A Tripartite Array of Transcription Factor Binding Sites Mediates cAMP Induction of Phosphoenolpyruvate Carboxykinase Gene Transcription and Its Inhibition by Insulin

(Received for publication, February 27, 1998, and in revised form, May 13, 1998)

David Yeagley, Joyce M. Agati, and Patrick G. Quinn

From the Department of Cellular and Molecular Physiology, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

Transcription of the phosphoenolpyruvate carboxykinase (PEPCK) gene is induced upon activation of protein kinase A by cAMP and phosphorylation of Ser-133 in the transcription factor, cAMP-response element binding protein (CREB), and this induction is inhibited by insulin. We show here that insulin does not act by dephosphorylating CREB or by affecting heterologous kinases that phosphorylate Ser-129 or Ser-142 in CREB. In addition, insulin inhibition of minimal PEPCK promoter activity induced by CREB-GAL4 + protein kinase A was equivalent to inhibition of basal transcription, and thus cAMP-independent. On the other hand, nearly complete insulin inhibition is observed with the full PEPCK promoter (−600/+69), indicating that other factors are involved. The additional promoter elements required for induction by protein kinase A lie within −271 nucleotides of the start site and correspond to putative binding sites for activator protein-1 and CAAT/enhancer-binding protein (C/EBP), first identified by Roesler et al. (Roesler, W. J., McFie, P. J., and Puttick, D. M., (1993) J. Biol. Chem. 268, 3791–3796). This tripartite array of binding sites for CREB, C/EBP, and activator protein-1 (AP-1) factors forms a cAMP response unit that, together with the minimal promoter, can mediate both induction by cAMP and inhibition by insulin. Thus, for the PEPCK gene with a single CREB site, the CREB-CBP-RNA polymerase II complex cannot mediate either induction by cAMP or inhibition by insulin.

Both cAMP and insulin alter the activities of protein kinases and phosphatases, exerting their acute effects through changes in the phosphorylation state of a variety of regulatory molecules in the cell (1–6). The enzymatic activity of the catalytic subunit of protein kinase A (PKAc) is restrained by its association with two regulatory subunits in the PKA holoenzyme. Binding of cAMP to the regulatory subunits of PKA induces a conformational change resulting in the release of free, active PKAc (7, 8), some of which is translocated to the nucleus where it phosphorylates promoter-associated CREB and induces gene transcription (9). Insulin stimulates the tyrosine kinase activity of its receptor and, in different cell types, activates a variety of kinase signaling cascades, including those characterized by Ras/mitogen-activated protein kinase, phosphatidylinositol 3-kinase, glycogen synthase kinase-3, protein kinase B, p70S6 kinase, and protein kinase C isoforms (10–21).

In general, glucagon (acting through cAMP) and insulin have counter-regulatory effects on hepatic glucose metabolism (reviewed in Granner and Pilkis (22)). The first committed step in gluconeogenesis and glycolysis is catalyzed by phosphoenolpyruvate carboxykinase (PEPCK) and glucokinase, respectively. Control of both enzymes is achieved by induction and repression of transcription of their respective genes, which is oppositely regulated by cAMP and insulin (22). This is essential for maintaining blood glucose levels within narrow limits. Lack of control by insulin contributes to hyperglycemia and thus to many of the pathologies afflicting diabetic patients.

The PEPCK gene has provided a useful model system for studying the integration of multiple hormonal signals at the level of a single gene (23–28). Acute hormonal regulation of PEPCK is exerted entirely by regulating transcription (26). PEPCK transcription is induced by glucagon (acting via cAMP), glucocorticoids, thyroid hormone, and retinoic acid, and is inhibited by insulin and phospholipid esters, all of which are mediated through 600 base pairs of the PEPCK promoter (27–33). Insulin and cAMP enhance and repress the transcription of distinct genes in the same cells (22), e.g. insulin stimulates gene 33 and inhibits PEPCK transcription in nuclei from H4IIE cells (34), indicating that gene-specific mechanisms employing combinatorial regulation must be utilized. The combined input of signaling pathways modifies different transcription factors found in unique juxtaposition on the promoters of different genes to set the transcription rates of each gene to a different level (35–38).

