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cAMP Prevents Glucose-mediated Modifications of Histone H3 and Recruitment of the RNA Polymerase II Holoenzyme to the L-PK Gene Promoter

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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 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. 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 of phosphorylated 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 all altered the acetylation and methylation of histones on 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.

Keywords

acetylation; methylation; promoter; transcriptional activation

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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 [1; 2]. 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 [2]. 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) [3; 4; 5; 6; 7]. ChREBP activates these target genes by binding to a carbohydrate response element (ChoRE) as a heterodimer with its partner Max-like protein X (Mlx) [8; 9]. Glucose metabolism may control nuclear entry of ChREBP [10], 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 [2;6; 11].

CBP is a transcriptional coactivator to a wide array of transcription factors and possesses intrinsic histone acetyltransferase (HAT) activity [12; 13]. 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 [14; 15]. Acetylation of histone H3 lysines and H4 lysines counteracts the compact structure of chromatin by relaxing the interaction between histone proteins and DNA [16]. Furthermore, methylation of histone H3-K9 is associated with transcriptional repression because it creates a recognition site for the binding of heterochromatin proteins [17; 18; 19]. 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 co-regulatory proteins [20], including CBP, and also preventing association of the N-terminal tail of histone H3 with the heterochromatin forming complexes [21].

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 [22; 23]. 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 [24; 25].

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 subsequently control recruitment of the Pol II holoenzyme to the L-PK gene promoter. We have shown here that glucose increased the acetylation of histone H3 on 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.

RESULTS

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 [1; 2; 6] Exposure of the highly glucose-sensitive 832/13 rat insulinoma cell line [26] 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 (Fig. 1A).We previously demonstrated that glucose-stimulated assembly 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 [2]. To further examine the role of ChREBP and CBP in the regulation of the L-PK gene, we employed a dominant-negative ChREBP protein [27], which was expressed via recombinant adenovirus (Fig. 1B). This produced a 63% decrease in the glucose-stimulated expression of the L-PK gene when compared to the GFP adenoviral control (Fig. 1C). Suppression of endogenous CBP levels by siRNA duplexes targeting the coding region of the CBP gene produced a 62% depletion of CBP compared to the control duplex siScramble (Fig. 1D). This decrease in CBP abundance was sufficient to decrease the glucose-stimulated expression of the L-PK gene by 79% (Fig. 1E). Further, we have shown via chromatin immunoprecipitation (ChIP) assay 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 (Fig. 1F),while siRNA-mediated suppression of CBP abundance decreased the relative glucose-stimulated association of ChREBP with the L-PK promoter by 72% (Fig. 1G). 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 [2] 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.

cAMP abrogates the glucose-mediated association of Pol II with the L-PK gene promoter

Promoter occupancy of Pol II is necessary for initiation of gene transcription[28; 29; 30] ; 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 (Fig. 2A). 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 (Fig. 2A). In addition, we observed a1.8-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 (Fig. 1). 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 (Fig. 1B); and 2) depleting the abundance of CBP via siRNA duplex transfection (Fig. 1D). 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 (Fig. 2B). However, overexpression of dominant-negative

ChREBP diminished Pol II occupancy on the L-PK gene promoter by 42%, compared to GFP (Fig. 2B). 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 (Fig. 2C).

Because the glucose-mediated recruitment of Pol II to the L-PK promoter was dependent upon ChREBP and CBP (Figs. 2B & 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 co-occupancy of proteins at a given genomic region [31]. At 20 mM glucose we observed an 3.3-fold increase in promoter fragments recovered by immunoprecipitating CBP which was decreased by 64% in the presence of forskolin (Fig. 2D). 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 (Fig. 2D). From the cumulative findings shown in Fig. 2A–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.

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 [22; 24; 32]). 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 10 μM forskolin blocked this recruitment by 63% (Fig. 3A). 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 (Fig. 3B). From these experiments we conclude that regulation of the L-PK gene by glucose and cAMP involves the recruitment of Pol II and the corresponding changes in phosphorylation of its CTD at the L-PK gene promoter and coding region.

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

An increase in the acetylation of histones H3 and H4 produces an open chromatin conformation that promotes transcriptional activity [33; 34]. 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 (Fig. 4A). 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 of histone H4 on the promoter by 63% (Fig. 4B).

To determine if these changes in association of acetylated histones with the L-PK gene promoter 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 (Figs. 4C & D). Similar to the above Pol II data, enhancing the abundance of

wild-type ChREBP fails to further increase the acetylation of either histone on the L-PK promoter (Figs. 4C 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 (Fig. 4D and 5E).We conclude that acetylation of histones H3 and H4 plays 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.

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

Methylation of histone H3 on lysine 4 is characteristic of transcriptionally active genes [35], whereas inactive genes are generally associated with methylation of histone H3 on lysine 9 [36]. To determine whether 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 (Fig. 5A) or overexpression of dominant-negative ChREBP (Fig. 5B). 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 (Fig. 6C). Similar to forskolin treatment, adenoviral overexpression of the dominant-negative ChREBP blunted the glucose-dependent increase in dimethylated (64%) and trimethylated (75%) H3-K4 on the L-PK coding region (Fig. 5D).

Additionally, while 20 mM glucose did not impact H3-K9 methylation association 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 5E). Additionally, 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 (Fig. 5F). Taken together with the observations regarding acetylation status of histones associated with the L-PK gene promoter (Fig. 4), 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.

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 [2]. To further understand how glucose and cAMP control expression of the L-PK gene, we investigated histone modification patterns and RNA Pol II recruitment to the L-PK gene promoter in response to the regulatory signals glucose and cAMP. Several significant findings emerged: 1) cAMP opposed both the glucose-dependent recruitment and phosphorylation status of Pol II at the L-PK gene promoter and coding region; 2) glucose and cAMP altered 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 was 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 (Fig. 2B 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 [37]. 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 (Fig. 2D). 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) was absolutely required for glucose-mediated expression of the L-PK gene; 2) was indispensable for the acetylation of histones H3 and H4 on the L-PK promoter (Fig. 4E and 5F); and 3) was required for the recruitment of Pol II to the L-PK promoter (Fig. 2D).

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 [25; 38]. The glucose-dependent increase in Pol II $pCTD^{Ser5}$ recruitment on the L-PK promoter was directly linked to induction of L-PK gene expression, whereas Pol II $pCTD^{Ser2}$ correlated with elongation of the L-PK gene; cAMP dominantly opposed 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 [15]. 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 [39]. 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 [40]. In the case of the L-PK gene, we observed a higher degree of acetylated histone H3 and H4 on the L-PK promoter following glucose stimulation as compared to the coding region of this gene (Fig. 4A and B). However, there was a differentially regulated modification pattern between these two histones in response to glucose signaling. Although the acetylation of H3 and H4 on the promoter was diminished by cAMP treatment, only the acetylation of H3 was increased by glucose, suggesting that in the context of the L-PK promoter of acetylation of histone H3 rather than H4 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 was dependent upon ChREBP (Fig. 4C and D) and CBP (Fig. 4E 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 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 decreased the association of acetylated H3 and H4 with the L-PK promoter (Fig. 4E and 5F), therefore we suspect that the HAT activity of CBP may be involved in the control of expression of this gene.

Increases in dimethylated [41] and trimethylated H3-K4 [42] 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 (Fig. 5A). In contrast, alterations of the methylation status of histone H3 was 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 was glucose-mediated and dependent upon ChREBP (Fig. 5C and D). These ChREBP-dependent increases in H3-K4 methylation on 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 blunted the expression of the L-PK gene, in part, by increasing the methylation of H3-K9 on the coding region (Fig 5E). A similar increase in methylation of H3-K9 on the coding region was observed when dominant-negative ChREBP was overexpressed (Fig 5F). 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 [43]. 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.

MATERIALS AND METHODS

Cell culture, adenovirus, 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 [26]. Adenoviruses were transduced as previously described [2; 6]. The construction of an adenovirus expressing dominant negative ChREBP, and wild-type ChREBP, gifts from Dr. Howard Towle (University of Minnesota), was described previously [44].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, NR4A2, and cyclophilin have been described previously [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.

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 [45] 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).

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation assays were performed using the Millipore ChIP assay kit (Cat # 17–295), with modifications described previously [46]. Relative binding of each factor is reported after normalization to IgG control. The ChoRE-containing portion of the L-PK promoter and a fragment 5 kb 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 [2]. 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.

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 [31; 47]. The primers used to amplify the appropriate regions of the L-PK gene promoter and coding region by real-time RT-PCR have been reported previously [2].

Statistical analysis

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

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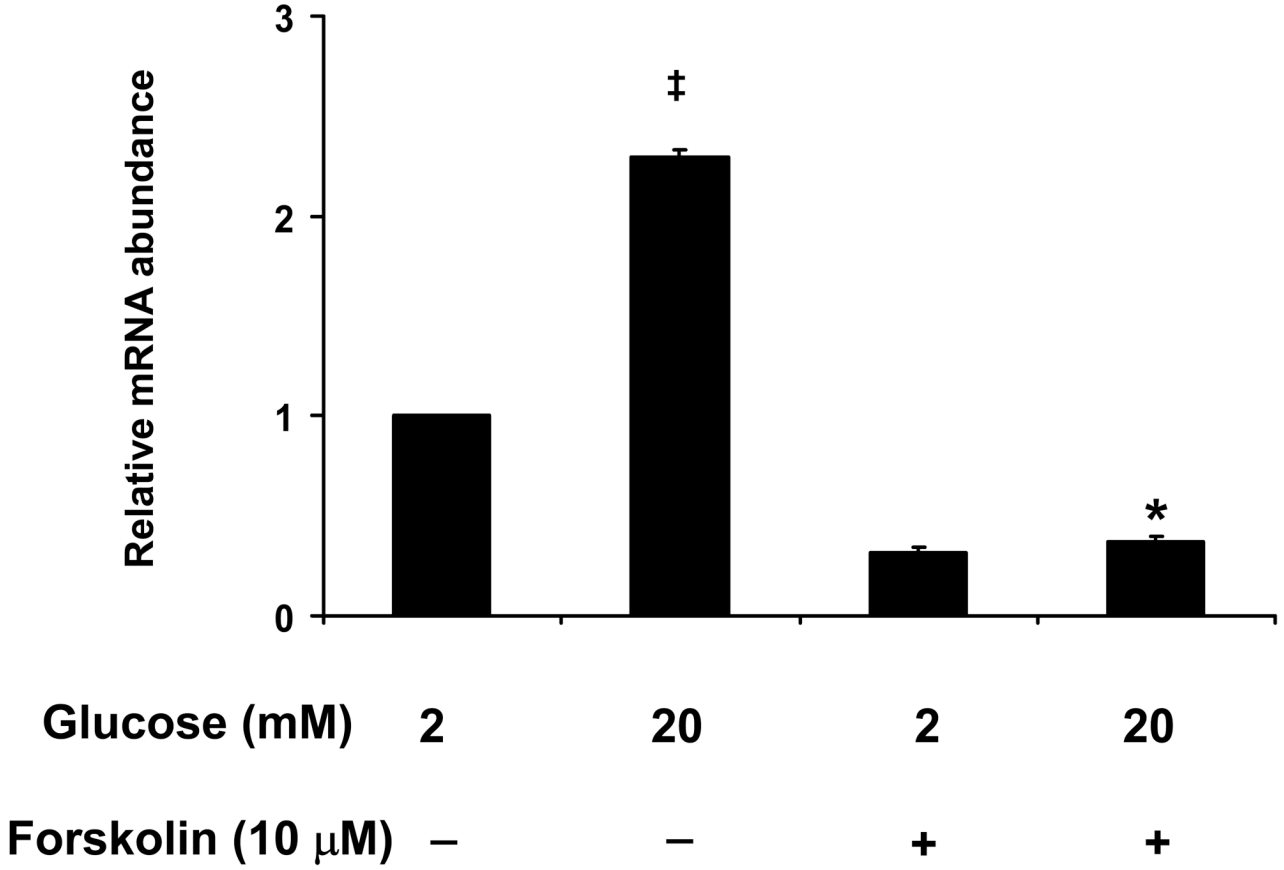
Figure 1A



