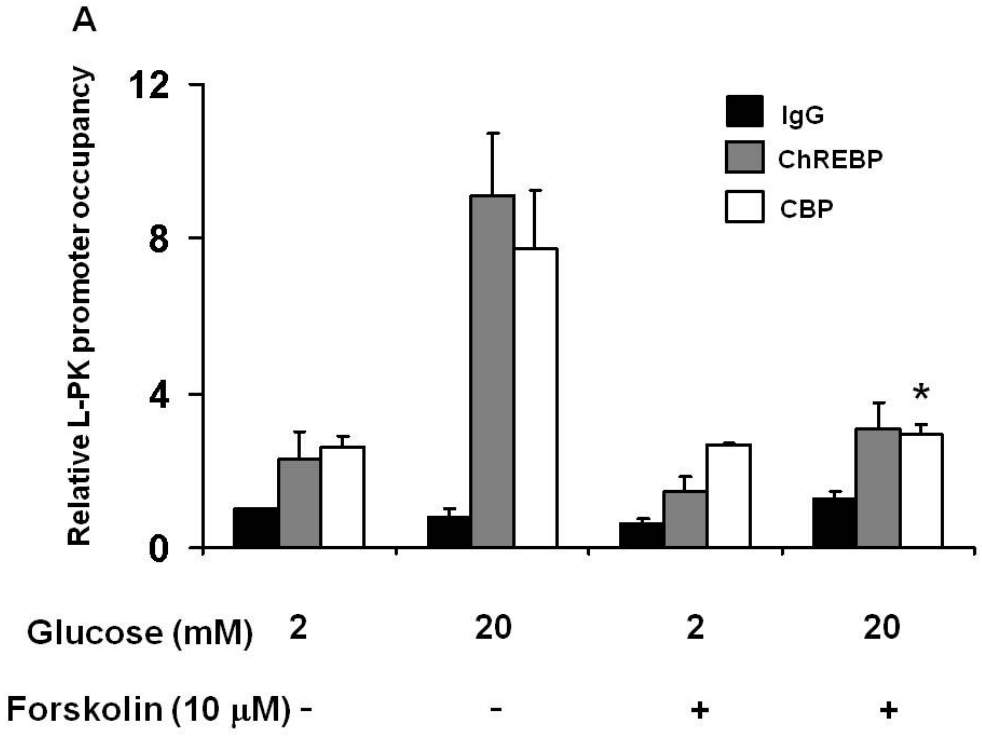

A
siScramble
siCBP # 1
siCBP # 2
CBP
265 kDa
β Actin
42kDa
B
Relative L-PK mRNA levels
siScramble
siCBP #1
siCBP #2
3
2
1
0
**
*
*
Glucose (mM) 2 20 2 20
Forskolin (10 μM) - - + +

Figure 5. siRNA-mediated suppression of CBP inhibits glucose-mediated induction of the L-PK gene. *A.* 832/13 cells were transfected with either siScramble or siRNA duplexes targeted to rat CBP mRNA for 48 h. Nuclear extracts were immunoblotted for CBP and β Actin as a loading control. The immunoblot is a representative of 2 independent experiments. *B.* 832/13 cells were transfected with the above duplexes for 48 h, followed by a 6 h culture with 2 mM or 20 mM glucose, in the presence or absence of 10 μM forskolin. L-PK mRNA levels were quantified by RT-PCR. Values represent the means ± SEM from three independent experiments. $^{**}P < 0.05$ vs. siScramble at 2 mM glucose, $^{*}P < 0.05$ vs. siScramble at 20mM glucose.

2.4.6 The glucose-mediated recruitment of CBP to the L-PK gene promoter is blocked by cAMP.

Because CBP is required for the glucose-mediated expression of the L-PK gene (Figure 5B), we next investigated whether glucose recruits CBP directly to the L-PK gene promoter and, in addition, if the cAMP-mediated deactivation of the L-PK gene was associated with loss of this coactivator molecule. Using chromatin immunoprecipitation, we observed a 2.7-fold increase in CBP occupancy on the ChoRE containing region of the L-PK gene promoter in response to increased (2 mM vs. 20 mM) glucose stimulation. Furthermore, forskolin treatment abolished this interaction (Figure 6A). Concomitant with loss of CBP on the L-PK gene promoter, in the presence of the cAMP signal we

readily detected CBP association with the NR4A2 gene promoter (Figure 6B). Taken together with the effects of siRNA-directed suppression of CBP, we conclude that the forskolin-mediated repression of L-PK gene transcription occurs, at least in part, due to a loss of CBP occupancy on the L-PK gene promoter.


A
Relative L-PK promoter occupancy
12
8
4
0
IgG
ChREBP
CBP
*
Glucose (mM) 2 20 2 20
Forskolin (10 μM) - - + +

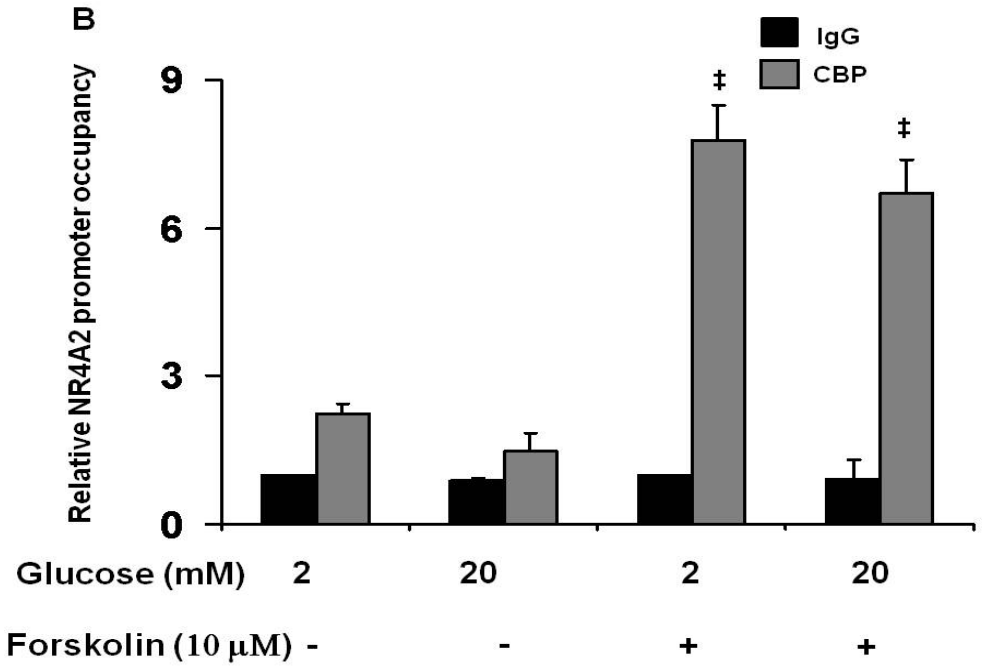
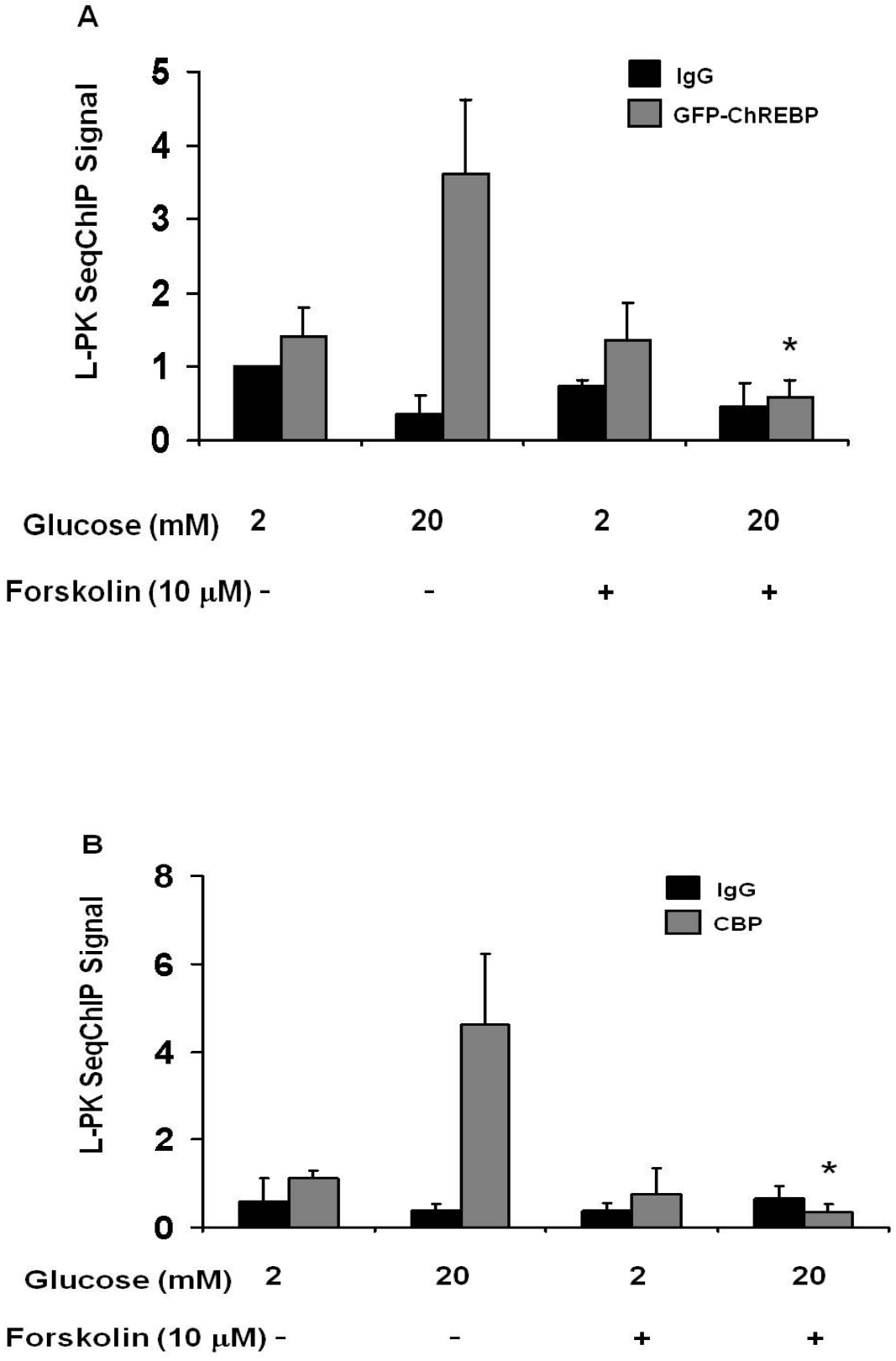

B
Relative NR4A2 promoter occupancy
9
6
3
0
IgG
CBP
‡
‡
Glucose (mM) 2 20 2 20
Forskolin (10 μM) - - + +

Figure 6. Glucose-dependent recruitment of CBP to the L-PK gene promoter is blocked by cAMP. 832/13 cells were cultured in 2 mM or 20 mM glucose in the presence or absence of 10 μM forskolin for 6 h. Relative promoter occupancy was determined by chromatin immunoprecipitation assay, using antibodies directed against ChREBP and CBP (A), or CBP alone (B), with IgG antisera serving as a control. The gene promoters for L-PK (A) and NR4A2 (B) were targeted for amplification. Data are means ± SEM from three independent experiments. *$P < 0.1$ vs. 20 mM glucose, ‡ $P < 0.05$ vs. 20 mM glucose.

2.4.7 ChREBP, HNF4α, and CBP co-occupy the LPK gene promoter in a glucose-dependent manner, and this complex is disrupted by cAMP.

Because CBP is required for glucose to induce the expression of the L-PK gene (Figure 5B) and glucose recruits CBP to the L-PK gene promoter (Figure 6A), we next examined whether CBP, ChREBP and HNF4α are present together in this context. To test this hypothesis, sequential ChIP (SeqChIP) analysis was performed. This assay provides information regarding co-occupancy of multiple proteins within a specific genomic region (51). In the first SeqChIP experiment, there was a 2.6-fold increase in promoter fragments recovered by immunoprecipitating overexpressed GFP-tagged ChREBP (Figure 7A), followed by eluting with GFP peptide and a subsequent second IP of the eluate using CBP antisera (Figure 7B). There was a 4.1-fold increase in CBP SeqChIP

signal at 20 mM glucose. When forskolin was added, there was a 12.8-fold decrease in CBP SeqChIP signal in the context of the ChoRE of L-PK gene promoter (Figure 7B). In the next experiment, we first immunoprecipitated GFP-tagged ChREBP (Figure 7C), followed by a second IP with antisera against HNF4α (Figure 7D). There was an approximate 1.6-fold increase in HNF4α SeqChIP signal, while forskolin diminished this recovery by 3-fold. We conclude that forskolin disrupts the ability of glucose to promote a complex containing CBP, ChREBP and HNF4α on the L-PK gene promoter, thus terminating transcription of this gene.


A
L-PK SeqChIP Signal
5
4
3
2
1
0
IgG
GFP-ChREBP
*
Glucose (mM) 2 20 2 20
Forskolin (10 μM) - - + +
B
L-PK SeqChIP Signal
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6
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2
0
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CBP
*
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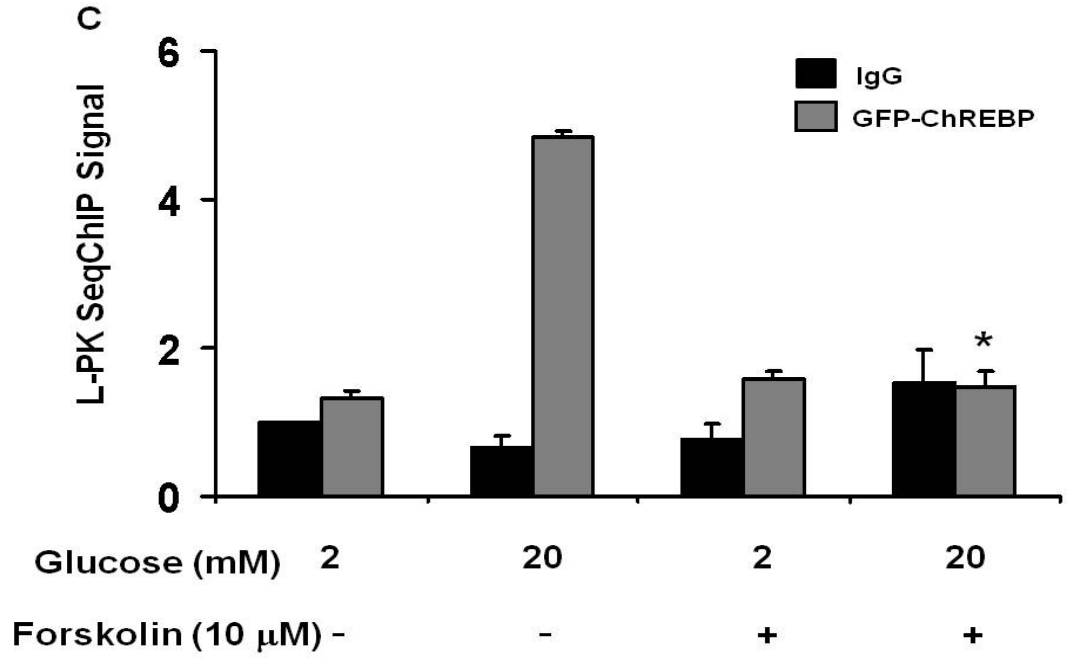
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Glucose (mM) 2 20 2 20
Forskolin (10 μM) - - + +

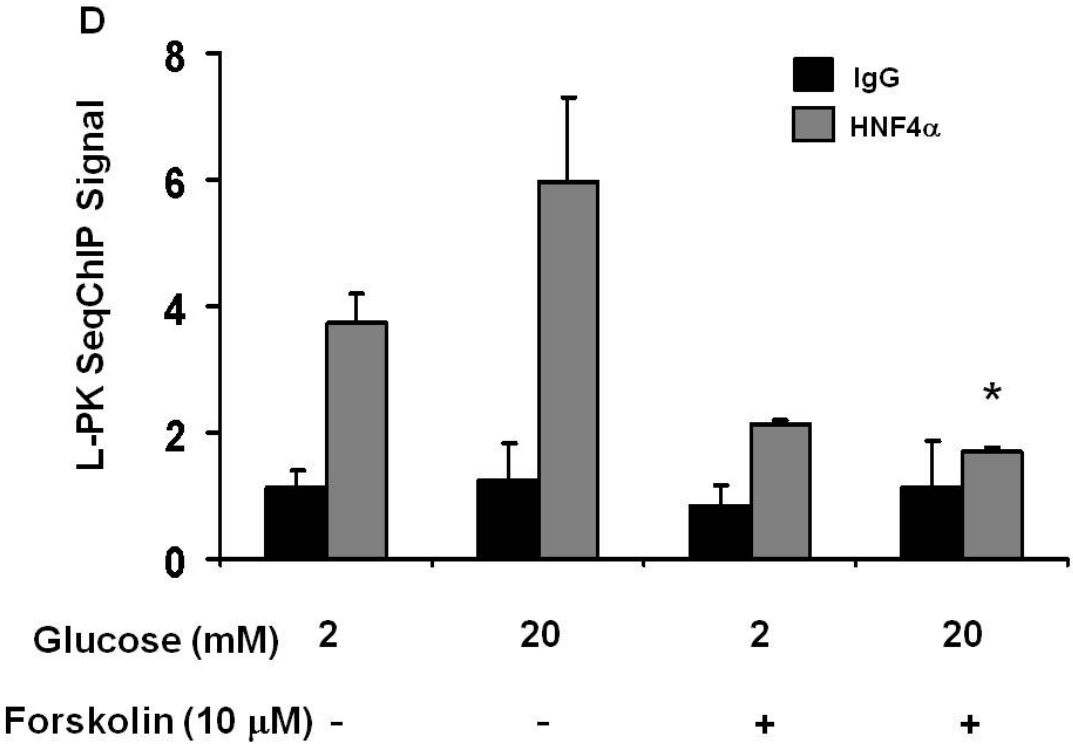

D
L-PK SeqChIP Signal
8
6
4
2
0
IgG
HNF4α
*
Glucose (mM) 2 20 2 20
Forskolin (10 μM) - - + +

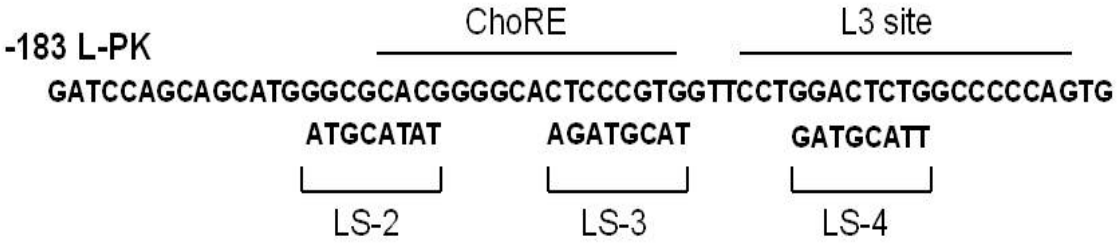
Figure 7. CBP, ChREBP and HNF4α become co-resident on the L-PK gene promoter in a glucose-dependent manner and this complex is disrupted by cAMP. 832/13 cells were transduced with GFP-ChREBP adenovirus, cultured for 24 h, followed by treatment with 2 mM or 20 mM glucose in the presence or absence of 10 μM forskolin for 6 h. Subsequently, GFP-ChREBP was immunoprecipitated with either anti-IgG or anti-GFP antibodies (A). CBP was then immunoprecipitated from the resulting eluate using anti-CBP with anti-IgG antisera used as a control (B). In a separate experiment performed under the same conditions, GFP-ChREBP was immunoprecipitated using IgG control or GFP antibodies (C), followed by a second immunoprecipitation of the eluate with either IgG or HNF4α antibodies (D). For all immunoprecipitates, the ChoRE-containing region of the L-PK gene promoter was targeted for amplification via real-time RT-PCR (A-D). Shown are means ± SEM from three independent experiments. *$P < 0.05$ vs. 20 mM glucose.

2.4.8 Increasing CBP abundance enhances glucose-mediated activation and abrogates the repression of the L-PK gene by cAMP.

Since CBP is recruited to the L-PK gene promoter in a glucose-dependent manner and its occupancy is lost under conditions where cAMP is elevated (Figure 6A), we hypothesized that CBP levels may be a limiting factor in regulation of this gene, as has been suggested for other genes regulated at the transcriptional level (134). Therefore, we examined both wild-type and mutant L-PK gene promoter activity following CBP

overexpression. Four L-PK-CAT reporter constructs were used for this experiment: wild-type plus three mutant promoter constructs (two different ChoRE site mutations and an L3 site mutation) (Figure 8A) (89). These four constructs allow us to further examine whether the transcription of the L-PK gene requires recruitment of the complex containing ChREBP, HNF4α and CBP to the L-PK promoter as demonstrated by sequential ChIP (Figure 7A). First, glucose-induced wild-type L-PK promoter activity 3-fold; when CBP was overexpressed glucose-mediated induction of promoter activity was augmented to 5.3-fold (Figure 8B). Additionally, increasing CBP abundance was sufficient to overcome the forskolin- mediated repression of the wild-type L-PK gene promoter (Figure 8B). Next, when either E-box in the ChoRE site was mutated (mutants LS-2 and LS-3) the glucose-induction of promoter activity was reduced to 1.5-fold, and no potentiation of this response was seen with CBP overexpression. Additionally, in the presence of ChoRE site mutants CBP overexpression was no longer able to overcome L-PK promoter activity repression by cAMP. Lastly, when the HNF4α binding site, L3, was mutated (LS-4 mutant) the glucose-induction of promoter activity was completely abolished in the presence or absence of CBP overexpression. Taken together with the above sequential ChIP data these findings illustrate binding of ChREBP, HNF4α and CBP to the L-PK promoter as a complex. We've shown a clear dependence on CBP to regulate expression of the L-PK gene by both glucose and cAMP and this regulation is lost when ChREBP or HNF4α is blocked from accessing the promoter.

A


-183 L-PK
ChoRE
L3 site
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ATGCATAT
AGATGCAT
GATGCATT
LS-2
LS-3
LS-4

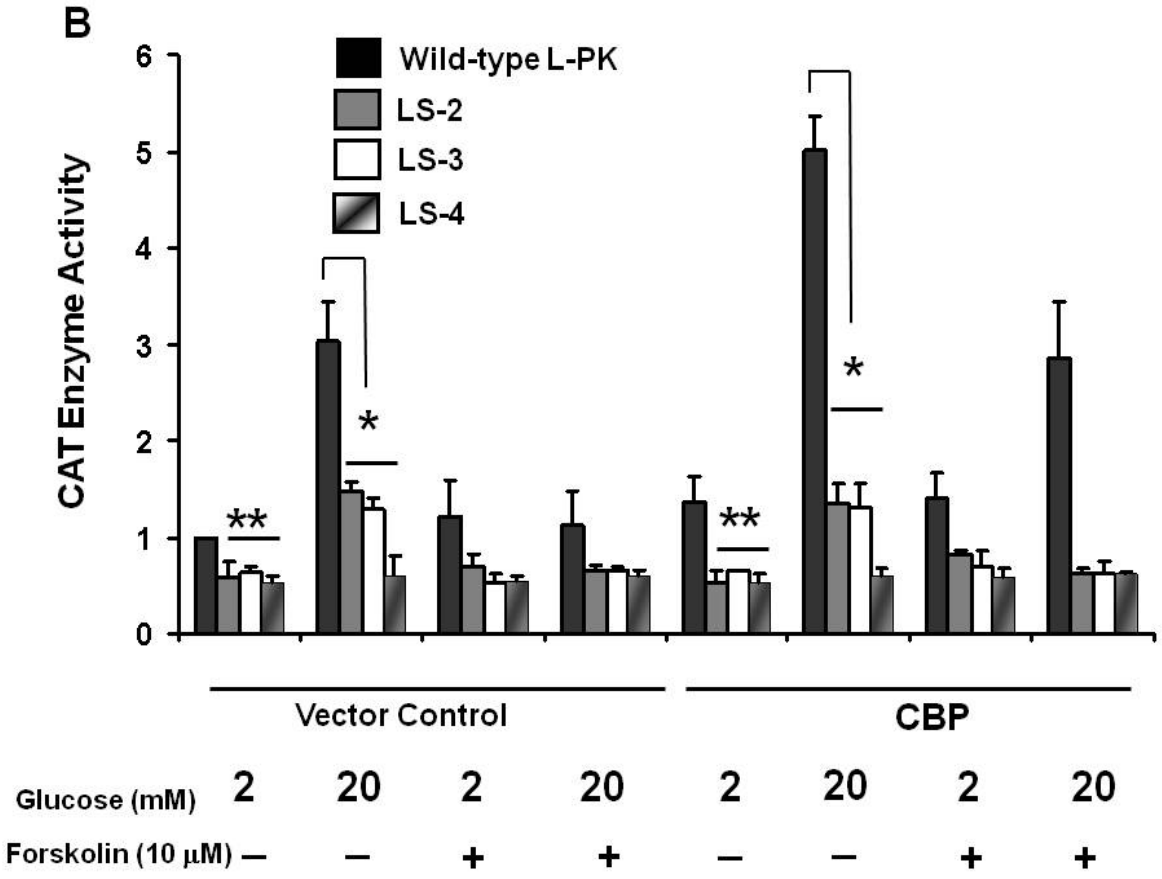
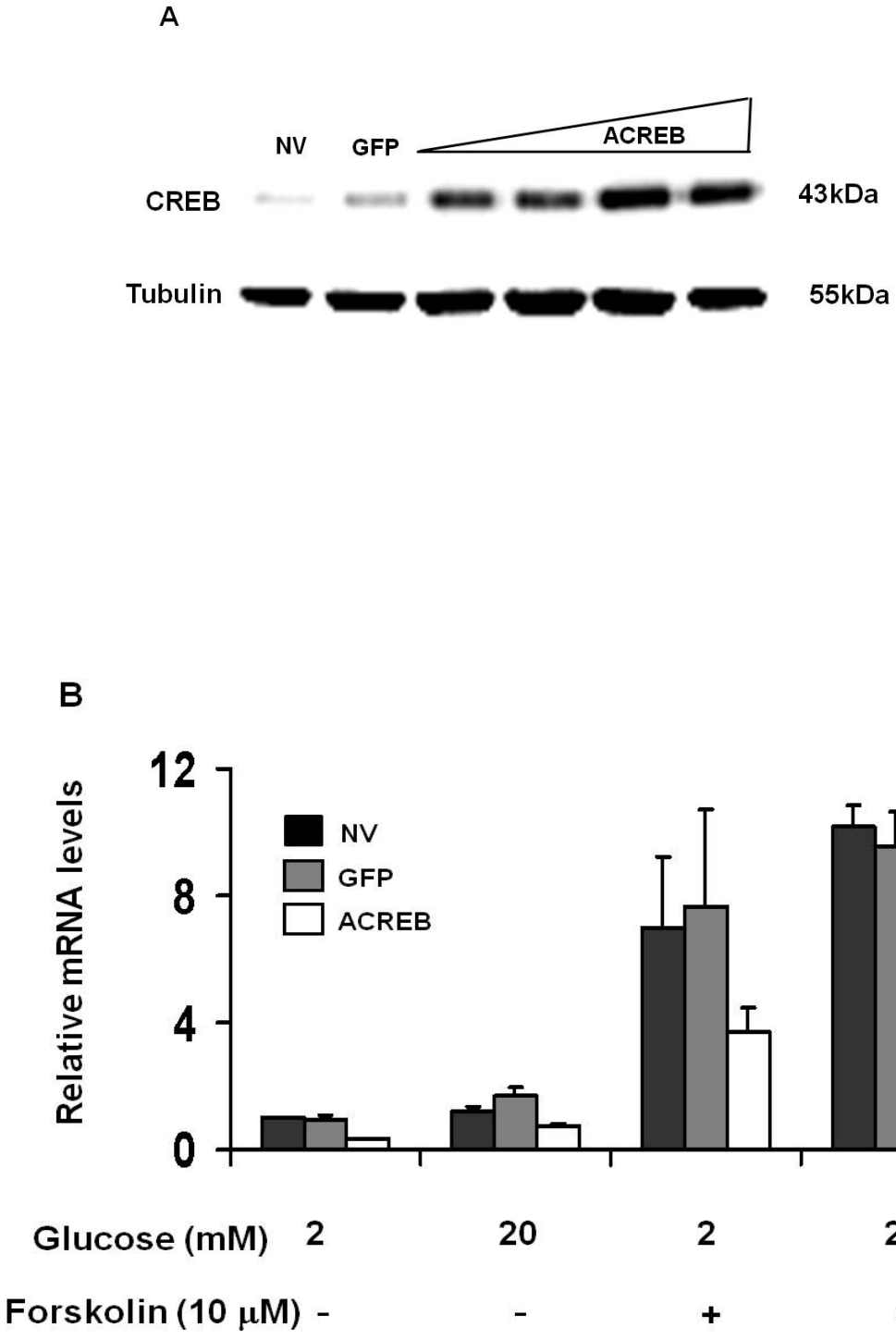


Figure 8. **Increasing CBP abundance enhances glucose-mediated activation and abrogates the repression of the L-PK gene by cAMP.** *A.* linker scanning mutations in the -183 to -125 region of the L-PK promoter were as described under "Materials and Methods". *B.* 832/13 cells were co-transfected for 18 h with 1 μg pLPK-CAT (the wild-type L-PK promoter driving expression of the CAT gene), 1 μg pLPK- LS-2 (L-PK promoter with mutation in ChoRE, 1 μg pLPK- LS-3 (L-PK promoter with mutation in ChoRE) or 1 μg pLPK- LS-4 (L-PK promoter with mutation in L3 site) and either control pUC19 (1 μg) or pRc/RSV-CBP (1 μg). Cells were treated with 2 mM or 20 mM glucose in the presence or absence of 10 μM forskolin for 6h. *B.* CAT enzyme activity was then

measured by ELISA assay. Data are means ± SEM from three independent experiments. **$P < 0.05$ vs. Wild-type at 2 mM glucose, *$P < 0.05$ vs. Wild-type at 20 mM glucose, ‡ $P < 0.05$ vs. Vector Control at 20 mM glucose.