We previously showed that multiple CREB binding sites ligated to a minimal PEPCK promoter could mediate induction by PKA and that this could be at least partly inhibited by insulin (39). We hypothesized that insulin disrupted interactions between CREB, CREB binding protein (CBP) and the RNA polymerase II complex to repress cAMP-inducible PEPCK expression. Similar results were reported by Blenis and Montminy and colleagues (40), who provided evidence that insulin stimulates activation of the Ras/mitogen-activated protein kinase pathway, leading to the phosphorylation of pp90rsKII and its binding to CBP, thus preventing induction of a PEPCK promoter (−134/+69) linked to chloramphenicol acetyltransferase. Other possibilities for insulin inhibition include: 1) de-
phosphorylation of the PKA site in CREB; 2) a change in phosphorylation of CREB by other kinases that modify CREB activation when (in)activated by insulin; or 3) the involvement of other transcription factors in the opposing regulation of PEPCK gene expression by cAMP and insulin.

With regard to the first, there are conflicting reports regarding the phosphatase responsible for dephosphorylation of CREB, PP1, or PP2a (41–43). A probable explanation for the discrepancies in these reports is that CREB is inactivated by distinct phosphatase activities in different cell types, which may or may not be regulated by insulin. Insulin inhibition of PP2a activity has been shown to contribute to CREB activation in some cells (44), but phosphorylation of the PKA site in CREB is unaffected by insulin in hepatoma cells (40).

With regard to the second possibility, there are two intriguing reports of heterologous kinases affecting induction by CREB. First, GSK-3 was shown to phosphorylate Ser-129 in CREB, contributing to its activation (45), and also to be phosphorylated and inactivated by insulin (46–48), acting through protein kinase B (15). Thus, phosphorylation of CREB by GSK-3 would not be carried out in insulin-treated cells, and CREB would remain incompletely activated, leading to a decline in transcription. Second, Sun et al. (49) showed that phosphorylation of Ser-142 in CREB by CamK II is inhibitory, even in the presence of PKA. This result suggested that modification of Ser-142 or a similar site by an insulin-stimulated kinase could account for the observed inhibition of the PEPCK gene, even if no change was observed in phosphorylation of the PKA site.

Concerning the third possibility, Roessler and colleagues have demonstrated that several promoter elements in addition to the CRE are required for induction of PEPCK gene expression in the HepG2 cell model (50–54). They used a combination of DNA binding and functional assays to demonstrate that induction by cAMP requires three C/EBP and one AP-1 site, located between –355 and –225 in the PEPCK promoter, in addition to a promoter region containing the CAAT and CRE elements of the PEPCK gene (–113/–69). Although induction of PEPCK fusion genes by cAMP is robust in HepG2 cells, the endogenous PEPCK gene is not expressed, and cAMP-induced activity of exogenous PEPCK fusion genes is not inhibited by insulin in these cells (55). In the present study, we examined the possibility that one or more of these heterologous elements is required, in addition to the CRE, for opposing regulation by cAMP and insulin of PEPCK fusion genes in the insulin-responsive H4IIe cell line.

The present study provides evidence that phosphorylation of CREB by PKA is necessary and sufficient for induction when multiple CREB binding sites are present and that P-CREB persists in insulin-treated cells. Phosphorylation of CREB on Ser-129 or Ser-142 (the GSK-3 and CamK II regulatory sites) can fine-tune induction, but it is not involved in inhibition by insulin. On the other hand, opposing regulation of PEPCK gene transcription by cAMP and insulin requires DNA elements that may bind C/EBP and AP-1 family transcription factors in addition to the CRE. Together with the minimal PEPCK promoter (–40/+1), a tripartite cAMP response unit (CRU), comprised of binding sites for CREB, and an upstream element containing putative C/EBP and AP-1 sites, is sufficient to mediate both induction by cAMP and inhibition of cAMP-inducible gene transcription by insulin.

**EXPERIMENTAL PROCEDURES**

**P-CREB Analysis by Western Blot**—Nuclear extracts were prepared from H4IIe cells after 30 min of hormone treatment, a time at which transcription induction of PEPCK by cAMP in these cells is maximal. Cells were treated with 0.1 mM 8-(4-chlorophenylthio)cAMP and/or 10 nM insulin, as indicated. The nuclear extracts were prepared by a modification of the method of Hurst et al. (56), increasing the EDTA to 1 mM and including NaF (20 mM), NaV (1 mM), and okadaic acid (1 μM), to inhibit nuclear protein kinase and phosphatase activities. Aliquots of the nuclear extracts were loaded onto two gels in parallel, which were electrophoresed and transferred to polyvinylidene difluoride membranes. One of these was probed with anti-PKA-phospho-CREB antibody and the other with anti-CREB antibody, and the blots were developed with chemiluminescent reagents and exposed to film. The epitatepurified PKA-phospho-CREB antibody was a generous gift from David Ginty, Johns Hopkins University.