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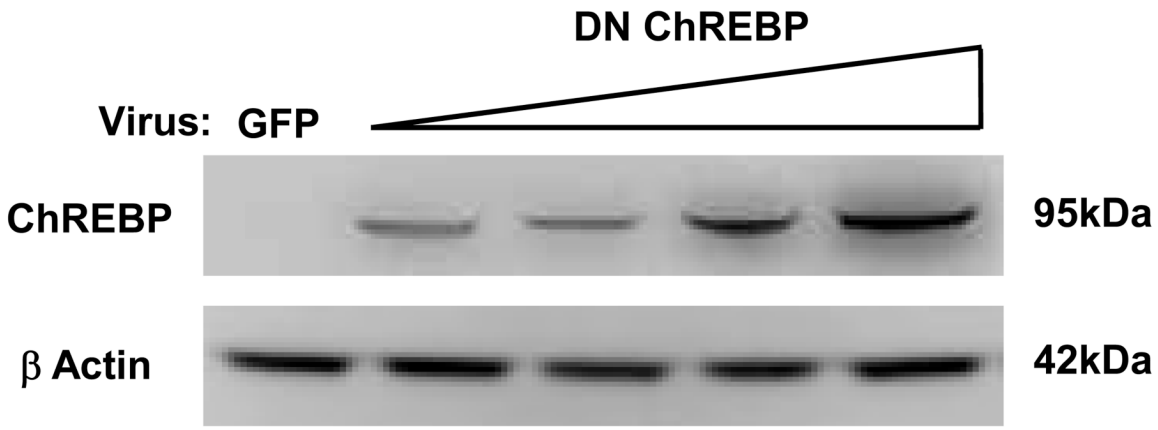
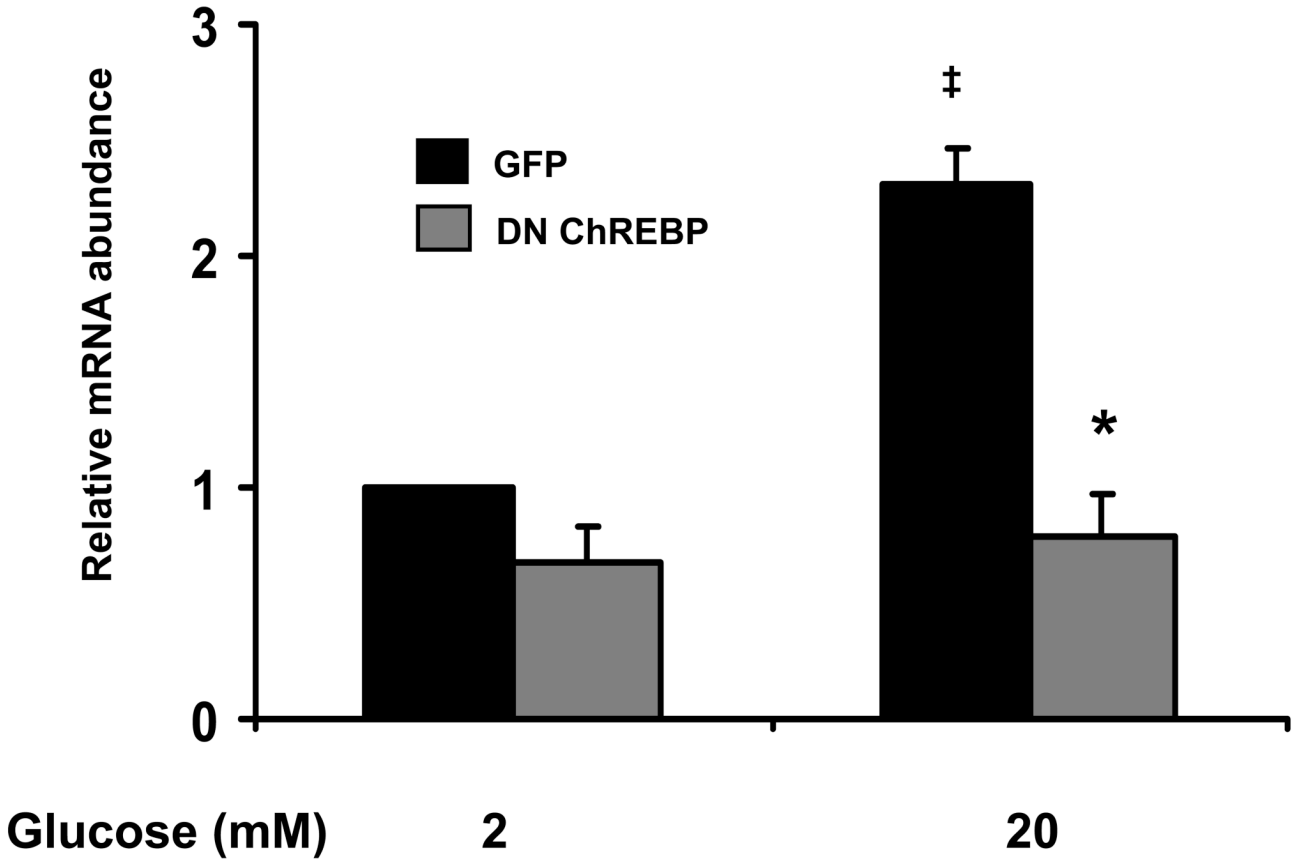


Figure 1C



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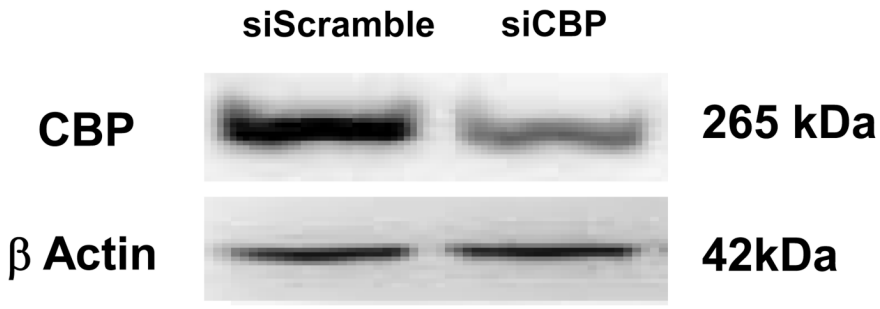

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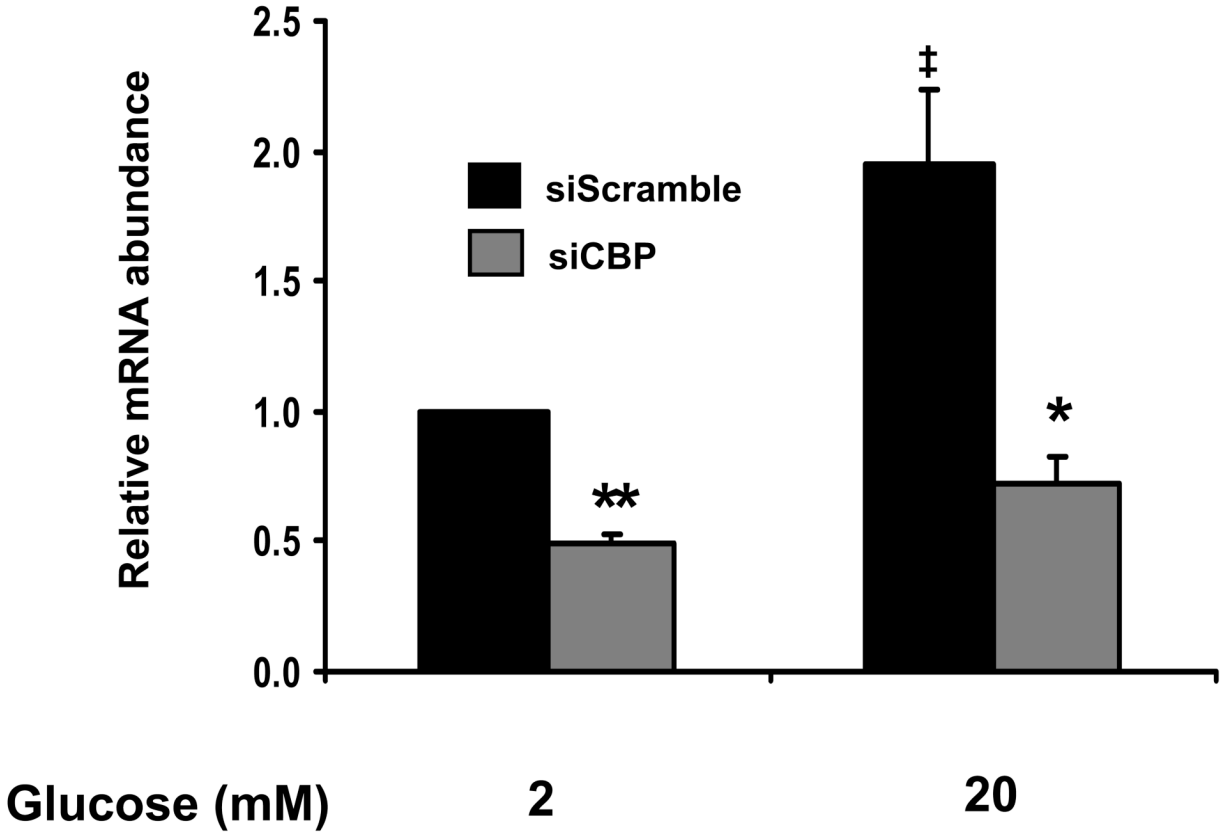

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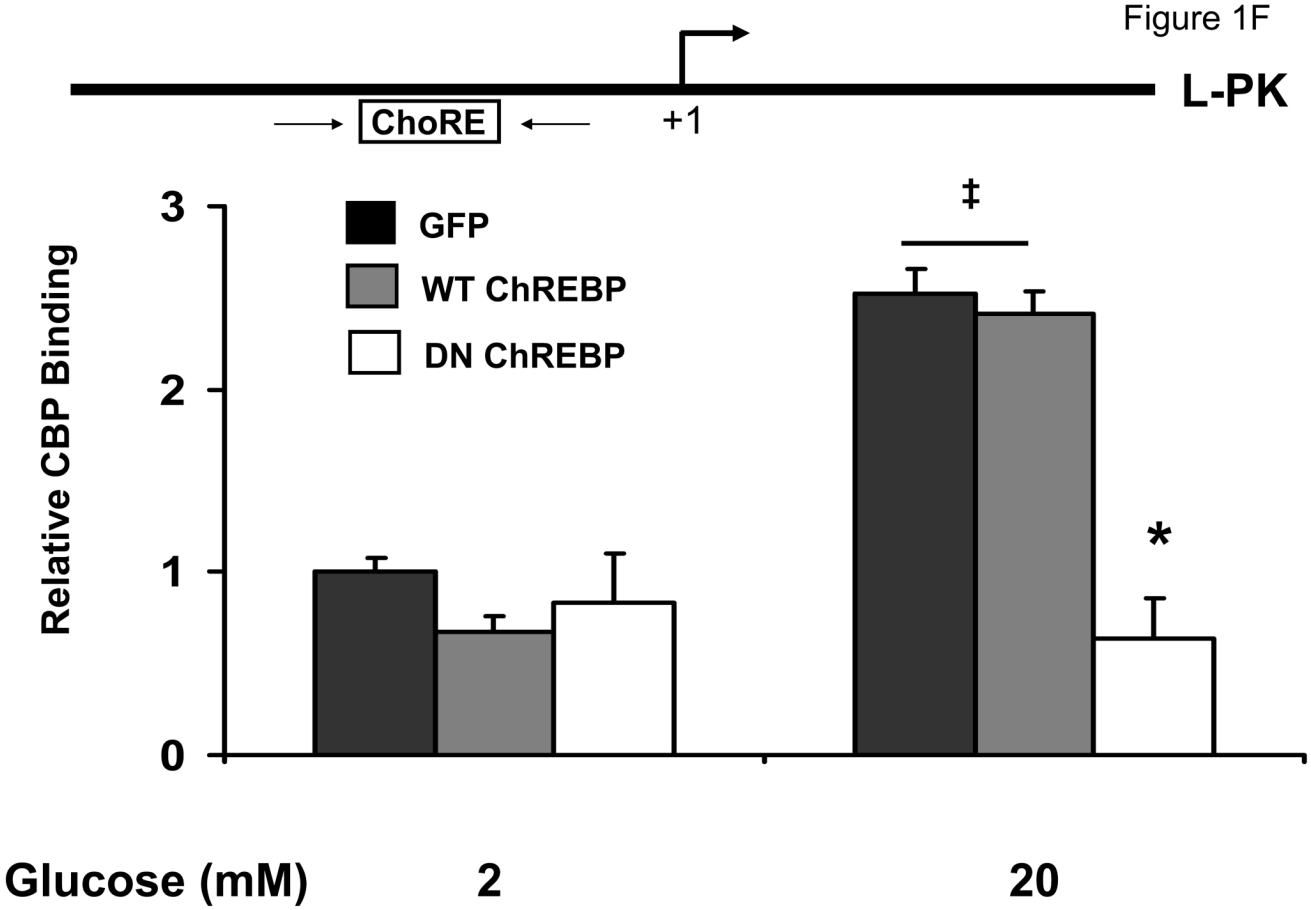