2.4.9 Dominant-negative CREB does not prevent the cAMP-mediated repression of the L-PK gene.

The promoter of the L-PK gene lacks a consensus CRE (29) but is clearly responsive to cAMP generating signals ((100) and Fig. 1). Because cAMP can repress the PPAR-γ gene in an indirect fashion requiring CREB (57), we sought to determine whether CREB regulates the L-PK gene in a similar manner. To test this hypothesis, a mutant form of the CREB transcription factor containing a serine-133 to alanine mutation, which prevents it from being phosphorylated and activated in response to increases in cAMP (65), was overexpressed via recombinant adenovirus in 832/13 cells (Figure 9A). While the cAMP-mediated induction of the c-fos gene was clearly inhibited by this maneuver (Figure 9B), the ability of forskolin to repress glucose-stimulated expression of the L-PK gene was unaffected (Figure 9C). We conclude that the cAMP repression of the L-PK gene is mediated in a CREB-independent manner.


A
NV
GFP
ACREB
CREB
43kDa
Tubulin
55kDa
B
Relative mRNA levels
12
8
4
0
NV
GFP
ACREB
*
Glucose (mM) 2 20 2 20
Forskolin (10 μM) - - + +

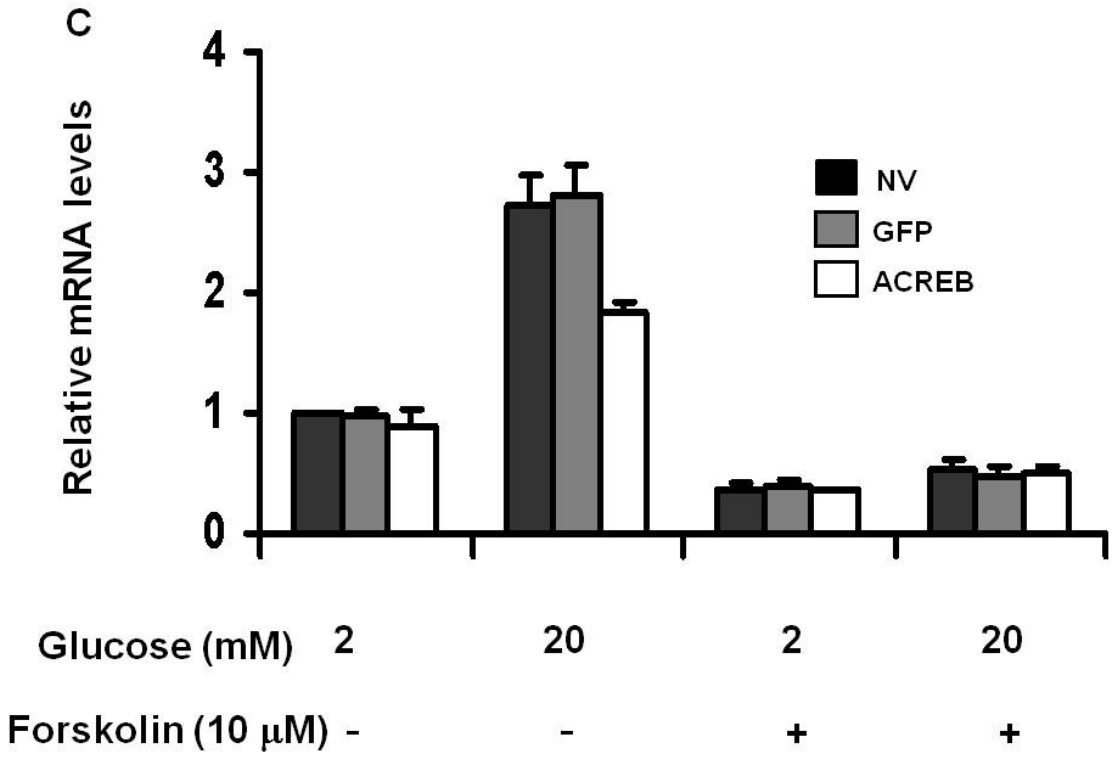
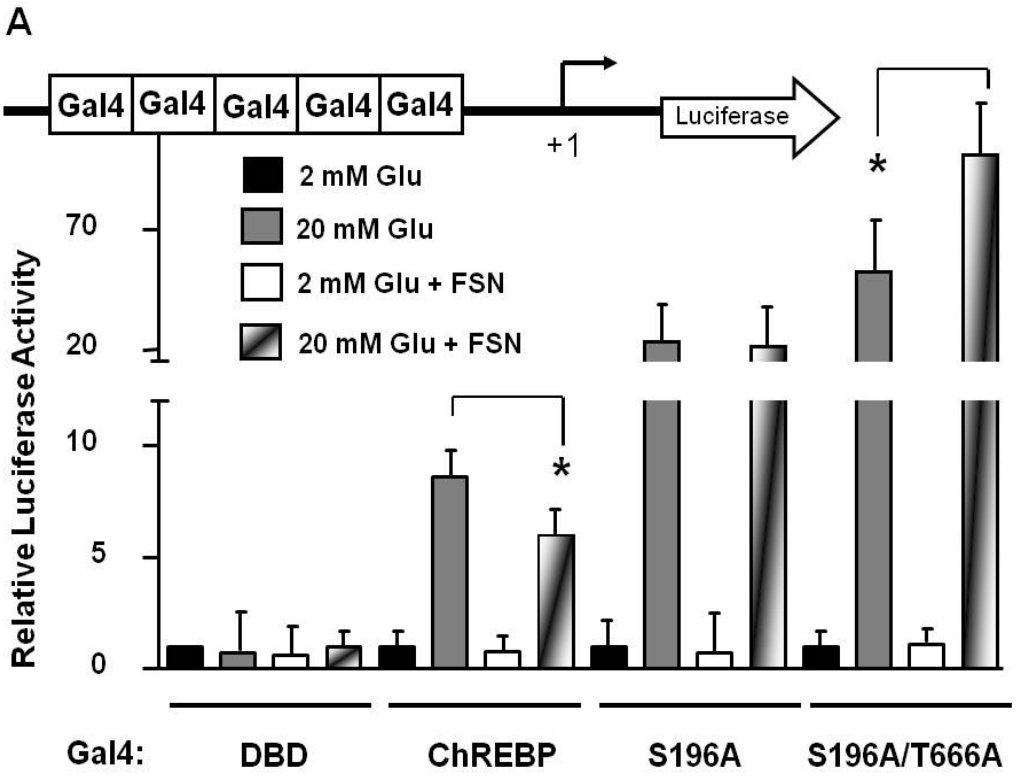


Figure 9. Dominant-negative CREB expression does not alleviate the cAMP-mediated repression of the glucose-stimulated L-PK gene. 832/13 rat insulinoma cells were transduced with either AdCMV-GFP or AdCMV-ACREB for 24 h. *A.* Nuclear extracts were immunoblotted for CREB and tubulin abundance. Following 24 h viral transduction, cells were treated with 2 mM or 20 mM glucose for an additional 6 h in the presence or absence of 10 μM forskolin. Relative levels of (B) c-fos (control) and (C) L-PK mRNA were determined by real-time RT-PCR. Data are expressed as means ± SEM from three independent experiments. * $P < 0.05$ vs. GFP at 20 mM glucose.

2.4.10 cAMP decreases wild-type and phospho-mutant ChREBP transactivation on the L-PK promoter.

We examined ChREBP transactivation capacity using glucose and cAMP in combination with two different promoter contexts. In the first series of experiments, a promoter luciferase construct containing five Gal4 DNA binding regions in tandem (pG5-luc) was used. In this experiment, there is an 8-fold activation of Gal4-ChREBP fusion construct by glucose whereas forskolin repressed transactivation of the wild-type factor by 31% (Figure 10A). However, when the PKA phosphoacceptor sites at either S196 or at both S196 and T666 in ChREBP were removed, the factor was no longer responsive to forskolin repression in this context (Figure 10A). Moreover, it was even more glucose responsive, which is in agreement with previous reports (86). However, when we performed similar experiments in the context of the L-PK gene promoter, wherein the 17 nucleotides of the ChoRE were replaced with the 17 bases of the Gal4 sequence. In this experiment, there is a 3.7-fold activation of Gal4-ChREBP fusion construct by glucose, whereas forskolin repressed transactivation of the wild-type factor by 68% (Figure 10B). Note that mutation of the PKA sites in ChREBP was unable to prevent the forskolin repression. We conclude that in the context of the L-PK gene promoter, repression by cAMP agonists is mediated largely by factors other than, but potentially associated with ChREBP.


A
Gal4
Gal4
Gal4
Gal4
Gal4
+1
Luciferase
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20 mM Glu
2 mM Glu + FSN
20 mM Glu + FSN
Relative Luciferase Activity
70
20
10
5
0
*
*
Gal4:
DBD
ChREBP
S196A
S196A/T666A

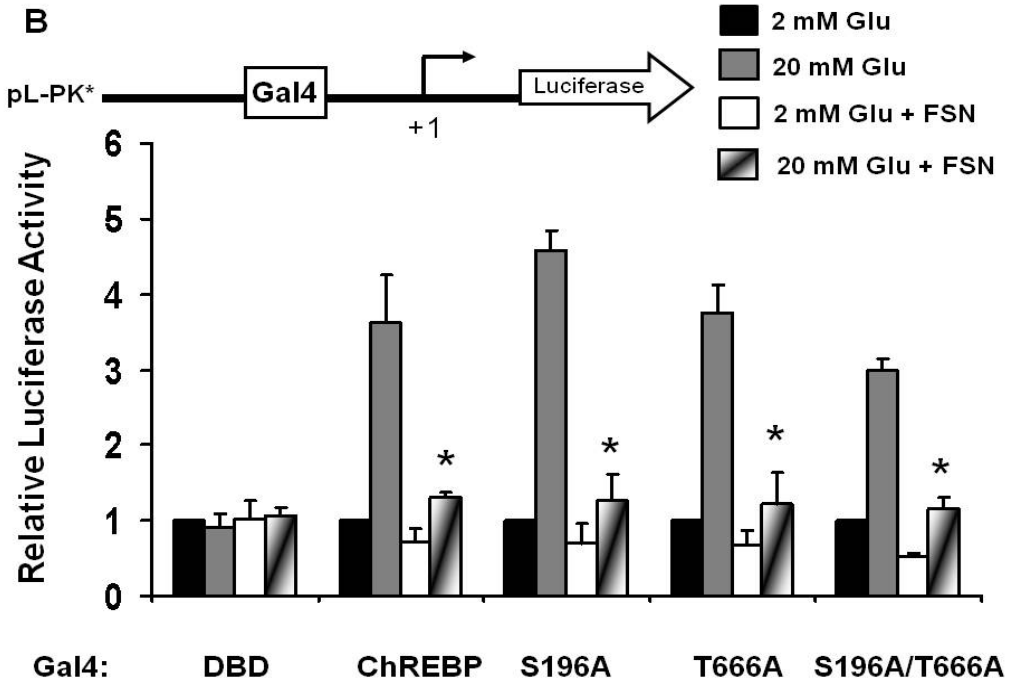
B
pL-PK*
Gal4
+1
Luciferase
2 mM Glu
20 mM Glu
2 mM Glu + FSN
20 mM Glu + FSN
Relative Luciferase Activity
6
5
4
3
2
1
0
*
*
*
*
Gal4:
DBD
ChREBP
S196A
T666A
S196A/T666A

Figure 10. cAMP-mediated decrease of ChREBP transactivation on the L-PK gene promoter. *A.* pG5-luc plasmid (with 5 tandem Gal4 DNA binding sites, 1 μg) was co-transfected (1 μg) with plasmids encoding GAL4-DBD, wild-type GAL4-ChREBP, or the indicated GAL4-ChREBP phosphorylation mutants into 832/13 cells for 18 h. Transfected cells were treated with 2 mM or 20 mM glucose in the presence or absence of 10 μM forskolin for 6 h. *B.* 832/13 cells were co-transfected for 18 h with (1 μg) plasmids encoding GAL4-DBD, wild-type GAL4-ChREBP, or the indicated GAL4-ChREBP phosphorylation mutants and pL-PK* (the wild-type L-PK promoter reporter with its ChoRE replaced with a Gal4 DNA binding site, 1 μg). Cells were treated for 6 h with 2 mM or 20 mM glucose in the presence or absence of 10 μM forskolin. Results are shown as relative luciferase reporter activity for three independent experiments. Values are means ± SEM. *$P < 0.05$ vs. 20 mM glucose for each respective group.

2.5 DISCUSSION

The L-type pyruvate kinase (L-PK) gene is an excellent model to study the reciprocal effects of cAMP and glucose, as glucose increases and cAMP decreases the expression of this gene (100). While the promoter elements that confer regulation by these opposing stimuli have been characterized, less is known about the assembly and disassembly of transcription factors and other regulatory molecules in response to glucose or cAMP-derived signals. The glucose-responsive transcription factor ChREBP is a key regulator of the L-PK gene (28, 33, 69, 160, 167). However, confusion exists regarding the

mechanism of transcriptional regulation by ChREBP. The original model for ChREBP-mediated regulation of the L-PK gene states that glucose promotes induction of this gene by opposing cAMP-stimulated phosphorylation (69); however, recent studies show that this model is incomplete and warrants revision (28, 38, 86, 87, 151). Therefore, in the current study, we examined the factors involved in both the activation of the L-PK gene in response to glucose and the repression by cAMP. The following key findings emerged: 1) suppression of the glucose-stimulated L-PK gene by cAMP is mediated via a PKA-dependent pathway (Figures 1D and E); 2) siRNA-mediated suppression of ChREBP, HNF4α or CBP completely prevented glucose-stimulated induction of the L-PK gene (Figures 2C, 2D and 5B); 3) decreased expression of the L-PK gene by cAMP coincides with decreased CBP, HNF4α, and ChREBP association with the L-PK gene promoter (Figures 4 and 6A); 4) overexpression of either wild-type or phospho-mutant ChREBP is neither sufficient to activate the L-PK gene under low glucose conditions nor can they override the cAMP-mediated repression of the gene (Figure 3B); 5) augmenting CBP abundance enhances the glucose-stimulated induction and is sufficient to overcome repression of the L-PK gene promoter activity by cAMP (Figure 8).

cAMP mediates its repressive effects on glucose-induced L-PK gene expression in a PKA-dependent pathway; however the mechanism is CREB-independent because when a mutant form of the CREB transcription factor containing a serine to alanine mutation, which prevents it from being phosphorylated and activated in response to increases in cAMP, was overexpressed via recombinant adenovirus the ability of forskolin to repress glucose-stimulated expression of the L-PK gene was unaffected (Figure 9). Furthermore, cAMP represses glucose-stimulated expression of the L-PK

gene by potentially controlling ChREBP at many levels. In addition to the cAMP-dependent decreased recruitment of ChREBP to the L-PK gene promoter (Figure 4), forskolin treatment resulted in a modest, but significant nuclear exclusion of ChREBP (unpublished results). When ChREBP was tethered in an L-PK promoter context via Gal4 fusion constructs, forskolin decreased ChREBP transactivation, both in the presence or absence of the PKA-phosphoacceptor sites (Figure 10B). However, we note that when similar experiments were performed with Gal4-ChREBP chimeras using a multimerized Gal4 DNA binding site promoter, removal of PKA-phosphoacceptor sites in ChREBP completely alleviated the forskolin-mediated repression of transactivation capacity by ChREBP (Figure 10A). Because ChREBP was sensitive to PKA-mediated phosphorylation in an artificial multimerized GAL4 promoter context, but not in the context of the L-PK gene promoter, we speculate that when a complex assembles on the L-PK gene promoter in response to elevated glucose concentrations, it masks the PKA sites in ChREBP, rendering the factor refractory to modulation by PKA. However, when a cAMP-derived signal releases CBP from the complex and certain proteins are recycled off the promoter (e.g., CBP to NR4A2), this potentially reveals the PKA sites in ChREBP, allowing phosphorylation and shuttling from the nucleus to the cytoplasm, as per previous models (69).

Additionally, the glucose-dependent increase in HNF4α recruitment to the L-PK gene promoter is blocked by cAMP treatment (Figure 4), but without a cAMP-mediated change in HNF4α nuclear abundance (unpublished observations). Phosphorylation of the putative PKA sites in HNF4α does not mediate the repressive effects of cAMP on transactivation of the L-PK gene (53). Therefore, the exclusion of HNF4α from the L-

PK gene promoter by cAMP cannot be explained by HNF4α phosphorylation-dependent events. We hypothesize that the exclusion is potentially due to another PKA-dependent phosphorylation event (perhaps via putative phosphoacceptor sites in CBP), ultimately leading to decreased stability of the complex containing ChREBP, HNF4α, and CBP. Depletion of ChREBP, HNF4α, and CBP led to a statistically significant decrease in basal expression of the L-PK gene. It is unknown whether these factors contribute indirectly or directly to expression of the L-PK gene at non-stimulatory glucose concentrations.

Since coordinated regulation of gene transcription requires assembly of multi-regulatory complexes on specific gene promoters concomitant with disassembly on other promoters and because coactivator molecules are often limiting (62, 134), they provide an attractive target upon which to finely tune the control of gene expression in response to multiple signals. We have shown that loss of CBP occupancy on the L-PK gene promoter in response to forskolin coincides with loss of L-PK mRNA levels concomitant with gain of CBP occupancy on the NR4A2 gene promoter and a corresponding increased expression of this gene (Figures 1C, 6A and B). This demonstrates a selective and coordinated regulation of gene transcription in response to a change in signals, as has been suggested for other systems (88, 93). Because of the absolute requirements for ChREBP and HNF4α (Figures 2B and D) and in addition, the recruitment of the co-activator CBP (Figure 6A), we propose a heretofore undescribed mechanism of both the glucose-mediated induction of the L-PK gene and its repression by cAMP. In this model, CBP is the limiting factor in the regulation of the L-PK gene. Initially, a rise in glucose metabolism promotes the association of ChREBP, HNF4α, and CBP with the L-PK gene

promoter, increasing transcription of the gene. However, when cAMP levels become elevated, this multi-regulatory complex is disrupted, CBP is recruited to other gene promoters (e.g., NR4A2) and unbound ChREBP is potentially shuttled to the cytoplasm. Based on an earlier report demonstrating association of HNF4α with CBP via GST pulldown analysis (168) and our current results above, we suspect that recruitment of CBP to the L-PK gene promoter likely stabilizes the complex assembled in response to glucose and that cAMP represses the gene by interfering with these protein-protein interactions. Additionally, putative PKA site(s) in CBP may be responsible for disruption of the complex at some genes (including L-PK) concomitant with assembly at others (e.g., NR4A2).

Moreover, siRNA-mediated suppression of CBP abundance diminishes the glucose-induction of L-PK mRNA, while enhancing CBP abundance both potentiates the glucose-mediated induction of promoter activity and prevents cAMP-mediated repression of such activity; we interpret these findings to indicate that CBP is a bona fide coactivator of the L-PK gene. We've clearly shown via Sequential ChIP (Figures 7A-D) that ChREBP, CBP and HNF4α co-reside as a complex on the L-PK promoter in a glucose-dependent manner, and that cAMP-mediated repression of the L-PK gene disrupts this complex assembly; however, whether CBP binds to ChREBP or HNF4α, or both, has yet to be elucidated.

We have thus demonstrated that a complex requiring ChREBP, HNF4α, and CBP is necessary for the activation of the L-PK gene in response to glucose. In addition, we report here for the first time that the coactivator CBP is recruited to the L-PK gene promoter in response to glucose; further, decreasing the association of this coactivator

with the L-PK gene promoter, either by siRNA technology or by increasing cAMP levels, blocks glucose-induced expression of the L-PK gene. Thus, a complex composed of ChREBP, HNFα, and CBP is a focal point for the opposing physiological signals, glucose and cAMP.

3.0 cAMP PREVENTS GLUCOSE-MEDIATED MODIFICATIONS OF HISTONE H3 AND RECRUITMENT OF THE RNA POLYMERASE II HOLOENZYME TO THE L-PK GENE PROMOTER *

3.1 ABSTRACT

Glucose and cAMP reciprocally regulate expression of the L-type pyruvate kinase (L-PK) gene by controlling the formation of a complex containing Carbohydrate Response Element Binding Protein (ChREBP) and the coactivator CREB Binding Protein (CBP) on the L-PK promoter. However, the role of post-translational histone modifications on the opposing effects of glucose and cAMP on the L-PK gene are unknown. Using the highly glucose-sensitive 832/13 rat insulinoma cell line, we demonstrated that glucose also regulates acetylation and methylation of various histone residues at the L-PK gene promoter. These glucose-dependent histone modifications correlated with an increase in the recruitment and phosphorylation of RNA Polymerase II (Pol II) on the L-PK gene promoter.

* This chapter is originally published: Burke, S.J., Collier, J.J. and Scott, D.K. (2009) cAMP prevents Glucose-Mediated Modifications of Histone H3 and Recruitment of the RNA Polymerase II Holoenzyme to the L-PK Gene Promoter. *Journal of Molecular Biology*.

Conversely, the cAMP agonist forskolin prevented glucose-mediated expression of the L-PK gene by decreasing the acetylation of histones H3 and H4 on the promoter, decreasing the methylation of H3-K4 on the coding region and increasing the methylation of H3-K9 on the coding region. These changes induced by cAMP culminated with a decrease in the glucose-dependent recruitment and phosphorylation of Pol II to the L-PK gene promoter. Furthermore, maneuvers that interfere with the glucose-dependent assembly of ChREBP and CBP on the L-PK promoter, such as: 1) increasing intracellular cAMP levels; 2) overexpression of a dominant-negative form of ChREBP; or 3) siRNA-mediated suppression of CBP abundance altered the association of acetylated and methylated histones with the L-PK promoter, which decreased Pol II recruitment and subsequently inhibited transcriptional activation of the L-PK gene. We conclude that the effects of glucose and cAMP are mediated in part by epigenetic modulation of histones.

3.2 INTRODUCTION

L-type pyruvate kinase (L-PK) encodes a key regulatory enzyme of the glycolytic pathway whose expression is tightly regulated, positively by glucose and negatively by cAMP (15, 100). We have recently described a glucose-sensing complex containing Carbohydrate Response Element Binding Protein (ChREBP) and CREB binding protein (CBP) that is required for the activation of the L-PK gene by glucose; cAMP represses L-PK gene transcription by interfering with the assembly of this complex on the L-PK gene

promoter (15). However, the signaling mechanisms and requisite post-translational modifications linking the assembly/disassembly of this complex to the L-PK promoter, and the induction and repression by glucose and cAMP, respectively, have not been elucidated.

ChREBP, a basic helix-loop-helix leucine zipper (bHLH-LZ) transcription factor, controls the expression of glycolytic (e.g. L-PK) and lipogenic enzyme genes (e.g. ACC and FAS) (28, 33, 69, 160, 167). ChREBP activates these target genes by binding to a Carbohydrate Response Element (ChoRE) as a heterodimer with its partner Max-like protein X (Mlx) (96, 136). Glucose metabolism may control nuclear entry of ChREBP (38), allowing this factor to associate with other transcriptional regulators necessary for full activation of the L-PK gene. ChREBP assembles on to the prototypical glucose responsive L-PK gene as part of a complex containing HNF4α and CBP, and once bound to DNA, glucose is required for transactivation.

CBP is a transcriptional coactivator to a wide array of transcription factors including those necessary for the expression of the L-PK gene (15). In addition, CBP possesses intrinsic histone acetyltransferase (HAT) activity (4, 117). Acetylation and methylation of histone N-terminal tail regions determine whether a gene will be actively transcribed by controlling the accessibility of chromatin to various transcriptional regulators (84, 138). Acetylation of histone H3 lysines and H4 lysines counteracts the compact structure of chromatin by relaxing the interaction between histone proteins and DNA (61). Furthermore, methylation of histone H3-K9 is associated with transcriptional repression because it creates a recognition site for the binding of heterochromatin proteins (5, 82, 109). Methylation of H3-K9 also promotes transcription repression by

blocking the acetylation of this same residue by histone acetyltransferases (HATs). By contrast, methylation of H3-K4 promotes remodeling of chromatin to the active state by attracting various coregulatory proteins (157), including CBP, and also preventing association of the N-terminal tail of histone H3 with the heterochromatin forming complexes (170).

These histone modifications render DNA accessible to transcription factors and also provide a platform for RNA Polymerase II (Pol II) to dock on the promoter. The C-terminal domain (CTD) of the largest subunit of Pol II consists of tandem heptapeptide repeats which can be modified by phosphorylation of serines at position 2 (Pol II $pCTD^{Ser2}$) or 5 (Pol II $pCTD^{Ser5}$) of the heptapeptide repeats (34, 35)**.** Pol II undergoes a cycle of phosphorylation and dephosphorylation during transcription, with serine 5 being phosphorylated during initiation and promoter clearance, and serine 2 phosphorylation occurring predominantly during the elongation phase of transcription (58, 104).

In the current study, we tested the hypothesis that glucose-mediated regulation of the L-PK gene by ChREBP and CBP requires histone modifications that control recruitment of Pol II holoenzyme to the L-PK gene promoter. We have shown here that glucose increased the association of acetylated histone H3 with the L-PK gene promoter region containing the ChoRE which led to recruitment of Pol II. Importantly, we observed decreased acetylation of histones H3 and H4 and promoter occupancy of Pol II by: 1) increasing intracellular cAMP levels; 2) overexpression of a dominant-negative ChREBP; or 3) siRNA-mediated suppression of CBP abundance. Additionally, elevations in cAMP or overexpression of a dominant-negative ChREBP blocked the glucose-

mediated increase in methylation of H3-K4, and increased methylation of H3-K9, on the coding region of the L-PK gene, ultimately repressing the expression of the L-PK gene.

3.3 MATERIALS AND METHODS

3.3.1 Cell culture, RNA isolation and measurement of RNAs by RT-PCR

INS-1- derived 832/13 insulinoma cells, a gift from Dr. Christopher Newgard (Duke University Medical Center), were cultured as described previously (60).Total RNA was isolated from 832/13 cells using TRI-reagent (Molecular Research Center; Cincinnati, OH). iScript (Bio-Rad; Hercules, CA) was used for first-strand synthesis of cDNA using 0.5 μg of RNA. For real-time PCR measurements of relative mRNA abundance, 2.5% of the total RT reaction was used with SYBR green master mix (Quanta Biosciences; Gaithersburg, MD). Real-time PCR was performed using the Applied Biosystems Prism 7300 detection system and software. The forward and reverse primer sequences used for L-PK and cyclophilin have been described previously (15). Relative mRNA levels of individual genes are reported after normalization to cyclophilin mRNA levels.

3.3.2 siRNA-mediated suppression of gene expression

Abundance of CBP was decreased by transfecting a pre-annealed duplex from Ambion (Austin, TX) (CBP: siRNA ID # 199670; Austin, Tx) into 832/13 cells using Dharmafect

reagent 1 (Dharmacon; Lafayette, CO) according to the manufacturer's suggested protocol. Suppression of CBP expression was confirmed via real-time PCR (not shown) and subsequent detection of protein levels by immunoblotting analysis following 48 h of duplex exposure. Densitometry was performed using Versadoc imaging system software (Bio-rad).

3.3.3 Isolation of nuclear protein and immunoblotting

The NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce; Rockford, IL) was used to prepare nuclear and cytosolic fractions. Whole cell lysates were prepared using Mammalian- Protein Extraction Reagent, M-PER, buffer (Pierce). The protein concentration was determined using the BCA assay (Pierce). Immunoblotting was performed as previously described (26) with modifications. Electrophoretic transfer from SDS-PAGE gels to polyvinylidine fluoride membranes were performed using the iBlot™ Dry Blotting System (Invitrogen; Carlsbad, CA). SNAP i.d. Protein Detection System (Millipore; Billerica, MA) was used for blocking and incubation with primary and secondary antibodies according to manufacturer's instructions. Antibodies used for detection of CBP were from Santa Cruz Biotechnology (Santa Cruz, CA), ChREBP from Novus Biologicals (Littleton, CO), and β Actin from Sigma (St. Louis, MO).