**H4IIe Cell Culture and Transfection Analyses**—H4IIe cells were grown and transfected as described previously (39, 55). In brief, the cells were transfected in solution with 20 μg of luciferase (firefly) reporter plus 2 μg of each expression plasmid plus 2 μg of pRL-SV (renilla luciferase, Promega Corp.) reporter to correct for differences in transfection efficiency. Half of the cells were seeded into each of two 60-mm dishes, one of which served as a control while the other was treated with 10 nM insulin. Where indicated, cells were cotransfected with an expression vector for the catalytic subunit of PKA, obtained from R. Maurer, Oregon Health Sciences University (57). After 4 h, the cells were treated with 20% MeSO for 3 min, washed in phosphate-buffered saline, and then medium with or without 10 nM insulin was added for the remaining 20 h. Cells were harvested by trypsin/EDTA, lysed, and luciferase activities for the firefly and renilla luciferase reporters were measured with the Dual Luciferase Kit of Promega, using an ALI Monolight 3020 dual injector luminometer. PEPCK promoter firefly luciferase activity was corrected for renilla luciferase activity in the same sample. All figures represent several transfection experiments, each normalized to the untreated control and the data combined for analysis. The number of experiments for each figure is indicated in the figure legends.

**Expression Vectors**—The pGL3 expression vector has been described in detail previously (58). CRG-S129A and CRG-S142A were created by mutating Ser-129 and Ser-142, respectively, to alanine, using site-directed primers and the Chameleon Mutagenesis Kit of Stratagene Corp. A fragment containing the desired mutation was sequenced in its entirety and used to replace the corresponding fragment in wild type CRG. All expression vector sequences were verified to be correct by DNA sequencing of the final plasmids used for transfection experiments.

**Reporter Vectors**—The pQ-Luc luciferase reporter plasmid is based on the promoterless pGL3-basic vector encoding firefly luciferase, obtained from Promega Corp. and modified to accept PEPCK promoter fragments. An internal βgl II site (map position 36) was destroyed by digestion, filling in the ends, and religation. The polynucleotide of pQGL3 was replaced with the polynucleotide of pQ-CAT (58) to create pQ-Luc and promoter fragments were inserted in the HindIII and KpnI sites of pQ-Luc. The pQ-Luc and G4-PEPCK promoters were described previously (55), as was the 5XT promoter (39). PEPCK promoters terminating at –400, –300, –271, –200, and –134 nucleotides (27) were subcloned into pQ-Luc. Site-specific oligonucleotides were used to introduce a SaII site upstream of the A sites (–252, –202) 2 sites flanking the C site (–245/–252); and 3 SpeI sites introduced 3′ of P3 (–225) and 5′ of the CAAT box (–109) in –271-Luc, which has a SaII site immediately 5′ of the promoter region. The corresponding promoter elements were removed by digestion with the appropriate enzyme (SaII or SpeI) followed by religation of the deleted vector. A promoter fragment containing the desired mutation was sequenced in its entirety and used to replace the corresponding fragment in the wild type reporter.

**FIG. 1.** The PKA site in CREB remains phosphorylated in H4IIe cells treated with insulin. H4IIe cells were treated with nothing, 8-(4-chlorophenylthio)cAMP (1 mM), and/or insulin (10 nM), as indicated, for 30 min. Nuclear extracts were prepared, and equal aliquots were loaded onto replicate gels. Following electrophoresis, proteins were transferred to membranes and probed with antibodies that recognize either total CREB or PKA-phosphorylated CREB (PKA-P-CREB).

![Image](image-url)
The SG4LT-Luc reporter contains a single copy of a GAL4 DNA recognition sequence upstream of the minimal promoter region of PEPCK. Oligonucleotides containing the C site \((-248/-227)\), A site \((-265/-247)\) or both A and C sites \((-265/-227)\) were cloned upstream of the G4 site to create A-SG4LT-Luc, C-SG4LT-Luc, or AC-SG4LT-Luc. All promoter sequences were verified to be correct by DNA sequencing of the final plasmids.

**RESULTS**

**Regulation of Phosphorylation of the PKA Site in CREB—**

The simplest model for the inhibition of PKA-induced PEPCK transcription would involve terminating transcription activation by insulin-mediated dephosphorylation of Ser-133 in CREB, which is phosphorylated by PKA. To determine whether this occurred, nuclear extracts were prepared from H4IIE cells, and the extent of phosphorylation of the PKA site, Ser-133, in CREB was determined. Nuclear extracts were prepared after 30 min, a time at which transcription of the PEPCK gene is maximal (26). Blots containing identical samples of nuclear proteins, were analyzed with antibodies recognizing only PKA-

