Glucocorticoids Regulate Transcription of the Gene for Phosphoenolpyruvate Carboxykinase in the Liver via an Extended Glucocorticoid Regulatory Unit*

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The hepatic transcriptional regulation by glucocorticoids of the cytosolic form of phosphoenolpyruvate carboxykinase (PEPCK-C) gene is coordinated by interactions of specific transcription factors at the glucocorticoid regulatory unit (GRU). We propose an extended GRU that consists of four accessory sites, two proximal AF1 and AF2 sites and their distal counterpart dAF1 (−993) and a new site, dAF2 (−1365); together, these four sites form a palindrome. Sequencing and gel shift binding assays of hepatic nuclear proteins interacting with these sites indicated similarity of dAF1 and dAF2 sites to the GRU proximal AF1 and AF2 sites. Chromatin immunoprecipitation assays demonstrated that glucocorticoids enhanced the binding of FOXO1 and peroxisome proliferator-activated receptor-α to AF2 and dAF2 sites and not to dAF1 site but enhanced the binding of hepatic nuclear transcription factor-4α only to the dAF1 site. Insulin inhibited the binding of these factors to their respective sites but intensified the binding of phosphorylated FOXO1. Transient transfections in HepG2 human hepatoma cells showed that glucocorticoid receptor interacts with several non-steroid nuclear receptors, yielding a synergistic response of the PEPCK-C gene promoter to glucocorticoids. The synergistic stimulation by glucocorticoid receptor together with peroxisome proliferator-activated receptor-α or hepatic nuclear transcription factor-4α requires all four accessory sites, i.e. a mutation of each of these markedly affects the synergistic response. Mice with a targeted mutation of the dAF1 site confirmed this requirement. This mutation inhibited the full response of hepatic PEPCK-C gene to diabetes by reducing PEPCK-C mRNA level by 3.5-fold and the level of circulating glucose by 25%.

Transcription of the gene for PEPCK-C (EC 4.1.1.32) is acutely controlled in a tissue-specific manner by diet and hormones (1, 2). The interaction between glucagon (acting via cAMP) and glucocorticoids that stimulate gene transcription and insulin, which inhibits this process, determines the level of hepatic PEPCK-C. Glucocorticoids stimulate transcription of this gene in the liver and kidney cortex (3) and inhibits it in adipose tissue (4); metabolic acidosis induces PEPCK-C gene transcription in the kidney cortex but has no effect in the liver (3). A number of transcription factors have been implicated in this complex, tissue-specific regulation of PEPCK-C gene transcription (5–8).

Glucocorticoids play a particularly important role in coordinating the control of PEPCK-C gene transcription in a tissue-specific manner. Olswang et al. (9) reported that glucocorticoids repressed PEPCK-C gene transcription in adipose tissue by interfering with the DNA binding of members of the C/EBP family of transcription factors to specific sites in the gene promoter. Glucocorticoids are also known to inhibit the transcription of the gene encoding C/EBPα in adipocytes (10). The induction of renal PEPCK-C gene transcription by glucocorticoids requires an intact and occupied hepatic nuclear factor 1 (HNF-1) binding site, which is the only renal-specific binding site identified to date in the PEPCK-C gene (7); this site is not required for PEPCK-C gene transcription in either the liver or adipose tissue (6, 11, 12).

An important advance in understanding the mechanisms by which glucocorticoids alter the regulation of PEPCK-C gene transcription stems from the work of Granner and co-workers (13), who have identified a region of the PEPCK-C gene promoter, which they termed the glucocorticoid regulatory unit (GRU). This region of the gene promoter extends from approximately −455 to −321 and contains three accessory protein binding domains (14), one of which (AF2) overlaps a site in the promoter that is involved in the repression of PEPCK-C gene transcription by insulin (15). The AF1 site binds HNF-4α (16), chicken ovalbumin upstream transcription factor (COUP-TF) (17), peroxisome proliferator activated receptor (PPARγ) (18), retinoic acid receptor (RARα) (19), and retinoid X receptor (RXRα) (20), whereas AF2 binds members of the Forkhead family of transcription factors, including FOXO1 (21) and HNF-3β (Foxa2) (22). Recently Stoffel and co-workers (23) reported that the AF2 site in the PEPCK-C gene promoter preferentially binds FOXO1 and only minimally HNF-3β. It has been proposed that C/EBPβ interacts at this site with other transcription factors as part of a nucleoprotein complex to regulate PEPCK-C gene transcription (24).