3.3.4 Chromatin immunoprecipitation assays

Chromatin immunoprecipitation assays were performed using the Millipore ChIP assay kit (Cat # 17-295), with modifications described previously (110). Relative binding of each factor was reported following normalization to control IgG. The ChoRE-containing portion of the L-PK promoter and a fragment downstream of the L-PK transcriptional start site were targeted for amplification. Forward and reverse primers used to amplify the L-PK gene promoter, downstream region of the L-PK gene and CRE-containing region of the NR4A2 promoter were described previously (15). Antibodies used for immunoprecipitation of acetylated histones H3 (K9 and K14) (Cat # 06-599) and H4 (K5, K8, K12 and K16) (Cat # 06-866), mono/di/trimethyl histone H3-K4 (Cat # 05-791), dimethyl histone H3-K4 (Cat # 07-030), trimethyl histone H3-K4 (Cat # 17-614), dimethyl histone H3-K9 (Cat # 17-648) and Pol II (Cat # 05-623) were from Millipore; Pol II pCTD^{Ser5} (Cat # ab5131) and Pol II pCTD^{Ser2} (Cat # ab5095) were from Abcam (Cambridge, MA); CBP (Cat # sc-369) and rabbit IgG (Cat # sc2027) from Santa Cruz Biotechnology; and ChREBP (Cat # NB400-135) from Novus Biologicals.

3.3.5 Sequential Chromatin Immunoprecipitation Assay

CBP was immunoprecipitated with anti-CBP (Cat # sc-369) antibody; the eluate was then subsequently immunoprecipitated with a Pol II antibody per a previously described method (51, 165). Primers were used to amplify the appropriate regions of the L-PK gene promoter and coding region by real-time RT-PCR.

3.3.6 Statistical analysis

Data analyses were performed using a one-way ANOVA (*P* values <0.05). A tukey post hoc test was used to determine differences within the ANOVA. Data are expressed as means ± SEM.

3.4 RESULTS

3.4.1 Expression of the L-PK gene requires ChREBP and CBP.

The L-PK gene is induced by glucose and repressed by cAMP agonists, even in the presence of stimulatory concentrations of glucose (15, 28, 100). Exposure of highly glucose-sensitive 832/13 rat insulinoma cell line to 20 mM glucose for 6 h resulted in a 2.3-fold increase in L-PK mRNA levels, relative to 2 mM glucose. Co-treatment with 20 mM glucose and 10 μM forskolin, an activator of adenylate cyclase, renders the L-PK gene completely refractory to glucose stimulation (Figure 11A). We previously demonstrated that glucose-stimulated recruitment of a complex containing ChREBP, HNF4α and CBP is required for expression of the L-PK gene and furthermore, that cAMP blocks the induction by glucose by preventing assembly of this complex on the L-PK gene promoter (15). To further examine the role of ChREBP and CBP in the regulation of the L-PK gene, we employed a dominant-negative ChREBP protein (95), which was expressed via recombinant adenovirus (Figure 11B). This produced a 63%

decrease in the glucose-stimulated expression of the L-PK gene when compared to the GFP adenoviral control (Figure 11C). Suppression of endogenous CBP levels by siRNA duplexes targeting the coding region of the CBP gene produced a 62% depletion of CBP abundance compared to the control duplex siScramble (Figure 11D). This decrease in CBP abundance was sufficient to decrease the glucose-stimulated expression of the L-PK gene by 79% (Figure 11E). Further, we have shown that the glucose-mediated increase in recovery of CBP on the L-PK promoter region spanning the ChoRE is abrogated when dominant-negative ChREBP is overexpressed (Figure 11F), while siRNA-mediated suppression of CBP abundance decreased the relative glucose-stimulated association of ChREBP with the L-PK promoter by 72% (Figure 11G). No change in relative occupancy of either ChREBP or CBP compared to IgG control with either glucose or cAMP was detected on the L-PK coding region (data not shown). Taken together, these observations confirm our previously published studies (15) and establish that each of these factors, ChREBP and CBP, are required for the other's ability to bind to the L-PK gene promoter.

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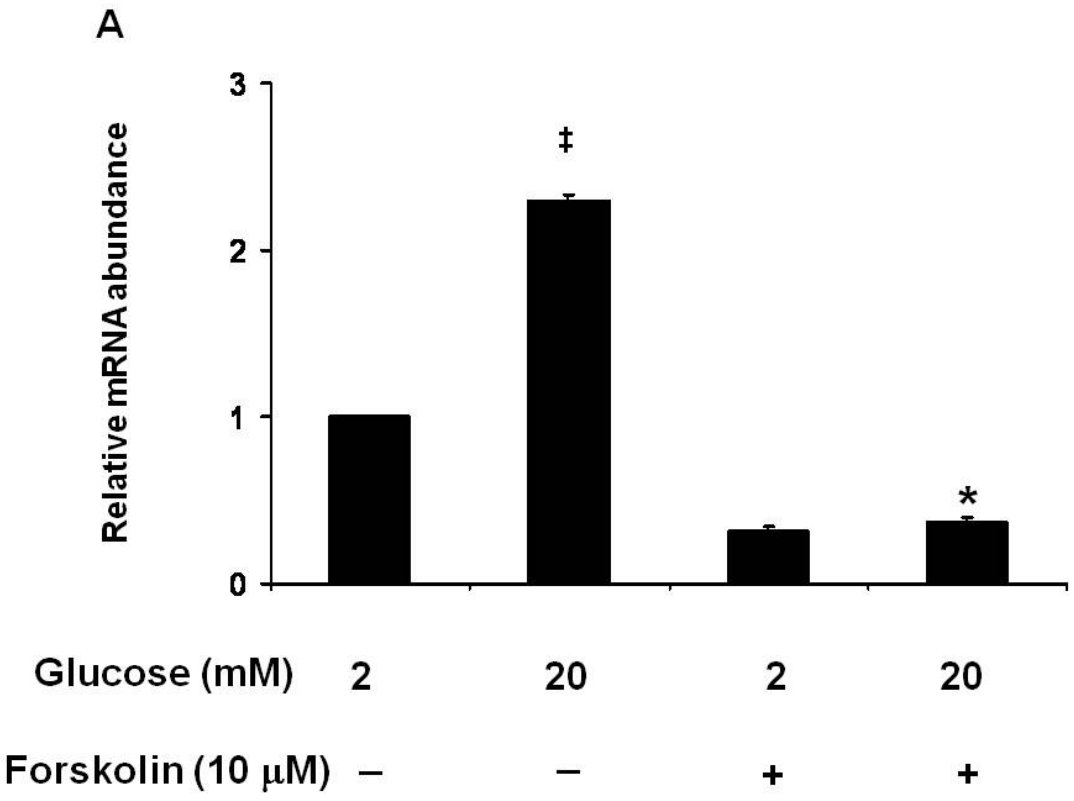
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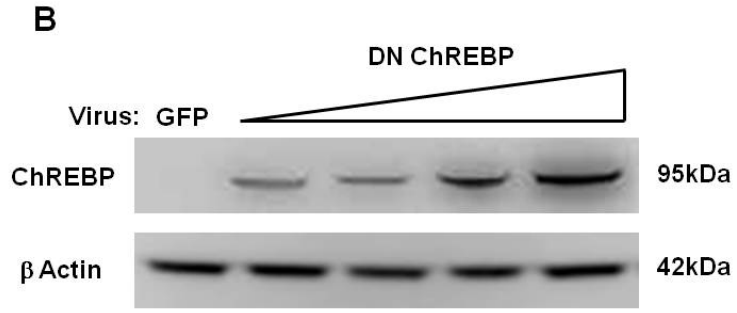
3
2
1
0
‡
*
Glucose (mM) 2 20 2 20
Forskolin (10 μM) – – + +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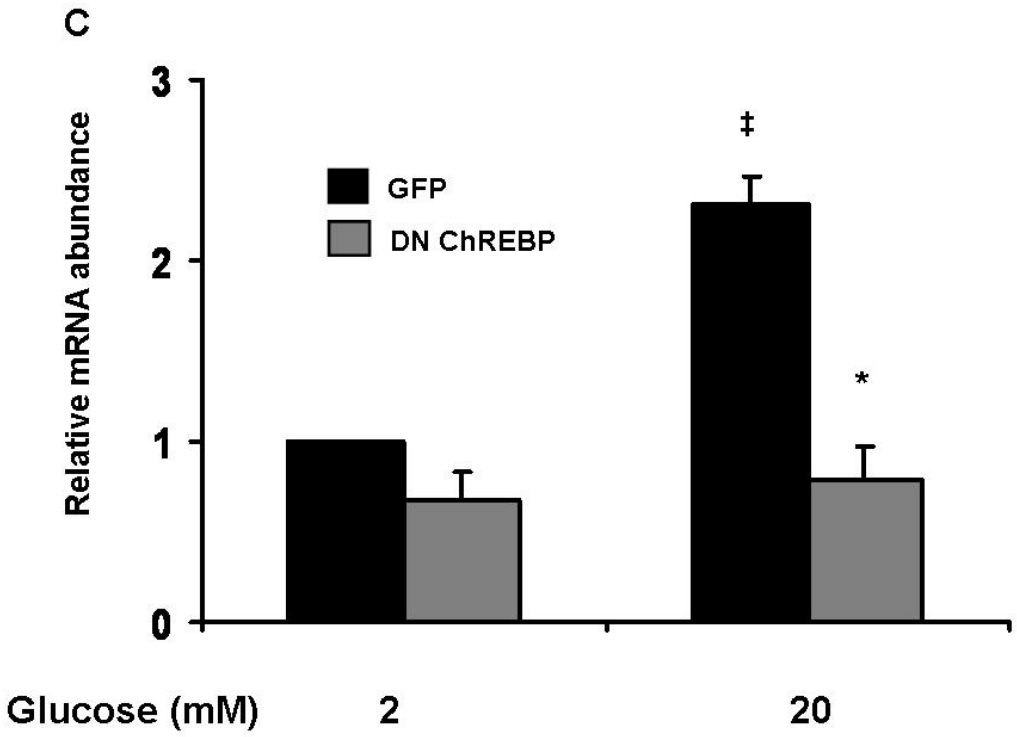
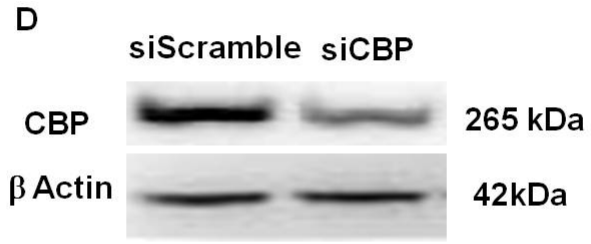
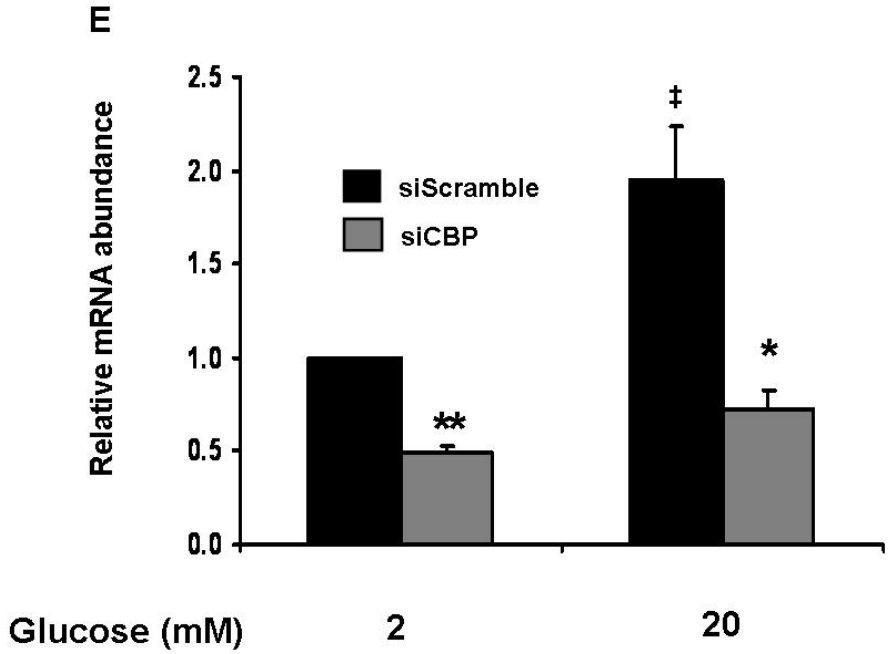
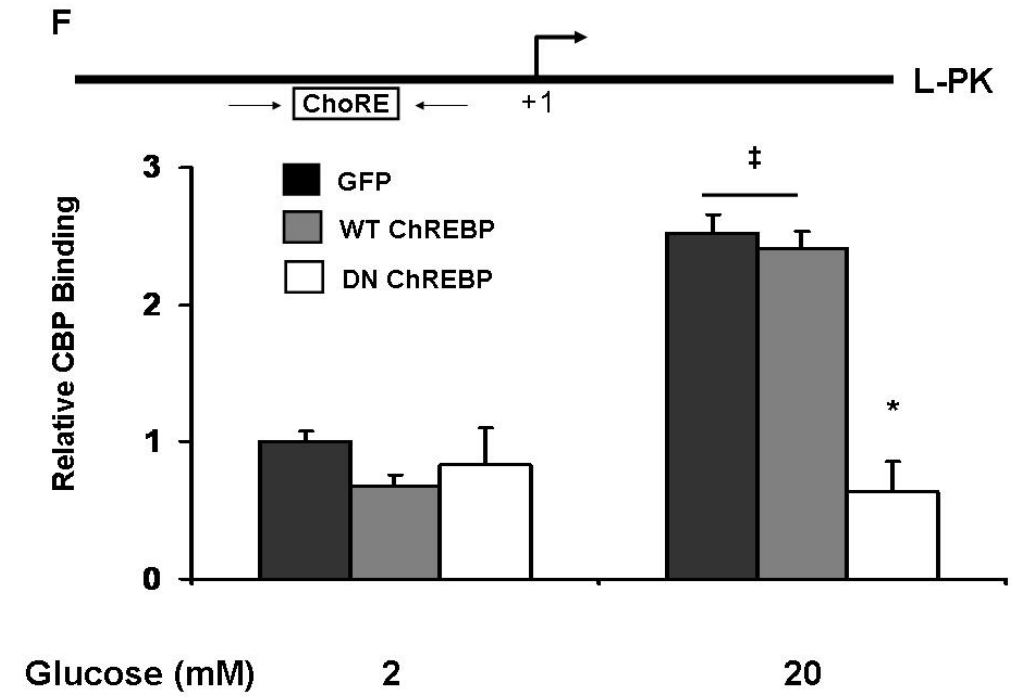
Virus: GFP
ChREBP
95kDa
β Actin
42kDa


C
Relative mRNA abundance
3
2
1
0
GFP
DN ChREBP
‡
*
Glucose (mM)
2
20


D
siScramble
siCBP
CBP
265 kDa
β Actin
42kDa


E
Relative mRNA abundance
2.5
2.0
1.5
1.0
0.5
0.0
siScramble
siCBP
**
‡
*
Glucose (mM)
2
20


F
L-PK
ChoRE
+1
Relative CBP Binding
GFP
WT ChREBP
DN ChREBP
‡
*
Glucose (mM)
2
20

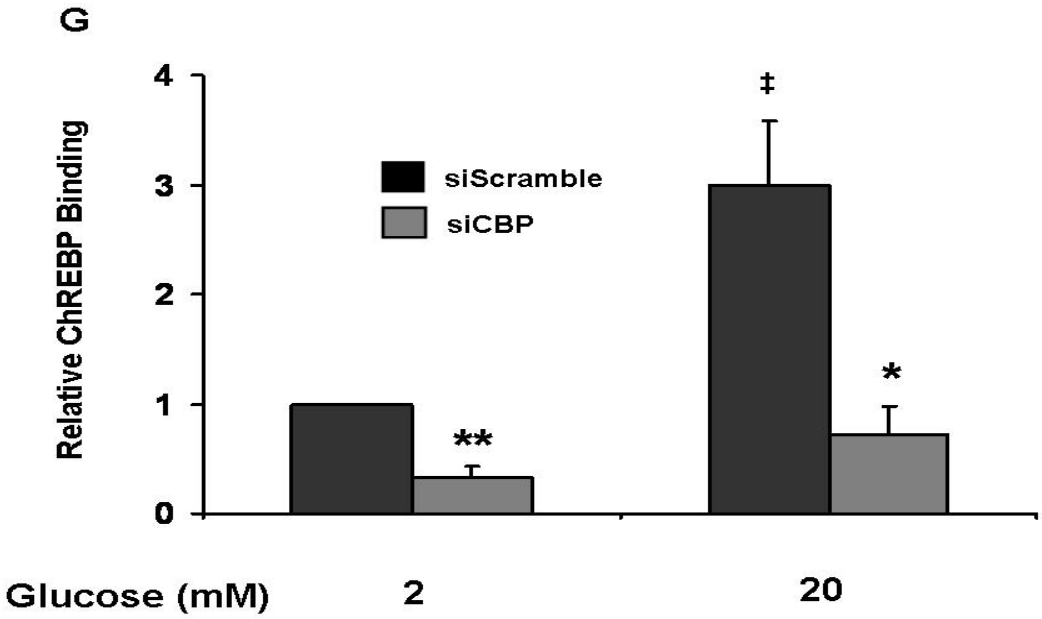

G
Relative ChREBP Binding
siScramble
siCBP
‡
**
*
Glucose (mM)
2
20

Figure 11. Expression of the L-PK gene requires ChREBP and CBP. *A.* 832/13 cells were treated with 2 or 20 mM glucose in the presence or absence of 10 μM forskolin for 6 h. (B-C, F) 832/13 cells were transduced with adenovirus expressing either GFP, wild-type ChREBP or a dominant- negative ChREBP protein for 24 h. *B*. Cell lysates were harvested, the nuclear fraction was isolated and immunoblots performed with antibodies for either ChREBP or β Actin serving as a loading control. The immunoblot is a representative of 2 independent experiments. *C,F.* 24 h following viral transduction, cells were then treated with 2 or 20 mM glucose for 6 h, (C) total RNA was isolated and relative abundance of L-PK mRNA was examined and (F) chromatin immunoprecipitation assays was performed using an antibody against CBP; a segment of the L-PK gene promoter containing the ChoRE site was targeted for amplification. Data are means ± SEM from three individual experiments. **P* < 0.05 vs. 20 mM glucose, ‡ *P* < 0.01 vs. 2 mM glucose. (D-E, G) 832/13 cells were transfected with either a negative siRNA control (siScramble) or an siRNA duplex targeting the coding region of CBP. *D*. After a 48 h incubation with siRNA duplexes, nuclear extracts were prepared, and immunoblots performed with antibodies against CBP with β-Actin as a loading control. The immunoblot is a representative of 2 independent experiments. *E,G.* Following exposure to siRNA duplexes for 48 h, cells were treated with 2 or 20 mM glucose (E) total RNA was isolated and relative abundance of L-PK mRNA was examined via RT-PCR and (G) chromatin immunoprecipitation assays were performed using an antibody against ChREBP with the L-PK gene promoter targeted for amplification via RT-PCR. Data represent means ± SEM from three individual experiments. **P* < 0.01 vs. 20 mM glucose, ***P* < 0.05 vs. siScramble at 2 mM glucose, ‡ *P* < 0.05 vs. 2 mM glucose.

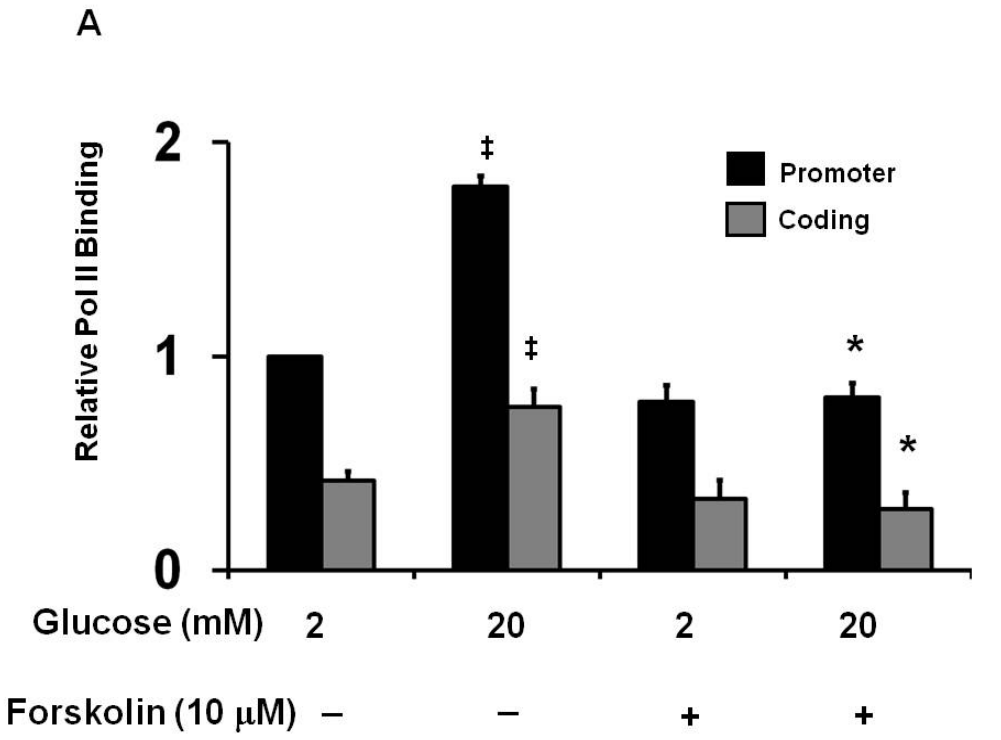
3.4.3 cAMP abrogates the glucose-mediated association of Pol II with the L-PK gene promoter and coding region.

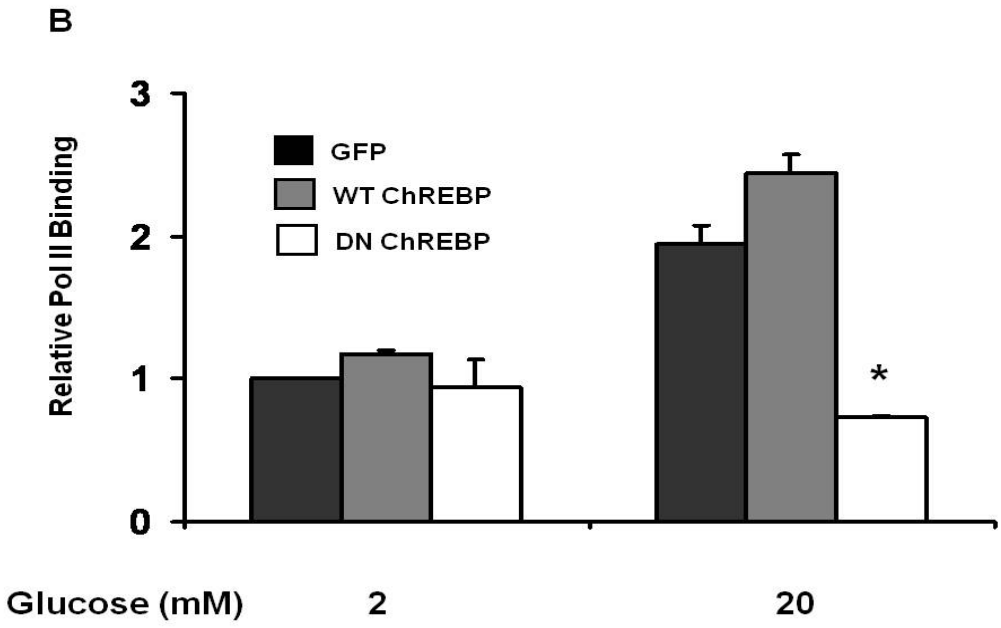
Promoter occupancy by Pol II is necessary for initiation of gene transcription (30, 74, 169); therefore, we determined whether glucose-mediated induction and cAMP-directed repression of the L-PK gene correlated with Pol II association and disassociation at the L-PK gene promoter. Raising the glucose concentration from 2 mM to 20 mM for 6 h produced a 1.8-fold increase in Pol II recruitment to the region of the L-PK promoter containing the ChoRE site (Figure 12A). By contrast, cells treated simultaneously with 10 μM forskolin and 20 mM glucose displayed a 53% decrease in Pol II binding to the same promoter region (Figure 12A). In addition, we observed a 1.8-fold increase in occupancy of Pol II on the coding region, which was blunted 69% in the presence of forskolin.

ChREBP and CBP occupancy on the L-PK promoter is necessary for the glucose-mediated induction of the L-PK gene (Figure 11). To determine whether the glucose-dependent recruitment of Pol II to the L-PK promoter also requires these transcription factors, we employed two strategies to interfere with their binding to the L-PK promoter: 1) overexpression of a dominant-negative ChREBP via recombinant adenovirus (Figure 11B); and 2) depleting the abundance of CBP via siRNA duplex transfection (Figure 11D). 832/13 cells transduced with GFP adenovirus displayed a 2-fold increase in Pol II recruitment to the L-PK promoter in response to glucose and overexpression of wild-type ChREBP did not increase the glucose-dependent recruitment of Pol II compared to control GFP (Figure 12B). However, overexpression of dominant-negative ChREBP diminished Pol II occupancy on the L-PK gene promoter by 42%, compared to GFP

(Figure 12B). In addition, decreasing CBP abundance blunted the glucose-mediated association of Pol II with the L-PK promoter by 60%, as compared to cells transfected with siScramble (Figure 12C).

Because the glucose-mediated recruitment of Pol II to the L-PK promoter was dependent upon ChREBP and CBP (Figures 12B and C), we next examined whether Pol II was also present on the promoter as part of a complex containing these two transcriptional regulators. To test this hypothesis, we performed a sequential ChIP (SeqChIP) analysis, which provides information regarding co-occupancy of proteins at a given genomic region (51). At 20 mM glucose we observed a 3.3-fold increase in promoter fragments recovered by immunoprecipitating CBP which was decreased by 64% in the presence of forskolin (Figure 12D). A subsequent second immunoprecipitation of the eluate using a Pol II antibody revealed a 2.8-fold increase in Pol II SeqChIP signal at 20 mM glucose. This signal was decreased 61% with the addition of forskolin (Figure 12D). From the cumulative findings shown in Figure 12A-D we conclude that Pol II is recruited to the L-PK gene promoter in a glucose-dependent manner, is part of the complex required for maximal transcription of the L-PK gene by glucose, and that forskolin-mediated repression of the L-PK gene occurs, at least in part, due to a loss of Pol II occupancy on the L-PK gene promoter.