**Fig. 2. Analysis of CRG phosphorylation site mutants.** A, the relevant portions of the luciferase reporter and CREB expression vectors used are shown. The phosphorylation sites within KID that were mutated to alanines and the kinases known to phosphorylate them are shown. B, H4IIE cells were cotransfected with either PEPCK-Luc or G4-PEPCK-Luc + CRG expression vectors in the absence and presence of a PKAc expression vector, as described under “Experimental Procedures.” Each precipitate was split into two dishes, and half of them were treated with 10 nM insulin for the final 20 h of the experiment. The results shown represent four independent experiments. C, H4IIE cells were cotransfected with 5XGT-Luc + CRG expression vectors and treated as above. The results shown represent five independent experiments.
phospho-CREB or all forms of CREB (Fig. 1). The amount of total CREB was unchanged by hormonal treatment, as expected. In contrast, the amount of PKA-phospho-CREB was enhanced by treatment with cAMP (ca). Furthermore, CREB remained phosphorylated in cells treated with CAMP and insulin (ca/I). Thus, insulin inhibition of cAMP-induced PEPCPK expression does not require dephosphorylation of the PKA site and inactivation of CREB.

Effects of Phosphorylation of Heterologous Sites in CREB—Two other phosphorylation sites in CREB are candidates for regulation by insulin. First, Ser-129 in CREB is subject to competitive phosphorylation by GSK-3, following phosphorylation of Ser-133 by PKA, and Fiol et al. (45) reported that phosphorylation of both sites was required for full activation of CREB. Since GSK-3 has been shown to be inhibited by insulin (46–48), its inactivation could result in inefficient activation of CREB. Second, Sun et al. (49) reported that phosphorylation of Ser-142 in CREB by Cam KII had a dominant negative effect, preventing activation by CREB, even when Ser-133 is phosphorylated. Insulin could inhibit induction by targeting Ser-142 in CREB. To determine whether phosphorylation at either of these sites is involved in regulation by insulin, the corresponding serines were changed to alanines by site-directed mutagenesis and the mutant CRGs were tested by cotransfection of H4IIe cells with reporter plasmids containing either the complete G4-PEPCK promoter or the minimal CREB promoter, 5XGT-Luc (Fig. 2A).

CRG, CRG-S129A and CRG-S142A all showed induction by PKA, whereas CRG-S133A did not, with either the G4-PEPCK promoter (Fig. 2B) or the 5XGT promoter (Fig. 2C). Induction by CRG-S129A was slightly attenuated, consistent with a small contribution of phosphorylation of Ser-129 to activation in H4IIe cells. Induction by CRG-S142A was slightly enhanced, which is consistent with a negative influence of phosphorylation at that site. However, neither site dramatically affected insulin inhibition of induction by PKA. Finally, the KID domain of CREB, which contains Ser-133, was sufficient to mediate robust induction by PKA with either promoter, although total activity was quite low for 5XGT, due to the absence of other factors supporting basal transcription. Again, insulin only partially inhibited induction mediated by PKA + KID-G4 in 5XGT-Luc, whereas induction was completely inhibited in G4-PEPCK.

The extent of inhibition for each promoter was calculated and is presented in Table I. The extent of inhibition of PKA-induced transcription of 5XGT-Luc was indistinguishable from that of basal transcription for either promoter (−insulin/+ insulin = 1.2–2). In contrast, insulin inhibited PKA-induced transcription of G4-PEPCK-Luc to a greater extent (−insulin/+ insulin = 3–4), similar to the wild type PEPCPK promoter (−insulin/+ insulin = 4.1). Thus, insulin inhibition of 5XGT-Luc is cAMP-independent. These results indicate that CREB is both necessary and sufficient for induction when multiple binding sites are present, but that induction by CREB alone is not effectively inhibited by insulin. Therefore, the P-CREB-CBP-RNA polymerase II complex cannot be targeted directly by insulin, as we (39) and others (40) have proposed. Other factors, in addition to CREB, must be required for opposing regulation of PEPCPK gene transcription by cAMP and insulin.

Additional Promoter Sequences Involved in Regulation—To determine what elements in addition to the CRE of the PEPCPK promoter are required for opposing regulation by cAMP and insulin, we analyzed promoter sequences 3′ and 5′ of the minimal promoter. Since ATF/CRE sites promote extension of the RNA polymerase II complex footprint downstream of the transcription initiation site (59), we tested sequences 3′ of the minimal promoter (−40/+1) for their possible involvement in opposing regulation by cAMP and insulin. Replacement of the CRE in PEPCPK with a GAL4 site in G4-PEPCK (−600/+69) confers opposing regulation in the presence of CRG (Fig. 3). In contrast, multiple GAL4 sites ligated to either the minimal promoter (−40/+1) or the 3′ extension of the minimal promoter (−40/+69) permitted induction by PKA, but not efficient inhibition by insulin. Thus, sequences downstream of the minimal promoter that are contacted by the polymerase complex do not play a role in insulin regulation.