Despite the key role played by the GRU in the control of PEPCK-C gene expression, there is evidence that it is not sufficient to entirely explain the effects of glucocorticoids on transcription of this gene. A segment of the PEPCK-C gene promoter from −540 to +73 bp was shown to be sufficient to confer full hepatic expression of a transgene in mice (25–27). However, sequences upstream of position −540 play regulatory roles beyond the basal expression of the gene. For example, in a

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2 The abbreviations used are: PEPCK-C, the cytosolic form of phosphoenolpyruvate carboxykinase; HSS, hypersensitive site; GR, glucocorticoid receptor; GRE, GR response element; GRU, glucocorticoid response unit; RAR, retinoic acid receptor; RXR, retinoid X receptor; PPAR, peroxisome proliferator-activated receptor; PPARα, PPARγ, PPARδ; RXRα, RXRβ, RXRγ; RT, reverse transcriptase; CAT, chloramphenicol acetyltransferase; FOXO1, Forkhead box class 1-α, Forkhead box receptor; Foxa2, Forkhead box class A-2; HNF-3β, hepatocyte nuclear factor 3β; HNF-4α, hepatocyte nuclear factor 4α; COUP-TF I and II, chicken ovalbumin upstream transcription factor I and II, respectively; CHIP, chromatin immunoprecipitation.
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fashion similar to the endogenous PEPCK-C gene, a transgene driven by a region of the gene promoter from −2000 to +73 is not expressed in the fetal liver (26, 27), whereas a transgene driven by segment of the gene promoter from 540 to +73 is expressed (28). These findings are supported by results from transient transfection experiments using Hepa 1C1C7 mouse hepatoma cells, which mimic the fetal liver. In these experiments the activity of a segment of the PEPCK-C gene promoter from −600 to +73 has a rate of transcription that is 5-fold higher than that of the counterpart 2000-bp gene promoter (28). Finally, glucocorticoids failed to stimulate transcription from a 500-bp segment of the rat PEPCK-C gene promoter in HepG2 cells (29), although glucocorticoids were shown to induce the expression of the endogenous gene in these cells (30). This suggested that the GRU extends upstream of the region that was described originally.

This notion is further supported by the discovery of hypersensitive sites (HSS) in the rat PEPCK-C gene promoter (31). A HSS locus was identified that is composed of two adjacent sub-sites, one of which mapped to positions −999 to −987; this coincides with PPAR binding domain in the PEPCK-C gene promoter (18). The second site was specific to PEPCK-C-expressing hepatoma cells and mapped to position −1400 (31). Subsequently it was found in committed and differentiated adipocytes (32) but not in kidney cells (33). In the current study we have further characterized both sub-sites of the HSS B, at −993 and at −1400 of the PEPCK-C gene promoter and have found that together these sites take part in an extended GRU. This extended GRU is liver-specific and plays an important role in the regulation of PEPCK-C gene transcription by glucocorticoids. The glucocorticoid receptor (GR) in the presence of glucocorticoids interacts with non-steroid nuclear receptors, leading to a synergistic liver-specific stimulation of PEPCK-C gene expression, which requires all four accessory sites. This is supported by the demonstration that diabetic mice with a mutation in the dAF1 site in the extended GRU of the PEPCK-C gene promoter do not have the same level of blood glucose as mice with the intact gene promoter.

EXPERIMENTAL PROCEDURES

Materials

Dulbecco’s modified Eagle’s medium, F-12, and fetal calf serum were purchased from Biological Industries, Kibutz Beit Haemek, Israel. Bio-synthetic human insulin was obtained from Novo Nordisk (Denmark). Dexamethasone, the synthetic glucocorticoid hormone, was purchased from Biological Industries, Kibutz Beit Haemek, Israel. Biologicals. Dexamethasone, the synthetic glucocorticoid hormone, was purchased from Bio-products. Dexamethasone, the synthetic glucocorticoid hormone, was purchased from Biological Industries, Kibutz Beit Haemek, Israel. Glucocorticoid Regulation of PEPCK-C Gene Transcription

were generated as described previously (34). All mice were maintained on a commercial chow diet ((catalog #TK2018SC+F) purchased from Harlan Teklad (Madison, WI) in the SPF (specific pathogen-free) unit of the animal facility at the Hebrew University-Hadassah Medical School, Jerusalem. The mutant mice used in the present work were backcrossed into the C57Bl background (seven generations) to achieve a more homogeneous genetic background of the population.