A
Relative Pol II Binding
2
1
0
‡
‡
*
*
Promoter
Coding
Glucose (mM) 2 20 2 20
Forskolin (10 μM) – – + +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Relative Pol II Binding
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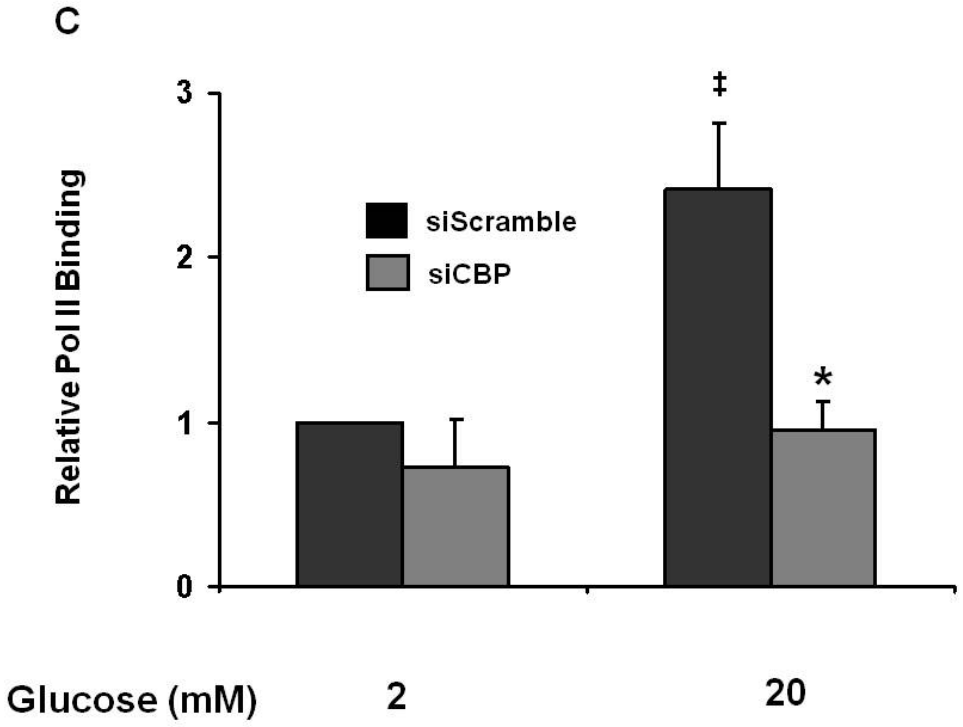
2
1
0
GFP
WT ChREBP
DN ChREBP
*
Glucose (mM) 2 20


C
Relative Pol II Binding
3
2
1
0
siScramble
siCBP
‡
*
Glucose (mM)
2
20

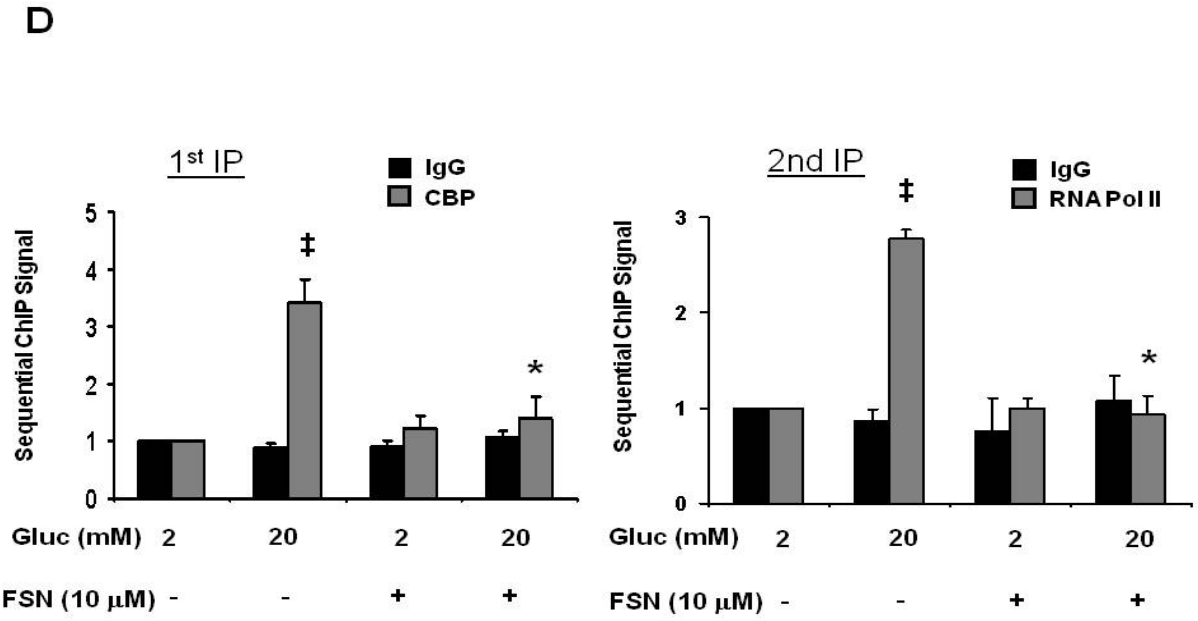
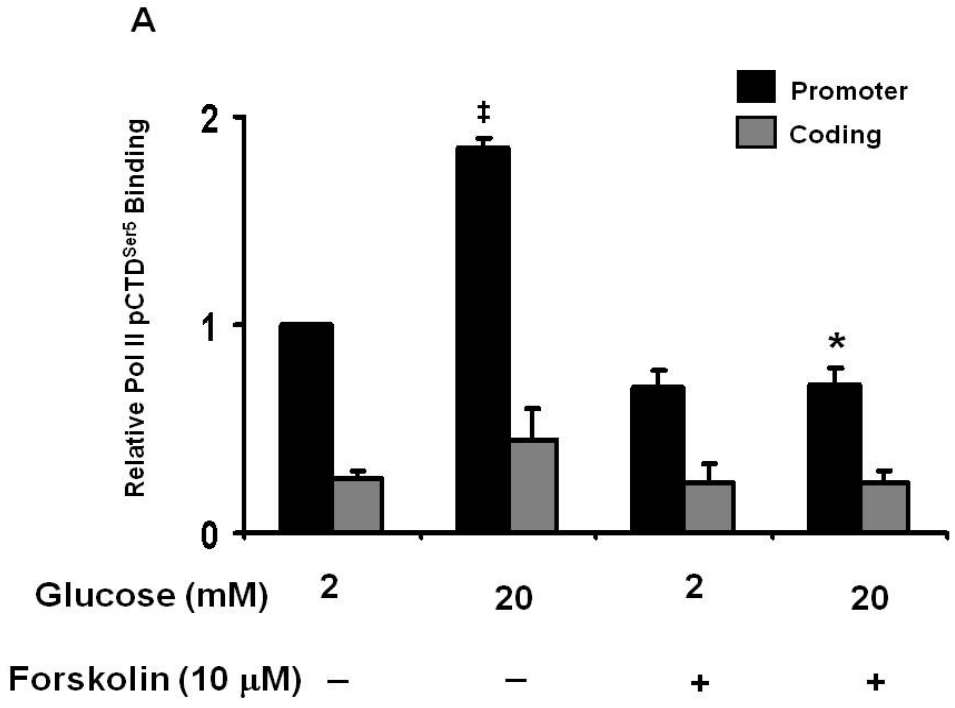

D
1st IP
IgG
CBP
Sequential ChIP Signal
5
4
3
2
1
0
‡
*
Gluc (mM) 2 20 2 20
FSN (10 μM) - - + +
2nd IP
IgG
RNA Pol II
Sequential ChIP Signal
3
2
1
0
‡
*
Gluc (mM) 2 20 2 20
FSN (10 μM) - - + +

Figure 12. cAMP diminishes the glucose-stimulated recruitment of Pol II the L-PK gene promoter. *A.* 832/13 cells were incubated in 2 or 20 mM glucose in the presence or absence of 10 mM forskolin for 6 h. *B*. Cells were transduced with the indicated adenoviruses for 24 h, followed by a 6 h incubation in 2 or 20 mM glucose. *C.* Cells were transfected with an siScramble or siCBP duplex for 48 h, then cultured in 2 or 20 mM glucose for 6 h. Using an anti-Pol II antibody, relative binding of Pol II was determined via ChIP assay. Gene promoter and coding region for L-PK was targeted for amplification. Data are expressed as means ± SEM from four to five independent experiments. ‡ $P < 0.05$ vs. 2 mM glucose, *$P < 0.05$ vs. 20 mM glucose. *D.* Cells were treated as in (A), CBP was immunoprecipitated with anti-CBP antibody, with anti-IgG antisera serving as a control. Pol II was subsequently immunoprecipitated from the resulting eluate using a Pol II antibody, again anti-IgG was used as a control. Values are means ± SEM from four individual experiments. ‡ $P < 0.01$ vs. 2 mM glucose, *$P < 0.01$ vs. 20 mM glucose.

3.4.4 cAMP opposes the glucose-stimulated recruitment of Pol II pCTD^{Ser5} to the promoter, and Pol II pCTD^{Ser2} to the coding region of the L-PK gene.

Phosphorylation of Pol II CTD at Serine 5 and Serine 2 accompanies initiation of transcription and elongation, respectively (for review see (32, 34, 58)). Using chromatin immunoprecipitation with antibodies specific for either Pol II pCTD^{Ser5} or Pol II pCTD^{Ser2}, we observed a 54% increase in Pol II pCTD^{Ser5} recovery on the L-PK gene

promoter in cells treated with 20 mM glucose and forskolin blocked this recruitment by 63% (Figure 13A). Similarly, we observed a glucose-induced increase (63%) in Pol II pCTD^{Ser2} recovery on the L-PK coding region; this association with the coding region was abrogated by 50% in the presence of forskolin (Figure 13B). From these experiments we conclude that regulation of the L-PK gene by glucose and cAMP involves corresponding changes in phosphorylated Pol II at the L-PK gene promoter and coding region.


A
Promoter
Coding
Relative Pol II pCTD^{Ser5} Binding
2
1
0
‡
*
Glucose (mM) 2 20 2 20
Forskolin (10 μM) – – + +

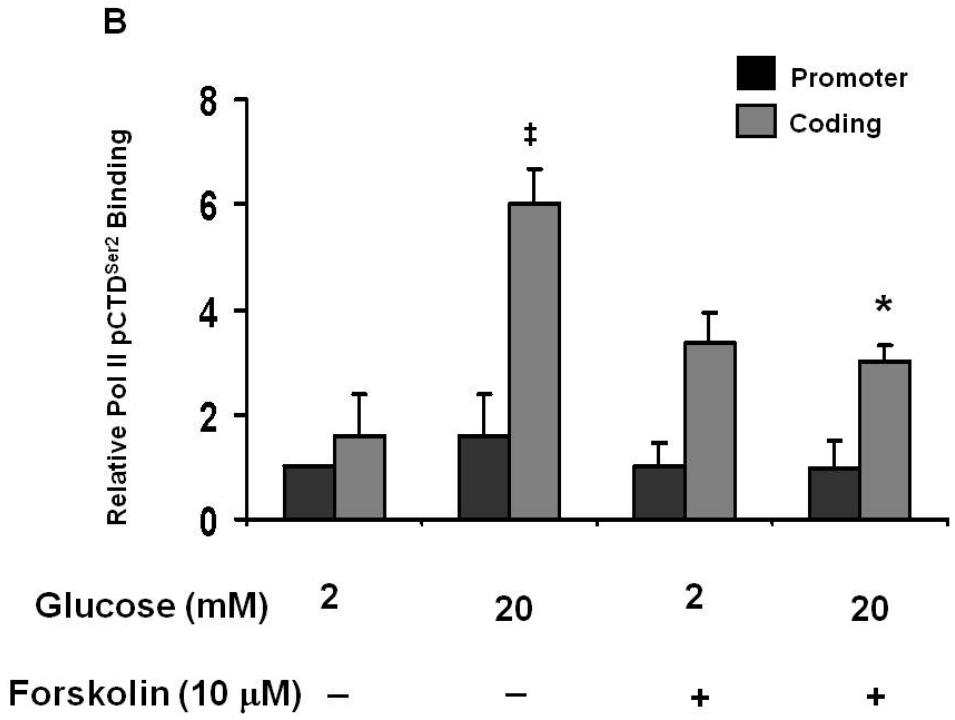
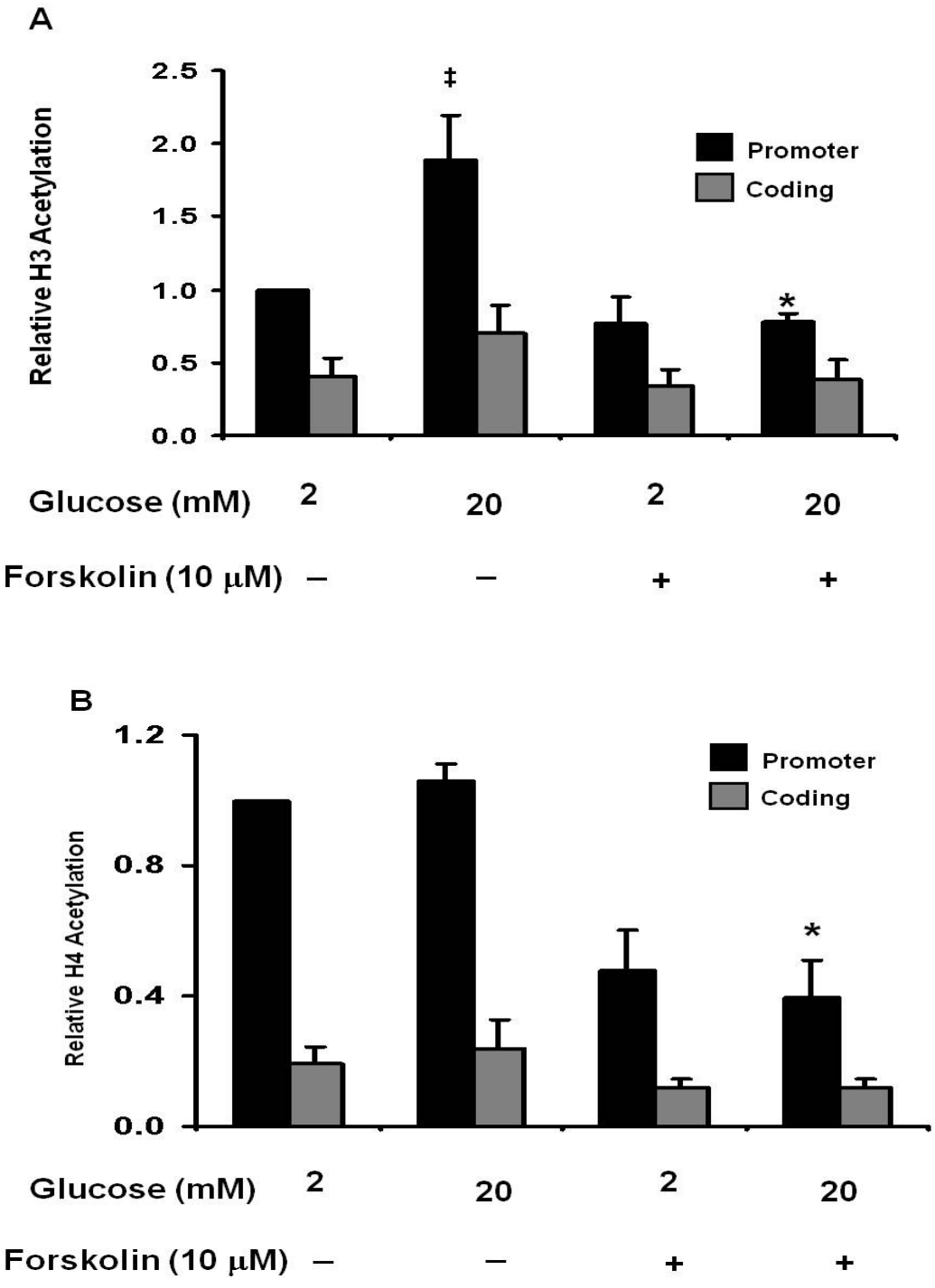

B
Promoter
Coding
Relative Pol II pCTD^{Ser2} Binding
8
6
4
2
0
‡
*
Glucose (mM) 2 20 2 20
Forskolin (10 μM) – – + +

Figure 13. cAMP abrogates the glucose-mediated recruitment of Pol II pCTD^{Ser5} to the promoter, and Pol II pCTD^{Ser2} to the coding region of the L-PK gene. Cells were treated with 2 or 20 mM glucose in the presence or absence of 10 μM forskolin for 6 h. Chromatin immunoprecipitation assays was performed using antibodies against Pol II pCTD^{Ser5} (A), or Pol II pCTD^{Ser2} (B). The L-PK promoter and coding region were targeted for amplification via real-time RT-PCR. Data shown are means ± SEM from five independent experiments. ‡ $P < 0.05$ vs. 2 mM glucose, *$P < 0.05$ vs. 20 mM glucose.

3.4.5 cAMP decreases the acetylation of histones H3 and H4 associated with the L-PK gene promoter.

An increase in acetylation of histones H3 and H4 produces an open chromatin conformation that promotes transcriptional activity (25, 55). Therefore, we sought to determine whether glucose and cAMP altered acetylation patterns of core histones H3 and H4 associated with the L-PK promoter. Relative abundance of acetylated histone H3, compared to IgG control, is significantly higher on the L-PK promoter than the coding region at non-stimulatory glucose concentrations. Glucose signaling induced a 53% increase while cAMP elevation decreased acetylation of H3 (58%) on the L-PK promoter region containing the ChoRE site (Figure 14A). Interestingly, although 20 mM glucose does not promote an increase in acetylation of histone H4 on the L-PK gene promoter compared to 2 mM, forskolin treatment decreases acetylation on the promoter by 63% (Figure 14B).

To determine if the changes in acetylation of histones on the L-PK gene are dependent upon formation of the glucose-sensing complex, we expressed a dominant-negative ChREBP or suppressed abundance of CBP with siRNA duplexes. When cells are exposed to 20 mM glucose concomitant with overexpression of dominant-negative ChREBP, there is a diminution of acetylated histones H3 (47%) and H4 (38%), as compared to GFP control (Figures 14C and D). Similar to the above Pol II data, enhancing the abundance of wild-type ChREBP failed to further increase the acetylation of either histone on the L-PK promoter (Figures 14C and D). Moreover, diminishing CBP abundance decreased the acetylation of histones H3 and H4 on the L-PK promoter by 67% and 66% respectively, as compared to cells transfected with siScramble (Figures 14E and F).We conclude that acetylation of histones H3 and H4 play a key role in coordinately regulating expression of the L-PK gene and that acetylation of these histones associated with the L-PK gene promoter is dependent upon ChREBP and CBP.


A
Relative H3 Acetylation
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2.0
1.5
1.0
0.5
0.0
‡
*
Promoter
Coding
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Forskolin (10 μM) – – + +
B
Relative H4 Acetylation
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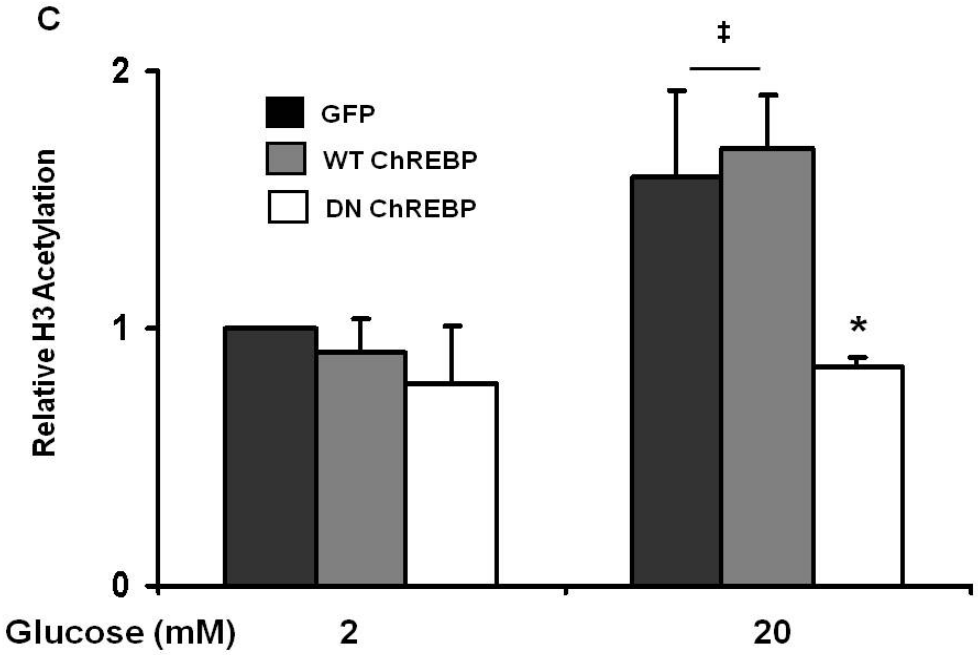
0.4
0.0
*
Promoter
Coding
Glucose (mM) 2 20 2 20
Forskolin (10 μM) – – + +


C
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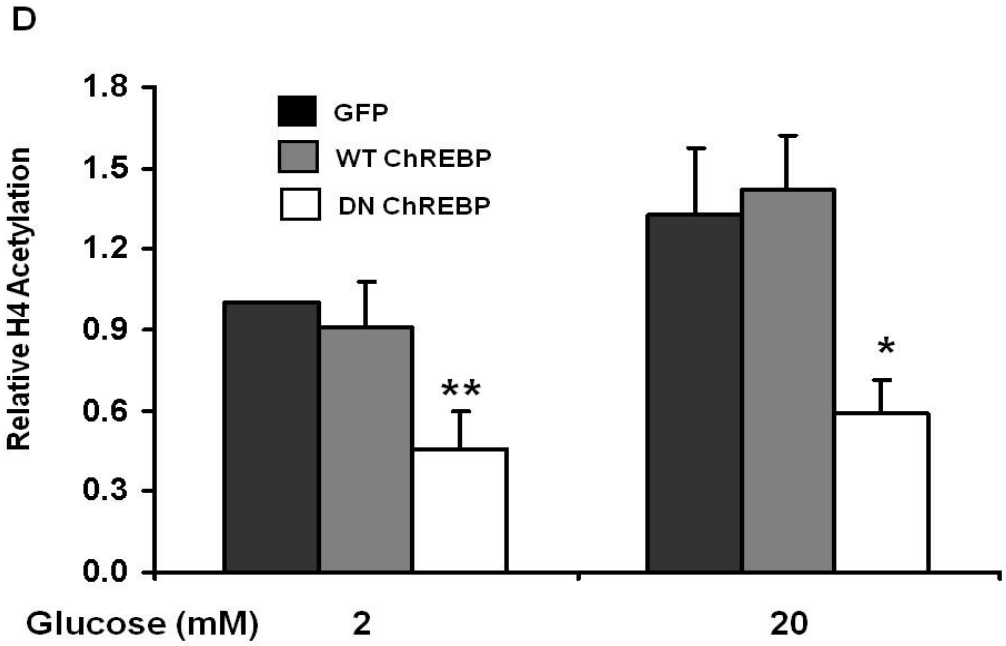
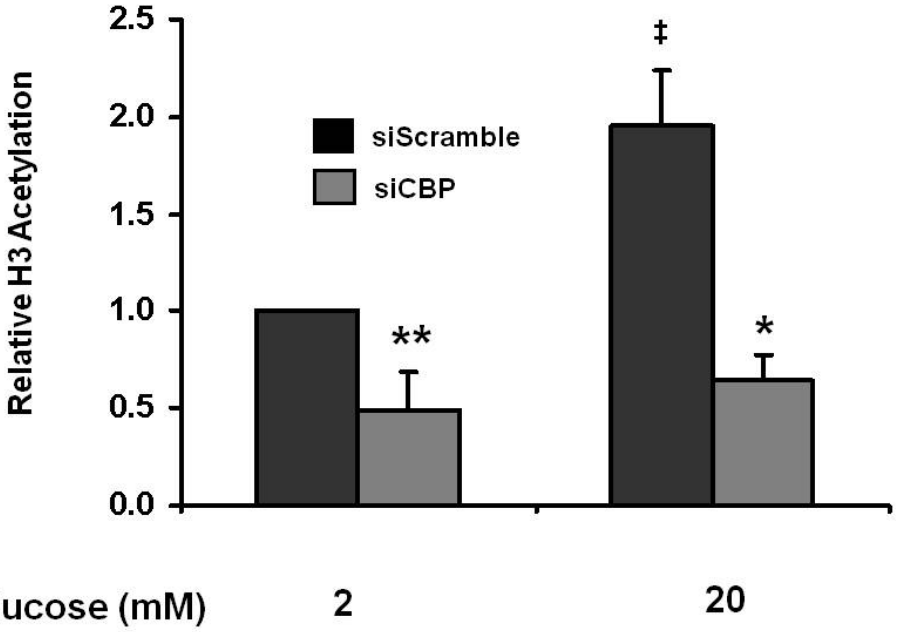
2
1
0
GFP
WT ChREBP
DN ChREBP
Relative H3 Acetylation
*
Glucose (mM)
2
20


D
1.8
1.5
1.2
0.9
0.6
0.3
0.0
GFP
WT ChREBP
DN ChREBP
Relative H4 Acetylation
**
*
Glucose (mM)
2
20

E


Relative H3 Acetylation
2.5
2.0
1.5
1.0
0.5
0.0
siScramble
siCBP
**
‡
*
Glucose (mM)
2
20

F

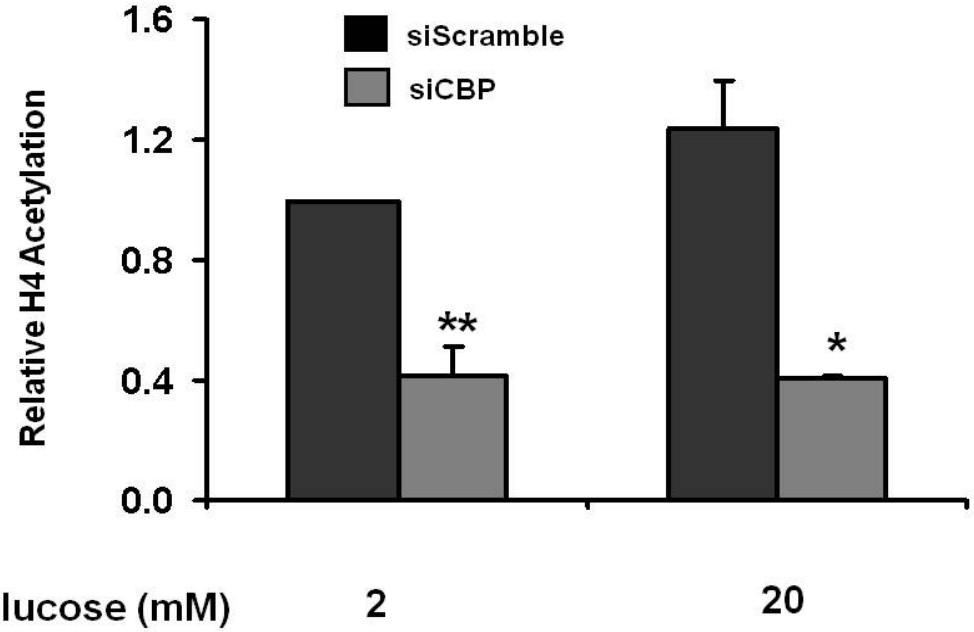

Relative H4 Acetylation
1.6
1.2
0.8
0.4
0.0
siScramble
siCBP
**
*
Glucose (mM)
2
20

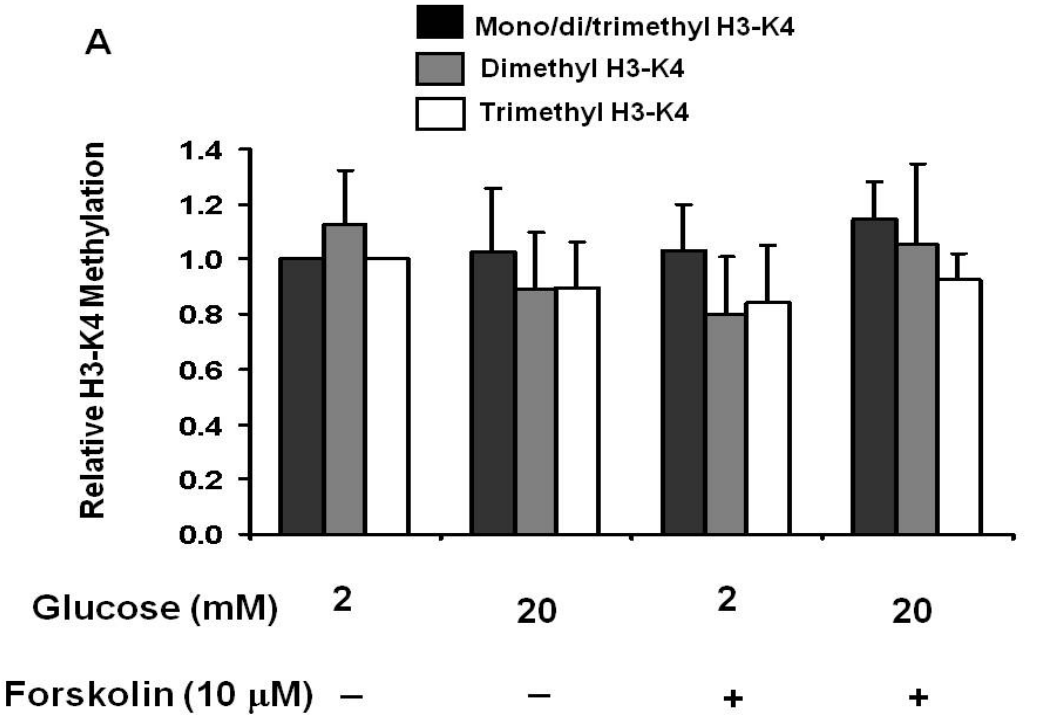
Figure 14. cAMP decreases the acetylation of histones H3 and H4 associated with the L-PK gene promoter. *A, B.* 832/13 cells were cultured in 2 or 20 mM glucose in the presence or absence of 10 μM forskolin for 6 h. *C, D.* Cells were transduced with adenoviruses expressing GFP, wild-type ChREBP or dominant-negative ChREBP for 24 h, followed by culture in 2 or 20 mM glucose for an additional 6 h. *E, F.* Cells were transfected with an siScramble or siCBP duplex for 48 h, then cultured for 6 h in 2 or 20 mM glucose. Relative promoter and coding region occupancy was determined by chromatin immunoprecipitation assay, using antibodies against either acetylated histones H3 (A, C, E) or H4 (B, D, F). The L-PK promoter and a segment of the L-PK coding region downstream of the transcriptional start site were targeted for amplification via real-time RT-PCR. Data represent means ± SE from three to five independent experiments. ‡ $P < 0.05$ vs. 2 mM glucose, ** $P < 0.05$ vs. controls at 2 mM glucose, * $P < 0.05$ vs. 20 mM glucose samples.