Given that the minimal promoter did not support opposing regulation, we analyzed 5′ truncations of the PEPCPK promoter for regulation by cAMP and insulin (Fig. 4). PEPCPK promoter elements within footprint P4 (−355/−300) that bind C/EBP and within footprint P3 (−271/−225) that bind AP-1 and C/EBP family members are required for induction by PKA in H4IIe cells, PEPCPK promoters terminating at −600, −400, −300, and −271 all supported induction by PKA and complete inhibition by insulin. Promoters terminating at −200 or −134 did not support induction by PKA, nor does a promoter terminating at −271 with a mutation in the CRE (data not shown). Thus, promoter elements in addition to the CRE and within −271 are necessary for induction by PKA.

Binding Sites for AP-1, C/EBP, CREB, and the Polymerase Complex Are Required for Regulation by Both Pka and Insulin—PEPCPK fusion gene expression is robustly induced by PKA in HepG2 cells, but is not inhibited by insulin, as it is in H4IIe cells (55), so it was not clear whether the same promoter
Fig. 4. Analysis of the role of 5′-PEPCK promoter sequences in regulation by cAMP and insulin. A map of the PEPCK promoter showing the relation of 5′-promoter end points to known regulatory elements is shown above the figure. H4IIE cells were cotransfected with the indicated luciferase reporters in the absence and presence of a PKAc expression vector, as described under “Experimental Procedures.” All PEPCK promoter sequences had +69 as the 3′-end point and the 5′-end point is indicated below the figure. Each precipitate was split into two dishes, and half of them were treated with 10 nM insulin for the final 20 h of the experiment. The results shown represent three (−200 and −134) to eight (all others) independent experiments.

DISCUSSION

The data presented here demonstrate that the P-CREB/CBP/polymerase complex is not sufficient to mediate insulin inhibition of cAMP-induced PEPCK gene transcription, as we (39) and others (40) previously proposed. Although CREB is necessary for induction of PEPCK gene transcription by PKA, it is not sufficient, as the single CRE in PEPCK does not mediate induction alone. Furthermore, even when present in several copies, CREB binding sites can mediate only induction by cAMP and not repression by insulin. Rather, an element containing putative binding sites for AP-1 and C/EBP, together with the single CREB binding site of the PEPCK gene, comprise the CRU required for induction by PKA. No additional promoter sequences other than the polymerase binding component of the minimal promoter are required. Thus, this tripartite array of transcription factors is targeted for opposing regulation by cAMP, acting through CREB, and by insulin, acting through a different factor associated directly or indirectly with the CRU.

There is considerable recent evidence for protein phosphatases playing a regulatory role in gene expression, particularly for genes involved in cell cycle progression (60–63). In addition, there have been reports of the involvement of specific phosphatases in dephosphorylation of CREB, the differences apparently arising from the cell type under study (41–43). The simplest mechanism for inhibiting PKA-induction would involve insulin activation of a CREB phosphatase. However, we show here that CREB remains phosphorylated in H4IIE cells treated with cAMP and insulin, in which transcription has been inhibited. This observation is in agreement with that of Nakajima et al. (40), although our other data presented here (Fig. 4–6) and elsewhere (64) do not support the model proposed in that report.

The finding that CREB is not dephosphorylated at the PKA site prompted us to investigate the contribution of other potentially regulatable phosphorylation sites, Ser-129 and Ser-142. The modest decrease in PEPCK activation seen with CRG-S133A is in agreement with the observation of Sun et al. (49). The increased activation seen with CRG-S142A is consistent with constitutive phosphorylation at this site restraining the activation potential of CREB. However, neither mutation (S129A, S142A) had any effect upon insulin inhibition. Thus, although these alternative phosphorylation sites in CREB can fine tune the extent of induction by PKA, they can not account for inhibition of PKA-activated PEPCK gene transcription by insulin.