Transgenic mice were genotyped by PCR, with rat PEPCK-C-specific primers to exon 9 (the 5’ primer: 5’-CTTGTCACGAAAGCTTCA-3’ and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ primer: 5’-GGTCCGACATCCCTCCTCAG) and to exon 10 (the 3’ prime...
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Evidence for an Extended GRU in HepG2 Cells—The endogenous PEPCK-C gene is not only expressed in HepG2 cells but is inducible by glucocorticoids (30). Therefore, the failure of the hormones to induce the 500-bp segment of the rat PEPCK-C gene promoter (29) strongly suggested that sequences outside this segment were required. We assessed whether the GRU in the PEPCK-C gene promoter extends upstream of the originally described site by comparing the effect of GR on transcription from the pck-2000-CAT gene both in chimeric genes that were driven by segments from -500 to +73 (pck-500-CAT) and -2000 to +73 (pck-2000-CAT) of the gene promoter. The GR in the presence of dexamethasone stimulated transcription of the pck-2000-CAT gene in HepG2 cells from 3- to 5-fold but had no effect on transcription of pck-500-CAT gene (Fig. 1). Because both pck-500-CAT and pck-2000-CAT have recognition sites for the PPAR and/or α, a non-stereoid nuclear receptor (18), we tested the response of both gene promoters to this receptor. Unlike GR, the heterodimer of PPARα with RXRa (PPAR/RXR) stimulated transcription from both gene promoters about 8-10-fold (Fig. 1). When GR was transfected together with PPAR/RXR, transcription of the chimeric pck-2000-CAT gene but not the pck-500-CAT gene was stimulated synergistically (Fig. 1). Thus, although pck-500-CAT failed to respond to GR in the presence of its ligand either when present alone or together with PPAR/RXR, the pck-2000-CAT gene not only responded to the stimulation by GR but also responded synergistically to the combination of GR and PPAR/RXR.

Footprinting Analysis of the Liver-specific HSS Site B—To identify putative upstream regulatory sites within the PEPCk-C gene promoter (−2000 to +73) that are responsive to glucocorticoids, we focused on the HSS B, previously described in this region (31). The HSS B locus comprises a non-liver-specific site centered at position −993 and a liver-specific site at position −1400 of the PEPCk-C gene. The non-liver-specific site contains a recognition site for PPARγ or PPARα (18), which is required for the expression of a transgene driven by the rat PEPCk-C gene promoter in adipose tissue of transgenic mice (49). This site was specfically mutated in the genome of mice (34), resulting in a total lack
of expression of the gene for PEPCK-C in the white adipose tissue but normal expression in the liver and kidney.

There is a high degree of sequence identity within the liver-specific region of HSS B (upper panel of Fig. 1) between the PEPCK-C gene promoter from the rat and the mouse (Fig. 2c). DNase I footprinting of this region demonstrated a binding site centered at position −1365 of the PEPCK-C gene that was protected by nuclear proteins isolated from rat liver but not from spleen (Fig. 2a). This site was partially protected by nuclear proteins from the liver of 19-day-old fetuses and from 3T3-F442A mouse adipocytes but not proteins from the kidney. To assess the affinity of nuclear proteins to the protected site, the ratio of the densities of a band inside and a band outside the protected region was set for footprinting performed in the absence of nuclear proteins. The ratio between the same bands using nuclear proteins from the adult liver (which expresses PEPCK-C) was 3.5, whereas the ratio obtained using spleen nuclear proteins was of 8.2 (close to the value obtained without nuclear proteins). Nuclear proteins from the fetal liver gave a ratio of 4.4, from the adipocytes a ratio of 5, and from the kidney a ratio of 7.2 (Fig. 2b). These results demonstrate not only that the site is specific for PEPCK-expressing tissues (it is absent in nuclear proteins isolated from the spleen), but also it had a differential affinity for binding nuclear proteins from tissues that express PEPCK-C, with the highest affinity exhibited by the liver and the lowest by the kidney (Fig. 2b). These results agree with those reported for the sub-site of HSS B in PEPCK-C gene promoter where DNase I sensitivity in the liver (31) and preadipocytes (32), but not in the kidney (33), was detected.

The sequence of the protected region around −1365 of the PEPCK-C gene promoter, which was identified by DNase I footprinting, has considerable similarity to the AF2 sequence in the GRU (13); on the basis of this similarity we termed this site the distal AF1 (dAF1). Likewise, the non-liver-specific HSS B sub-site, which co-localized with the PPARα (18, 49) and is similar to the AF1 site in the GRU (13), was termed the distal AF1 (dAF1) site.