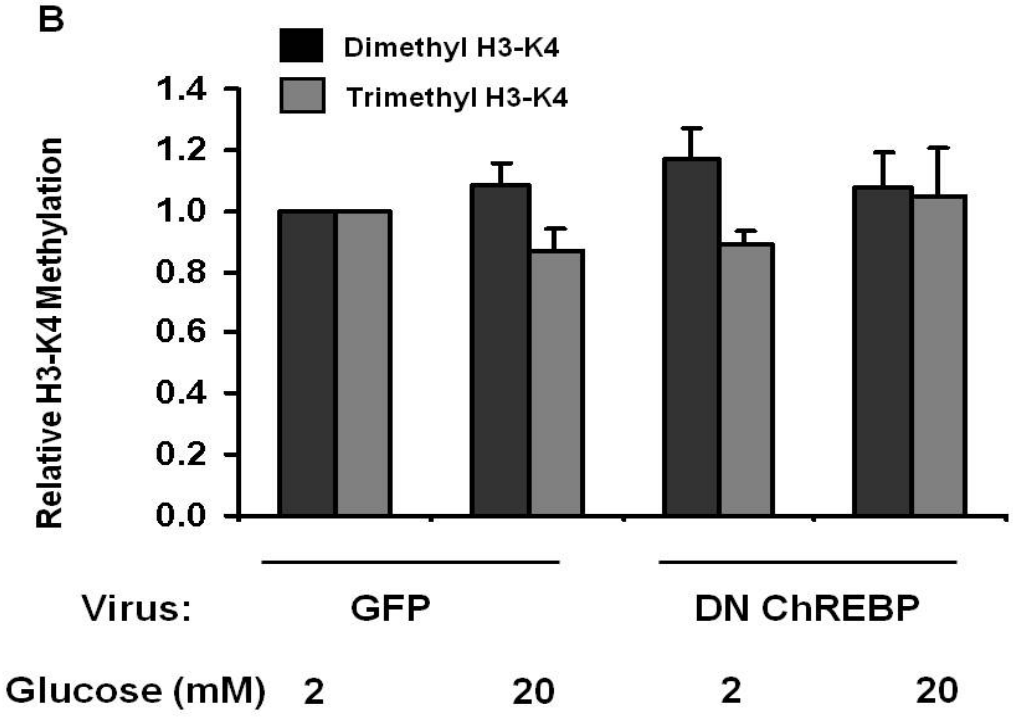
3.4.6 Glucose and cAMP produce opposing effects on the methylation status of histone H3 on the L-PK promoter and coding region.

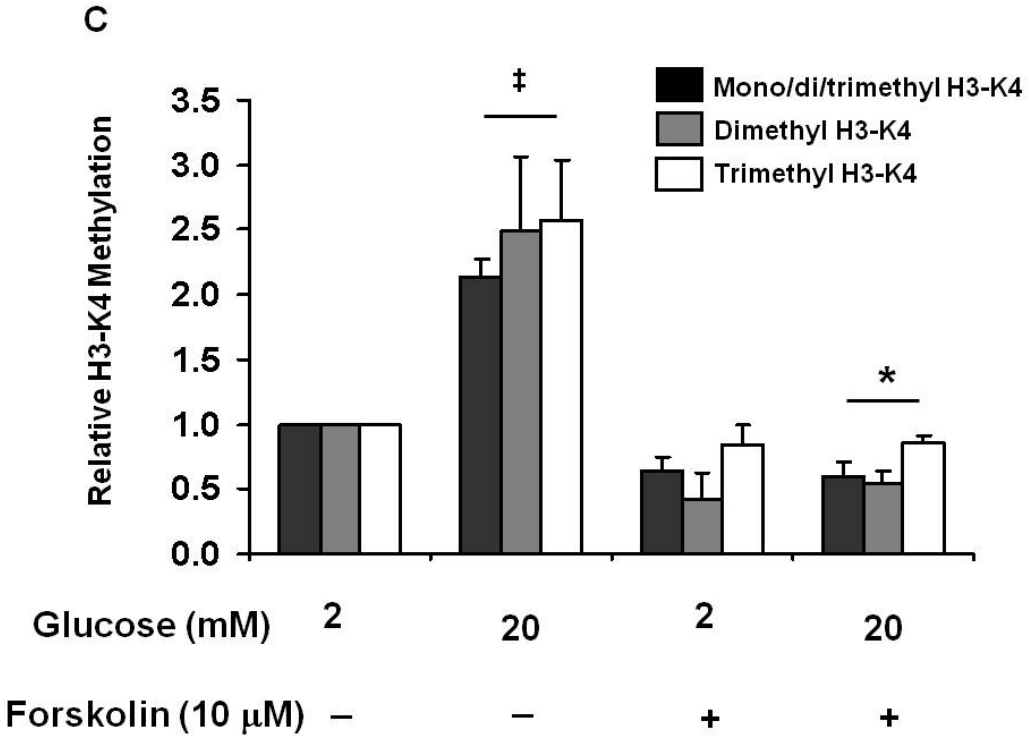
Methylation of histone H3 on lysine 4 is characteristic of transcriptionally active genes (139), whereas inactive genes are generally associated with methylation of histone H3 on lysine 9 (115). To determine whether histone H3 methylation is linked to glucose-dependent activation and cAMP-directed repression of L-PK gene expression, we performed ChIP assays with antibodies that detect histone H3 methylated at lysine 4 or 9. Relative levels of H3-K4 methylation (mono/di/trimethyl, dimethyl H3-K4 and trimethyl

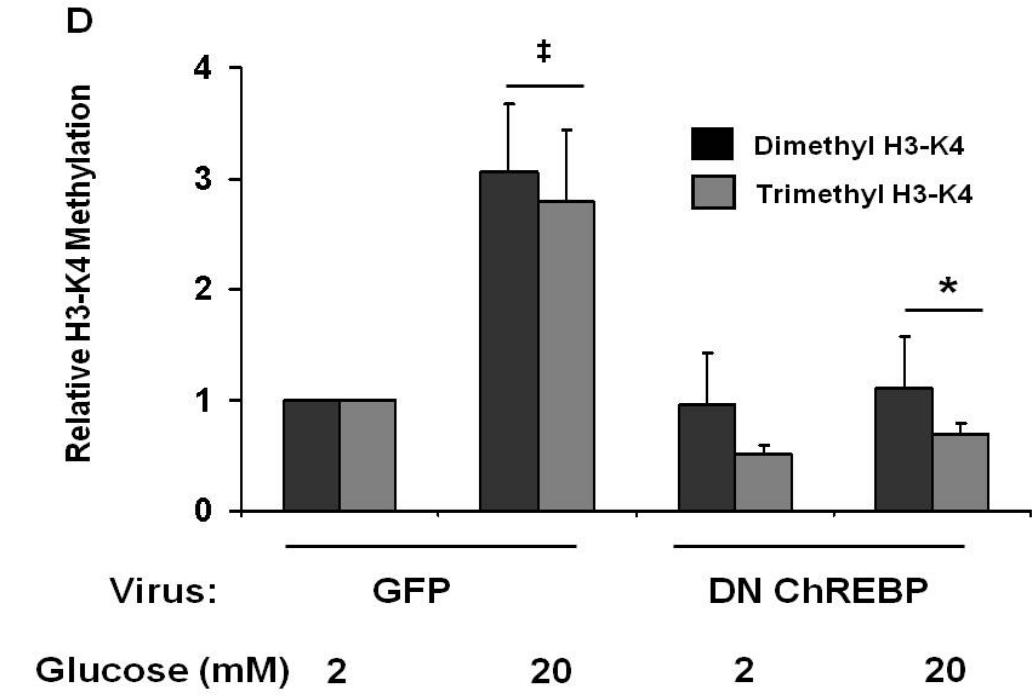
H3-K4) were unchanged on the L-PK promoter region spanning the ChoRE site by either glucose or cAMP treatment (Figure 15A) or overexpression of dominant-negative ChREBP (Figure 15B). However on the coding region, there is a glucose-dependent increase in methylation of H3-K4 (2.5- and 2.6-fold for dimethylation and trimethylation, respectively), which is blunted 78 and 67% (dimethylation and trimethylation, respectively) by cAMP (Figure 15C). Similar to forskolin treatment, adenoviral overexpression of the dominant-negative ChREBP blunted the glucose-dependent dimethylation (64%) and trimethylation (75%) of H3-K4 on the L-PK coding region (Figure 15D).

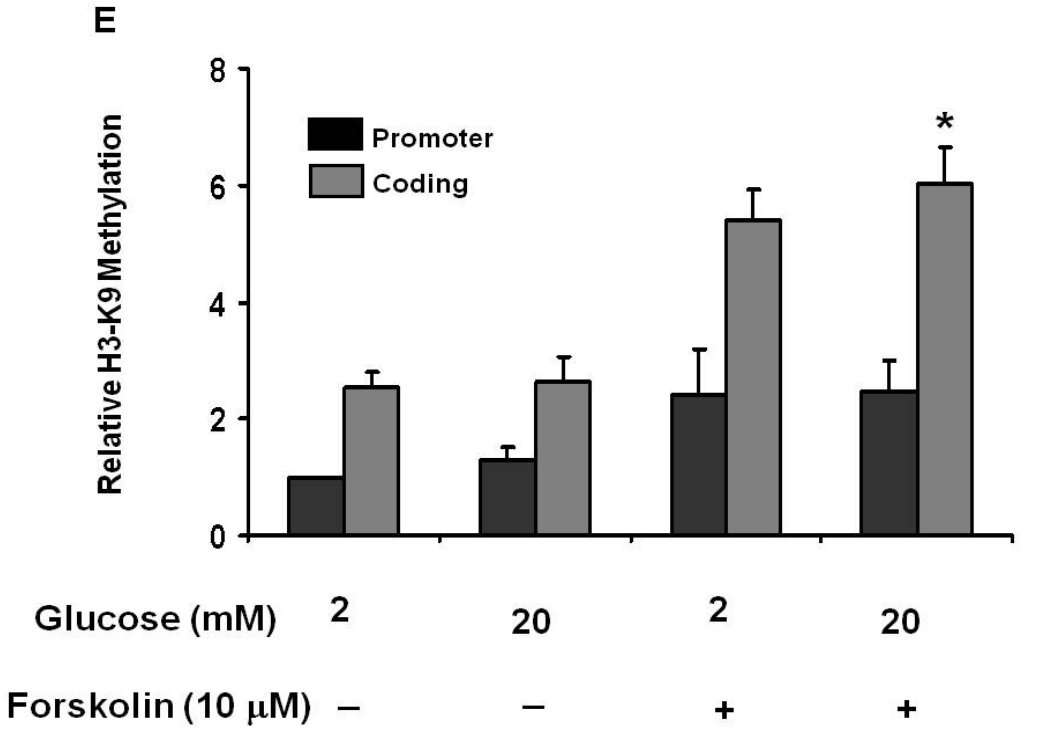
Additionally, while 20 mM glucose did not impact H3-K9 methylation at either the L-PK promoter or coding region, a 2.3-fold increase in H3-K9 methylation was observed on the coding region of the L-PK gene in the presence of cAMP (Figure 15E). Furthermore, a 2.3-fold increase in methylation of H3-K9 on the L-PK coding region was observed following dominant-negative ChREBP overexpression compared to the GFP control (Figure 15F). Taken together with the observations regarding acetylation status of histones associated with the L-PK gene promoter (Figure 14), we conclude that there is an important role for histone modifications (e.g., acetylation and methylation) in regulating transcriptional activation of the L-PK gene by glucose and cAMP.


A
Mono/di/trimethyl H3-K4
Dimethyl H3-K4
Trimethyl H3-K4
Relative H3-K4 Methylation
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Glucose (mM) 2 20 2 20
Forskolin (10 μM) – – + +


B
Dimethyl H3-K4
Trimethyl H3-K4
Relative H3-K4 Methylation
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Virus: GFP DN ChREBP
Glucose (mM) 2 20 2 20


C
Relative H3-K4 Methylation
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
‡
*
Mono/di/trimethyl H3-K4
Dimethyl H3-K4
Trimethyl H3-K4
Glucose (mM) 2 20 2 20
Forskolin (10 μM) – – + +


D
Relative H3-K4 Methylation
4
3
2
1
0
‡
*
Dimethyl H3-K4
Trimethyl H3-K4
Virus: GFP DN ChREBP
Glucose (mM) 2 20 2 20


E
Relative H3-K9 Methylation
Promoter
Coding
*
0
2
4
6
8
Glucose (mM) 2 20 2 20
Forskolin (10 μM) – – + +

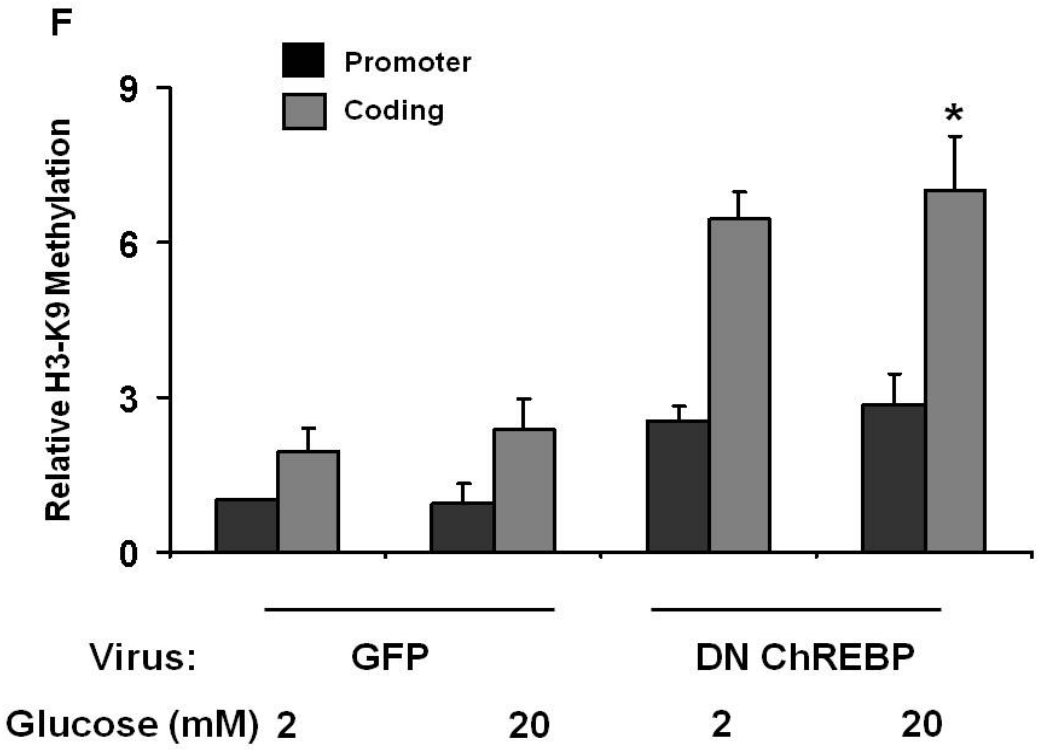

F
Relative H3-K9 Methylation
Promoter
Coding
*
0
3
6
9
Virus: GFP DN ChREBP
Glucose (mM) 2 20 2 20

Figure 15. Glucose and cAMP signaling alter the methylation status of histone H3 on the L-PK promoter and coding region. *A, C, E.* 832/13 cells were treated with 2 or 20 mM glucose in the presence or absence of 10 μM forskolin for 6 h. *B, D, F.* Recombinant adenoviruses expressing GFP, wild-type ChREBP or dominant-negative ChREBP were transduced into cells for 24 h, cells were then cultured in 2 or 20 mM glucose for an additional 6 h. Chromatin immunoprecipitation assays were employed to determine relative promoter and coding region occupancy of methylated histone H3-K4 (A-D) and H3-K9 (E-F). The L-PK promoter and a segment of the L-PK coding region were targeted for amplification by RT-PCR. Data represent means ± SE from three to five independent experiments. ‡ $P < 0.05$ vs. 2 mM glucose, *$P < 0.05$ vs. 20 mM glucose samples.

3.5 DISCUSSION

Glucose and cAMP regulate the expression of the L-PK gene by promoting the assembly and disassembly, respectively, of a glucose-sensing complex composed of ChREBP, HNF4α and CBP (15). To further understand how glucose and cAMP control expression of the L-PK gene, we investigated histone modification patterns and Pol II recruitment to the L-PK gene promoter in response to the regulatory signals glucose and cAMP. Several significant findings emerged: 1) cAMP opposes both the glucose-dependent recruitment and phosphorylation status of Pol II at the L-PK gene promoter and coding region; 2) glucose and cAMP alter the pattern of modified histones (including acetylation and

methylation) on the L-PK promoter and coding region; and 3) this signal specific histone modification pattern and recruitment of Pol II to the L-PK promoter is dependent upon ChREBP and CBP.

ChREBP and CBP are required for full glucose-mediated expression of the L-PK gene; we now further extend these prior findings to include the glucose-stimulated recruitment of Pol II to the L-PK promoter and its exclusion by elevations in intracellular cAMP. Overexpression of either a mutant form of ChREBP or an siRNA-mediated decrease in the abundance of CBP blunted the ability of Pol II to bind to the L-PK promoter. The diminished Pol II at the L-PK promoter (Figures 12B and C) may be interpreted as follows: the complex containing ChREBP and CBP initially assembles on the L-PK promoter and Pol II is subsequently recruited. We suspect that formation of the glucose-sensing complex on the L-PK promoter may allow the coactivator CBP to form a bridge with the basal transcriptional apparatus, thus physically assisting the recruitment of Pol II, as has been previously described in other model systems (21). In this regard, we have shown via sequential chromatin immunoprecipitation that CBP and Pol II were present within the same region of the L-PK promoter at the same time point (Figure 12D). Additionally, it is probable that CBP, via its HAT activity, may transfer acetyl groups to histones H3 and H4, thereby allowing for an open chromatin formation, and thus providing a platform on the promoter onto which the Pol II holoenzyme can dock. In support of this idea, we demonstrated using siRNA duplexes that CBP: 1) is absolutely required for glucose-mediated expression of the L-PK gene; 2) is indispensable for association of acetylated histones H3 and H4 with the L-PK promoter (Figures 14E and F); and 3) is required for the recruitment of Pol II to the L-PK promoter (Figure 12D).

In addition to recruitment of Pol II to the L-PK gene promoter, we examined the phosphorylation state of this enzyme. Phosphorylation of Pol II CTD Ser^5 residues correlates with transcription initiation and promoter clearance; it also coordinates transcription with RNA processing as the phosphorylated CTD provides a platform for recruiting factors involved in 5' capping, splicing, and 3' polyadenylation to the nascent transcript (104). The glucose-dependent increase in Pol II $pCTD^{Ser5}$ recruitment on the L-PK promoter is directly linked to induction of L-PK gene expression, whereas Pol II $pCTD^{Ser2}$ correlates with elongation of the L-PK gene; cAMP dominantly opposes these glucose-dependent phosphorylation events, which we propose prevents transcriptional initiation and elongation.

Histone modifications contribute to the activation or repression of transcription by determining the accessibility of DNA to transcription factors. Transcriptionally active genes are located in regions of 'open' chromatin that are hyperacetylated on lysines of histones H3 and H4. Hypo- or de-acetylation of these histones leads to a 'closed' chromatin structure, and thus is associated with inactive genes (138). Methylation of histone H3 can have distinctive roles in transcriptional regulation depending on the specific lysine residue methylated. Methylation of H3-K4 is associated with 'open' chromatin, thus actively transcribed genes, whereas methylation of H3-K9 is observed in areas of 'closed' chromatin and thereby inactive genes (81). Distinctions emphasizing important differences in the relative acetylation of H3 versus H4 have led to the suggestion that these two histones can be differentially regulated (153). In the case of the L-PK gene, we observed a higher degree of acetylated histone H3 and H4 associated with the L-PK promoter following glucose stimulation as compared to the coding region of

this gene (Figures 14A and B). However, there is a differentially regulated modification pattern between these two histones in response to glucose signaling. Although the acetylation of H3 and H4 on the promoter is diminished by cAMP treatment, only the acetylation of H3 is increased by glucose, suggesting that in context of the L-PK promoter, acetylation of histone H3 rather than H4 potentially plays a more important role in determining accessibility of DNA to the trans-acting factors required for glucose-mediated induction of this gene. Furthermore, in this study, we have shown that acetylation of histones H3 and H4 on the L-PK promoter are dependent upon ChREBP (Figures 154 and D) and CBP (Figures 14E and F). We interpret these data to mean that assembly/disassembly of a complex containing ChREBP and CBP on the L-PK promoter, events controlled by glucose and cAMP, is necessary for the altered association patterns of acetylated histones with this promoter region. Transfer of acetyl groups to several different lysines on the N-terminal tail of core histones is catalyzed by histone acetyltransferases (HATs); silencing of CBP decreases the association of acetylated H3 and H4 with the L-PK promoter (Fig. 14E and 5F), therefore we suspect that the HAT activity of CBP may be involved in the control of expression of this gene.

Increases in acetylation of both dimethyl (17) and trimethyl H3-K4 (129) have been shown at promoters of active genes; however no changes in methylation status of these residues were seen on the L-PK promoter in the presence of either the glucose or cAMP signal (Figure 15A). In contrast, alterations of the methylation status of histone H3 is observed on the L-PK coding region. Increases in total methylation, in addition to specific dimethylation and trimethylation modifications, of histone H3 at lysine 4 is glucose-mediated and dependent upon ChREBP (Figures 15C and D). These ChREBP-

dependent increases in H3-K4 methylation with the coding region may support recruitment of the glucose-sensing complex to the L-PK promoter and facilitate transcriptional elongation. Similar to methylation of histone H3 on lysine 4, methylation of histone H3 at lysine 9 appears to be regulated on the L-PK coding, but not promoter, region. cAMP blunts expression of the L-PK gene, in part, by increasing the methylation of H3-K9 on the coding region (Figure 15E). A similar increase in methylation of H3-K9 on the coding region was observed when dominant-negative ChREBP was overexpressed (Figure 15F). Since expression of the dominant-negative ChREBP mimics the effect of increasing intracellular cAMP, it is entirely possible that the cAMP-directed increase in H3-K9 methylation may be dependent upon the loss of ChREBP from the L-PK promoter.

A group of DNase hypersensitive sites in the proximal L-PK promoter in adult rat livers correlate with the transcriptional activity of the L-PK gene; intensity is severely weakened during periods of prolonged fasting (11). It was suggested that histone modifications responsible for altering the chromatin structure may explain the variations in hypersensitive regions of the L-PK promoter. In the current study, we have clearly shown that changes in acetylation and methylation status on the L-PK promoter and coding region in response to glucose and cAMP correlate with induction and repression, respectively, of this gene, which is in agreement with the prior study. We suspect that these chromatin modifications alter nucleosome positioning around the L-PK gene promoter and are thus part of the mechanism underlying regulation by glucose and cAMP.

Taken together, the data presented here support the conclusion that acetylation and methylation of histones determine the accessibility of DNA to the factors that recruit the Pol II holoenzyme and ultimately regulate expression of the L-PK gene. Furthermore, we have demonstrated a dependence on ChREBP and CBP for these modifications. Although we have focused on acetylation of histone H3 and H4, and methylation of H3-K4 and H3-K9, it is possible that other modifications induced by glucose and/or cAMP are necessary for maximal activation and repression of the L-PK gene. We have shown in this study that glucose- mediated expression of the L-PK gene correlated with increased H3 acetylation on the promoter and H3-K4 methylation on the coding region. In contrast, cAMP-dependent inactivation of the L-PK gene was characterized by H3 and H4 hypo- or de-acetylation and decreased H3-K4 methylation on the promoter; in addition, cAMP increased H3-K9 methylation on the coding region. We conclude that assembly and disassembly of ChREBP and CBP on the L-PK promoter, by glucose and cAMP, respectively, regulate histone modifications at the promoter and coding regions of the L-PK gene.

4.0 SUMMARY AND FUTURE DIRECTIONS

4.1 SUMMARY

A post-prandial rise in circulating glucose levels induces the expression of genes that promote glucose metabolism via glycolysis (L-PK) and fatty acid biosynthesis (ACC and FAS) (27, 148). Conversely, during the fasted state, a rise in hormones that promote increased intracellular cAMP levels prevents the induction of these metabolic enzyme genes (13, 100). Therefore, the L-PK gene is an ideal candidate to study transcriptional regulation by two opposing stimuli, glucose and cAMP.

We determined that two primary DNA-binding transcription factors, ChREBP and HNF4α, are both equally required to mediate the glucose-induction of the L-PK gene, and that glucose signaling promoted an increased recruitment of both of these factors to their respective response elements in the proximal L-PK promoter. Furthermore, we demonstrated that agonists promoting increased intracellular cAMP levels abrogate binding of both of these factors to the L-PK gene. Although ChREBP and HNF4α are known promote induction of the L-PK gene by glucose (1, 28, 33, 69, 159, 160) the mechanism is unknown. Therefore, our studies complement and extend the findings of

both our laboratory and other laboratories. However, prior to the studies presented herein, the mechanism of cAMP-directed repression of this gene had never been studied.

Although sequence-specific transcription factors that mediate induction of the L-PK gene by glucose have themselves been studied, the coactivator necessary to provide maximum inductive capacity to the gene has never been established. Based on previous reports that CBP interacts with HNF4α *in vitro* (168), we postulated that CBP may function as a coactivator to the L-PK gene. Therefore, we tested this hypothesis directly and subsequently demonstrated that CBP functions as a bona fide coactivator of the L-PK gene.

Having established that glucose induces expression of the L-PK gene by recruiting a complex of ChREBP, HNF4α and CBP to the L-PK promoter, and that cAMP represses expression of the gene by inhibiting association of this complex with the promoter [Chapter 2], we next set out to establish how occupancy of this complex on the promoter enabled transcriptional initiation of the L-PK gene. We discovered that occupancy of a complex containing ChREBP, HNF4α and CBP on the L-PK promoter in response to glucose was associated with and required for an increase in histone H3 acetylation surrounding the ChREBP and HNF4α response element binding sites. Concomitantly, occupancy of this complex promoted an increased association of Pol II with the L-PK promoter that corresponded with increased expression of the gene. cAMP signaling mediated deacetylation of histones H3 and H4 in a complex-dependent manner, decreased recruitment of Pol II to the promoter, and subsequently inhibited expression of the L-PK gene.

The pattern of methylation of the N-terminal tail of histone H3 varied in response to glucose and cAMP signaling on the coding region of the L-PK gene; no change was observed on the promoter. Glucose promoted an increase in methylation of histone H3 at lysine 4 in a ChREBP-dependent manner, which was opposed by cAMP. We propose that glucose mediates this modification as a mechanism for providing DNA access to Pol II during the elongation phase of transcription. cAMP inhibits transcription by not only opposing the methylation of histone H3 at lysine 4, but also by promoting an increase in methylation at lysine 9. In summary, glucose and cAMP promote opposing alterations in histone modifications that render DNA accessible or inaccessible, respectively, to Pol II during both initiation and elongation of the L-PK gene [Chapter 3].

Based on our findings, we propose the following model (Figure 16) to explain the molecular events that promote induction and repression of the L-PK gene following exposure to glucose and cAMP, respectively.

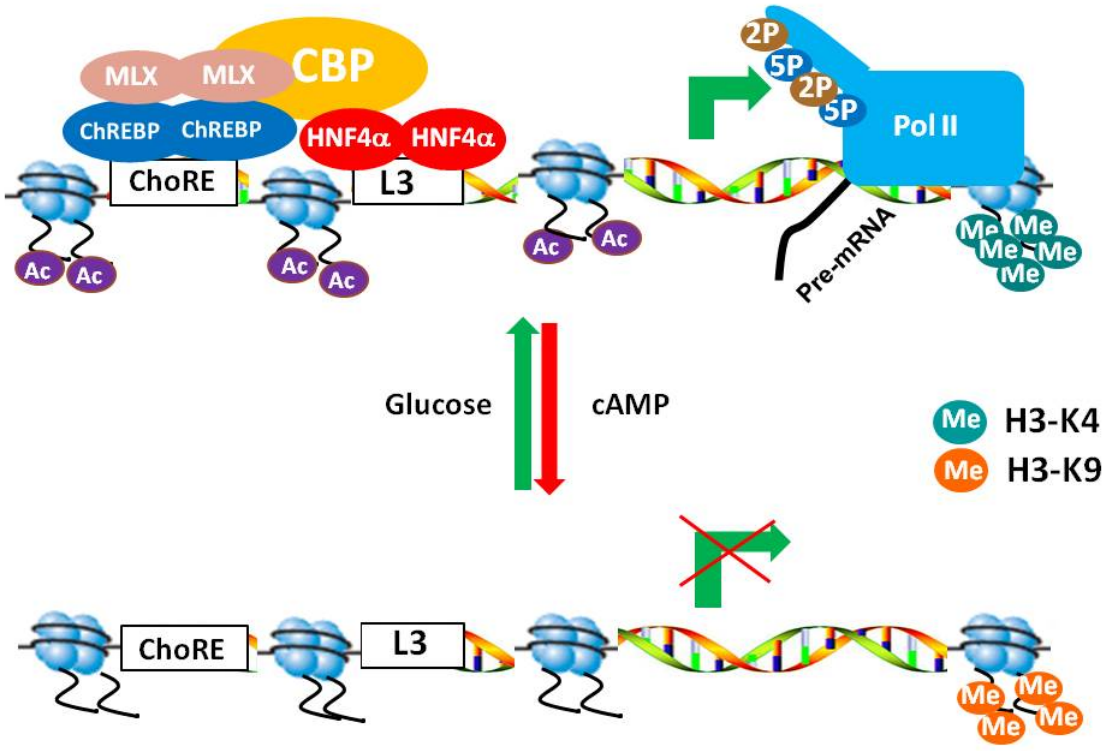


Figure 16. Regulation of the L-type Pyruvate Kinase gene by Glucose and cAMP.

