Elements of the Glucocorticoid and Retinoic Acid Response Units Are Involved in cAMP-mediated Expression of the PEPCK Gene*

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Although many genes are regulated by the concerted action of several hormones, hormonal signaling to gene promoters has generally been studied one hormone at a time. The phosphoenolpyruvate carboxykinase (PEPCK) gene is a case in point. Transcription of this gene is induced by glucagon (acting by the second messenger, cAMP), glucocorticoids, and retinoic acid, and it is dominantly repressed by insulin. These hormonal responses require the presence of different hormone response units (HRUs), which consist of constellations of DNA elements and associated transcription factors. These include the glucocorticoid response unit (GRU), cAMP response unit (CRU), retinoic acid response unit (RARU), and the insulin response unit. HRUs are known to have functional overlap. In particular, the cAMP response element of the CRU is also a component of the GRU. The purpose of this study was to determine whether known GRU or RARU elements or transcription factors function as components of the CRU. We show here that the glucocorticoid accessory factor binding site 1 and glucocorticoid accessory factor binding site 3 elements, which are components of both the GRU and RARU, are an important part of the CRU. Furthermore, we find that the transcription factor, chicken ovalbumin upstream promoter transcription factor (COUP-TF), and two coactivators, cAMP response element-binding protein and steroid receptor coactivator-1, participate in both the cAMP and glucocorticoid responses. This provides a further illustration of how the PEPCK gene promoter integrates different hormone responses through overlapping HRUs that utilize some of the same transcription factors and coactivators.

Glucose homeostasis is exquisitely controlled by the opposing actions of a variety of hormonal signals. Much of this control is achieved through the regulation of hepatic genes that encode the glycolytic and gluconeogenic enzymes. For example, glucagon (acting via the cAMP/PKA signaling pathways), glucocorticoids, thyroid hormones, and retinoic acid induce the phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32) gene, which encodes a rate-determining enzyme in gluconeogenesis. Insulin dominantly inhibits both the basal and hormone-induced expression of this gene (1–4). The hormonal regulation of PEPCK gene expression is mediated by the actions of a diverse array of transcription factors that bind to several DNA elements in the PEPCK gene promoter. The complex of factors and DNA elements required for a given hormonal response is known as a hormone response unit (HRU) (5).

The DNA elements that comprise the PEPCK glucocorticoid response unit (GRU) are illustrated at the top of Fig. 2A. This GRU consists of elements located between −467 and −300, including two tandem glucocorticoid receptor binding elements and several flanking accessory factor elements that are essential for a full response. These elements include gAF1, gAF2, and gAF3, which bind HNF-4/COUP-TF, HNF-3β, and COUP-TF, respectively (6–10). C/EBPβ also serves as a glucocorticoid response accessory factor at the CRE (6, 11). The PEPCK promoter also contains a retinoic acid response unit (RARU) that consists of two retinoic acid response elements (RARE1 and RARE2) that bind retinoic acid receptor heterodimers. RARE coincides with gAF1 of the GRU, whereas RARE2 coincides with gAF3 (12, 13).

Induction of PEPCK gene transcription by glucagon (cAMP) is mediated by a group of promoter elements and their respective transcription factors, known collectively as the cAMP response unit (CRU) (see Fig. 2A). The PEPCK gene CRU consists of several known DNA elements located between positions −300 and −86 relative to the transcriptional start site, including the CRE and the P3 and P4 elements. P3(I) and P4(II) are essential for a full cAMP/PKA response (2, 4, 14–16). These elements are located between −300 and −200 and contain binding sites for the AP-1 and C/EBP family members. The CRE is located between −93 and −86 and binds to members of the CREB/cAMP response element modulator, C/EBP, and AP-1 families of transcription factors in vitro (11, 17, 18). The CRE has three functions: 1) it binds CREB as part of the basal promoter; 2) it is essential for the cAMP response, in which case CREB is phosphorylated on glucocorticoid response unit; CRU, cAMP response unit; RARU, retinoic acid response unit; RA, retinoic acid; gAF, glucocorticoid accessory factor binding site; SRC, steroid receptor coactivator; DDME, DDDME, Dulbec-co’s modified Eagle’s medium; COUP-TF, chicken ovalbumin upstream promoter-transcription factor; HNF, hepatic nuclear factor; CRE, cAMP response element; CREB, cAMP response element-binding protein; CBP, CREB-binding protein; ChIP, chromatin immunoprecipitation; DBD, DNA binding domain; G4, Gal4; USF, upstream stimulatory factor; C/EBP, C/EBP enhancer-binding protein; RAR, retinoic acid receptor; RARE, retinoic acid response element; Luc, luciferase; PBS, phosphate-buffered saline; RRX, retinoid X receptor; G6Pase, glucose 6-phosphatase; AP-1, activator protein-1. * This work was supported in part by National Institutes of Health Grants DK02887 (to M. W.-L.), DK35107 and DK07061 (to D. K. G.), and DK20593, the Vanderbilt Diabetes Research and Training Center, and the Veterans Administration Research Service, and the Vanderbilt University School of Medicine Medical Scientist Training Program GM07347 (to D. T. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. ‡ To whom correspondence should be addressed: Dept. of Molecular Physiology & Biophysics, 707 Light Hall, Vanderbilt University School of Medicine, Nashville, TN 37232-0615. Tel.: 615-322-7004; Fax: 615-322-7236; E-mail: daryl.granner@mcmail.vanderbilt.edu. † The abbreviations used are: PKA, protein kinase A; PEPCK, phosphoenolpyruvate carboxykinase; HRU, hormone response unit; GRU, cAMP response unit; RARU, retinoic acid response unit; RA, retinoic acid; gAF, glucocorticoid accessory factor binding site; SRC, steroid receptor coactivator; DDME, Dulbecco’s modified Eagle’s medium; COUP-TF, chicken ovalbumin upstream promoter-transcription factor; HNF, hepatic nuclear factor; CRE, cAMP response element; CREB, cAMP response element-binding protein; CBP, CREB-binding protein; ChIP, chromatin immunoprecipitation; DBD, DNA binding domain; G4, Gal4; USF, upstream stimulatory factor; C/EBP, C/EBP enhancer-binding protein; RAR, retinoic acid receptor; RARE, retinoic acid response element; Luc, luciferase; PBS, phosphate-buffered saline; RRX, retinoid X receptor; G6Pase, glucose 6-phosphatase; AP-1, activator protein-1.
serine 133 by PKA; and 3) it is necessary for a full glucocorticoid response (2, 6, 14, 19).