The finding that CREB binding sites ligated to the minimal PEPCK promoter do not support opposing regulation by cAMP and insulin, but only induction by PKA and basal inhibition by insulin, led us to reinvestigate the promoter requirements for regulation. Analysis of 5′-truncation mutations of the PEPCK promoter showed that the sequences required for regulation lie within −271 nucleotides of the start site. The elegant work of Roesler et al. (50–54, 65, 66) showed that three putative C/EBP

Drawer
sites plus one AP-1 site are required, in addition to the CRE-containing region (−113/+69) of the PEPCK promoter, for induction by PKA in HepG2 cells. Two of the C/EBP sites lie within a footprint designated P4 (−330/−269) and one lies within footprint P3 (−264/−230), as does the AP-1 site (53, 66). In the insulin-responsive H4IIE cell line, we found no requirement for the C/EBP sites within P4, either for induction by PKA or for inhibition by insulin, although they appear to modestly augment overall expression. However, we identified a requirement for the A and C sites of P3 for opposing regulation of PEPCK gene transcription by cAMP and insulin in H4IIE cells. Deletion of either of these sites was as detrimental to induction by cAMP (and inhibition by insulin) in H4IIE cells as deletion of the single CRE site in the PEPCK promoter. In addition, we show that the only requirement for regulation beyond the minimal promoter region (−40/+1) is the AC region and the CRE, which together comprise a CRU. Like the complex GRU of the PEPCK gene, the array within the CRU contains binding sites for both general (CREB, AP-1) and liver-enriched (C/EBP) transcription factors and a signal-specific switch, in this case CREB, which is functionally analogous to the glucocorticoid receptor in regulation through the GRU.

Previous studies reported that multiple copies of CREB binding sites together with a minimal PEPCK promoter (5XGT) could mediate induction by PKA and at least partial inhibition by insulin (39). Similar findings were reported by Nakajima et al.
Inhibition of cAMP-induced transcription may result from modification of a crucial CRU component, CREB, AP-1, C/EBP, and CRE sites in the promoter, of which CREB is targeted by PKA. This unique array of factors may be recognized by a co-activator (CoA-X). Insulin inhibits basal transcription independently of its inhibition of cAMP-induced transcription. Inhibition of cAMP-induced transcription may result from modification of a crucial CRU component, CREB, AP-1 (7), and/or C/EBP (7), or from disruption of a higher order complex, such as interaction with one or more co-activators used by the CRU.

(40), who argued that insulin targeted CBP by activating pp90Rsk, which binds to CBP and is proposed to prevent induction. They used an H4IIE cell line stably transfected with a PEPCK promoter containing only 134 nucleotides of 5′-promoter sequence. We, like Roesler et al. (50), see no induction with this promoter (cf. Fig. 4). It must be noted that our previous report, showing insulin inhibition of the 5XGT promoter, was done with the less sensitive chloramphenicol acetyltransferase reporter, with which it was impossible to reliably ascertain the effects of insulin on basal transcription (39). In the present study, using the more sensitive luciferase reporter, we observed that inhibition by insulin was no greater for PKA-induced transcription of 5XGT-Luc than it was for basal transcription. In contrast, when CRG was analyzed with a complete PEPCK promoter, insulin nearly completely inhibited induction by PKA. This result is evident in analysis of both wild-type and heterologous phosphorylation site mutants of CRG (cf. Fig. 2, Table I). In light of these findings, our original hypothesis, that insulin targets the P-CREB-CBP-polymerase complex directly, appears to be untenable.

Importantly, our results and those of Roesler et al. (50, 51) suggest that the prevailing model for induction of gene expression by cAMP (i.e. that PKA-phospho-CREB binds CBP, which serves as a bridging factor to the polymerase complex) (67, 68) either is not generally applicable or is incomplete. Phosphorylation of CREB on Ser-133 by cAMP is necessary and sufficient for binding of CBP in vitro (69). In addition, CBP has been shown to interact with TFIIB (67) and RNA helicase A (68) in vitro and has been proposed to act as a bridging factor between CREB and the polymerase complex (67, 68). However, in the case of PEPCK transcription, a single binding site for CREB is insufficient by itself for induction, although it is required. Similar results have been described for cAMP-mediated induction of the aromatase gene in gonads by CREB and SF-1 (70) and for induction of the α-glycoprotein hormone gene by CREB interacting with other factors (38). For PEPCK, sites for heterologous factors are required in addition to the CREB site, but these other factors can not mediate induction by PKA in the absence of CREB (cf. Fig. 6, CRG-S133A).