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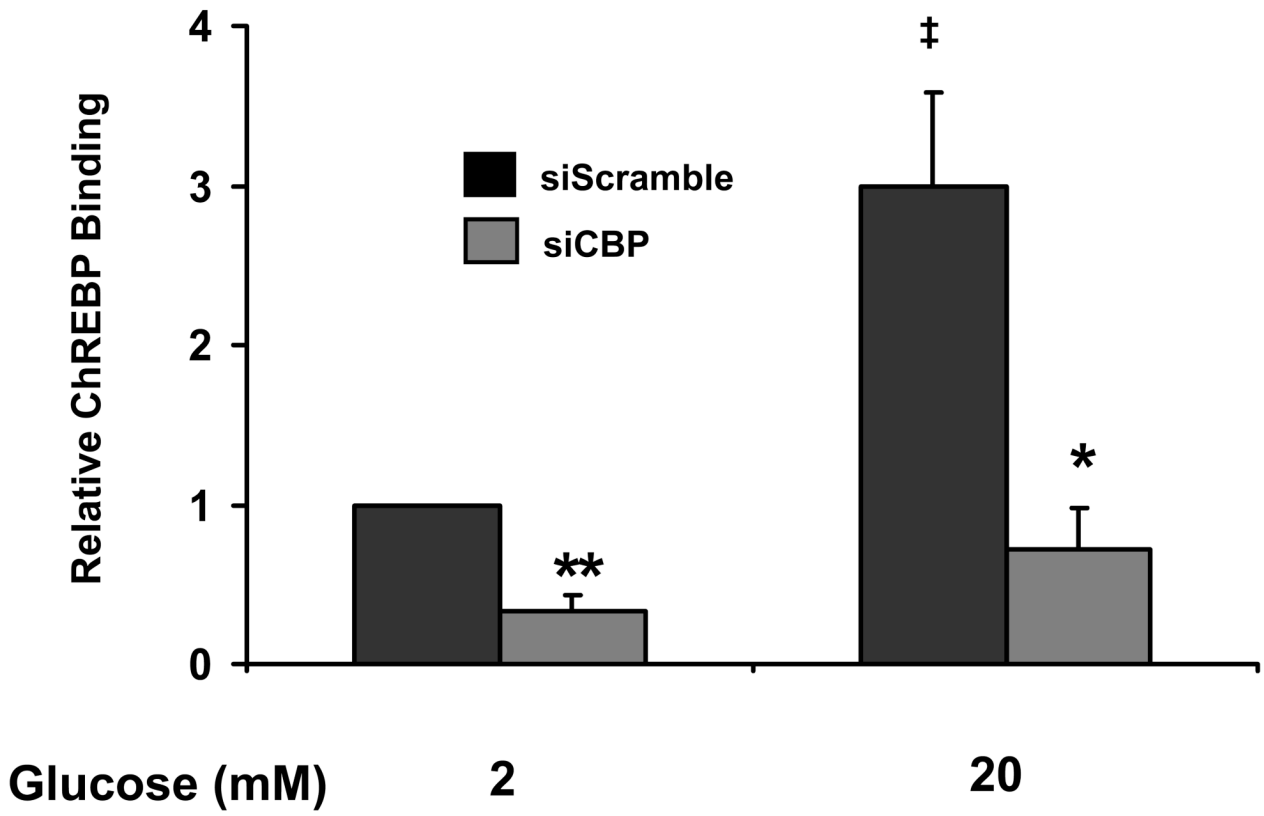
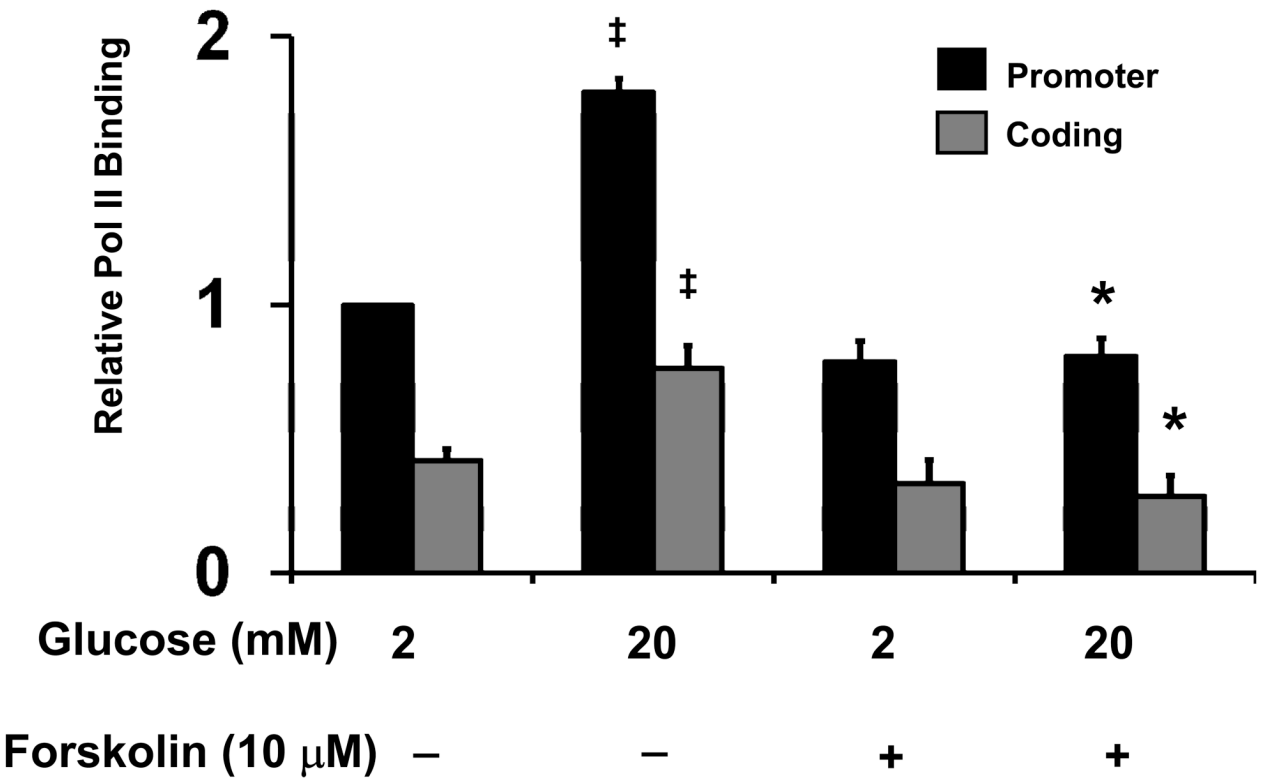


FIG 1. Expression of the L-PK gene requires ChREBP and CBP

A. 832/13 cells were treated for 6 h with 2 or 20 mM glucose in the presence or absence of 10 μM forskolin. Total RNA was isolated, and RT-PCR was performed using primers specific for L-PK. Values are means ± SEM from three independent experiments. **P* 0.001 vs. 20 mM glucose. (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 and a portion of the coding region were targeted for amplification. Data are means ± SEM from three individual experiments. **P* < 0.05 vs. 20 mM glucose, ‡*P* < 0.01 vs. 20 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 and coding region targeted for amplification via RT-PCR. Data represent means ± SEM from three individual experiments. **$P < 0.05$ vs. siScramble at 2 mM glucose, *$P < 0.001$ vs. 20 mM glucose, ‡ $P < 0.05$ vs 20 mM glucose.

Figure 2A



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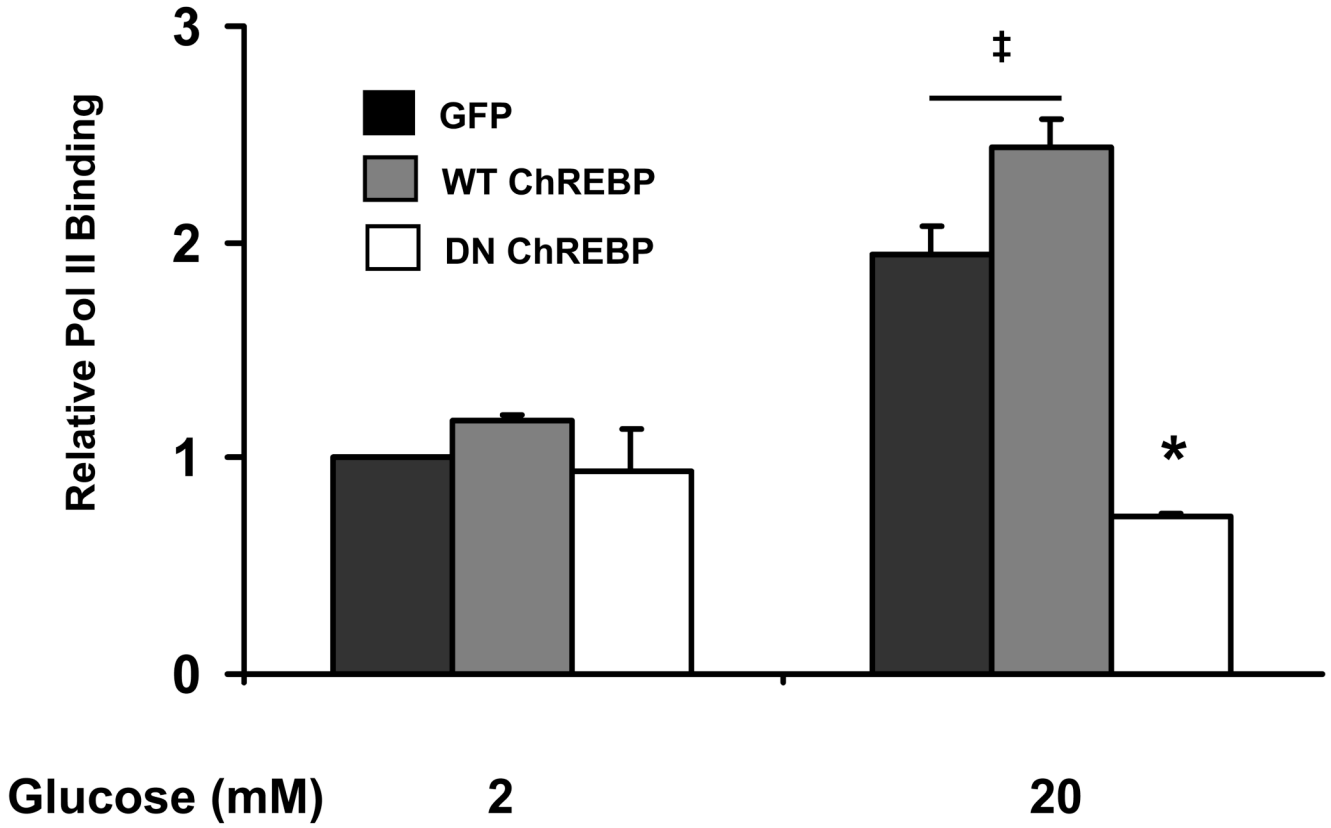