A complex containing ChREBP, HNF4α and the coactivator CBP assembles on the promoter of the L-PK gene in response to glucose to promote expression of this gene. Conversely, an increase in intracellular cAMP levels prevents the recruitment of this complex to the L-PK promoter, thus inhibiting expression of this gene. Furthermore, assembly of this complex on the promoter in response to glucose, alters the pattern of H3 acetylation on the promoter, and methylation on coding region, and recruits Pol II to facilitate transcriptional regulation; cAMP opposes the glucose-mediated modification of histones and recruitment of Pol II to repress expression of the L-PK gene.

4.2 FUTURE DIRECTIONS

Although our work has provided a much clearer insight into the mechanism of regulation of the L-PK gene by glucose and cAMP, there are many questions that remain unanswered.

The intermediates/metabolites generated by glucose metabolism that control factor association with the L-PK promoter in response to glucose and cAMP signaling are unknown. One study demonstrated using a non-metabolizable glucose analog, 2-deoxyglucose, that the glucose induction of the L-PK gene requires phosphorylation of the hexose, but does not require further metabolism of glucose through the glycolytic pathway. 2-deoxyglucose can be become phosphorylated to 2-deoxyglucose-6-phosphate and can partly mimic the glucose induction of the L-PK gene (100). However, the metabolite(s) required to mediate these effects have yet to be determined.

Chan and colleagues determined that ChREBP contains a glucose-sensing module. This module contains two domains: a low-glucose inhibitory domain (LID) that inhibits ChREBP transactivation at low glucose concentrations, and a glucose-response activation conserved element (GRACE) that mediates transactivation at stimulatory glucose concentrations (86). Whether or not other factors that promote expression of the L-PK gene contain a similar glucose-sensing module and whether this module is influenced by cAMP is unknown. While we have shown that the cAMP-mediated repression of the L-PK gene is mediated via the PKA pathway, the exact phosphorylation events necessary to mediate disassembly of the glucose-sensing complex have yet to be determined.

Furthermore, the temporal pattern of recruitment/exclusion of ChREBP, HNF4α and CBP on the L-PK promoter in response to glucose and cAMP is unknown. These factors may assemble prior to binding. Alternatively, ChREBP and HNF4α may be recruited by glucose and the coactivator CBP subsequently binds after these factors associate with their cognate response elements. Similarly, exploring the temporal pattern of histone modifications and Pol II recruitment would determine whether histone modifications allow accessibility of DNA to Pol II, or whether binding of Pol II stimulates histone modifications. In the latter case, this may provide access to other factors, such as p-TEFb, or factors that bind the phosphorylated CTD of Pol II.

Numerous lines of evidence have been provided demonstrating that CBP functions to coactivate expression of the L-PK gene; however, whether HAT activity of CBP directly acetylates histones H3 and H4 on the L-PK promoter region examined has not yet been determined. Furthermore, coactivators and corepressors exist as large multi-regulatory complexes that respond to various stimuli by altering transcriptional programs. Therefore, CBP may be just one of a large number of coactivators/ corepressors that control activation and repression in response to glucose and cAMP, respectively. The roles of ATP-remodeling complex proteins, HDACs, histone demethylases, HMTs, and HATs other than CBP, in the initiation and elongation of transcription remain to be determined.

Finally, the transcriptional regulation of the L-PK gene in response to glucose and cAMP has been studied by a number of laboratories for almost 20 years. However, the role of this enzyme in pancreatic β-cell biology has never been examined. As a rate-limiting glycolytic enzyme, L-PK is perfectly situated to contribute significantly to β-cell

glucose-stimulated insulin secretion. Elucidating the potential role of L-PK in β–cell function may be beneficial for developing therapeutic strategies for treatment of both Type 1 and Type 2 diabetes.

APPENDIX A: REGULATION OF THE ACC GENE BY GLUCOSE AND cAMP: ROLE OF CHREBP, C/EBPβ AND CBP

A.1 ABSTRACT

Acetyl CoA Carboxylase α (ACC) is a key enzyme of fatty acid metabolism that converts acetyl CoA to malonyl CoA. In the pancreatic β-cell, the expression of the ACC gene is tightly regulated, positively by glucose and negatively by cAMP; however, the protein factors controlling the expression of this gene are largely unknown. Using the highly glucose and cAMP-sensitive 832/13 rat insulinoma cell-line, we demonstrated that the glucose-mediated induction of the ACC gene is independent of two transcription factors required for the glucose responsiveness of other metabolic enzyme genes; Hepatic Nuclear Factor 4 α (HNF4α) and the insulin-responsive factor, Sterol Response Element Binding Protein 1c (SREBP-1c), are necessary for the glucose induction of L-type Pyruvate Kinase (L-PK) and Fatty Acid Synthase (FAS), respectively.

We demonstrated that two transcription factors, Carbohydrate Response Element Binding Protein (ChREBP) and C/AAT Enhancer Binding Protein β (C/EBPβ), are both individually required for the glucose-stimulated induction of the ACC gene; however, overexpression of either of these factors failed to potentiate the glucose induction or

alleviate the repression by cAMP of ACC gene expression. Furthermore, we elucidated that the coactivator CREB Binding Protein (CBP) was also required for the glucose-mediated induction of the ACC gene.

Glucose signaling promoted an increase in the recruitment and phosphorylation of RNA Polymerase II (Pol II) on the ACC PII gene promoter and coding region, whereas cAMP blunted both recruitment and phosphorylation of Pol II. Recruitment of Pol II to the ACC gene promoter in response to glucose was dependent upon CBP, but not ChREBP or C/EBPβ.

We conclude that cAMP and glucose regulation of the ACC gene requires ChREBP, C/EBPβ and CBP. The expression of the ACC gene occurs, in part, due to recruitment of Pol II to the ACC PII promoter and coding region, in a CBP-dependent manner; cAMP represses expression of the gene by disrupting the glucose-dependent recruitment of Pol II. Finally, induction of the ACC gene was shown to be completely insulin-independent.

A.2 INTRODUCTION

The Acetyl Co-A Carboxylase α (ACCα) gene encodes a pivotal regulatory enzyme of fatty acid biogenesis catalyzing the conversion of acetyl co-A to malonyl CoA. Expression of the ACCα is induced by glucose and repressed by cAMP-generating agents including forskolin (13), yet the signaling pathway responsible for the induction and repression of ACCα gene expression remains unresolved.

The ACCα gene is transcribed from two different promoters, termed PI and PII producing transcripts that are differentially spliced to produce a minimum of five mRNAs that vary in their 5' untranslated regions (90); only PII transcripts appear to be generated in β cells (72). Analysis of the PII promoter by Kim revealed a glucose responsive element that may confer the inductive properties of glucose to this gene (36). This glucose responsive sequence however is dissimilar from the conserved ChoRE sequence known to bind the basic Helix-Loop-Helix Leucine Zipper (bHLH-LZ) transcription factor Carbohydrate Response Element Binding Protein (ChREBP) on glucose responsive promoters in primary hepatocytes and β cells (116).

ChREBP has been well characterized as an essential factor in the induction of glucose responsive genes including L-PK and FAS (28, 33, 69, 167). ChREBP mediates transcriptional activation of target genes by binding to the ChoRE with its heterodimerization partner Mlx (94, 96, 136). Furthermore, in addition to ChREBP, a second transcription factor, Sterol Response Element Binding Protein-1c (SREBP-1c), has been demonstrated to mediate the induction of lipogenic gene expression (3, 49, 71, 98, 137). A number of groups present evidence that simultaneous binding of ChREBP to the ChoRE, and SREBP-1c binding to a sterol response element (SRE) is essential for a synergistic induction of lipogenic gene expression (41, 75).

The basic region leucine zipper (bZIP) transcription factor CCAAT/enhancer binding protein beta (C/EBPβ) mediates transcription of its target genes by binding as a homodimer or heterodimer (with other members of the C/EBP family) to a CCAAT box, or as a homodimer to chimeric CRE/CEBP or CRE sites (47). bZIP proteins mediate

their biological role by interacting with genomic DNA to recruit coactivators, such as CREB Binding Protein (CBP), to promote transcription.

CBP is known to coactivate a wide array of genes including the glucose-responsive glycolytic enzyme gene L-type Pyruvate Kinase (L-PK) (15). CBP mediates its coactivator function two-fold: 1) CBP recruit RNA Polymerase II (Pol II) to direct transcriptional initiation; and 2) CBP contains intrinsic histone acetyltransferase (HAT) activity, therefore can covalently modify histones to provide accessibility to factors required for transcription (21).

From these studies we conclude that the glucose-mediated induction of the ACC gene requires the transcription factors ChREBP and C/EBPβ, in addition to the coactivator CBP. Recruitment of Pol II to both the ACC PII promoter and coding region in response to glucose requires CBP. cAMP inhibits transcription of the ACC gene by blunting the recruitment of Pol II to both the PII promoter and coding region. Finally, we elucidated that the induction of the ACC gene in β cells is completely independent of insulin.

A.3 MATERIALS AND METHODS

A.3.1 Cell Culture, RNA isolation and quantification by real-time RT-PCR

The INS-1-derived 832/13 rat insulinoma cell line was cultured as previously described (60). Total RNA was isolated from 832/13 cells using TRI-reagent (Molecular Research

Center) according to the manufacturer's protocol. iScript (Bio-Rad) was used for first-strand synthesis of cDNA using 0.5 μg -1 μg of RNA. 2.5% of the total RT reaction was used as input for PCR using SYBR green master mix with ROX (Bio-Rad), 0.5 μL each of forward and reverse primers, in a total volume of 25ul. Real-time PCR was performed using the Applied Biosystems Prism 7300 sequence detection system. Relative mRNA levels for individual genes were normalized to cyclophilin mRNA levels.

A.3.2 Isolation of nuclear proteins and immunoblotting

Isolation of nuclear proteins and immunoblotting has been previously described (15). The antibodies used for protein quantification were SREBP-1c: Cat # sc-8984 (Santa Cruz Biotechnology); C/EBPβ: Cat # sc-150 (Santa Cruz); ChREBP: Cat # NB400-135 (Novus Biologicals); HNF4α: Cat # MAB10090 (Millipore) and β Actin: Cat # 4967 and Tubulin: Cat # 2144 (Cell Signaling Technology).

A.3.3 siRNA-mediated suppression of gene expression

SREBP-1c, C/EBPβ, ChREBP, and HNF4α abundance was decreased by transfecting pre-annealed duplexes from Ambion into 832/13 cells using Dharmafect reagent 1 (Dharmacon) according to the manufacturer's protocol (SREBP-1c: siRNA ID # s134945 and s134943, C/EBPβ: siRNA ID # 201012 and 46760, ChREBP: siRNA ID # 57311 and 190928, and HNF4α: siRNA ID # 199193 and 51661). Decreased abundance of the targeted genes was analyzed by immunoblotting.

A.3.4 Chromatin immunoprecipitation (ChIP) assays

ChIP assays were then performed using a ChIP Assay Kit from Millipore (Cat # 17-295) with modifications as described previously (110). The ChoRE-containing portion of the ACC PI promoter, a section of the proximal ACC PII promoter and a fragment of the ACC coding region in exon 2 were targeted for amplification. Primers used to amplify the ChoRE-containing region of the PI promoter were as follows: 5'-ACAACTAGGGCCTGGAAGTG-3' and 5'-GGAGGCCTCTTTATAAGACT-3' (upstream and downstream, respectively). Primers used to amplify the ACC PII promoter from 300bp upstream of the transcriptional start site were: 5'-CTAAGCAAAGGGGTTGGTGA-3' and 5'-AGCCAAGAGGTGCAGTCAGT-3' (upstream and downstream, respectively). Primers used to amplify the fragment of coding region were as follows: 5-CTTGCGGAGAGAGGTGTAGG-3' and 5'-GAACGCCAAACGGAGAGTTA-3' (upstream and downstream, respectively). Relative binding of each factor is reported after normalization to IgG control. Antibodies used for immunoprecipitation of Pol II (Cat # 05-623) was from Millipore; Pol II pCTD^{Ser5} (Cat # ab5131) and Pol II pCTD^{Ser2} (Cat # ab5095) were from Abcam; and rabbit IgG (Cat # sc2027) was from Santa Cruz Biotechnology.

A.3.5 Statistical analysis

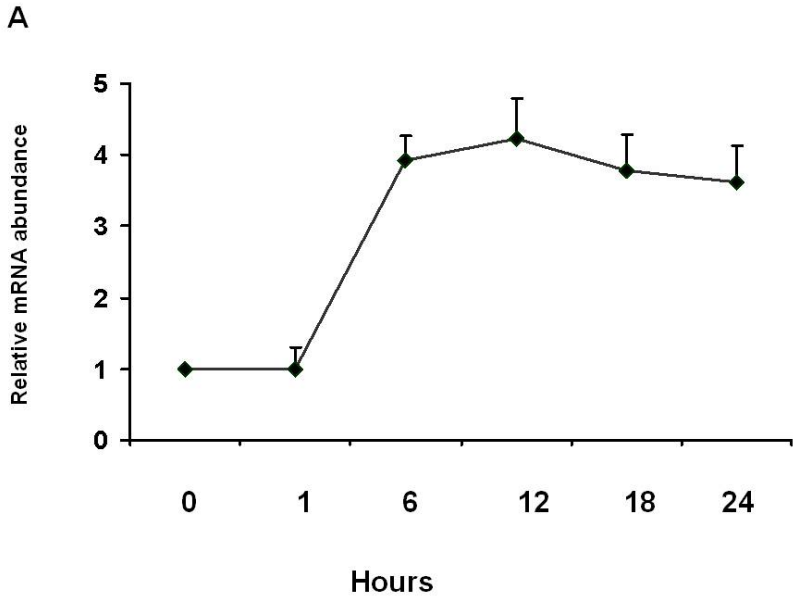
A one-way ANOVA plus Tukey post hoc test was performed to detect statistical differences, of which those <0.05 were considered statistically significant.

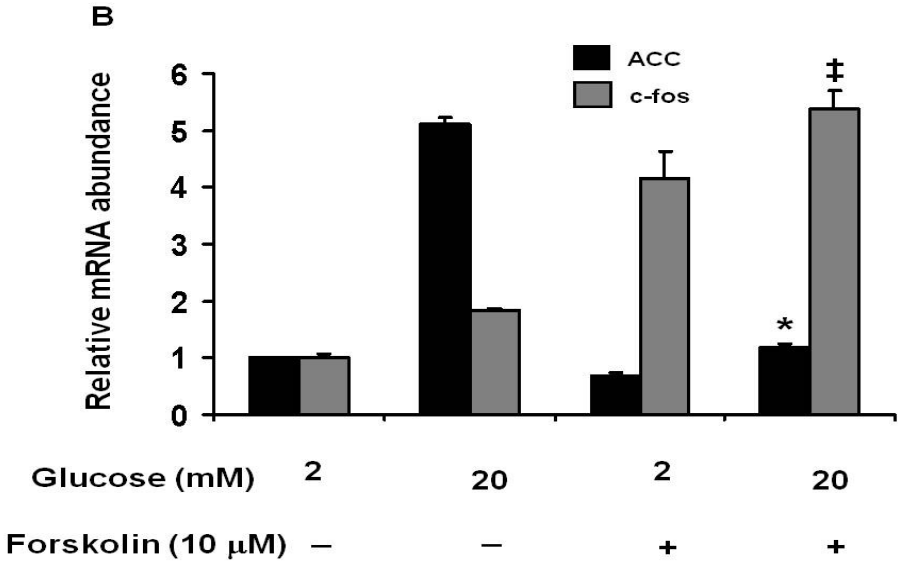
A.4 RESULTS

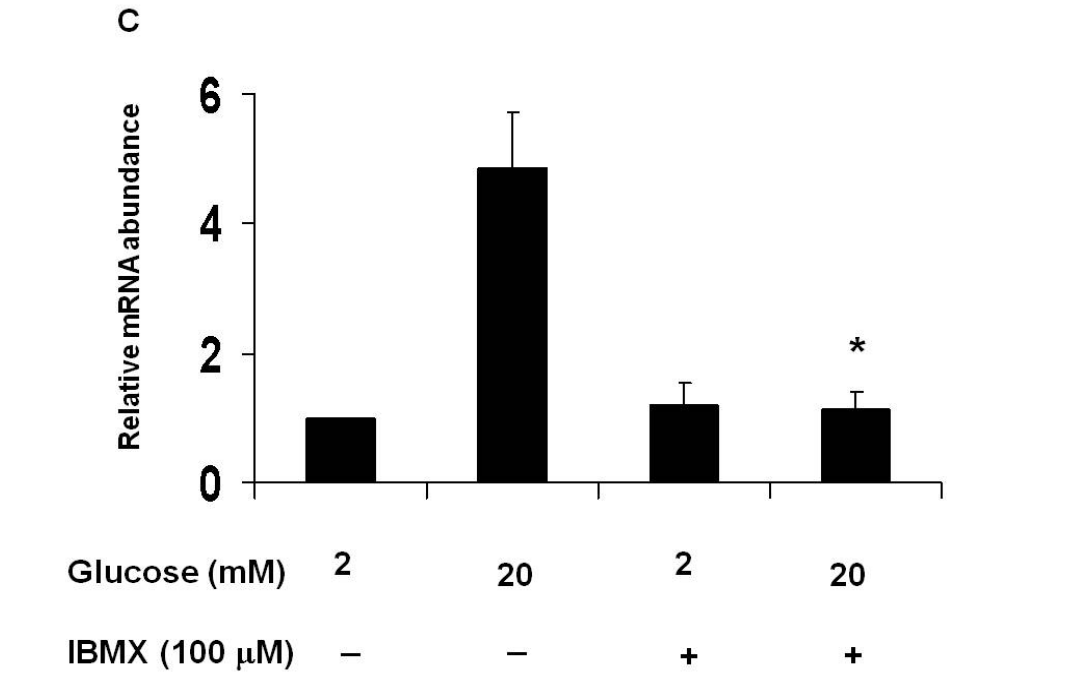
A.4.1 cAMP inhibits the glucose-mediated induction of the ACC gene.

Previous work has documented that glucose induces expression of the lipogenic enzyme gene Acetyl Co-A Carboxylase (ACC), while cAMP opposes this induction (12). To further investigate these molecular mechanisms, we have studied the regulation of this gene using the highly glucose-sensitive INS-1- derived 832/13 rat insulinoma cell line. We first examined the time frame of ACC mRNA induction in 832/13 cells treated with glucose (2 mM and 20 mM). Glucose induced expression of the ACC gene at 1 h; because expression levels reached a maximum at 6 h, all subsequent experiments were performed at this time-point (Figure 17A). 832/13 cells treated with 20 mM glucose for 6 h displayed an approximate 5-fold induction of ACC mRNA levels. Incubation of cells with 10 μM forskolin, a cAMP-generating agent, completely prevented glucose from stimulating expression of the ACC gene; in contrast forskolin induces a 5.6-fold expression in the control gene c-fos (Figure 17B). Additionally, a second cAMP-generating agent, IBMX (a phosphodiesterase inhibitor) also blunted the glucose

induction of the ACC gene, similar to treatment with forskolin (Figure 17C). In 832/13 cells treated with the transcriptional inhibitor, Actinomycin D, 10 μM forskolin did not have any negative impact on ACC mRNA stability (Figure 17D). From these data we can conclude that the cAMP-mediated repression of the glucose-stimulated ACC gene occurs primarily at the level of transcription.


A
Relative mRNA abundance
5
4
3
2
1
0
0
1
6
12
18
24
Hours


B
Relative mRNA abundance
ACC
c-fos
6
5
4
3
2
1
0
*
‡
Glucose (mM) 2 20 2 20
Forskolin (10 μM) – – + +


C
Relative mRNA abundance
6
4
2
0
*
Glucose (mM) 2 20 2 20
IBMX (100 μM) – – + +

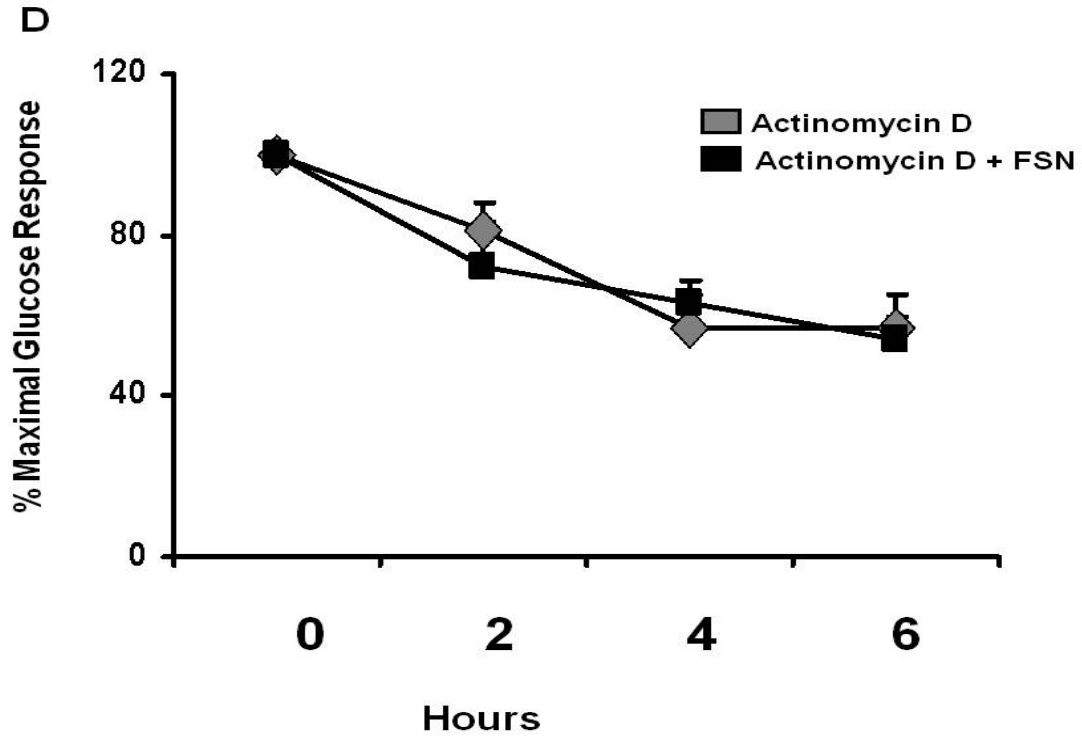
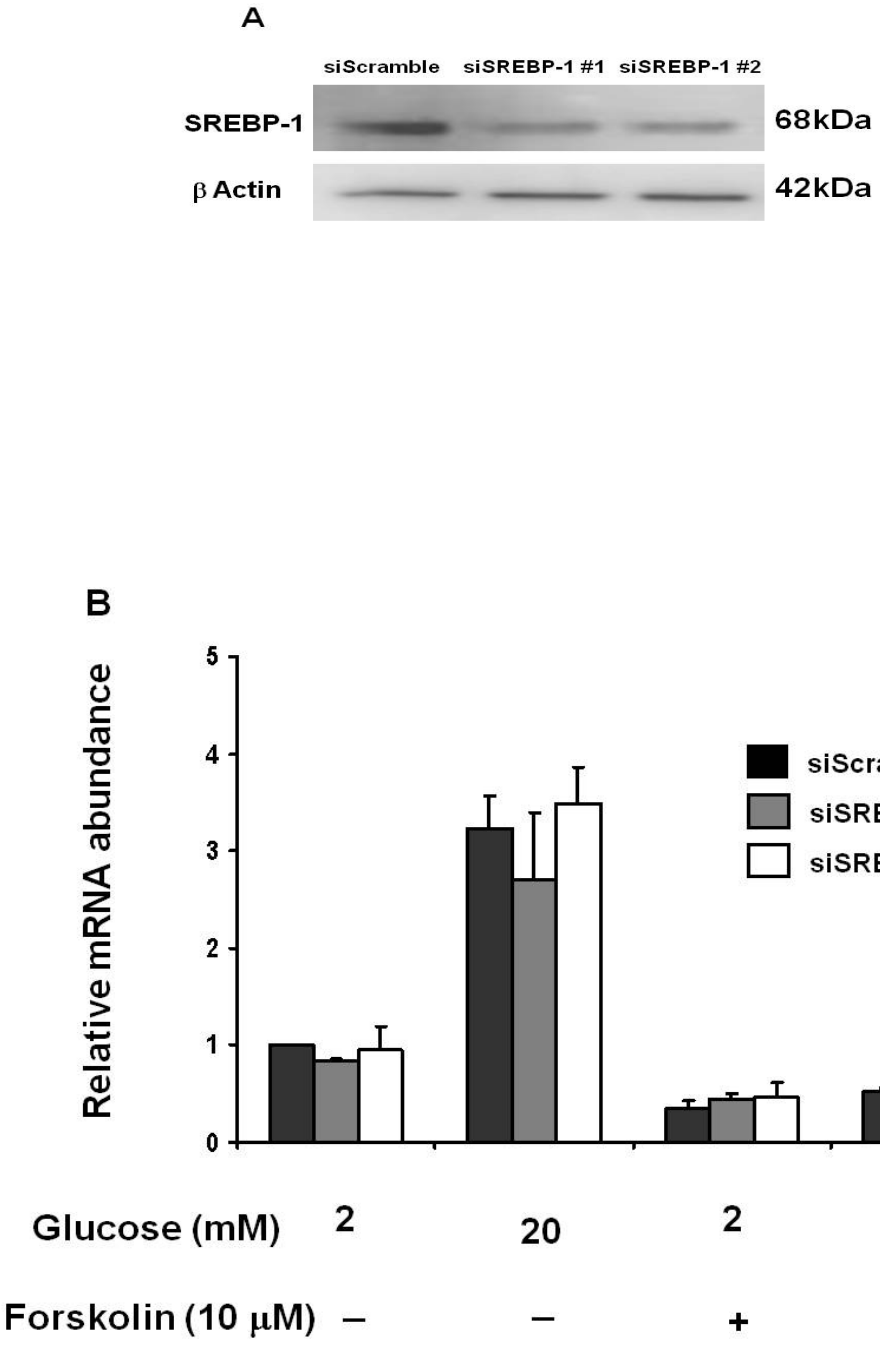

D
% Maximal Glucose Response
120
80
40
0
Actinomycin D
Actinomycin D + FSN
0 2 4 6
Hours

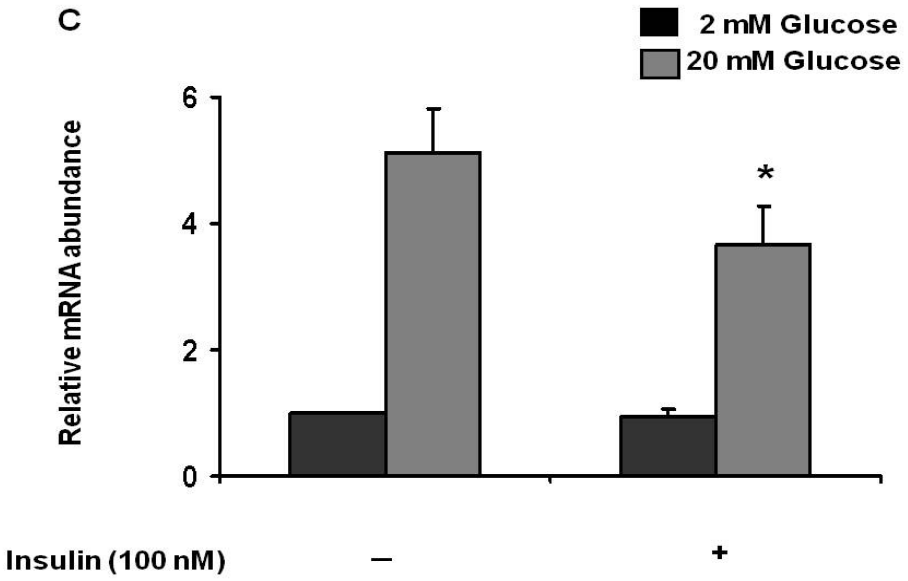
Figure 17. cAMP inhibits the glucose-mediated induction of ACC gene expression, but does not decrease ACC mRNA stability . *A.* 832/13 insulinoma cells were cultured in 2 and 20 mM glucose for up to 24 h. Results are shown as fold increase at 20 mM glucose. *B*, C. Cells were treated with 2 mM and 20 mM glucose in the presence or absence of 10 μM forskolin (B) or 100 μM IBMX (C) for 6 h. Total RNA was extracted and relative levels of ACC and c-fos mRNA were analyzed by real-time RT-PCR. **P* < 0.01 vs. 20 mM glucose, ‡ *P* < 0.05 vs. 20 mM glucose. *D*. 832/13 cells were treated with 20 mM glucose for 6h, and then incubated with 10 μM forskolin in the presence or absence of the transcriptional inhibitor Actinomycin D at 2 h increments for a period of 6 h. The stability of mRNA was assessed by measuring relative abundance of ACC mRNA levels at 0, 2, 4 and 6 h post glucose-stimulation. Data are expressed as means ± SEM from three to five independent experiments.