Any new model for PEPCK gene regulation, such as that shown in Fig. 7, must take into account: 1) that different mechanisms are employed for activation of basal and cAMP-induced transcription; 2) that different mechanisms are used by insulin for inhibition of basal and cAMP-induced transcription; and 3) that heterologous CRU-associated factors plus CREB are required for reversible regulation of PEPCK gene expression by cAMP and insulin. The constitutive activation domain of CREB is proposed to activate basal transcription by binding TFID (71), specifically TAF110 (72), the rate-limiting component in assembly of an active RNA polymerase II complex (73). It is clear from the current study that insulin inhibits basal transcription (and PKA-induced transcription of 5XGT-Luc) by a different mechanism than is utilized for inhibition of PKA-induced PEPCK transcription. As for induction by PKA, it is possible that heterologous factors could stabilize the association of CBP and/or another co-activator (CoA-X in Fig. 7) with the PEPCK promoter in a way in which a single dimer of CREB cannot, or that these heterologous components of the CRU may contribute to activation in some other way. The specificity of this unique regulatory array would explain how insulin can inhibit transcription of the PEPCK gene while stimulating the transcription of other genes, such as gene 33, in the same H4IIE cells (34). Although, it is not yet clear how insulin might target specific components of the CRU, these factors must cooperate in a unique way to confer induction by PKA and inhibition by insulin. The fact that CREB alone can mediate induction when present in multiple copies, but that it can not mediate inhibition by insulin, argues against the P-CREB-CBP component of the CRU being targeted by insulin. The precise nature of the CRU complex and its regulation by insulin remain to be determined. However, it is clear that the same constellation of transcription factors in the CRU that is required for induction by cAMP is also required for effective inhibition of this induction by insulin.

Finally, it is of interest that inhibition of glucocorticoid induction is thought to be mediated by competition between positively and negatively acting factors for binding to the insulin response sequence (31). We show here that the PKA induction and insulin inhibition observed with the wild type PEPCK promoter can be reconstituted with the minimal promoter plus the CRU. Thus, the unique combination of transcription factors bound to CRU (or GRU) elements are probably targeted by insulin to inhibit PEPCK expression. Insulin inhibition may be mechanism specific and involve modification of factors or complexes unique to the CRU or GRU. Alternatively, a crucial factor, common to both the CRU and GRU, may be modified by insulin to inhibit gluconeogenic hormone-induced transcription of the PEPCK gene. Only elucidation of the transcription factors within the CRU and GRU that are targeted by insulin will determine whether common or distinct mechanisms are employed by insulin for inhibition of cAMP- and glucocorticoid-induced PEPCK gene transcription.

Acknowledgments—We thank Justin Cho for valuable technical assistance, David Ginty for affinity-purified anti-PKA-phospho-CREB antibody, and David Spector for critical reading of the manuscript.