It is important that the action of these various hormones be integrated into a response that is appropriate for the particular physiologic situation. How such integration occurs at the molecular level has yet to be resolved, but the assembly of multiple HRUs in a single promoter may explain the phenomena of additivity, synergism, and dominant repression that are observed in the presence of different combinations of hormones. As the organization of the PEPCk promoter is further defined, it is also becoming apparent that many of the individual DNA elements are involved in multiple hormonal responses. As described above, both gAF1 and gAF3 from the GRU serve as RAREs in the RARU (12, 13). Furthermore, the gAF2 element, which is essential for the glucocorticoid response, is also part of the insulin response unit (20).

This study provides a more detailed understanding of the CRU and identifies elements common to the CRU, GRU, and RARU. We show here that a deletion of certain elements, specifically gAF1/RARE1 and gAF3/RARE2, greatly reduces the response of the PEPCk gene to cAMP. Furthermore, two coactivators, steroid receptor coactivator-1 (SRC-1) and CREB-binding protein, are utilized by both the CRU and GRU to drive PEPCk gene transcription.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs—** PEPCk gene promoter truncations were constructed by PCR amplification of the appropriate regions of the PEPCk gene promoter in PEPCk/Luc. The primers used to amplify in the 5'-directed mutation were as follows: 5'-GAAAACCTGGAGGCTCGCCTC-3' and 5'-CGGGGTACCCCGAGCCTATAGTTTGCATCAGC-3'. The QuikChange site-directed mutagenesis kit (Stratagene) was used to introduce two mutations into the resulting fragment into pGL3b at the corresponding restriction site.

The truncated PEPCk gene promoter from PEPCk/Luc was amplified by PCR with primers 5'-CGGGAATTCAGCTGTAGTTTGCATCAGC-3' and 5'-GGCTGGTACCGAGCCTATAGTTTGCATCAGC-3'. The PCR products were digested with restriction enzymes, and the resulting fragments were ligated into pGL3b at the corresponding restriction sites. The resulting plasmids were sequenced. The sequence of all subcloned fragments was verified by DNA sequencing.