A.4.2 Glucose-mediated induction of the ACC gene is independent of insulin and SREBP-1c.

The transcription factor sterol regulatory element binding protein-1c (SREBP-1c) has been implicated in mediating the insulin-dependent inductive effect on ACC gene expression in hepatocytes (41, 75). In an effort to determine whether this factor is also necessary for the glucose-stimulated upregulation of ACC mRNA levels in β cells, we decreased abundance of the transcription factor SREBP-1c via siRNA. 832/13 cells were transfected with siRNA duplexes targeting different exons of the coding region of SREBP-1c, both of which significantly reduced abundance of SREBP-1c (Figure 18A).

Suppression of SREBP-1c abundance had no negative impact on the ability of glucose to stimulate expression of the ACC gene, demonstrating that this factor is non-essential in this regard (Figure 18B). This led us to hypothesize that the induction of ACC expression is insulin-independent. To test this hypothesis, cells were treated with stimulatory concentrations of both glucose and insulin, however insulin failed to augment the glucose induction, in fact we observed a significant repressive effect on relative ACC expression (Figure 18C). These data are in contrast to the FAS gene, which was induced by both glucose and insulin, with the insulin induction of FAS expression requiring SREBP-1c (Figure 18D). Therefore, we conclude that the glucose-mediated induction of the ACC gene does not require SREBP-1c. Additionally, insulin alone or in combination with glucose is insufficient to induce or augment expression of the ACC gene, respectively.


A
siScramble
siSREBP-1 #1
siSREBP-1 #2
SREBP-1
68kDa
β Actin
42kDa
B
Relative mRNA abundance
0
1
2
3
4
5
siScramble
siSREBP-1c #1
siSREBP-1c #2
Glucose (mM)
2
20
2
20
Forskolin (10 μM)
–
–
+
+


C
2 mM Glucose
20 mM Glucose
Relative mRNA abundance
6
4
2
0
*
Insulin (100 nM)
–
+

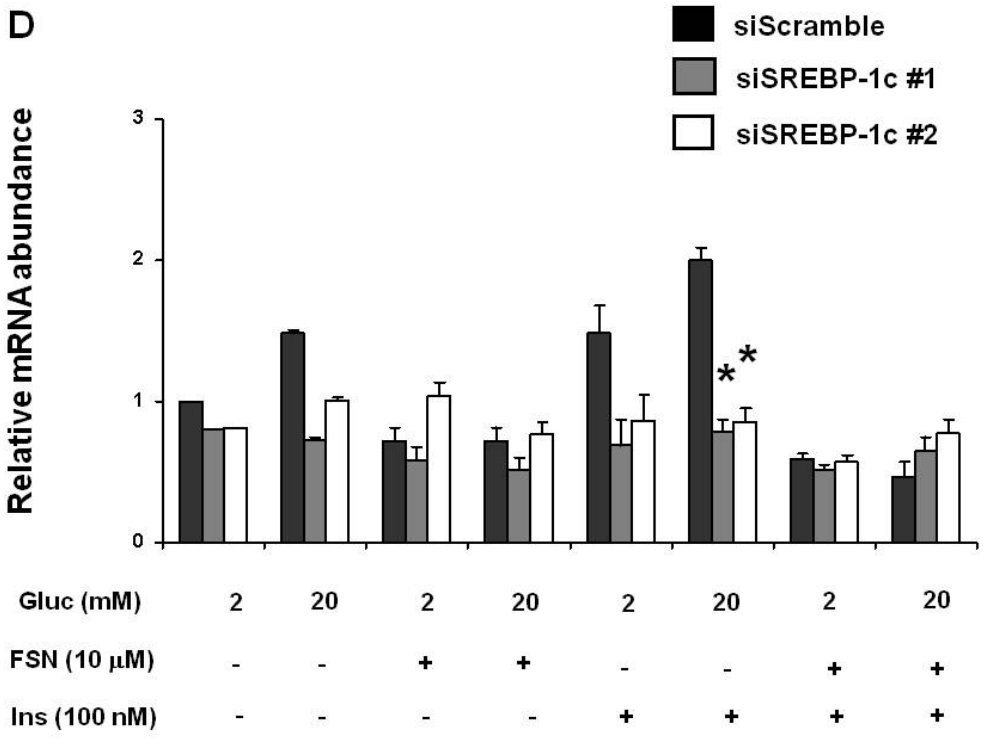
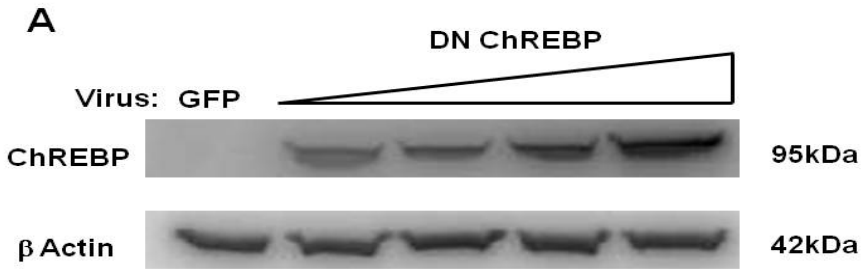

D
siScramble
siSREBP-1c #1
siSREBP-1c #2
Relative mRNA abundance
3
2
1
0
* *
Gluc (mM) 2 20 2 20 2 20 2 20
FSN (10 μM) - - + + - - + +
Ins (100 nM) - - - - + + + +

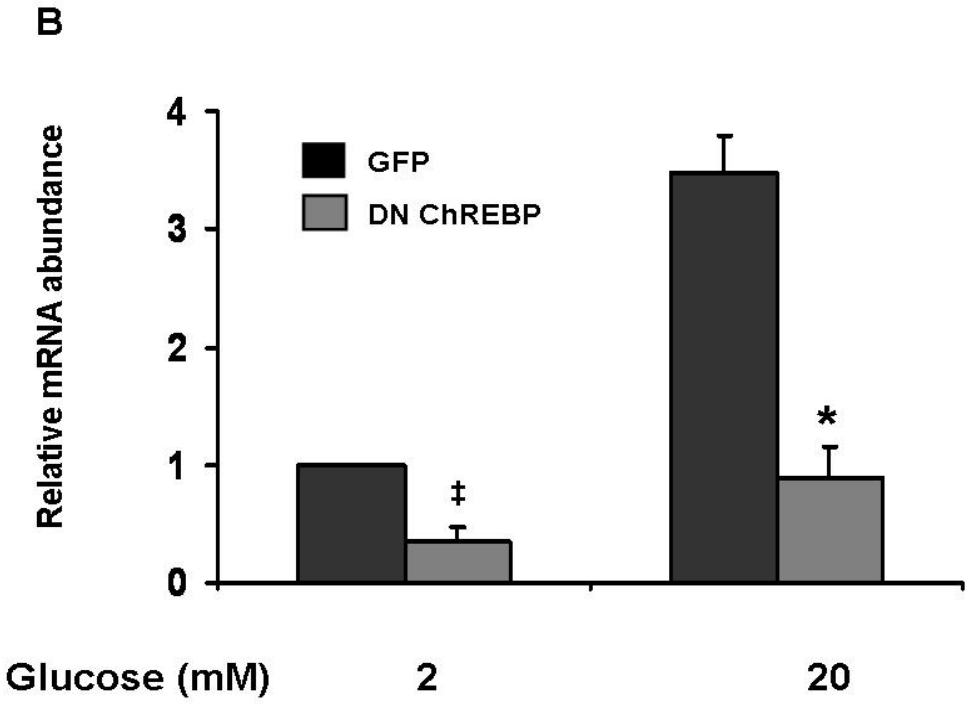
Figure 18. Glucose-mediated induction of the ACC gene is independent of insulin and SREBP-1c. 832/13 cells were transfected with either an siRNA control (siScramble) or two siRNA duplexes targeting different exons of the coding regions of SREBP-1c. *A.* Following 48 h of duplex transfection, nuclear extracts were prepared, and immunoblots performed with antibodies against SREBP-1 with β-Actin as a loading control. The immunoblots are a representative of 2 independent experiments. *B.* In a separate experiments, cells were transfected with duplexes for 48 h, followed by a 6 h incubation with 2 or 20 mM glucose in the presence or absence of 10 μM forskolin. *C.* 832/13 cells were cultured in 2 or 20 mM glucose in the presence or absence of 100 nM insulin for 6 h. *D.* Cells were treated as in (B) and also in the presence or absence of 100 nM insulin for 6 h. *B-D.* Relative abundance of ACC mRNA was examined. Data shown are means ± SEM from three independent experiments. **P* < 0.05 vs. 20 mM glucose.

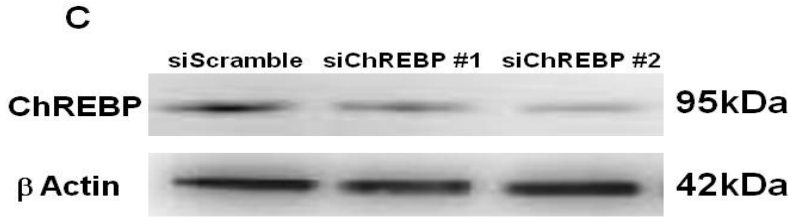
A.4.3 Glucose-mediated induction of the ACC gene requires ChREBP and C/EBPβ, but not HNF4α.

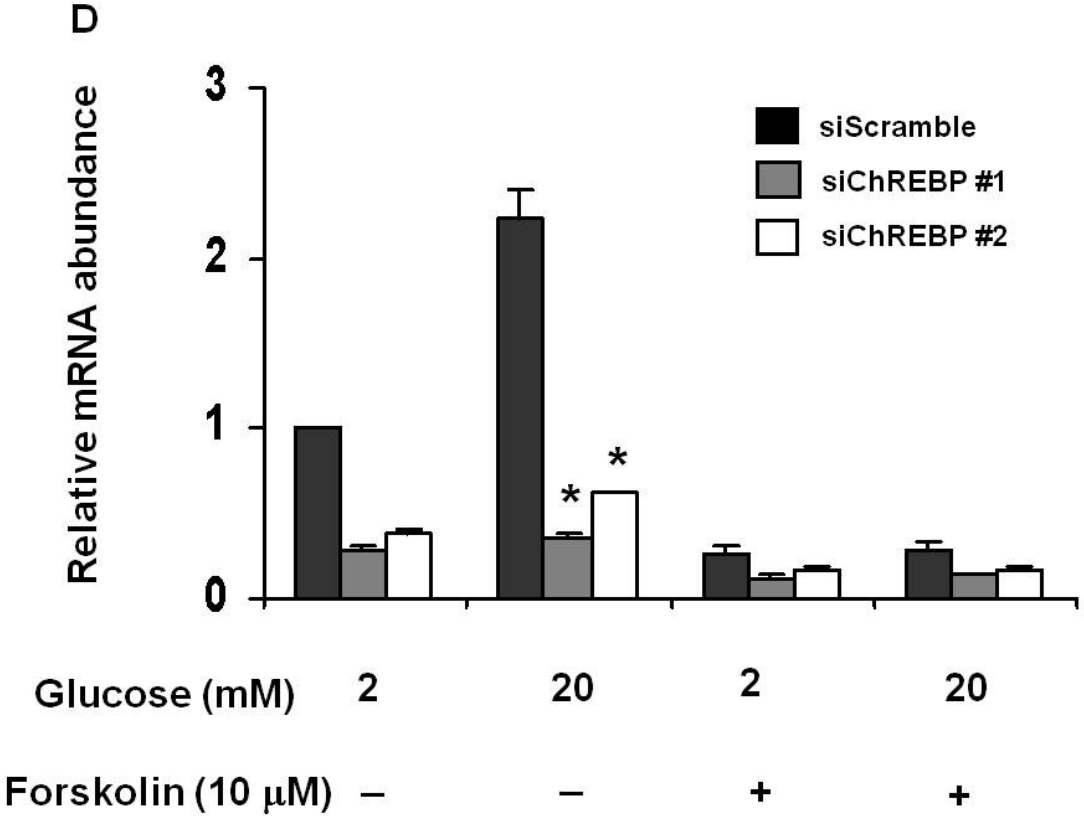
The ACC PI promoter has been reported to contain promoter elements for the transcription factors ChREBP and C/EBPβ (ChoRE and C/AAT box, respectively (116). Therefore, we sought to determine if these factors were required for the glucose-stimulated expression of the ACC gene in β cells. We employed two mechanisms to determine the potential involvement of ChREBP in mediating the glucose-induction of the ACC gene: 1) overexpression of a dominant-negative ChREBP via recombinant

adenovirus (Figure 19A); and 2) suppression of endogenous ChREBP levels using two siRNA duplexes that target different exons of the gene (Figure 19C). Both manipulations significantly reduced the expression of glucose-stimulated ACC mRNA levels (Figures 19B and D). Endogenous C/EBPβ levels were decreased by using two different siRNA duplexes targeted against two separate exons in the C/EBPβ gene (Figure 19E). Following depletion of C/EBPβ protein levels, glucose-mediated expression of the ACC gene was reduced by 44% and 40% by duplex 1 and 2, respectively (Figure 19F). HNF4α has been shown to mediate the glucose-induction of the L-PK gene; therefore, we suppressed abundance of this factor to determine if it was also required for the glucose response of the ACC gene. Suppression of the HNF4α abundance by siRNA duplexes (Figure 19G) failed to abrogate the glucose-mediated induction of the ACC gene. Therefore we can conclude that although ChREBP and C/EBPβ are essential components of the glucose-mediated induction of the ACC gene, this induction does not require HNF4α.

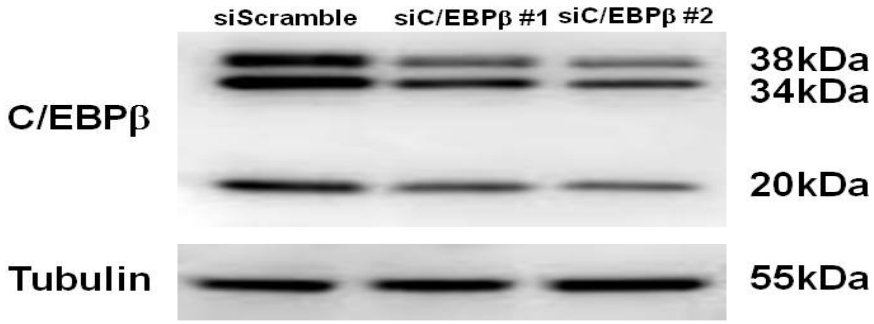

A
DN ChREBP
Virus: GFP
ChREBP
95kDa
β Actin
42kDa


B
Relative mRNA abundance
4
3
2
1
0
GFP
DN ChREBP
‡
*
Glucose (mM)
2
20

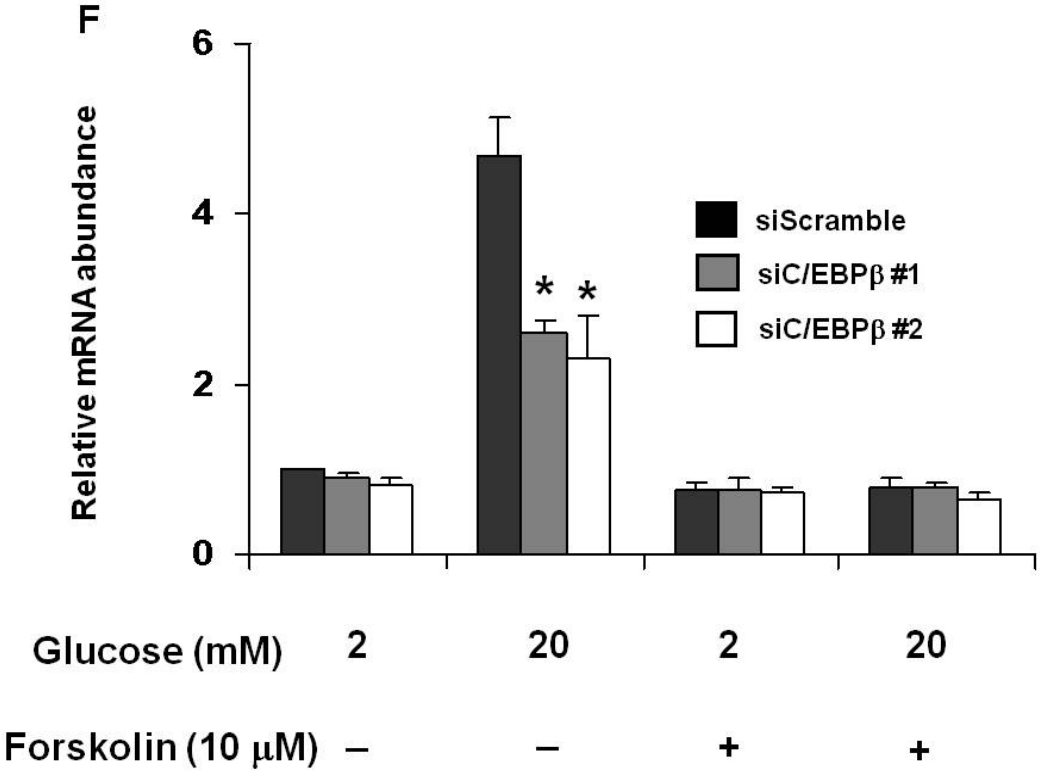

C
siScramble
siChREBP #1
siChREBP #2
ChREBP
95kDa
β Actin
42kDa

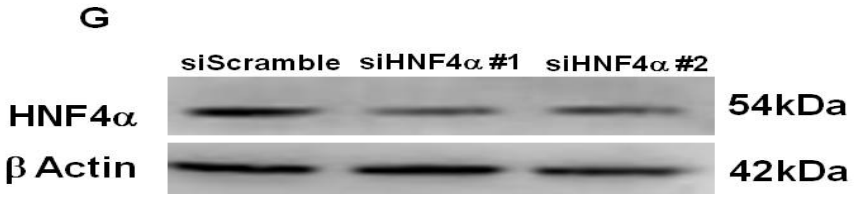

D
Relative mRNA abundance
3
2
1
0
siScramble
siChREBP #1
siChREBP #2
*
*
Glucose (mM)
2
20
2
20
Forskolin (10 μM)
–
–
+
+

E


siScramble
siC/EBPβ #1
siC/EBPβ #2
C/EBPβ
38kDa
34kDa
20kDa
Tubulin
55kDa

F


Relative mRNA abundance
6
4
2
0
siScramble
siC/EBPβ #1
siC/EBPβ #2
* *
Glucose (mM) 2 20 2 20
Forskolin (10 μM) – – + +


G
siScramble
siHNF4α #1
siHNF4α #2
HNF4α
β Actin
54kDa
42kDa

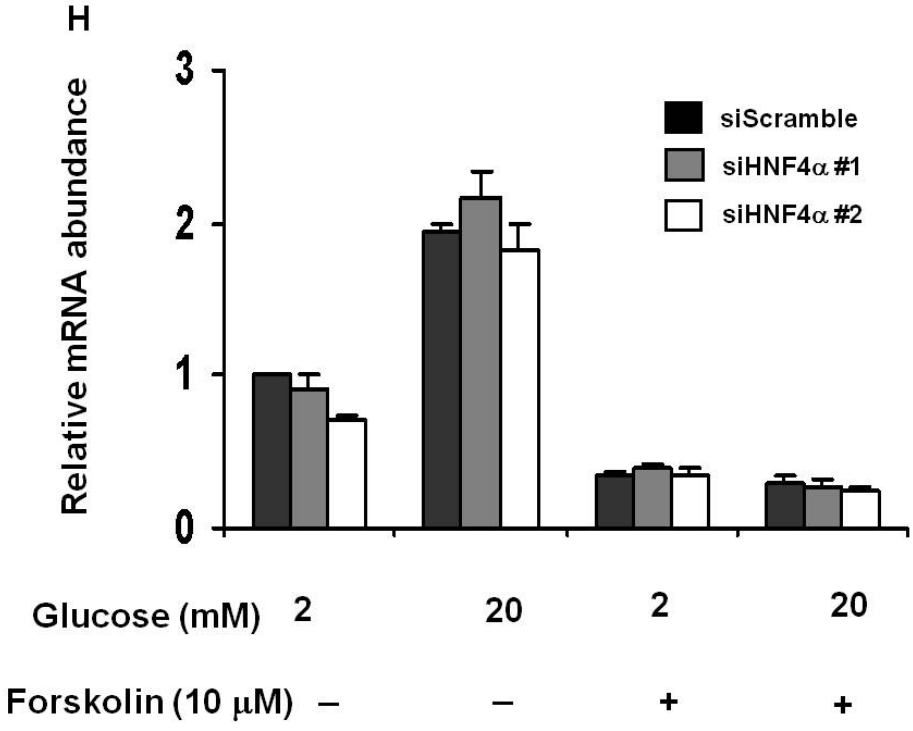
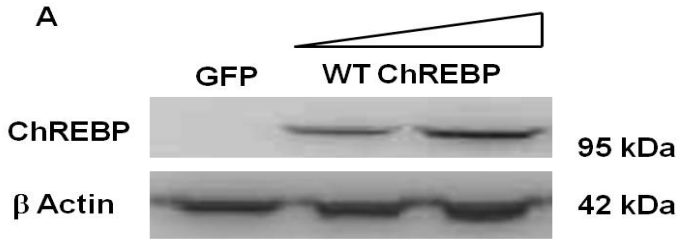
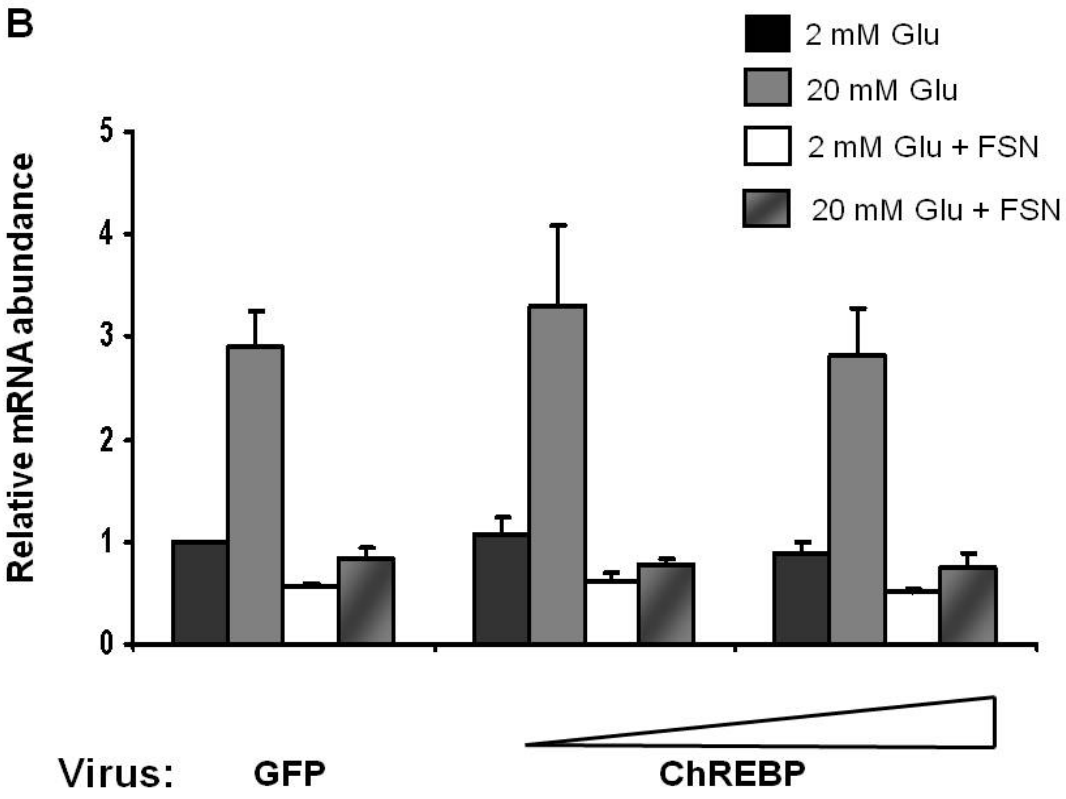

H
Relative mRNA abundance
3
2
1
0
siScramble
siHNF4α #1
siHNF4α #2
Glucose (mM) 2 20 2 20
Forskolin (10 μM) – – + +

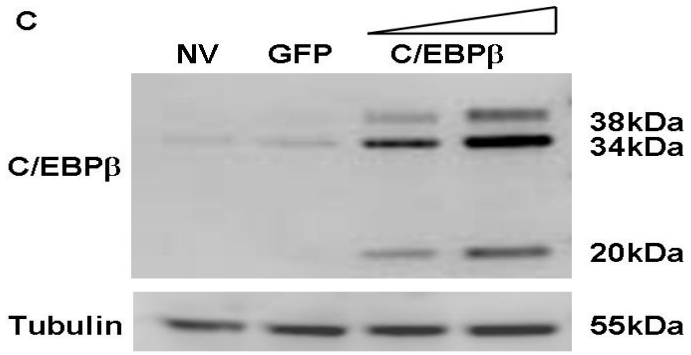
Figure 19. Glucose-mediated induction of the ACC gene requires ChREBP, C/EBPβ but not HNF4α. *A.* 832/13 cells were transduced with recombinant adenoviruses expressing GFP and a dominant-negative ChREBP, followed by culture of the cells for 24 h and preparation of nuclear extracts. The levels of ChREBP and β-actin protein levels were determined by immunoblotting. Shown is a representative of two independent immunoblots. *B.* 832/13 cells were transduced with the indicated adenoviruses, cultured for 24 h, and treated with 2 mM or 20 mM for 6 h. Relative ACC mRNA levels were examined by real-time RT-PCR. Values are expressed as means ± SEM. ‡ $P < 0.05$ vs. 20 mM glucose, *$P < 0.01$ vs. 20 mM glucose. *C, E, G.* 832/13 cells were transfected with either siScramble or two siRNA duplexes against different exons of the coding regions of ChREBP, C/EBPβ or HNF4α. Nuclear extracts were prepared following 48 h of duplex transfection, and immunoblots performed with antibodies against ChREBP (C), C/EBPβ (E) or HNF4α (G), with β-actin or tubulin as a loading control. The immunoblots are a representative of 2 experiments. *D, F, H.* Cells were transfected with duplexes for 48 h, followed by a 6 h incubation with 2 or 20 mM glucose in the presence or absence of 10 μM forskolin. Relative abundance of ACC mRNA was examined. Data shown are means ± SEM from three independent experiments. *$P < 0.05$ vs. siScramble for mRNA experiments.

A.4.4 Overexpression of either ChREBP or C/EBPβ fails to augment the glucose-induction or alleviate the cAMP repression of ACC gene expression.

The results of the previous experiments demonstrated that a decreased abundance of either ChREBP or C/EBPβ inhibits the ability of glucose to induce the expression of the ACC gene; therefore we hypothesized that in the presence of cAMP treatment, enhancing the abundance of either of these factors could be sufficient to rescue the inhibitory actions of cAMP on ACC gene expression. 832/13 cells were transduced with adenoviruses overexpressing either wild-type ChREBP (Figure 20A) or C/EBPβ (Figure 20B), with GFP serving as an adenoviral control in both experiments. Exposure of insulinoma cells to increased abundance of either wild-type ChREBP (Figure 20B) or C/EBPβ (Figure 20D) was not sufficient to either augment the glucose induction of the ACC gene, or to overcome the repression of this gene by cAMP. From these data we conclude that simply enhancing the abundance of either of these factors does not impact either induction or deactivation of the ACC gene by glucose and cAMP, respectively.