REFERENCES

1. Cohen, P., Campbell, D. G., Dent, P., Gomez, N., Lavoinne, A., Nakielny, S., Stokoe, D., Sutherland, C., and Traverse, S. (1992) Biochem. Soc. Trans. 20, 671–674.
2. Cohen, P. (1992) Trends Biochem. Sci. 17, 408–413
3. Denton, R. M., Tavare, J. M., Borthwick, A., Dickens, M., Diggle, T. A., Edgell, N. J., Hall, S. W., Igoe, J. M., Lynch, D. F., Moule, S. K., Schmitz-Pfeiffer, C., and Welsh, G. I. (1992) Biochem. Soc. Trans. 20, 659–664
4. Hunter, T. (1995) Cell 80, 225–236
5. Cross, D. A., Watt, P. W., Shaw, M., van der Kaay, J., Downes, C. P., Houlder, J. C., and Cohen, P. (1997) FEBS Lett. 406, 211–215
6. Mendez, R., Kullmorgen, G., White, M. P., and Rooth, R. E. (1997) Mol. Cell. Biol. 17, 5184–5192
7. Orkin, S. H., Puett, R. W., Fehling, H. J., Lin, J., Edgell, N. J., and McCarthy, D. (1978) J. Biol. Chem. 253, 3997–4003
8. Corbin, J. D., Cobb, C. E., Beebe, S. J., Granner, D. K., Koch, S. R., Gettys, T. W., Blackmore, P. F., Francis, S. H., and Wells, J. N. (1988) Adv. Second Messenger Phosphoprotein Res. 21, 75–86
9. Hagiwara, M., Brindle, P., Harrostunian, A., Armstrong, R., Rivier, J., Yue, W., Tsien, R., and Montminy, M. R. (1993) Mol. Cell. Biol. 13, 4852–4859
10. Hassar, V. R., Tasbar, B. E., Nadiv, O., Rothenberg, P., Roberts, C. T., LeRoith, D., Yarden, Y., and Zick, Y. (1992) J. Biol. Chem. 267, 17483–17486
11. Meule, S. K., Welsh, G. I., Edgell, N. J., Foulstone, E. J., Proud, C. G., and Denton, R. M. (1997) J. Biol. Chem. 272, 7713–7719
12. Burgering, B. M. T., Medema, R. H., Maassen, J. A., van der Wetering, M. L., van der Eb, A. J., McCormick, F., and Bos, J. L. (1991) EMBO J. 10, 1103–1109
13. Medema, R. H., Wubbolts, R., and Bos, J. L. (1991) Mol. Cell. Biol. 11, 5963–5967
14. Yamashita, D., Holt, K., and Pessin, J. (1993) J. Biol. Chem. 268, 14597–14600
15. Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovic, M., and Hemmings, B. A. (1995) FASEB J. 9, 1043–1051
16. Aoki, A. M., Tassaert, L., Coudwell, B., Cron, P., Mowrye, N., Cohen, P., and Hemmings, B. A. (1996) EMBO J. 15, 6541–6551
17. Nakano, H., Brewer, K. A., and Exton, J. H. (1993) J. Biol. Chem. 268, 13–16
18. Price, D. J., Gussalu, J. R., and Avruch, J. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 7944–7948
19. Myers, M. G., Backer, J. M., Sun, X. J., Shoelson, S. E., Hu, P., Schlessinger, J., Yoakim, M., Schaffhausen, B., and White, M. F. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 10350–10354
20. Rutter, G. A., White, M. R., and Tavar, J. M. (1995) Curr. Biol. 5, 890–899
21. White, M. F., and Kahn, C. R. (1994) J. Biol. Chem. 269, 1–4
22. Granner, D. M., and Pilkis, S. (1990) J. Biol. Chem. 265, 10173–10176
23. Wynshaw-Boris, A., Short, J. M., Loose, D. S., and Hanson, R. W. (1986) J. Biol. Chem. 261, 9714–9720
24. Hanson, R. W., and Gardner, A. J. (1972) Am. J. Clin. Nutr. 25, 1010–1021
25. Luscher, T., Wehrli, F. W., and Meisner, H. M. (1989) Proc. Natl. Acad. Sci. U.S.A. 79, 5137–5141
26. Sasaki, K., Cripe, T. P., Koch, S. R., Andreone, T. L., Petersen, D. D., Beale, E. G., and Granner, D. K. (1984) J. Biol. Chem. 259, 15242–15251
27. Quinn, P. G., Wong, T. W., Magnanu, M. A., Shabb, J. B., and Granner, D. K. (1988) Mol. Cell. Biol. 8, 3467–3475
28. Magnanu, M. A., Quinn, P. G., and Granner, D. K. (1987) J. Biol. Chem. 262, 14917–14920
29. Liu, J., and Hanson, R. W. (1991) Mol. Cell. Biochem. 104, 89–100
30. Short, J. M., Wynshaw-Boris, A., Short, H. P., and Hanson, R. W. (1986) J. Biol. Chem. 261, 9721–9726
31. O’Brien, R. M., and Granner, D. K. (1991) J. Biol. Chem. 266, 609–619
32. Chu, D. T., and Granner, D. K. (1980) J. Biol. Chem. 261, 16848–16853
33. Lucas, P. C., O’Brien, R. M., Mitchell, J. A., Davis, C. M., Imai, E., Forman, B. M., Samuels, H. H., and Granner, D. K. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 2184–2188
34. Chu, D. T., Davis, C. M., Chrapkiewicz, N. B., and Granner, D. K. (1988) Mol. Cell. Biol. 8, 13097–13101
35. Frankel, A. D., and Kim, P. S. (1991) Cell 65, 717–719
36. Mitchell, P. J., and Tjian, R. (1989) Science 245, 371–378
37. Saltman, A. G., and Weinmann, R. (1989) FASEB J. 3, 1723–1733
38. Budworth, P., Quinn, P., and Nilson, J. (1997) Mol. Endocrinol. 11, 1669–1680
39. Quinn, P. G. (1994) J. Biol. Chem. 269, 14375–14378
40. Nakajima, T., Uchida, C., Anderson, S. F., Lee, C. G., Harwitz, J., Parvin, J. D., and Montminy, M. (1997) Cell 90, 1107–1112
41. Chiriva, J., Kowk, R., Lamb, N., Hagiwara, M., Montminy, M., and Goodman, R. (1993) Nature 365, 855–859
42. Caralee, D., and Richards, J. (1997) Mol. Endocrinol. 11, 292–304
43. Xing, L., Gopal, V. K., and Quinn, P. G. (1998) J. Biol. Chem. 273, 17483–17486
44. Ferreri, K., Gill, G., and Montminy, M. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 1210–1213
45. Nakajima, T., Uchida, C., Anderson, S. F., Parvin, J. D., and Montminy, M. (1997) Genes Dev. 11, 730–747