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**Cell Culture and Transient Transfection—** H4IIE hepatoma cells were grown to confluence in Dulbecco’s modified Eagle medium (DMEM) containing 2.5% (v/v) newborn calf serum and 2.5% (v/v) fetal bovine serum. Cells were cross-linked with 1% formaldehyde (Fisher Scientific) for 10 min. The lysates were transferred to pre-chilled Eppendorf tubes containing 25-μm diameter glass beads (ratio of lysate to bead volume was 1:1), pre-washed with cell scraping buffer. To shear chromatin, the lysate/bead mixture was sonicated (Virtis; 2.5-mm tip) on ice for 12 × 10-s pulses at a setting of 3 (output of 4–5 watts), yielding an average fragment size of 150 bp in size. Sheared chromatin was centrifuged at 14,000 rpm for 10 min at 4 °C to remove debris, and the supernatant was divided into aliquots for subsequent 10-fold dilution in ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 1.2% DMEM, pH 8, 16.7 mM Tris-HCl, pH 8, with protease and phosphatase inhibitors). To provide a positive control for each condition, one undiluted aliquot was retained for further processing in parallel with all other samples at the reversal of cross-linking step. To reduce nonspecific background, 1 ml chromatin sample was pre-cleared with 10 μl of protein A/G PLUS-agarose slurry (Santa Cruz Biotechnology, Inc.), supplemented with 100 μg/ml sonicated salmon sperm DNA (Stratagene), for 1 h at 4 °C on a rotating wheel, after which the beads were pelleted, and the supernatant was transferred to a new tube. Chromatin complexes were immunoprecipitated for 12–18 h at 4 °C while rotating with amounts (5–10 μg) of primary antibody optimized for selective immunoprecipitation of signal or without antibody to provide negative controls. Immunocomplexes were collected with 40 μl of protein A/G-agarose + 100 μg/ml salmon sperm DNA, while rotating for 3 h at 4 °C, followed by centrifugation at 1000 × g for 1 min at 4 °C. The beads were washed for 5 min at 4 °C with 1 ml of each of the following buffers in succession: low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 ml DNA wash buffer, 0.1% SDS, 1% Triton X-100, 1.2% DMEM, pH 8, 20 mM Tris-HCl, pH 8, 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 1.2% DMEM, pH 8, 20 mM Tris-HCl, pH 8, 500 mM NaCl), LiCl wash buffer (0.25 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, pH 8, 10 mM Tris-HCl, pH 8), and twice with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). Chromatin complexes were eluted from the beads in three consecutive 30-min rotating incubations with 200 μl of elution buffer (1% SDS, 0.1 mM NaHCO3) at 22 °C. To reverse cross-linking and digest DNA present in the samples, NaCl (200 mM final concentration) and RNase mixture (Ambion) were added, and the samples were incubated at 65 °C for ≥6 h. To digest proteins, samples were incubated at 45 °C for 90 min after the addition of the following at their final concentrations: 10 mM EDTA, 40 mM Tris-HCl, pH 6.5, and 50 μg/ml protease K. Samples were extracted twice with phenol/chloroform/isooamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1). DNA was precipitated with 5 μg of glycogen (Promega) and 20 vol of 95% ethanol and collected by microcentrifugation for 30 min at 4 °C. Samples were resuspended in 100 μl of nuclease-free water (Promega) and stored at −80 °C for subsequent PCR analysis.

**RESULTS**

**CBP and SRC-1 Associate with the Endogenous PEPCk Gene Promoter in Response to cAMP—** CBP and SRC-1 are recruited to the PEPCk gene promoter in response to dexamethasone, a classic glucocorticoid. As shown previously (22), CBP typically causes a 2- to 3-fold induction of PEPCk mRNA levels in H4IIE cells, as confirmed in the experiment shown in Fig. 1A. We wanted to determine whether the same coactivators that associate with the PEPCk gene promoter after dexamethasone treatment also associate in response to CAMP. The ChIP assay was therefore used to directly identify proteins that interact with the PEPCk gene promoter in response to cAMP treatment of H4IIE cells in vivo (Fig. 1B). We found that CBP interacts with the PEPCk promoter after CAMP treatment. This result was expected, because CBP action is regulated by CAMP signaling pathways (27, 28). It was somewhat surprising to observe recruitment of SRC-1 to the PEPCk gene promoter in response to cAMP, however, because relatively few studies

*D. T. Duong, unpublished data.*
exist showing the involvement of this coactivator in cAMP action. SRC-1 is a coactivator with COUP-TF, which is an important mediator of the glucocorticoid response in the PEPCK gene, and CBP interacts with CREB on a variety of promoters. ChIP assays were therefore performed using a COUP-TF or CREB antibody. We found that COUP-TF is constitutively bound to the promoter, in agreement with previous electrophoretic gel shift mobility analysis of the PEPCK gene promoter (10). CREB is also bound to the promoter under basal conditions, and binding was not increased when cells were treated with cAMP, as unchanging bands were detected under these conditions (Fig. 1B). These data suggest that CREB and COUP-TF are constitutively bound to the PEPCK gene promoter, whereas CBP and SRC-1 are recruited in response to cAMP treatment. The next set of experiments were performed to specifically locate the region of the PEPCK promoter targeted by COUP-TF, CREB, CBP, and SRC-1.

**CREB and CBP Function at the CRE to Mediate the PKA Response**—The CRE is necessary for both cAMP- and glucocorticoid-mediated PEPCK gene expression, and CBP is likely to act through the proteins bound to this element. H4IIE rat hepatoma cells were transiently transfected with a construct that contains the region from −467 to +69 of the PEPCK gene promoter positioned upstream of the luciferase reporter gene (referred to as PEPCK/Luc). This construct typically gives a 2- to 4-fold induction when cotransfected with the catalytic subunit of PKA (2.5 µg) in the presence or absence of expression vectors encoding the catalytic subunit of PKA (2.5 µg) or CBP (10 µg) or an equivalent amount of plasmid DNA as a control. Cells were treated for 18 h in serum-free DMEM, and cell lysates were prepared for luciferase assays as described under “Experimental Procedures.” The results illustrated in panel A are normalized to the basal activity of PEPCK/Luc in the presence of G4DBD alone, and the data represent the mean of four experiments ± S.E.