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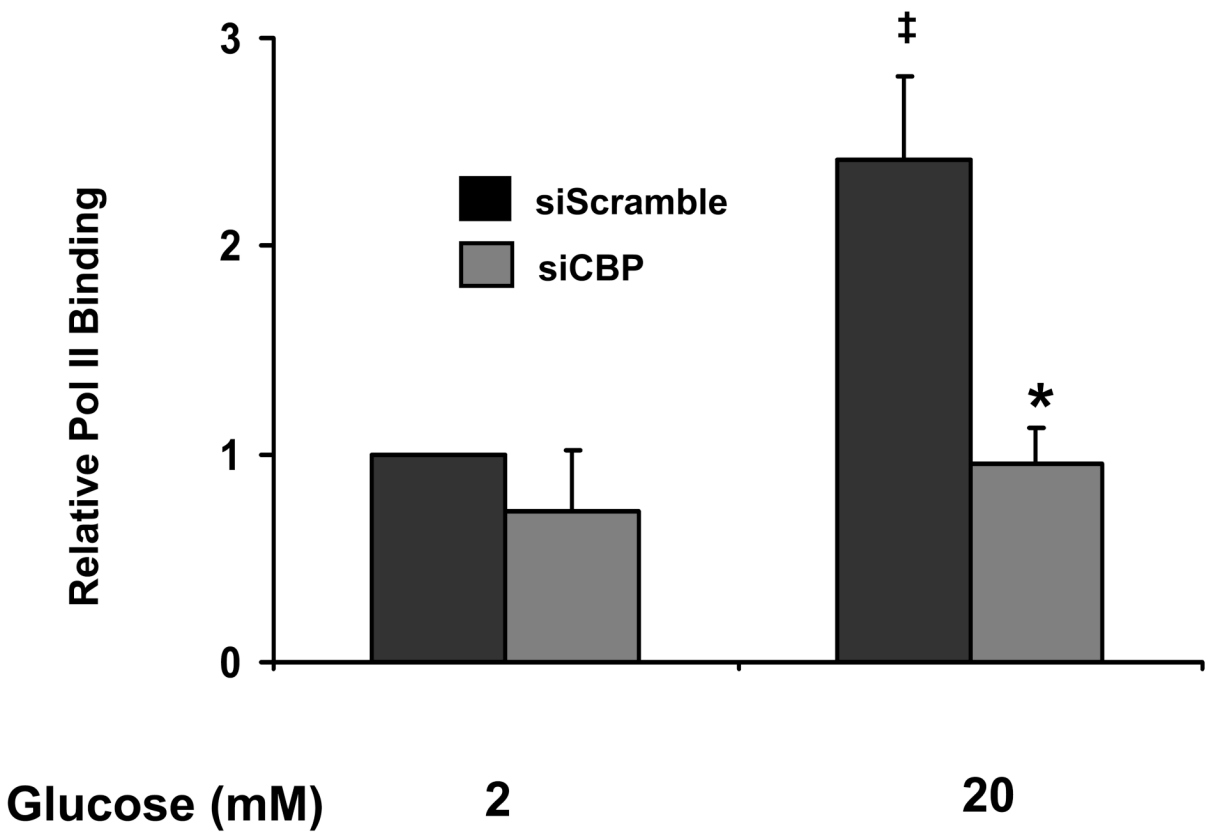

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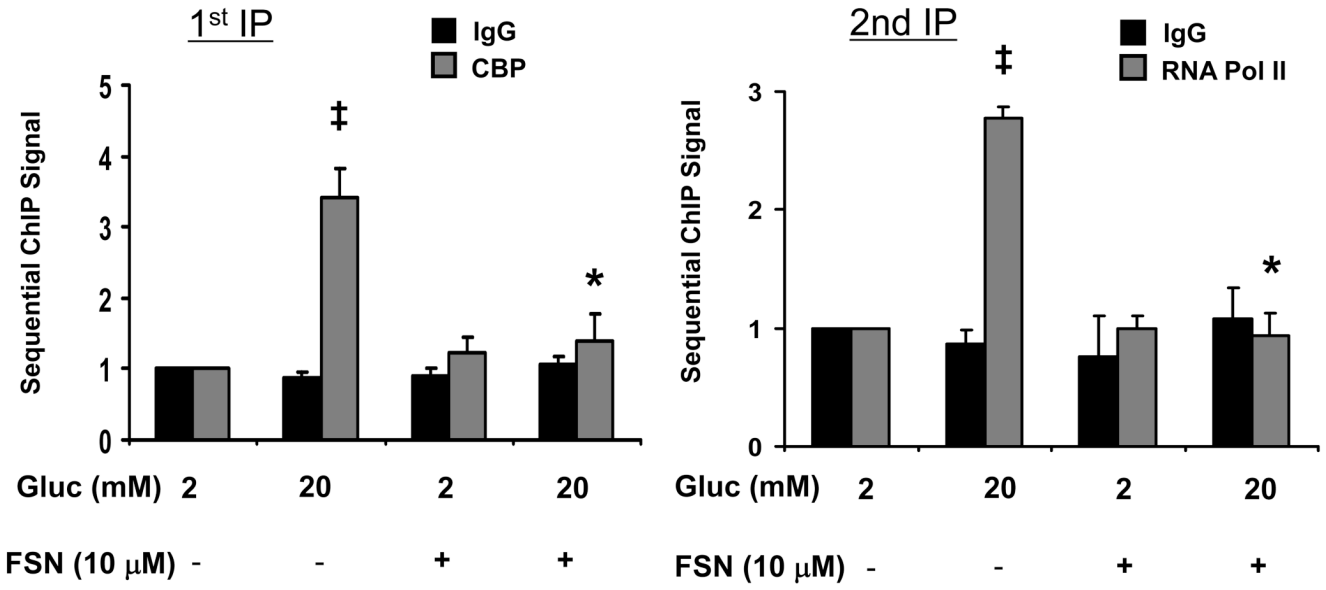
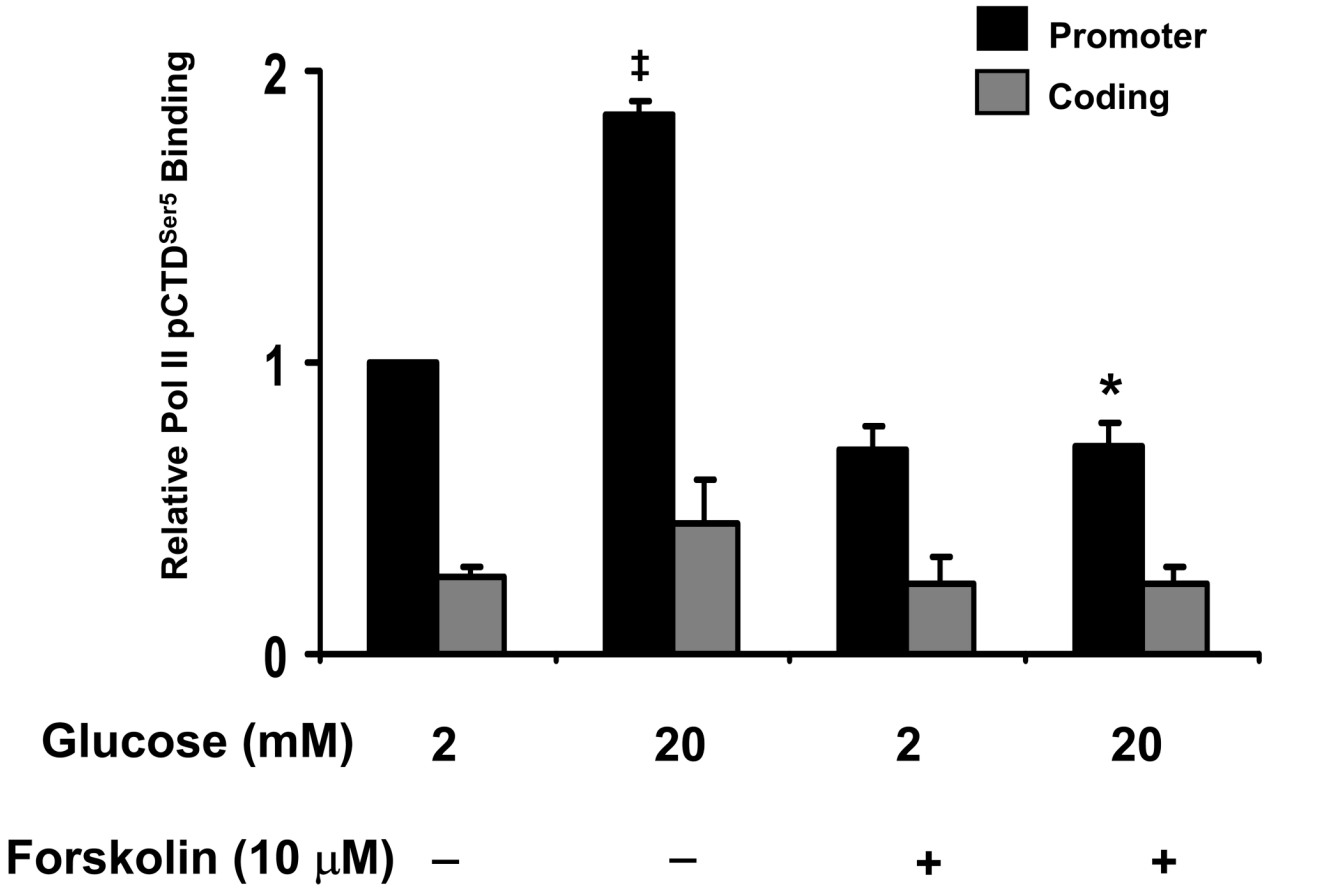


FIG 2. cAMP diminishes the glucose-stimulated recruitment of Pol II the L-PK gene promoter

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 promoter binding of Pol II was determined via ChIP assay. Gene promoter (A–D) and coding region (A) for L-PK was targeted for amplification, and the data were normalized to control IgG. Data are expressed as means ± SEM from four to five independent experiments. **P* < 0.05 vs. 20 mM glucose. *D*. Cells were treated as in (A), CBP was then 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. 20 mM glucose, ‡ *P* < 0.05 vs 20 mM glucose.