A
GFP
WT ChREBP
ChREBP
95 kDa
β Actin
42 kDa


B
2 mM Glu
20 mM Glu
2 mM Glu + FSN
20 mM Glu + FSN
Relative mRNA abundance
0
1
2
3
4
5
Virus:
GFP
ChREBP


C
NV
GFP
C/EBPβ
C/EBPβ
38kDa
34kDa
20kDa
Tubulin
55kDa

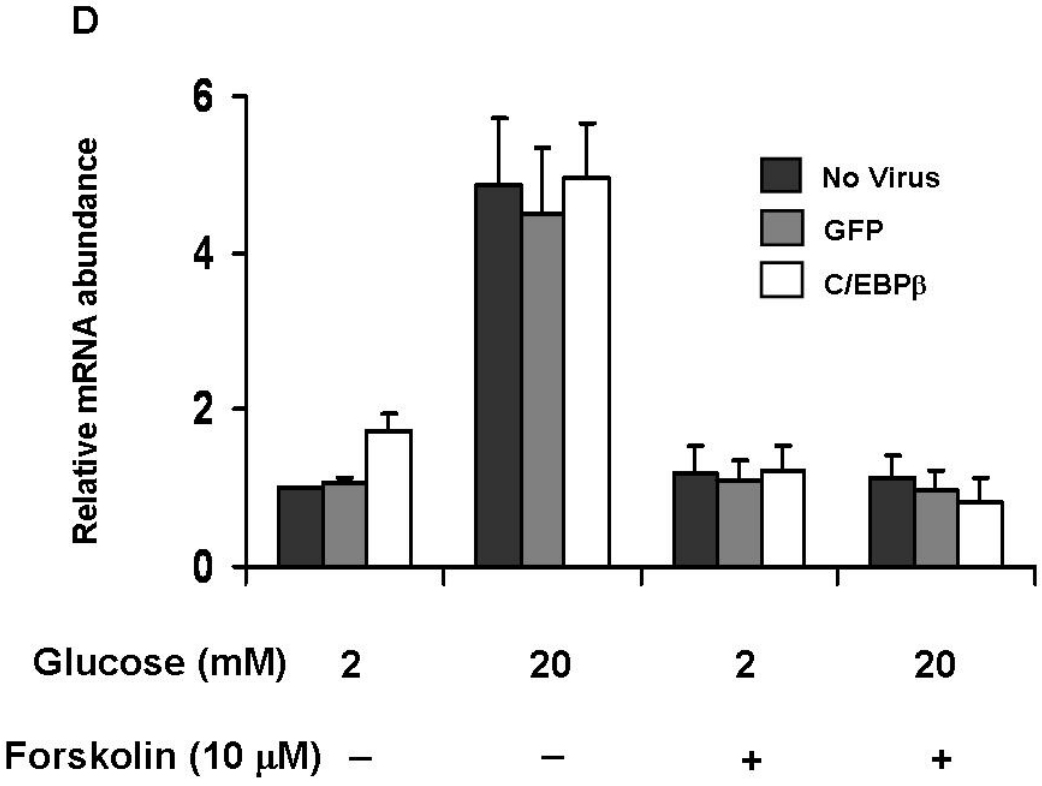
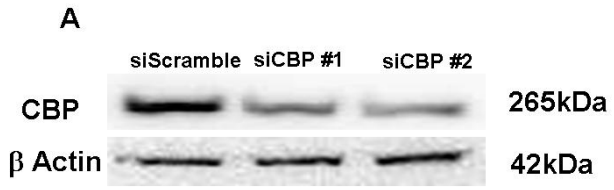

D
Relative mRNA abundance
6
4
2
0
No Virus
GFP
C/EBPβ
Glucose (mM) 2 20 2 20
Forskolin (10 μM) – – + +

Figure 20. Overexpression of either ChREBP or C/EBPβ fails to augment the glucose-induction or alleviate the cAMP repression of ACC gene expression. *A, C.* Cells were transduced for 24 h with adenoviruses expressing GFP and either wild-type ChREBP (A) or C/EBPβ (C). Nuclear extracts were harvested for immunoblotting. Shown are representatives of two blots. *B, D.* Following 24 h viral transduction, cells were treated with 2 or 20 mM glucose in the presence or absence of 10 μM forskolin for 6 h. Relative ACC mRNA abundance was examined via real-time RT-PCR. Data shown are means ± SEM.

A.4.5 Glucose-mediated induction of the ACC gene requires the coactivator CBP.

CBP is a transcriptional coactivator that interacts with a wide array of sequence-specific transcription factors to regulate induction of gene transcription (15). CBP interacts with ChREBP and HNF4α to coactivate expression of the glucose-responsive L-PK gene; therefore, we sought to determine whether CBP was similarly required for the glucose-mediated induction of the ACC gene. Indeed, siRNA-mediated suppression of endogenous CBP levels (Figure 21A) produced an 66 and 58% decrease in the glucose-stimulated expression of the ACC gene. Our data confirm a requirement for the coactivator CBP in promoting expression of the ACC gene in response to glucose signaling.


A
siScramble
siCBP #1
siCBP #2
CBP
265kDa
β Actin
42kDa

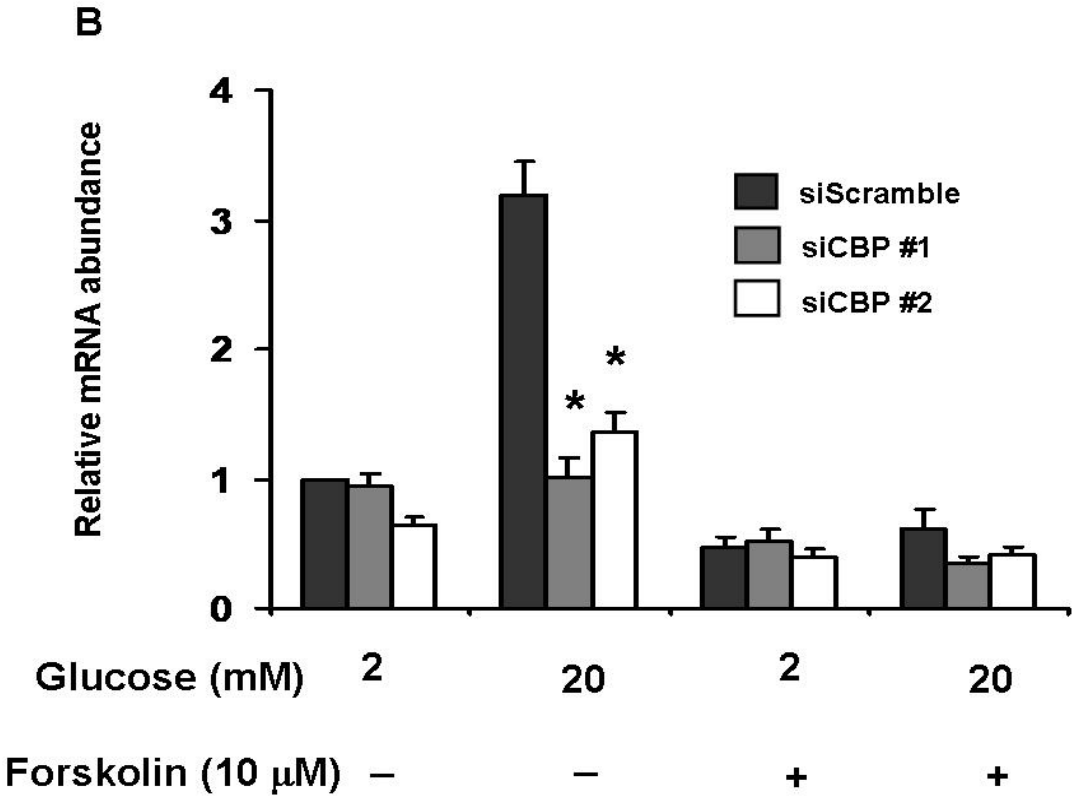
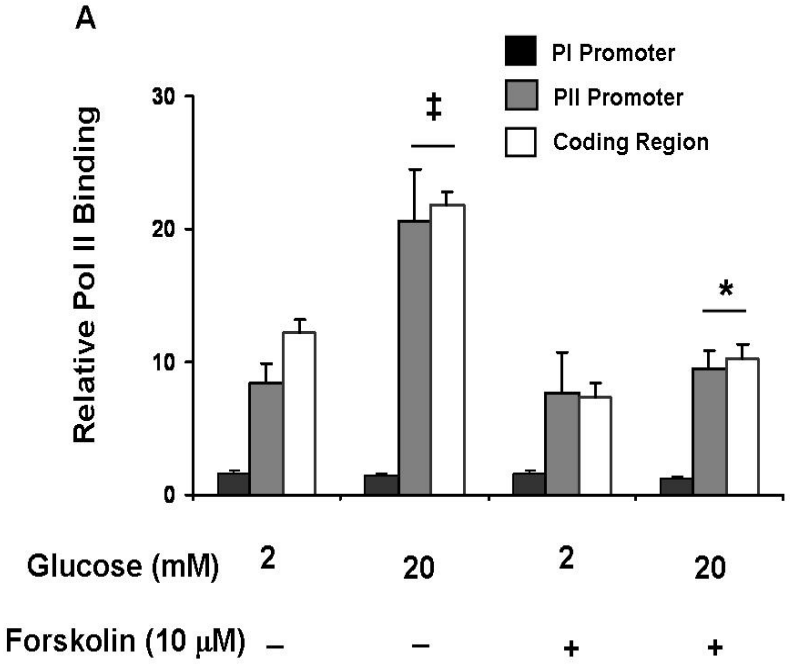

B
Relative mRNA abundance
4
3
2
1
0
siScramble
siCBP #1
siCBP #2
*
*
Glucose (mM)
2
20
2
20
Forskolin (10 μM)
–
–
+
+

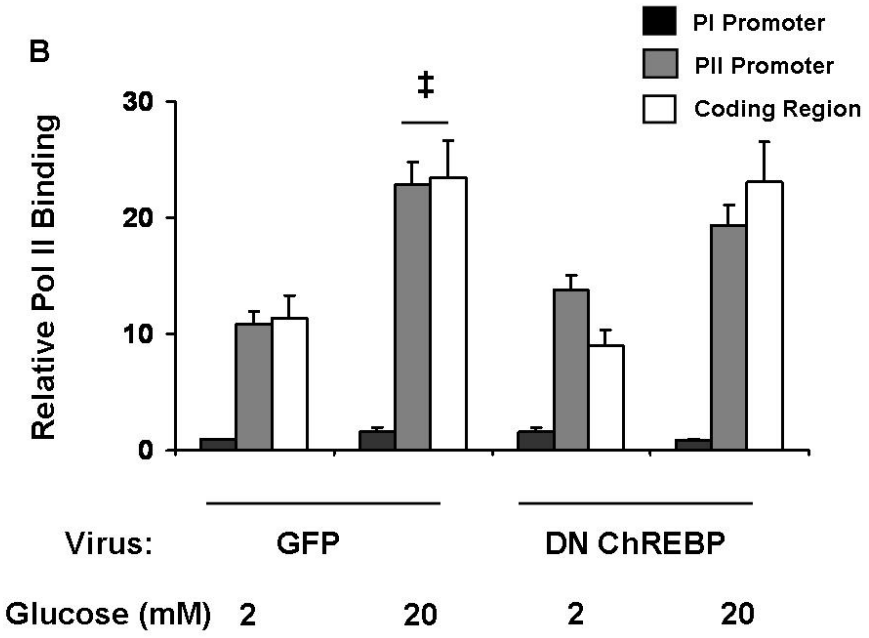
Figure 21. Glucose-mediated activation of the ACC gene requires the coactivator CBP. *A.* 832/13 cells were transfected with either an siScramble control or two siCBP siRNA duplex for 48 h. The abundance of CBP and β-actin were determined by immunoblotting. Shown is a representative of two independent immunoblots. *B.* Cells were transfected with the above duplexes for 48 h then cultured for a further 6 h in 2 or 20 mM glucose in the presence or absence of 10 μM forskolin. Relative mRNA abundance of L-PK was measured via RT-PCR. Data are expressed as means ± SEM. **P* < 0.01 vs. siScramble at 20 mM glucose.

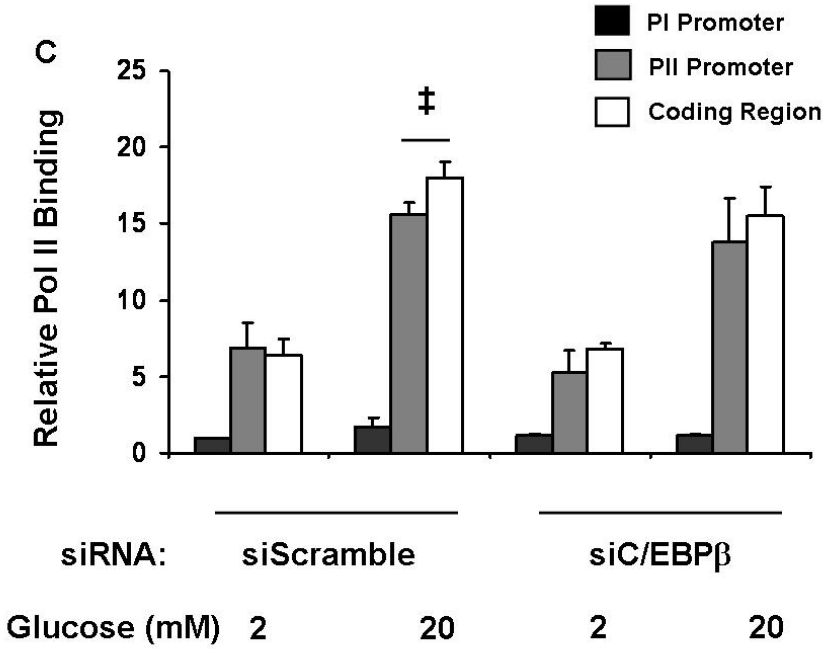
A.4.6 Glucose-mediated recruitment of Pol II to the ACC PII promoter and coding region requires CBP, but not ChREBP and C/EBPβ, and is blocked by cAMP.

Pol II regulates gene expression by promoting the initiation and elongation of transcription from gene promoters and coding regions, respectively (169). We examined whether glucose-mediated activation and cAMP-directed repression of the ACC gene promoted recruitment/de-recruitment of Pol II to the ACC promoter and coding region in response to these two signals. Recruitment of Pol II to the PI promoter was unaffected by treatment with either glucose or cAMP, as compared to IgG control (not shown). In contrast, stimulatory glucose concentrations resulted in 2.1 and 1.8-fold increase in recovery of Pol II on the PII promoter and coding region, respectively, as compared to basal glucose levels. Cells treated concomitantly with 20 mM glucose and 10 mM forskolin, resulted in a 51% decrease in Pol II promoter occupancy as compared to 20 mM glucose alone (Figure 22A).

We have elucidated that ChREBP, C/EBPβ and CBP are all required to mediate induction of the ACC gene, therefore, we hypothesized that the glucose-dependent recruitment of Pol II was mediated in a manner dependent on these factors. To test this hypothesis we: 1) overexpressed DN ChREBP via recombinant adenovirus; 2) suppressed expression of C/EBPβ and 3) CBP via siRNA duplex transfection, then examined the promoter occupancy of Pol II at 20 mM glucose compared to controls. Similar to Figure 22A, we observed a 1.9-fold increase in Pol II recruitment to the ACC promoter and coding region, respectively, when GFP was overexpressed in the presence of 20 mM glucose (Figure 22B). Overexpression of DN ChREBP at 20 mM glucose failed to abrogate the recruitment of Pol II to either the PII promoter or coding region of the ACC gene. Similarly, suppression of C/EBPβ abundance at 20 mM glucose did not result in a significant decrease in Pol II promoter or coding region occupancy, as compared to siScramble control (Figure 22C). Conversely, a decrease in CBP abundance at 20 mM glucose displayed a 54 and 43% decrease in Pol II recruitment on the promoter and coding region, respectively, compared to siScramble control (Figure 22D). We conclude that glucose stimulates expression of the ACC gene by promoting the recruitment of Pol II to the ACC PII promoter; this recruitment requires CBP, but not ChREBP and C/EBPβ. cAMP represses ACC gene expression by decreasing recruitment/ promoting de-recruitment of Pol II from the ACC PII gene promoter and coding region.


A
PI Promoter
PII Promoter
Coding Region
Relative Pol II Binding
30
20
10
0
‡
*
Glucose (mM) 2 20 2 20
Forskolin (10 μM) – – + +


B
PI Promoter
PII Promoter
Coding Region
Relative Pol II Binding
30
20
10
0
‡
Virus: GFP DN ChREBP
Glucose (mM) 2 20 2 20


C
PI Promoter
PII Promoter
Coding Region
Relative Pol II Binding
25
20
15
10
5
0
‡
siRNA:
siScramble
siC/EBPβ
Glucose (mM) 2 20 2 20

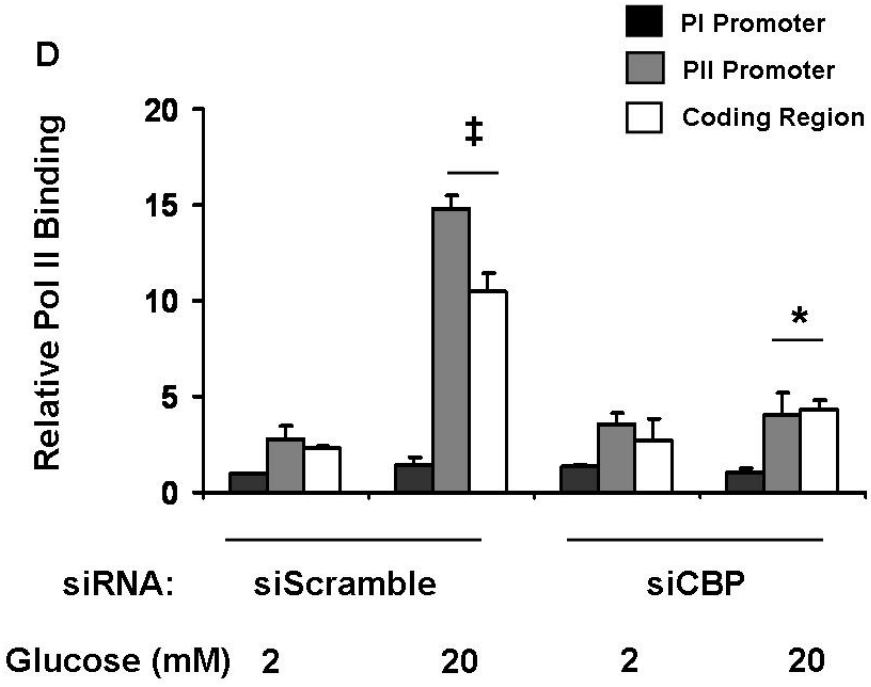
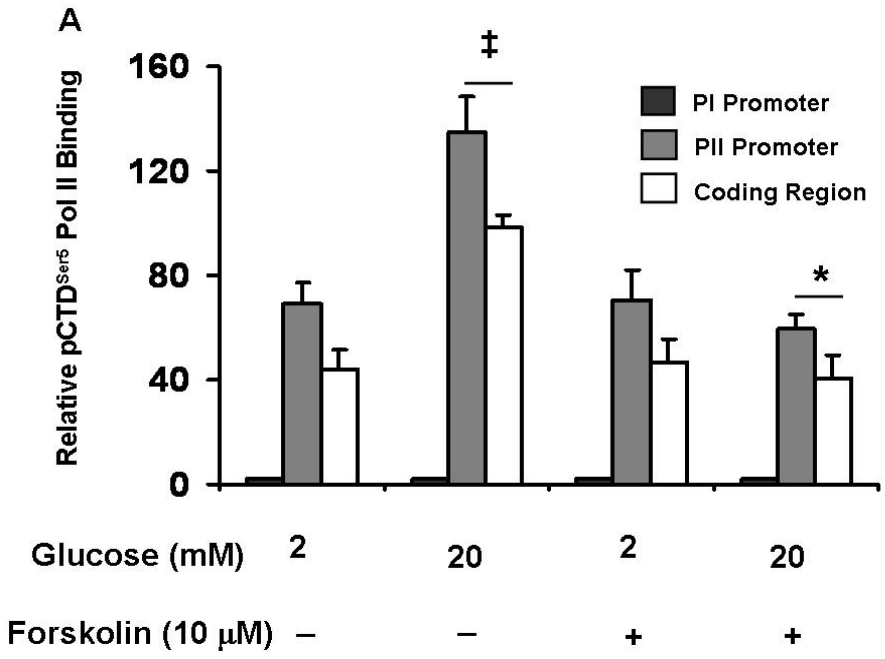

D
PI Promoter
PII Promoter
Coding Region
Relative Pol II Binding
20
15
10
5
0
‡
*
siRNA:
siScramble
siCBP
Glucose (mM) 2 20 2 20

Figure 22. Glucose-mediated recruitment of Pol II to the ACC PII promoter and coding region requires CBP, but not ChREBP and C/EBPβ, and is blocked by cAMP. *A.* 832/13 cells were treated with 2 or 20 mM glucose in the presence or absence of 10 μM forskolin for 6 h. *B, C, D.* Cells were transduced with viruses expressing GFP or DN ChREBP for 24 h (B), cells were transfected with siRNA duplexes targeting either an negative Scramble control or C/EBPβ (C) or CBP (D), then cultured in 2 or 20 mM glucose for 6 h. *A-D.* Relative promoter and coding region occupancy of Pol II was determined via ChIP assay. Results shown are from 3-5 independent experiments, and data are expressed as means ± SEM. ‡ $P < 0.05$ vs. 20 mM glucose *$P < 0.05$ vs. 20 mM glucose.

A.4.7 cAMP alleviates the glucose-mediated increase in occupancy of Pol II pCTD^{Ser5} and pCTD^{Ser2} on the ACC PII promoter and coding region.

Following recruitment to specific gene promoters, Pol II becomes phosphorylated on Ser 5 of its CTD to promote initiation of transcription. Similarly, phosphorylation of Pol II CTD at Ser 2 is essential for Pol II to switch between the initiation and elongation phases of transcription (30, 34, 35). Stimulation with 20 mM glucose generated a 1.4-fold increase in PII promoter occupancy compared to non-stimulatory glucose concentration (2 mM). We observed a higher occupancy of Pol II pCTDSer5 on the PII promoter than the coding region; however the increased occupancy on both the promoter and coding region is blunted in the presence of 10 mM forskolin (Figure 23A). Glucose signaling induced an increased association of Pol II pCTDSer2 with both the coding region and PII

promoter; however the relative occupancy was higher on the coding region than the PII promoter. The cAMP agonist forskolin decreased the association of Pol II pCTDSer2 with both the promoter and coding region. From the cumulative findings of Figure 22A and Figure 23, we have established that glucose promotes both the recruitment and phosphorylation of Pol II necessary for transcriptional initiation and elongation, and that cAMP represses transcription by opposing the recruitment and phosphorylation of Pol II.


A
Relative pCTD^Ser5 Pol II Binding
160
120
80
40
0
‡
*
PI Promoter
PII Promoter
Coding Region
Glucose (mM) 2 20 2 20
Forskolin (10 μM) – – + +

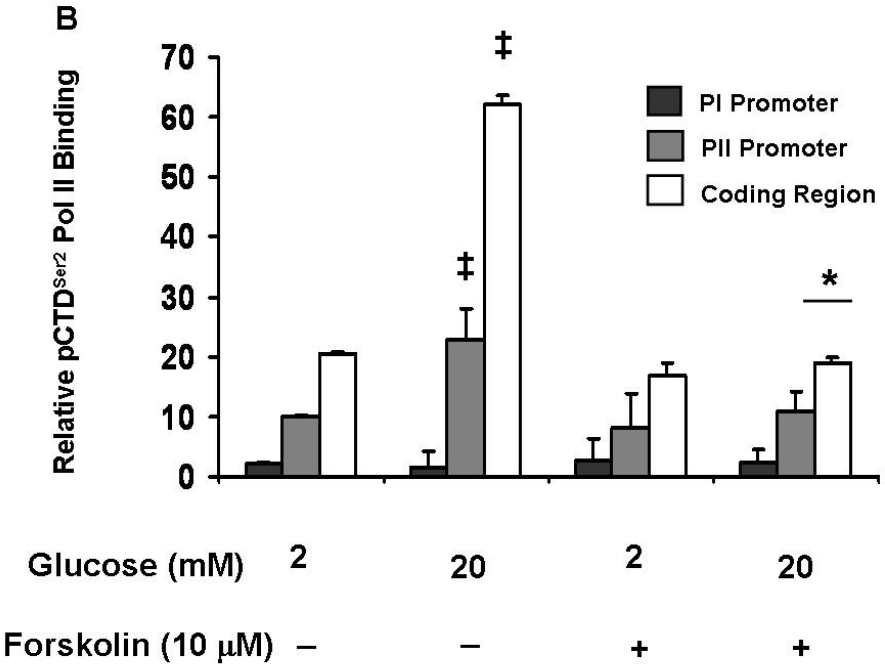

B
Relative pCTD^Ser2 Pol II Binding
70
60
50
40
30
20
10
0
‡
‡
*
PI Promoter
PII Promoter
Coding Region
Glucose (mM) 2 20 2 20
Forskolin (10 μM) – – + +

Figure 23. cAMP alleviates the glucose-mediated increase in occupancy of Pol II pCTD^{Ser5} and pCTD^{Ser2} on the ACC PII promoter and coding region. *A.* 832/13 cells were cultured for 6 h in 2 or 20 mM glucose in the presence or absence of 10 μM forskolin. Relative promoter and coding region occupancy was determined via ChIP assay using antibodies against Pol II pCTD^{Ser5} Pol II pCTD^{Ser2}. ACC PI and PII promoters and a segment of the coding region were targeted for amplification. Data are expressed as means ± SEM from 5 separate experiments ‡ $P < 0.05$ vs. 20 mM glucose *$P < 0.05$ vs. 20 mM glucose.

A.5 DISCUSSION

Acetyl CoA Carboxylase (ACC) functions as a key enzyme in the fatty acid biosynthetic pathway as it catalyzes the conversion of acetyl coA to malonyl coA. The ACC gene is reciprocally regulated by the opposing stimuli glucose and cAMP, yet the factors that regulate expression of this gene in response to these signaling inputs have not been elucidated in β cells of the pancreatic islets (13). Therefore, using the INS-1- derived 832/13 rat insulinoma cell line, we examined the mechanism by which glucose induces, while cAMP represses, expression of the ACC gene. Several key findings emerged from this study: 1) siRNA-mediated suppression of HNF4α and SREBP-1c abundance failed to block glucose-mediated induction of the ACC; 2) suppression of the abundance of ChREBP, C/EBPβ or CBP completely blunted the glucose-stimulated induction of the ACC gene; 3) enhancing the abundance of either ChREBP or C/EBPβ was insufficient to

augment the glucose-induction or alleviate the cAMP-repression of ACC gene expression; 4) glucose promoted recruitment of Pol II to the ACC PII promoter in a CBP-dependent manner, and this recruitment was blocked by elevations in cAMP levels; and 5) insulin was unable to either induce or augment expression of the ACC gene at non-stimulatory or stimulatory glucose concentrations, respectively.

Glucose-mediated induction of several key metabolic enzyme genes, L-PK and FAS, require the transcription factor HNF4α (1, 15); however, suppression of HNF4α abundance revealed that this factor is not required to mediate the glucose induction of the ACC gene. Similarly, in contrast to several reports highlighting that induction of the ACC gene in hepatocytes requires the bHLH-LZ transcription factor SREBP-1c (41), glucose-dependent induction of the ACC gene in β cells does not require SREBP-1c.

Glucose-directed induction of the ACC gene requires the transcription factors ChREBP and C/EBPβ. Response elements for these two factors, the ChoRE and CAAT box, respectively, are present in the ACC PI proximal promoter. Towle and colleagues demonstrated via ChIP assay that ChREBP is recruited to the ChoRE in the PI promoter in hepatocytes (116). However, similar experiments using our 832/13 β cell model showed no significant recruitment of ChREBP to the PI promoter ChoRE in response to glucose, as compared to IgG control (data not shown). Similarly, no alterations in C/EBPβ occupancy on the PI promoter CAAT box were observed with glucose treatment, compared to control (data not shown).

The ACC gene can be transcribed from two different promoters, PI and PII; Kim and coworkers identified that only PII transcripts are produced in β cells (36, 72, 90). We next focused our attention on the PII promoter; however, a detailed scan of the proximal

promoter revealed no consensus ChoRE or CAAT box sequences. Using primers that amplified the 'glucose-responsive' region of the PII promoter, ChIP assays failed to detect occupancy of either ChREBP or C/EBPβ in response to glucose at this promoter location, as compared to IgG control (data not shown). Therefore, although we can conclude that ChREBP and C/EBPβ are both individually required to mediate the glucose-stimulated induction of the ACC gene, whether they bind to an undetermined location in the ACC PI or PII promoter has not been established. An alternative explanation may be that these factors indirectly alter the expression of another undetermined factor that is, in turn, required for induction of the ACC gene.

Glucose promoted induction of the ACC gene by recruiting Pol II to the ACC PII promoter and coding region, and subsequently phosphorylating the CTD to facilitate transcription initiation and elongation, respectively. cAMP repressed expression of the ACC gene by abrogating the recruitment and serine phosphorylation of Pol II on both the PII promoter and coding region. In support of ACC transcripts being driven solely from the PII promoter in β cells, we observed no significant occupancy of Pol II on the ACC PI promoter, relative to IgG control. Suppressing abundance of the coactivator CBP blocked the glucose-mediated recruitment of Pol II to the PII promoter and coding region, correlating with a decrease in expression of the ACC gene. Similar experiments revealed that recruitment of Pol II to the PII promoter and coding region in response to glucose was not dependent upon either ChREBP or C/EBPβ.

In contrast to the FAS gene which can be induced or augmented by insulin at basal or stimulatory glucose concentrations, respectively, expression of the ACC gene in β cells does not require insulin. This finding illustrates a selective difference in the

regulation of the ACC gene between two tissues, hepatocytes and β cells, as numerous groups have shown an insulin-dependent component of the induction of the ACC gene in hepatocytes.

We have thus elucidated that the glucose-dependent induction of ACC gene expression in 832/13 cells requires ChREBP and C/EBPβ through an as yet undetermined mechanism. Glucose, via the coactivator CBP, recruits Pol II to the ACC PII promoter and coding region, to initiate expression of the ACC gene. cAMP opposes the induction of gene expression by inhibiting the recruitment of Pol II to both the PII promoter and coding region. Finally, we have determined that insulin is not required to regulate expression of the ACC gene in rat pancreatic β cells.

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