PKA-stimulated PEPCK gene expression was examined next. Replacement of the CRE with a Gal4 DNA binding element (referred to as (C/G)-PEPCK/Luc) resulted in a 50% reduction of the PKA response (Fig. 2B, compare lanes 2 and 4). The same
H4IIE cells were transiently transfected with (C/G)-PEPCK/Luc (5 μg) or constructs in which (C/G)-PEPCK/Luc was truncated to result was observed for the glucocorticoid response in earlier experiments, as either deletion of the CRE or its replacement with a Gal4 DNA binding element also reduces this response by 50% (6, 11). Others have shown that G4CREB (a chimeric construct in which the Gal4 DNA binding domain (G4DBD) is ligated to full-length CREB) is effective at restoring the PKA response when it binds to a Gal4 element that has replaced the CRE (29). This result is confirmed here, as G4CREB increased the PKA response ~3-fold over that mediated by the G4DBD alone (Fig. 2B, lanes 4 and 7). The activity mediated by G4CREB is higher than that mediated by PKA alone. Wilson et al. (30) showed recently that, besides G4CREB, G4C/EBPα and G4C/EBPβ also mediate this response (30). It is therefore possible that different combinations of these factors bind to the CRE to mediate the PKA response of the endogenous PEPCK gene. CBP is an important coactivator for glucocorticoid-mediated PEPCK gene expression, and it has been shown to drive PEPCK gene transcription by interacting with proteins bound to the CRE (22). The role of CBP at the CRE was therefore examined for the PKA response. CBP enhanced the PKA response of wild-type PEPCK/Luc by ~2-fold (Fig. 2A, lanes 2 and 4) and of G4CREB by 3- to 4-fold (Fig. 2B, compare lanes 6 and 8).

GRU/RARU Elements Are Necessary for the PKA Response

A G4CBP construct was cotransfected with (C/G)-PEPCK/Luc to further explore the role of CBP in PKA-mediated gene expression (Fig. 3A). G4CBP, in the absence of cotransfected PKA, increased reporter gene activity 7-fold (Fig. 3A, lanes 1 and 2). Cotransfection of PKA further increased the response to nearly 20-fold over the basal value (Fig. 3A, lane 3); thus CBP has substantial activity at the CRE, even in the absence of CREB binding at the CRE. This activity is much greater than the 3- to 4-fold response observed in the experiment illustrated in Fig. 2B.

CBP interacts with several coactivators to stimulate gene transcription. It is thus possible that CBP interacts with upstream coactivators to mediate the PKA response. A series of 5' deletions (to positions −299, −200, −120, and −100 relative to the transcription start site) of (C/G)-PEPCK/Luc was constructed to examine the role of upstream promoter elements/factors in the PKA response. Truncation of the promoter to −299, which removes the GRU elements gAF1 (RARE1 of the RARU), gAF2, and gAF3 (RARE2 of the RARU), nearly abolished the PKA response when G4CBP is bound to a Gal4 element at the CRE (Fig. 3A, lanes 4 and 5). Removal of the P3 and P4 regions by truncation to −200, or further deletion to −120, provides reporter gene constructs that exhibit no PKA response (Fig. 3A, lanes 6–9). Further truncation of the promoter to −100, which removes the CAAT box, an element that contains a binding site for the transcription factor NF1, resulted in a reporter gene with greatly decreased basal activity and no PKA responsiveness, even in the presence of G4CBP (Fig. 3A, lanes 10 and 11).

To further investigate the involvement of the GRU/RARU DNA binding elements, the context of the wild-type pro-

![Fig. 3. GRU and RARU elements are necessary for the PKA response](image-url)

—299, −200, −120, or −100. These various reporter constructs were transfected in the presence or absence of 1 μg of expression vectors encoding G4DBD or G4CBP or an equivalent amount of plasmid DNA (panel A). The data are normalized to the basal activity of (C/G)-PEPCK/Luc in the presence of G4DBD and represent the mean of four experiments ± S.E. Deletion constructs of PEPC/Luc were also made and are illustrated at the top of panel B. The GRU/RARU elements were added 5' of the −200 deletion construct (referred to as (GRU/RARU)/−200). These reporter constructs (5 μg) were transfected into H4IIE cells in the presence or absence of an expression vector encoding the catalytic subunit of PKA (2.5 μg) or an equivalent amount of plasmid DNA (panel B). The data are normalized to the basal activity of PEPC/Luc, and the data represent the mean of four experiments ± S.E.
motor, 5′ deletions (to −299, −200, and −120) were made in PEPCKLuc (Fig. 3B). In this circumstance, the PKA response was also inhibited by truncation of the PEPCKLuc promoter to −299 (Fig. 3B, compare lanes 1 and 2 with lanes 3 and 4). Truncation of the promoter to −200 and −120 also resulted in a lack of stimulation by PKA (Fig. 3B, lanes 5–8). In fact, there was a trend toward repression in the presence of the catalytic subunit of PKA (Fig. 3B). The role of the GRU/RARU elements was further verified by adding these elements (bases −467 to −300 relative to the transcription start site) immediately upstream of the −200 construct, referred to as (GRU/RARU)/−200. H4IIE hepatoma cells were transiently transfected with this construct, and a restoration of the PKA response was observed (Fig. 3B, lanes 9 and 10). These results confirm that some component of the GRU/RARU is necessary for full stimulation of PEPCKLuc by PKA.