Figure 3A



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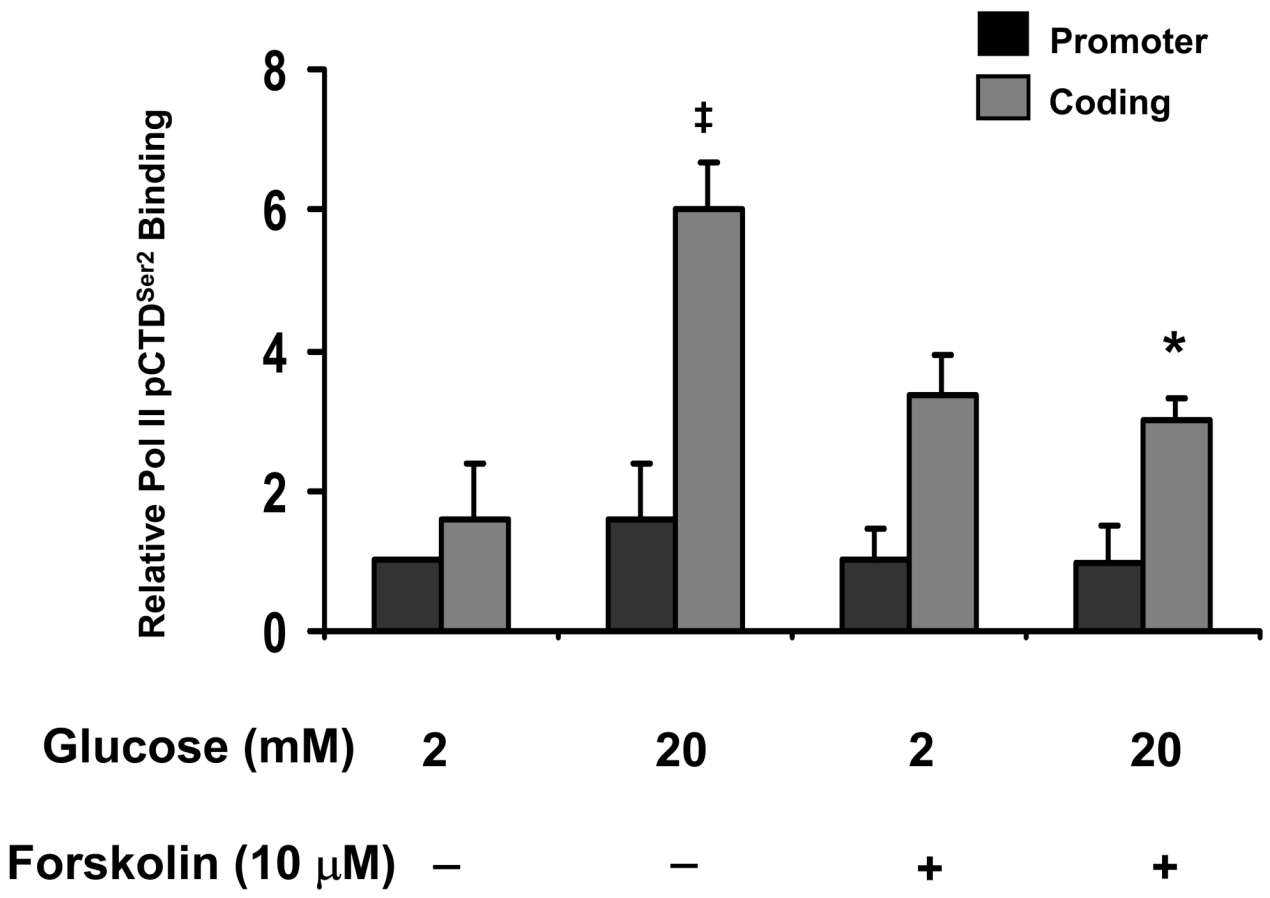
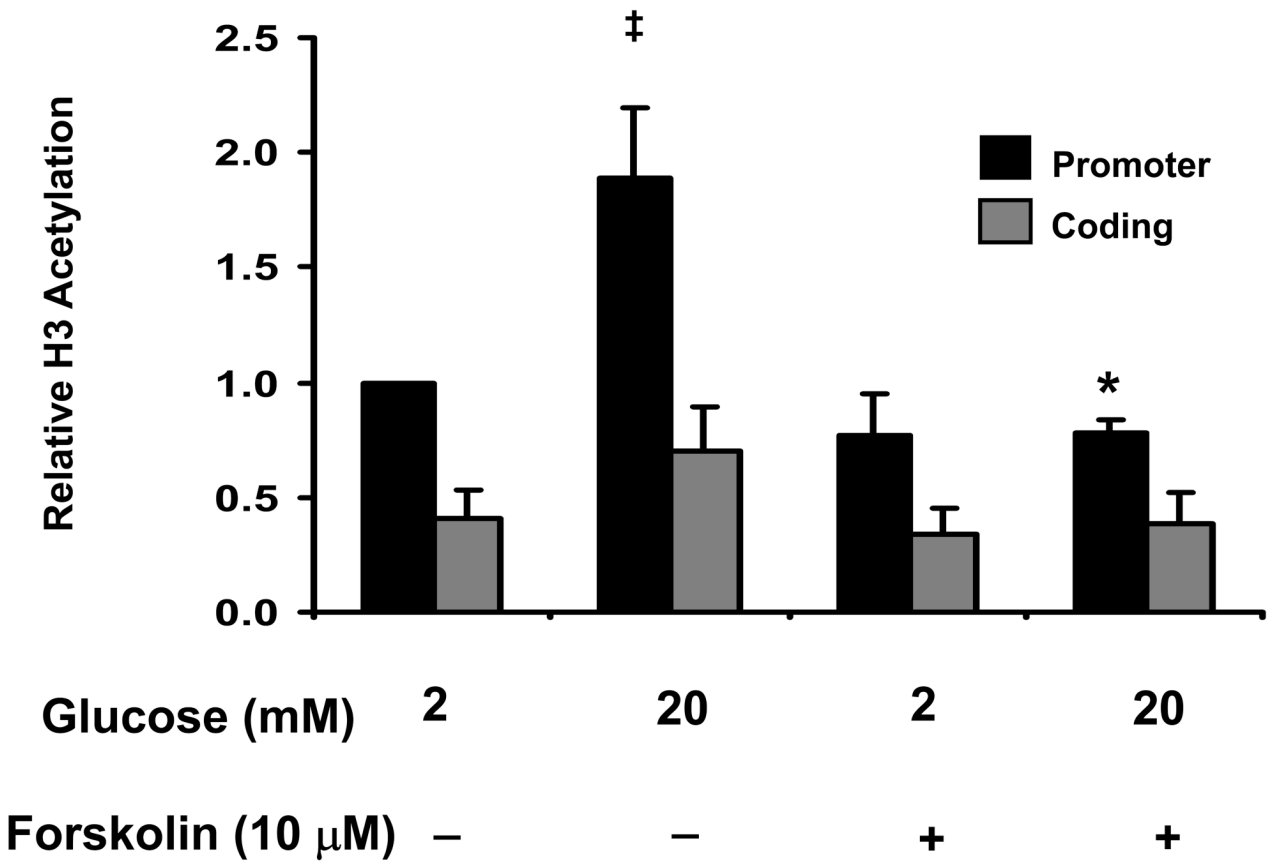


FIG 3. cAMP abrogates the glucose-mediated recruitment of Pol II pCTD^{Ser5} to the promoter, and Pol II pCTD^{Ser2} to the coding region of the L-PK gene promoter

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), and normalized to control IgG. 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. 20 mM glucose, ‡ $P < 0.05$ vs 20 mM glucose.

Figure 4A



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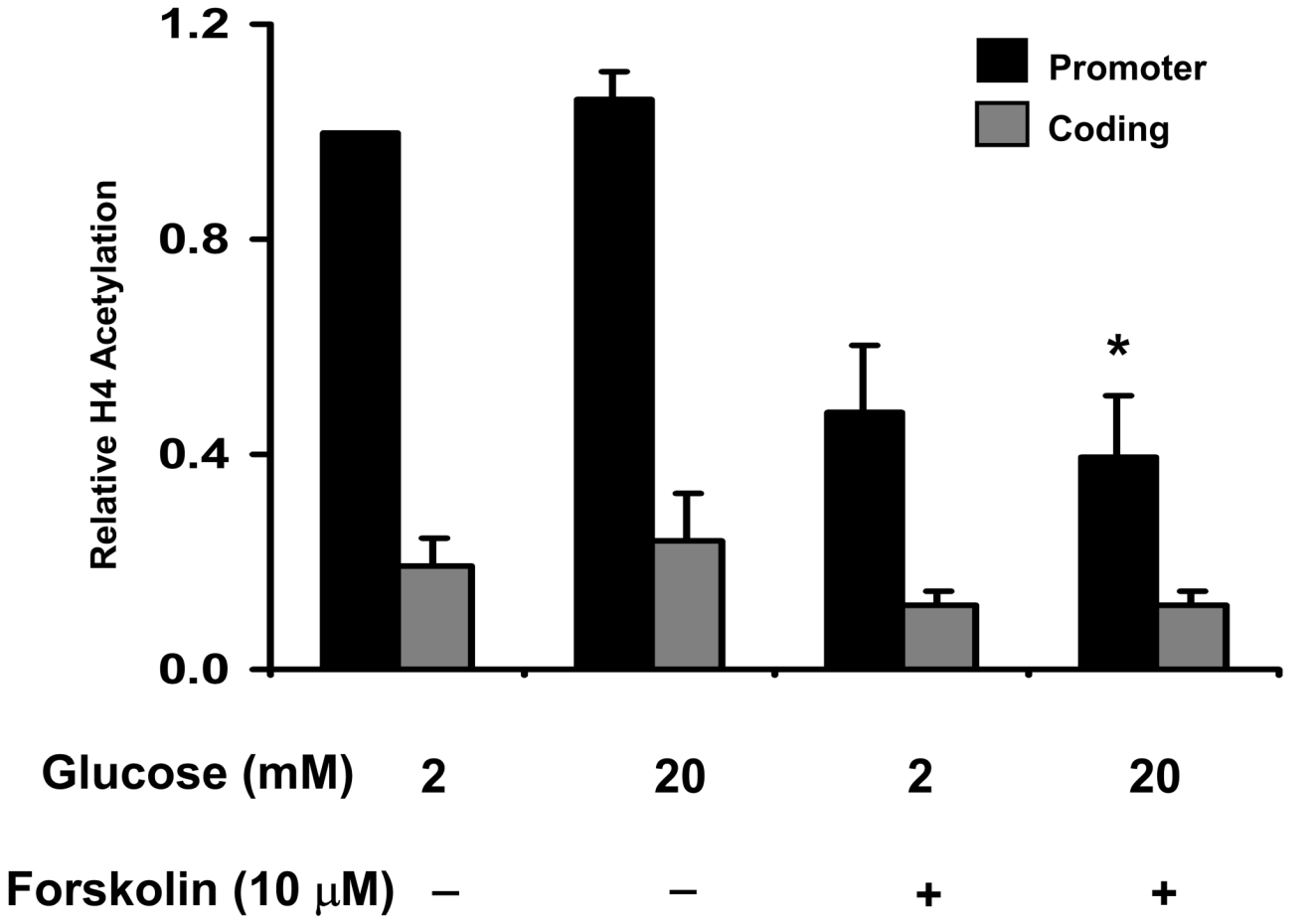