gAF1 and gAF3 of the GRU/RARU Mediate the PKA Response—In addition to the CRE, the GRU consists of the elements gAF1, gAF2, and gAF3 that bind to HNF-4/COUP-TF, HNF-3β, and COUP-TF, respectively (8–10). The RARU consists of two RAREs that are coincident with gAF1 and gAF3 and bind retinoic acid receptor (RAR)/retinoid X receptor (RXR) heterodimers (12, 13). The gAF elements were initially identified by deletion and mutational analysis, and the identity of the factors that bind these elements was later confirmed using an electrophoretic mobility gel shift assay, the Gal4 system, and the ChIP assay. A similar approach was used to evaluate the role of the GRU/RARU in the PKA response, starting with the Gal4 system. Each of the GRU or RARU elements was replaced by a Gal4 binding site, and these constructs were tested for PKA responsiveness. Replacement of gAF2 had no effect on the PKA response (Fig. 4A, compare lanes 1 and 2 with lanes 3 and 4). Mutation of either gAF1/RARE1 or gAF3/RARE2, however, significantly reduced this response (Fig. 4A, compare lanes 1 and 2 with lanes 5 and 6 or with lanes 7 and 8). Because replacement of gAF3/RARE2 with the Gal4 DNA binding site was particularly detrimental to the PKA response, we focused our attention on this element.

The gAF3/RARE2 element and an immediately adjacent E box bind COUP-TF and USF, respectively (Fig. 4B, top (10)). RARE2 is also a DR5-type RARE composed of three half-sites (α, β, and γ) (Fig. 4B, top). RARE2 binds an RAR/RXR heterodimer but only in the presence of retinoic acid (13). Because retinoic acid is not necessary for the PKA response, it is unlikely that RAR/RXR play a role in this response. We therefore wanted to determine whether COUP-TF or USF mediate the PKA response. Methylation interference studies show that COUP-TF makes contact with the γ half-site, and USF binds to the E box. Furthermore, mutations that prevent COUP-TF or USF binding to the PEPCK promoter show that the γ half-site is necessary for the glucocorticoid response, whereas the E box is not involved (10, 13). The mutations shown to prevent binding of COUP-TF or USF to the PEPCK promoter were therefore introduced into PEPCKLuc to examine for a possible role of these sites in the PKA response. Mutation of the γ half-site is referred to as AF3γm and prevents COUP-TF binding, whereas the E box mutation is referred to as AF3Em and prevents USF binding. In transient transfection assays of H4IIE hepatoma cells, a mutation of the E box had no effect on the PKA response (Fig. 4B, compare lanes 1 and 2 with lanes 3 and 4), but a mutation of the γ half-site completely abolished this response (Fig. 4B, lanes 5 and 6).

Treatment of H4IIE cells with cAMP did not cause additional protein complexes to bind to gAF3/RARE2 in electrophoretic mobility shift.

Fig. 4. gAF1 and gAF3 are essential for the PKA response. H4IIE cells were transiently transfected with 5 μg of PEPCKLuc or PEPCKLuc in which the gAF1, gAF2, or gAF3 elements were replaced by a Gal4 DNA binding element. These constructs were cotransfected with 2.5 μg of an expression vector encoding the catalytic subunit of PKA or an equivalent amount of plasmid DNA (panel A). The data are normalized to the basal activity of PEPCKLuc and represent the mean of eight experiments ± S.E. The PKA responses of gAF1/Gal4 and gAF3/Gal4 were significantly reduced (+, p < 0.05; **, p < 0.01 by Student’s t test). The sequence of the region containing the gAF3 element, with the sites for COUP-TF and USF binding as revealed by DNA methylation studies, is illustrated at the top of panel B. The γ half-site or the E box was mutated in PEPCKLuc, and 5 μg of each construct was transiently transfected into H4IIE cells with or without 2.5 μg of the catalytic subunit of PKA (panel B). The data are normalized to the basal activity of PEPCKLuc and represent the mean of eight experiments ± S.E.

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These data strongly suggest that COUP-TF, which is a member of the GRU, is involved in the PKA response. These observations are compatible with the ChIP data presented in Fig. 1.

**COUP-TF and SRC-1 Rescue the PKA Response**—COUP-TF, which was originally identified as a stimulatory transcription factor required for the expression of the chicken ovalbumin gene (31), also acts as a repressive factor in that it counteracts the positive transcriptional effects mediated by factors such as RAR/RXR, thyroid hormone receptor/RXR, 1,25-dihydroxyvita-

**Fig. 5. COUP-TF and SRC-1 rescue the PKA response.** H4IIE cells were transiently transfected with 5 μg of PEPCK/Luc or PEPCK/Luc with gAF3 mutated to the Gal4 DNA binding element (referred to as gAF3/G4) in the presence or absence of 2.5 μg of an expression vector encoding the catalytic subunit of PKA. In addition, 1 μg of an expression vector encoding either G4DBD or G4COUP-TF was cotransfected in these experiments (panel A). The data are normalized to the basal activity of PEPCK/Luc and represent the mean of four experiments ± S.E. Experiments identical to those performed in panel A were performed in panel B except that G4SRC-1 or G4SRC-1 Ala/Ala (a construct with serine residues 1179 and 1185 mutated to an alanine) was employed. The data are normalized to the basal activity of PEPCK/Luc and represent the mean of eight experiments ± S.E. The activity of G4DBD, G4SRC-1, and G4SRC-1 Ala/Ala on the E1b promoter was also examined by transfecting H4IIE cells with 1 μg of each construct and 5 μg of the 5× Gal4/E1b-Luc reporter gene construct. The data are normalized to the activity of 5× Gal4/E1b-Luc in the presence of G4DBD and represent the mean of four experiments ± S.E. (panel C).
Regulation of PEPCK Gene Expression by cAMP

...min D3 receptor/RXR, peroxisome proliferator-activated receptor/RXR, HNF-4, and estrogen receptor (32–35). In the context of the PEPCK gene, COUP-TF acts as a stimulatory factor at gAF3, and it is required for a complete glucocorticoid response. A chimeric protein in which the G4DBD is ligated to COUP-TF was used to determine whether COUP-TF can rescue the PKA response of PEPCK/Luc when gAF3/RARE2 is replaced by a Gal4 DNA binding element. Although the G4DBD did not mediate a PKA response when bound to gAF3/RARE2, G4COUP-TF rescued this response (Fig. 5A, compare lanes 3 and 4 with lanes 5 and 6).

SRC-1 is a coactivator with COUP-TF and is an important regulator of the glucocorticoid response of the PEPCK gene promoter (36–38). Furthermore, SRC-1 is regulated by cAMP, as 8-bromo-cAMP treatment increases the phosphorylation of SRC-1 on threonine 1179 and serine 1185, an effect that is inhibited by the PKA inhibitor (38). Phosphorylation of these sites is necessary for a functional cooperation between SRC-1 and CBP for coactivation of the progesterone receptor (39). A chimeric protein in which the G4DBD is ligated to wild-type SRC-1 was therefore tested at gAF3/RARE2 to determine whether SRC-1 can also rescue the PKA response. Although G4DBD did not mediate a PKA response when bound to gAF3/RARE2, G4SRC-1 rescued the PKA response (Fig. 5B, compare lanes 3 and 4 with lanes 5 and 6).

Experiments were also performed to determine whether the threonine 1179 and serine 1185 phosphorylation sites of SRC-1 are essential for the PKA response. H4IIE cells were cotransfected with a PEPCK/Luc reporter gene in which gAF3 is replaced by a Gal4 DNA binding element and with a G4SRC-1 construct in which residues 1179 and 1185 are mutated to alanine (referred to at G4SRC-1 Ala/Ala). A mutation of these two amino acids completely prevented responsiveness of the PEPCK gene to PKA (Fig. 5B, compare lanes 5 and 6 with lanes 7 and 8), suggesting that either the phosphorylation of these residues and/or the interaction of this region of SRC-1 with other factors is important. The expression of G4SRC-1 Ala/Ala and wild-type G4SRC-1 could not be tested in H4IIE because of the extremely low transfection efficiency of these cells. Instead, H4IIE cells were cotransfected with the 5x Gal4/E1b-Luc construct that has five Gal4 DNA binding elements upstream of the E1b promoter and luciferase reporter gene. Cells were cotransfected with G4DBD, G4SRC-1, or G4Ala/Ala. As shown in Fig. 5C, G4SRC-1 and G4SRC-1 Ala/Ala had comparable activity at this promoter, suggesting that these two chimeric proteins are expressed at comparable levels. The data also show that residues 1179 and 1185 of SRC-1 are specifically important for expression of the PEPCK gene promoter but not the E1b gene.