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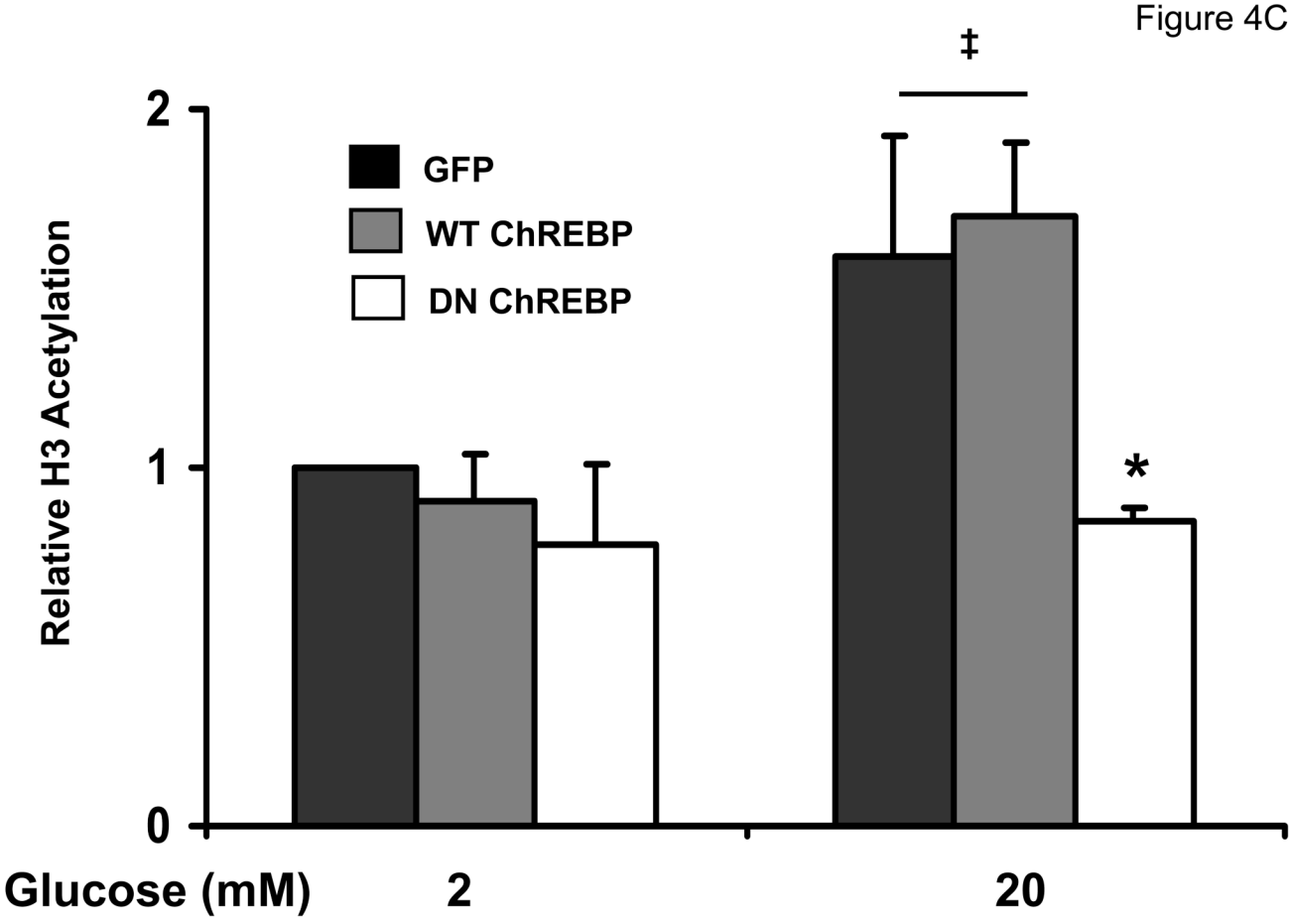

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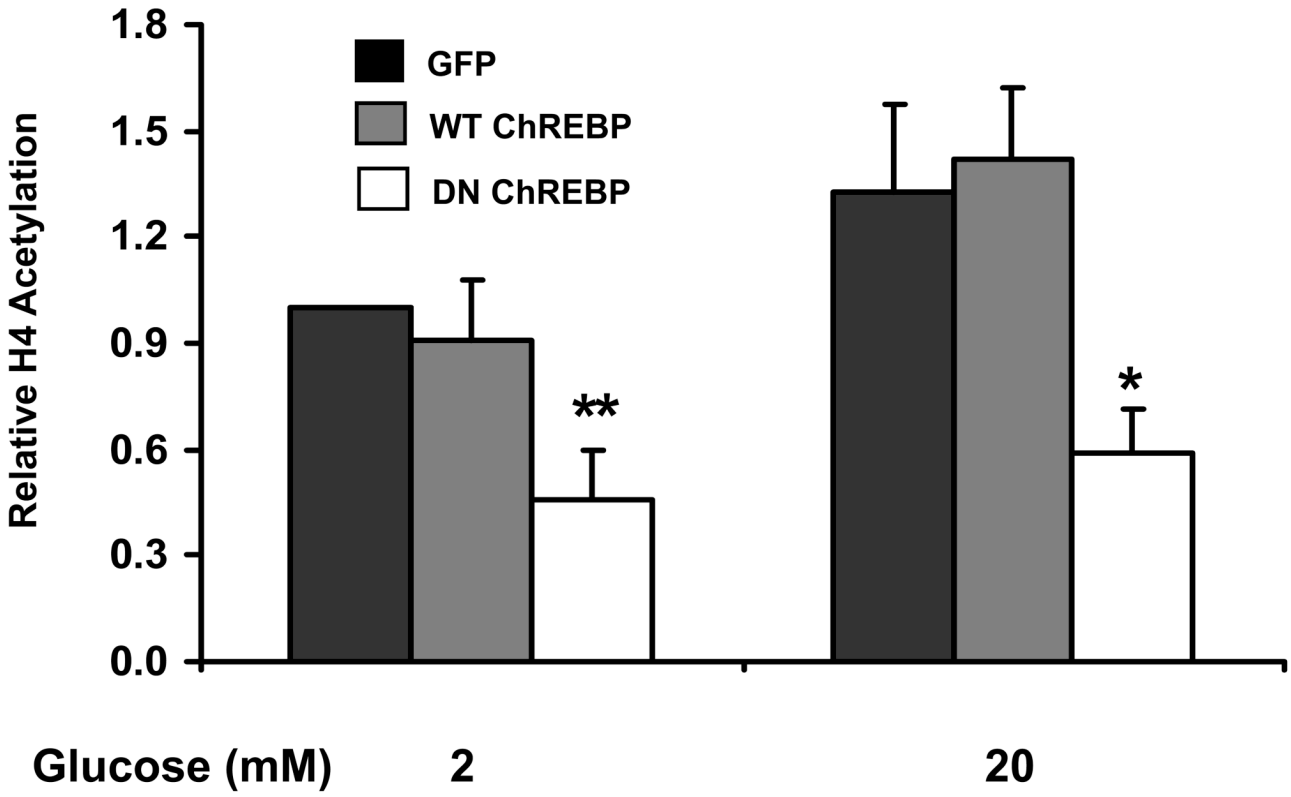

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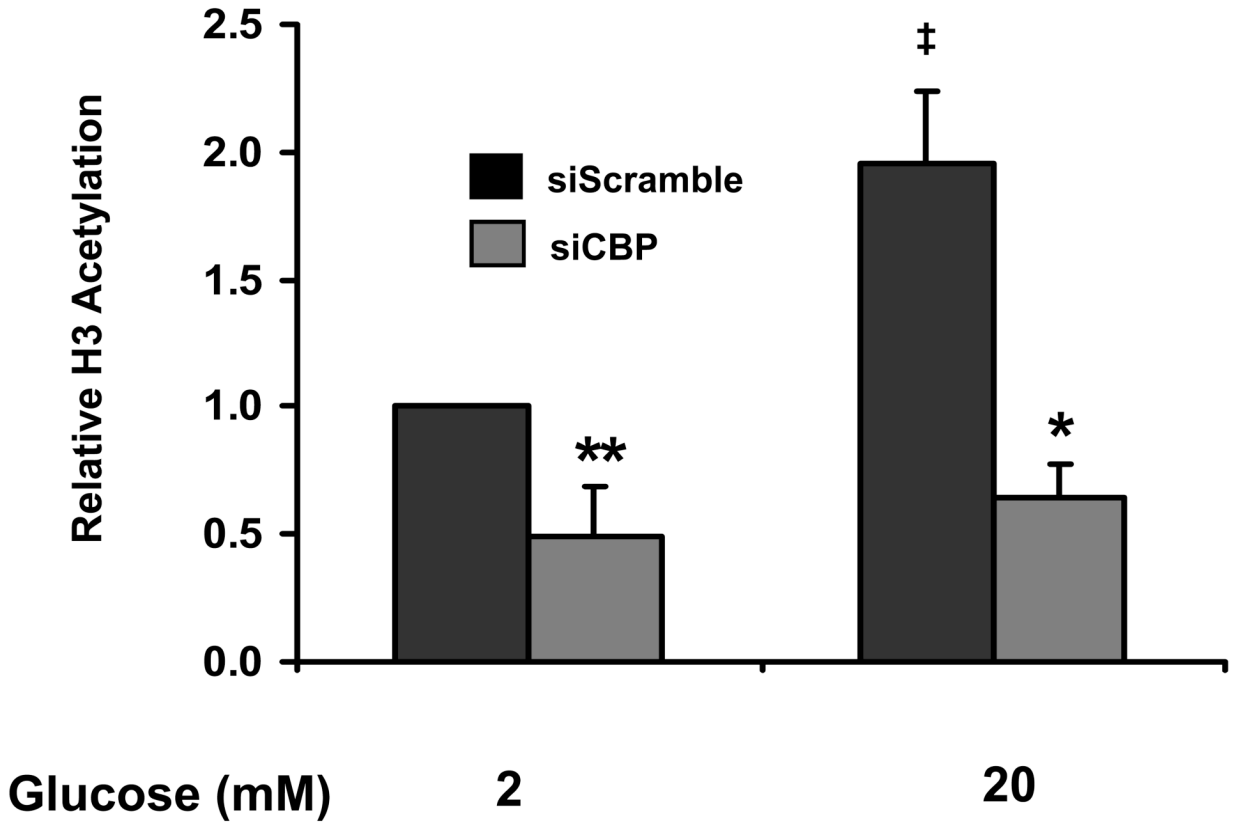

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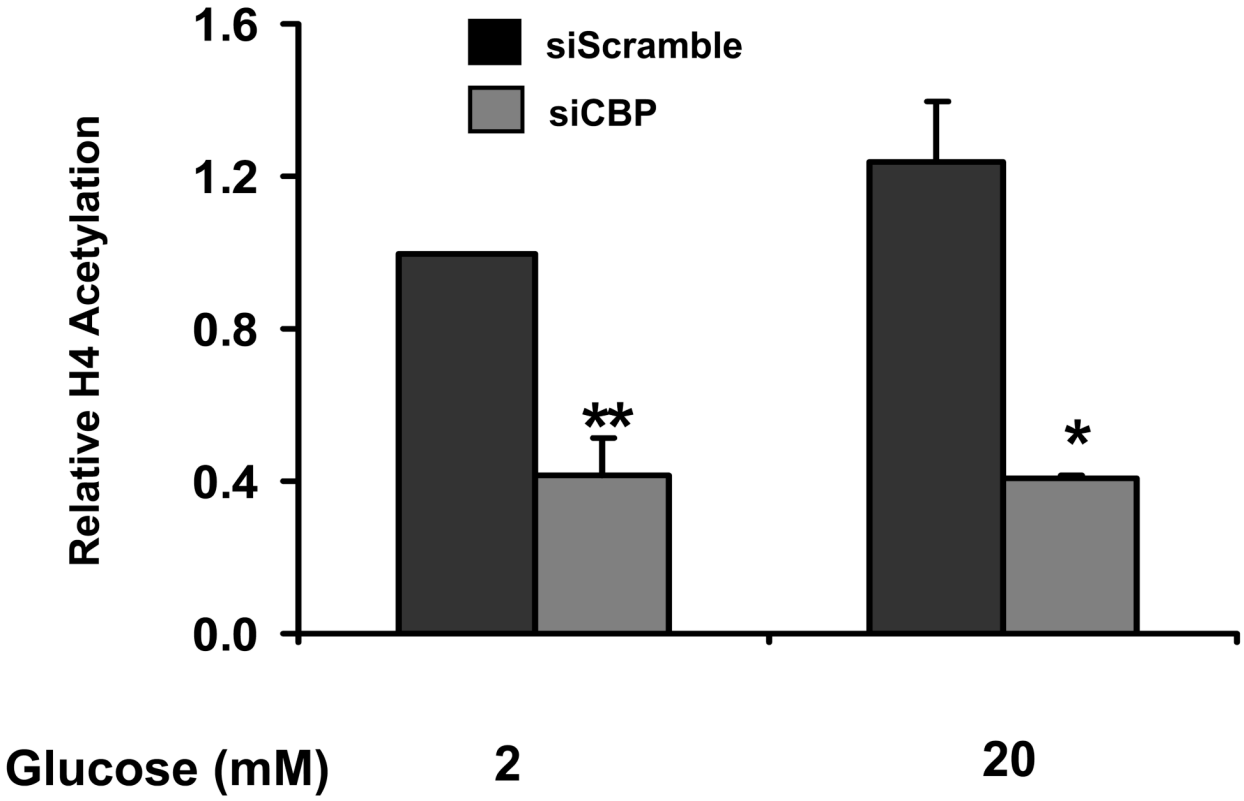
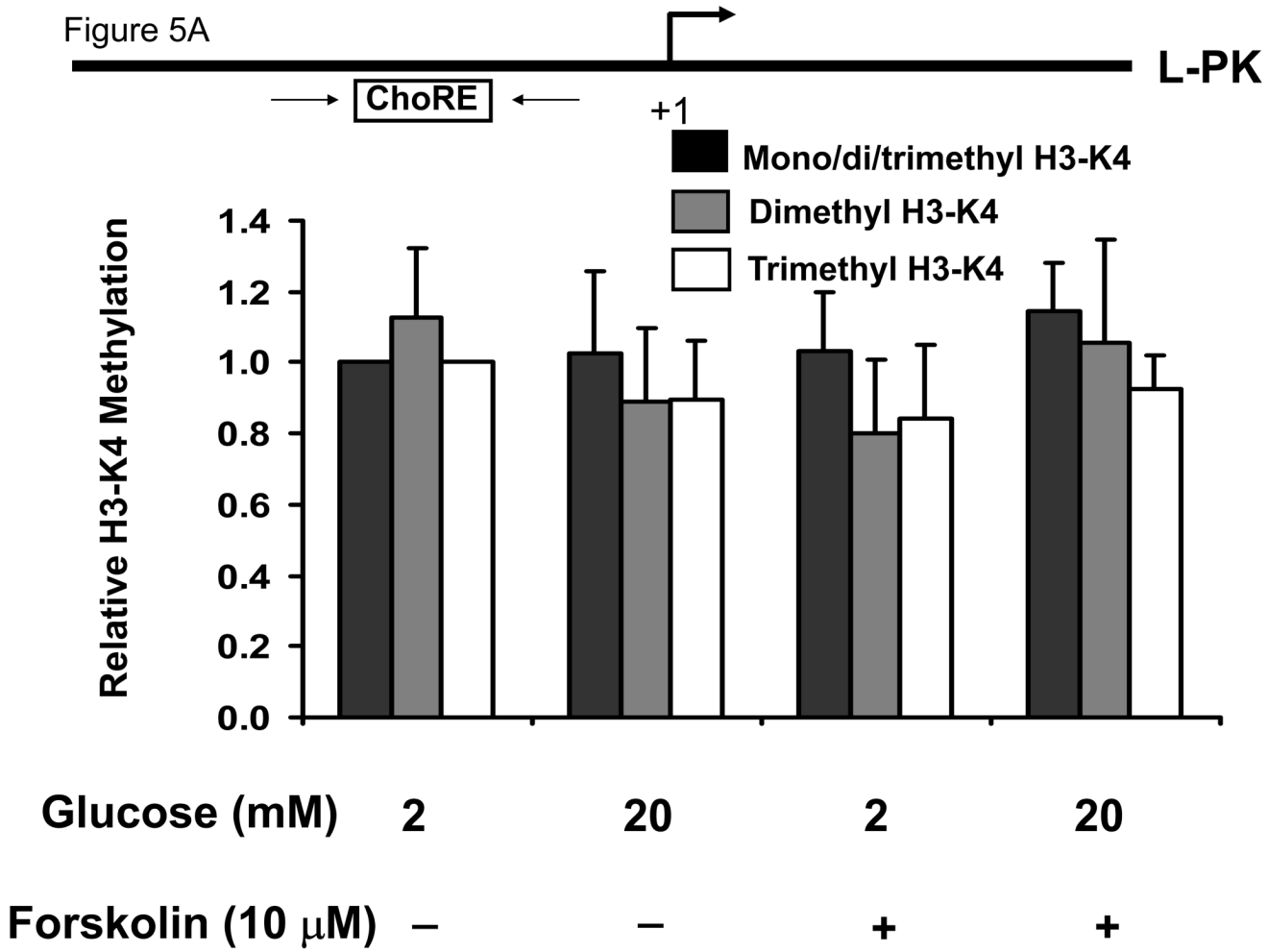


FIG 4. cAMP decreases the acetylation of histones H3 and H4 associated with the L-PK gene promoter

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 were determined by chromatin immunoprecipitation assay, using antibodies against either acetylated histones H3 (A, C, E) or H4 (B, D, F), and normalized to control IgG. Data represent means ± SE from three to five independent experiments. ***P* < 0.05 vs. 2 mM glucose control samples, **P* < 0.05 vs. 20 mM glucose control samples, ‡ *P* < 0.05 vs 20 mM glucose.



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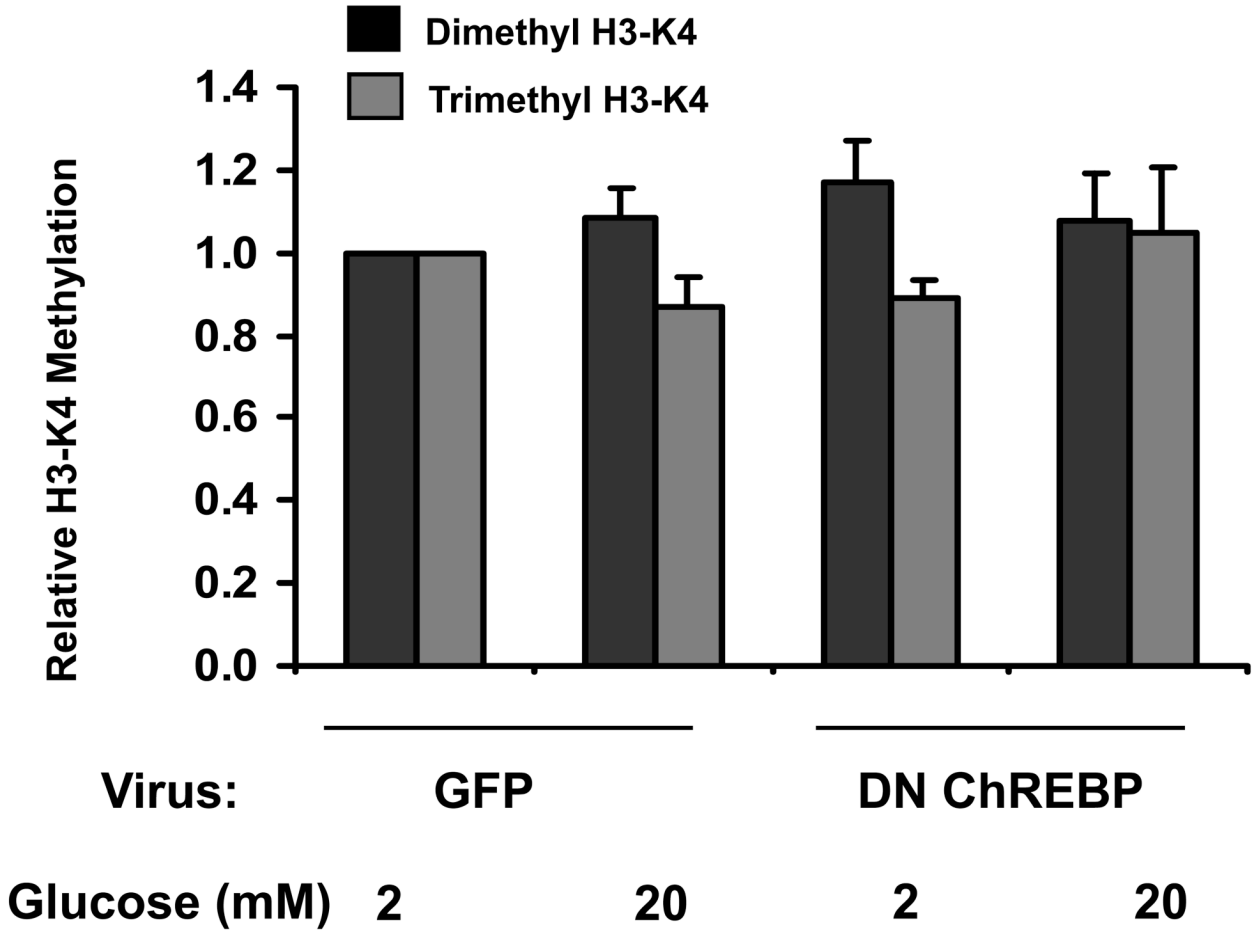

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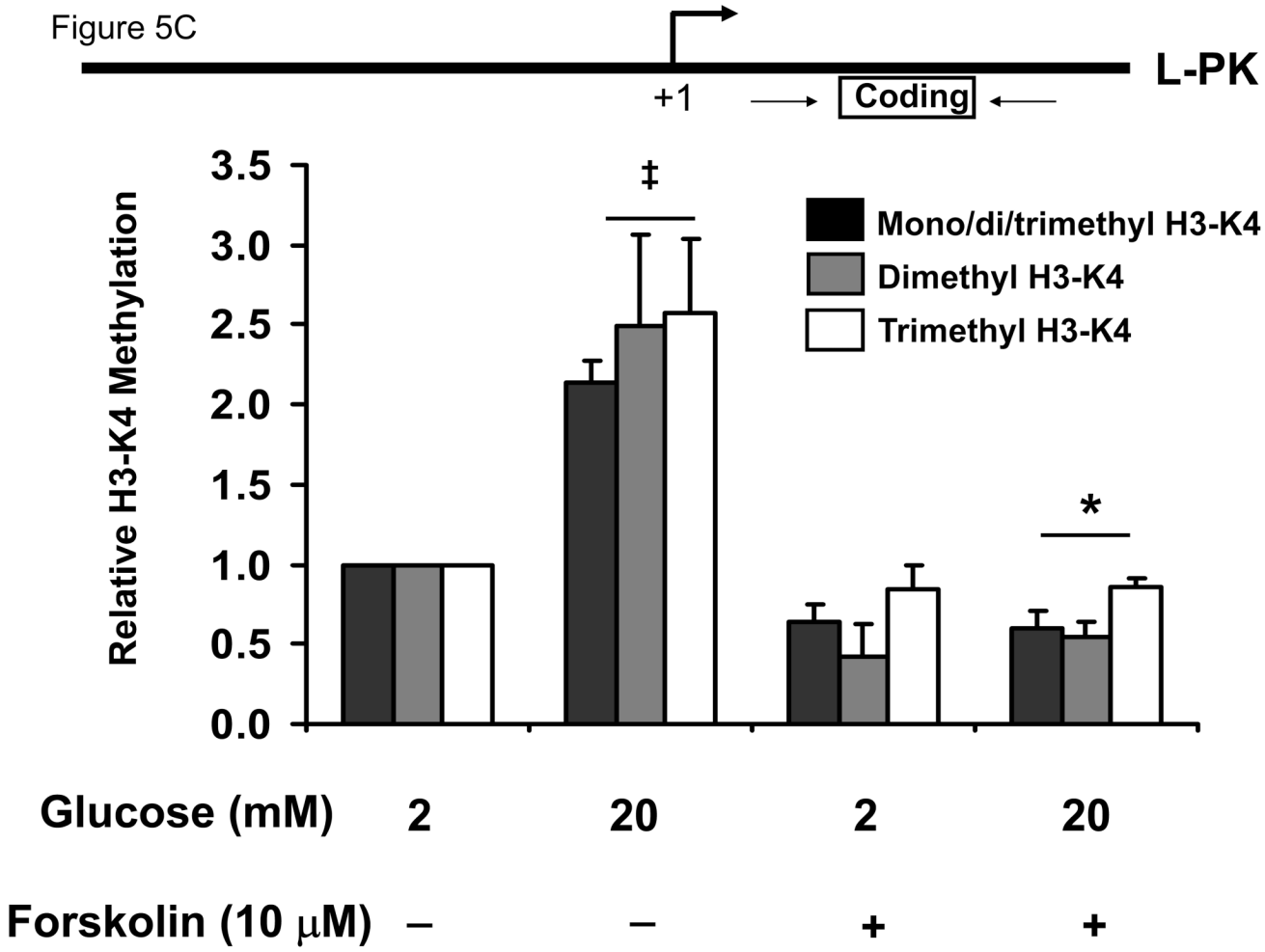