SRC-1 at gAF3/RARE2 Does Not Effectively Mediate the Retinoic Acid or Glucocorticoid Response—Because this is the first time that SRC-1 was shown to function at gAF3/RARE2 (Fig. 5B), we wanted to determine whether SRC-1 also plays a role at this element for the retinoic acid (RA) and glucocorticoid responses of the PEPCK gene. SRC-1 supports the glucocorticoid response when tethered to gAF1/RARE1 and gAF2 (36), so it is possible that it also functions at gAF3/RARE2. The role of SRC-1 for the RA response of the PEPCK gene has yet to be studied. Experiments were performed, therefore, to determine whether G4SRC-1 rescues either the RA or glucocorticoid responses when bound at gAF3/RARE2. G4SRC-1 Ala/Ala was also used to determine the role of residues 1179 and 1185 of SRC-1 for these responses. As expected, both the RA and glucocorticoid responses were markedly reduced when gAF3/RARE is mutated to a Gal4 DNA binding site (Fig. 6, compare lanes 2 and 5 and lanes 3 and 6). Unlike the PKA response, however, G4SRC-1 was unable to recover the RA response, which was somewhat surprising, because SRC-1 can coactivate with RAR/RXR heterodimers (40, 41) (Fig. 6, compare lane 8 with lane 2). G4SRC-1 caused a small increase of the glucocorticoid response when tethered to gAF3/RARE2 but did not recover the response to the full extent, as observed at gAF1/RARE1 and gAF2 (36) (Fig. 6, compare lane 9 with lane 3). Although not significant, mutation of residues 1179 and 1185 partially blunted the small recovery of the glucocorticoid response mediated by SRC-1 (Fig. 6, compare lanes 9 and 12), suggesting that these residues may play a role in the glucocorticoid response.

DISCUSSION

Transcription of the PEPCK gene is regulated by a number of hormones, including glucagon (acting via PKA), glucocorticoids, retinoic acid, and insulin (5, 42–44). Each hormone response is mediated by a set of cis-elements and associated transcription factors that are collectively termed a hormone response unit or HRU. In this study, we further describe the components of the PEPCK CRU. The CRU was originally defined as extending from −300 to −86 relative to the transcriptional start site (2). This region encompasses the CRE, which binds the CREB/cAMP response element modulator, C/EBP, and AP-1 families of transcription factors, and the P3(I) and P4(II) elements, which bind to members of the C/EBPs and AP-1 families of transcription factors (2, 4, 11, 15–18). We find here that the CRU requires the participation of additional upstream elements, as the deletion or mutation of components of the GRU/RARU severely restricts the PKA response. A mutation of the gAF1/RARE1 or gAF3/RARE2 elements, which are...
shared by both the GRU and RARU, significantly reduces the PKA response. On the other hand, a mutation of gAF2, which disables its ability to function in the GRU, has no effect on the PKA response.

Although a simple hormone response element may provide "on or off" control, a HRU provides a more flexible and versatile means of regulating gene transcription and thus may allow for a more precise control of a specific metabolic process. The sharing of components between several HRUs provides additional flexibility and regulatory control. Several genes, in addition to PEPCK, encode metabolic enzymes that integrate multiple hormone responses. For example, the tyrosine aminotransferase and insulin-like growth factor-binding protein 1 genes possess GRUs that contain an insulin response sequence that overlaps with the HNF-3 binding site, allowing insulin to repress these genes in a dominant manner over glucocorticoid-mediated activation (45–47). Mutation of two insulin response elements that bind to HNF-3 and NF1-like proteins prevents insulin-mediated repression of the cytosolic aspartate aminotransferase gene. Mutation of these elements also partially decreases the response of this gene to glucocorticoids (48).

Furthermore, the hepatic glucose 6-phosphatase (G6Pase) gene is composed of different overlapping HRUs that mediate its activation by cAMP and glucocorticoids and dominant repression by insulin (49–53). A CRE is an accessory factor element for both the CRU and GRU of hepatic G6Pase, whereas an HNF-1α DNA binding element is an accessory factor site for the hepatic G6Pase GRU and is also an accessory factor element for the G6Pase CRU in the kidney (49, 54, 55).

With regard to the PEPCK gene, gAF1 and gAF3 of the GRU are also RAREs that comprise a functional RARU. It is unlikely that RAR/RXR is involved in the PKA response, because retinoic acid is not present in our culture media. Furthermore, we have found that RAR is recruited to the PEPCK promoter only in the presence of retinoic acid.4 We find here that COUP-TF binding to gAF3/RARE2, which is essential for a full glucocorticoid response, is also required for a full PKA response. In addition, the coactivators SRC-1 and CBP are shared by both the PEPCK CRU and GRU. This overlapping structure of the PEPCK gene promoter is termed a metabolic control domain (5, 13, 56). Our current thinking is that the arrangement of several functionally interacting HRUs may explain the phenomena of additivity, synergism, and dominant repression observed in the presence of different combinations of hormones. The metabolic control domain may thus integrate the action of a variety of hormonal signals to provide a transcriptional response that is appropriate for a particular physiological process, which is gluconeogenesis in the case of the PEPCK gene.