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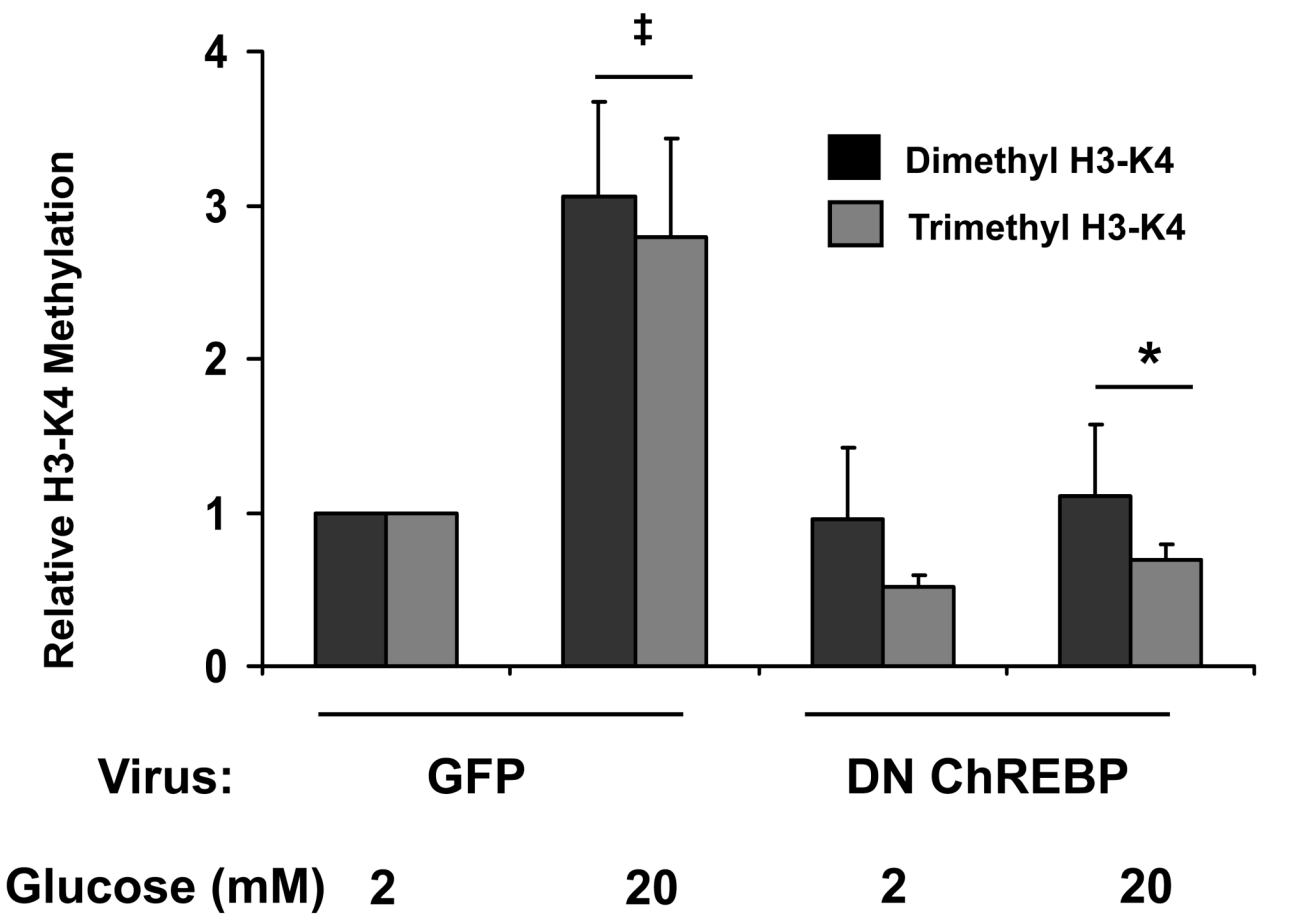

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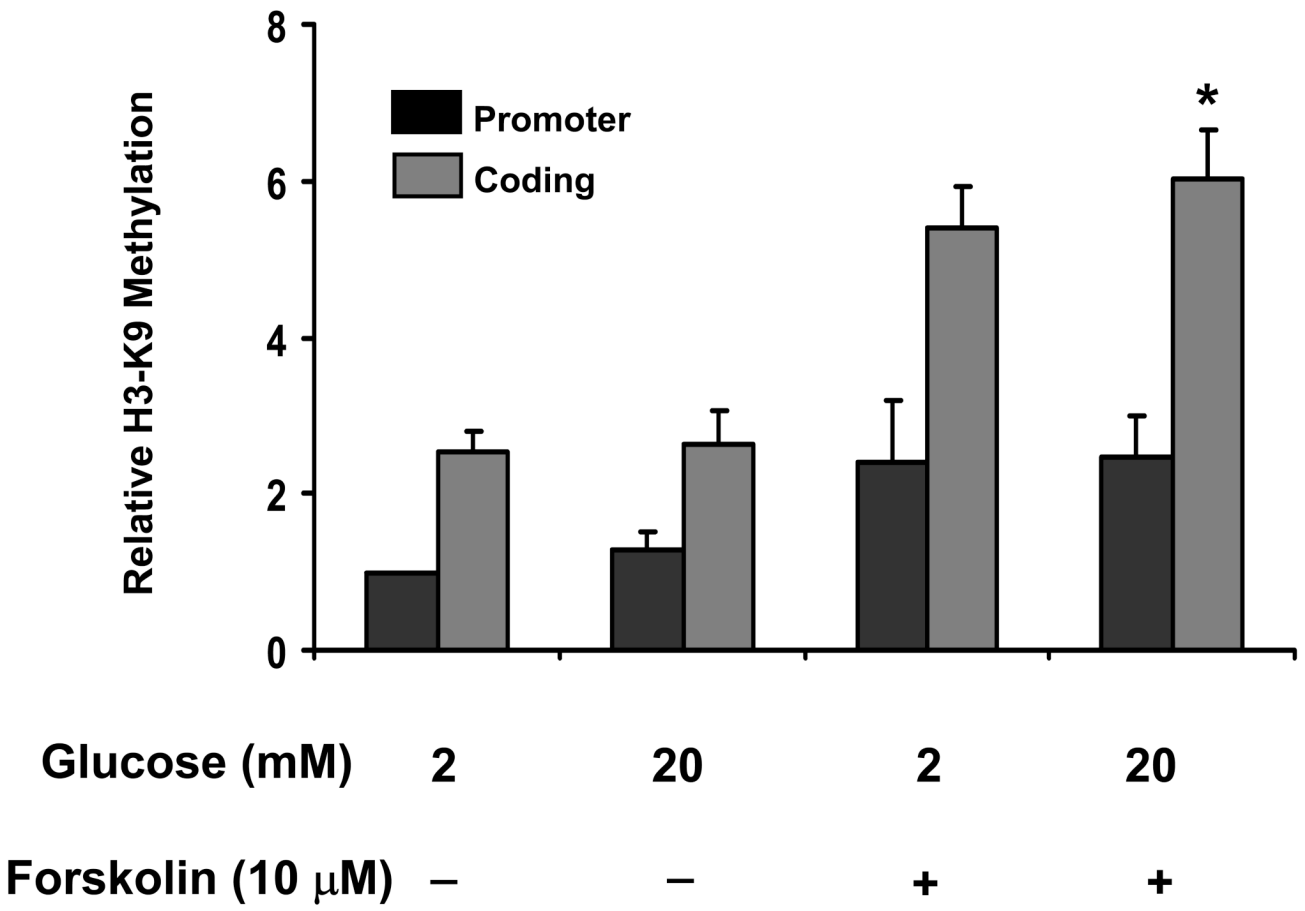

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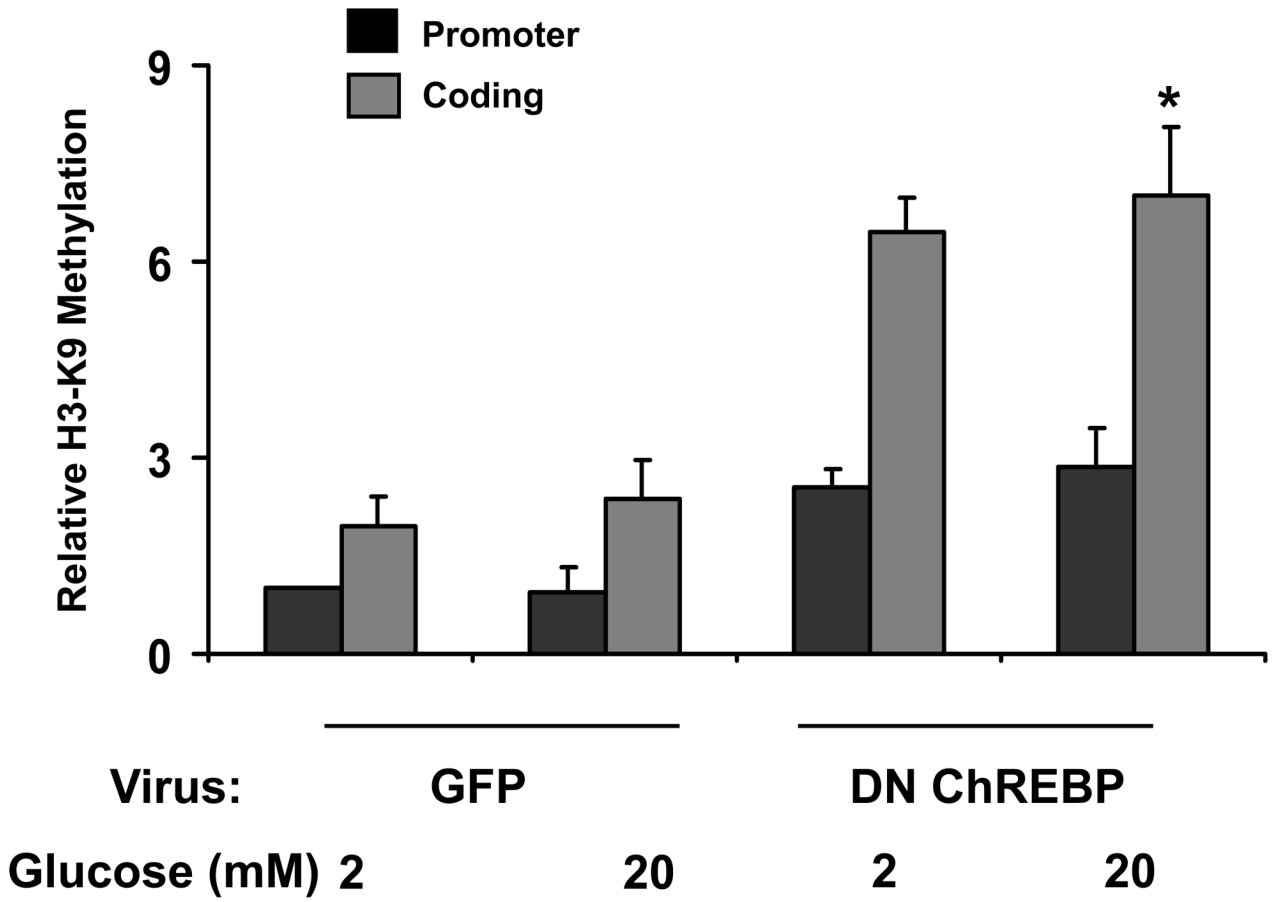

FIG 5. Glucose and cAMP signaling alter the methylation status of histone H3 on the L-PK promoter and coding region

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, C, 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 in the presence or absence of 10 μM forskolin for an additional 6 h. Chromatin immunoprecipitation assays were employed to determine relative promoter occupancy of methylated histone H3-K4 (A–D) and H3-K9 (E–F), and normalized to control IgG. 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. 20 mM glucose samples, ‡ *P* < 0.05 vs 20 mM glucose.