Different hormonal inputs have combinatorial effects on PEPCK gene expression. For instance, glucocorticoids and cAMP have additive effects, whereas glucocorticoids and retinoic acid have additive to synergistic effects on PEPCK gene transcription.5 The fact that both gAF1/RARE1 and gAF3/RARE2 are components of each of these three hormone responses suggests that the factors that bind to these sites, and their associated coregulators, play a central role in these combined stimulatory effects. Several mechanisms may explain how these hormones act together to enhance PEPCK gene expression. For example, it is possible that a greater number of CBP and SRC-1 molecules are recruited to the PEPCK gene promoter under combined treatment with dexamethasone and cAMP, although we have not observed an increase in the association of these coactivators with the PEPCK promoter under these conditions in the ChIP assay (data not shown). Alternatively, these hormones may cause a modification of the coactivators recruited to the PEPCK gene promoter. PKA signaling causes phosphorylation of SRC-1 at residues 1179 and 1185 (39), and both of these sites are important for the component of the PKA response that is mediated through gAF3/RARE2. Furthermore, SRC-1 potentiates the activity of COUP-TF when COUP-TF is bound to the DR4 element of the CYP1A1 gene promoter, and COUP-TF associates with SRC-1 in vivo (37). We are currently trying to determine whether a direct interaction between COUP-TF and SRC-1 is important for the PKA response of the PEPCK gene. Perhaps phosphorylation of SRC-1 is necessary for such an interaction. Furthermore, the phosphorylation of the coactivators that associate with gAF1/RARE1 and gAF3/RARE2 may lead to their interaction with the basal transcription machinery, thereby increasing the rate of transcription.

CBP is also recruited to the PEPCK gene promoter in response to glucocorticoid or cAMP treatment. We show here that CBP works at the CRE to mediate the PKA response. CBP most likely interacts with CREB at the CRE, although it is possible that other factors, such as C/EBPα, may mediate this response, as well. C/EBPβ, which also interacts with CBP, binds to the CRE to mediate the glucocorticoid response. This response is enhanced by CBP, which also suggests a role for this coregulator at the CRE (22). Little is known about phosphorylation of CBP, although it contains several PKA consensus phosphorylation sites. It is noteworthy that insulin, a repressor of PEPCK gene transcription, causes the dissociation of CBP from the PEPCK promoter, underscoring the importance of the interaction of this coactivator with the promoter for induction of PEPCK gene expression (11, 22).

Organization of a gene promoter into multiple, interacting HRUs may allow for a more precise regulation of gene transcription in the face of fluctuating environmental conditions. The PEPCK gene is used as a model system to study the integration of multiple hormones into a transcriptional response. The organization of its promoter into multiple HRUs, and the inherent redundancy found therein, may help prevent the dysregulation of this gene. For instance, deletion of gAF1, gAF2, gAF3, or the CRE still allows for 50% of the PEPCK response to glucocorticoid or cAMP treatment. We show here that CBP works at the CRE to mediate the PKA response. CBP most likely interacts with CREB at the CRE, although it is possible that other factors, such as C/EBPα, may mediate this response, as well. C/EBPβ, which also interacts with CBP, binds to the CRE to mediate the glucocorticoid response. This response is enhanced by CBP, which also suggests a role for this coregulator at the CRE (22).

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REFERENCES
1. Sasaki, K., Cripe, T. P., Koch, S. R., Androne, T. L., Petersen, D. D., Beale, E. G., and Granner, D. K. (1984) J. Biol. Chem. 259, 15242–15251
2. Liu, J. S., Park, E. A., Gurney, A. L., Roessler, W. J., and Hanson, R. W. (1991) J. Biol. Chem. 266, 19095–19102
3. Imai, E., Stromstedt, P. E., Quinn, P. G., Carlstedt-Duke, J., Gustafsson, J. A., and Granner, D. K. (1990) Mol. Cell. Biol. 10, 4712–4719
4. Hanson, R. W., and Roessler, L. (1997) Annu. Rev. Biochem. 66, 581–611
5. Lucas, P. C., and Granner, D. K. (1992) Annu. Rev. Biochem. 61, 1131–1173
6. Imai, E., Miner, J. N., Mitchell, J. A., Yamamoto, K. R., and Granner, D. K. (1993) J. Biol. Chem. 268, 3535–3556
7. Petersen, D. D., Magnuson, M. A., and Granner, D. K. (1988) Mol. Cell. Biol. 8, 96–104
8. Wang, J. C., Stromstedt, P. E., O’Brien, R. M., and Granner, D. K. (1996) Mol. Endocrinol. 10, 794–800
9. Hall, R. K., Sladek, F. M., and Granner, D. K. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 412–416
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