Electrophoresis Mobility Shift Assay—To begin characterizing the patterns of hepatic nuclear proteins that bind to the dAF1 and dAF2 sites, we performed mobility shift assays. A similar pattern of binding between the AF1 and dAF1 sites and between the AF2 and dAF2 sites of the PEPCK-C gene promoter to nuclear proteins was noted using electrophoresis mobility shift assay. There was an efficient competition obtained only between the analogous sites of each couple. Thus, the band shift of the dAF1 probe was equally competed by the same amount of nonradioactive self-competitor (dAF1 site) or its partner (AF1 site), whereas neither the dAF1 nor AF1 sites competed with binding to the dAF2 probe. Likewise, although the band shift of the dAF2 probe was efficiently competed by itself and by the AF2 sequence, neither dAF2 nor AF2 competed with the band shift of the dAF1 probe (Fig. 3, a and b, respectively). The similarity in protein binding between the dAF2 and AF2 sites implies that dAF2 site likely constitutes a binding recognition site for the HNF-3α (Foxa2) and FOXO1, members of the Forkhead gene family, as found for the AF2 site (50, 51) (23). The similarity between dAF1 and AF1 also suggests that non-steroid nuclear receptors bind to dAF1, as previously established for AF1 (51).

To assess the identity of hepatic proteins binding to dAF1 and dAF2, gel shift analysis was performed using specific antibodies. The dAF1 probe was supershifted by the addition of an antibody to HNF-4α to an extent that corresponded to its abundance in hepatic nuclei (52) (Fig. 3a). However, the antibody to PPARα did not affect the binding of proteins to the dAF1 site. Binding of members of the Forkhead family of transcription factors was demonstrated using the dAF2 site as a probe (Fig. 3b). The results showed diminished intensity of the bound lower band (relative to the upper band bound), with antibodies against
FOXO1 and phosphorylated FOXO1 (P-FOXO1) and the appearance of a weak but discrete super shift band with HNF-3β antibody (Fig. 3b).

ChIP Assay—The in vivo occupancy of regulatory sites of the extended GRU by transcription factors was assessed by a ChIP assay using isolated rat hepatocytes that were treated with hormones. We noted a similar binding pattern by transcription factors to both the AF2 and dAF2 sites (Fig. 4a-c) that differed from the binding pattern to dAF1 site (Fig. 4c). The addition of dexamethasone stimulated the occupation of both the AF2 and dAF2 sites by FOXO1 and PPARα (Fig. 4a, a and b) but failed to stimulate the occupation of dAF1 by either of these factors (Fig. 4c). The apparent effect of dexamethasone on the binding of PPARα was not expected and equally surprising was the lack of response of HNF-4α to the added hormone (Fig. 4a and b). We did observe a reciprocal effect of glucocorticoids on the binding pattern of these two factors to dAF1 (Fig. 4c). The hormones stimulated the binding of HNF-4α but had no effect on that of PPARα. Unlike the binding of factors described above, the addition of dexamethasone had no effect on the binding of HNF3β to either AF2 or dAF1 (Fig. 4a and c). A moderate stimulation of binding of this factor to the dAF2 was noted, but even then it seemed to be inferior to the stimulation of FOXO1 binding to this site (Fig. 4b). These results essentially corroborate those by Wolfrum et al. (23), who reported on binding of FOXO1 rather than HNF3β to the AF2 site.

The addition of insulin to the hepatocytes inhibited the effect of dexamethasone on FOXO1, PPARα, and HNF-4α (Fig. 4a–c). In contrast, insulin intensified the binding of P-FOXO1 to all three sites when added by itself or in the presence of dexamethasone (Fig. 4a–c). There are two exceptions; one is the stimulation by dexamethasone of the binding of P-FOXO1 to dAF1 (Fig. 4c), and the second is the intensified binding of HNF3β by insulin to dAF2 and dAF1 sites but in this case only in the presence of dexamethasone (Fig. 4a, b and c). Our data suggest that insulin globally inhibited the binding of all the factors stimulated by dexamethasone but intensified the binding of P-FOXO1.

**Transient Transfection Experiments**—We next determined whether other non-steroid receptors (besides PPAR), such as HNF-4α, RARα, and COUP-TF 1 and COUP-TF 2, could interact with GR to synergistically stimulate transcription from the PEPCK-C gene promoter (−2000 to +73) (Figs. 5 and 6). Synergistic stimulation was noted with HNF-4α and with RAR/RXR, but COUP-TF 1 and 2 inhibited both the basal level of gene transcription from the PEPCK-C gene promoter and the activation for transcription by HNF-4α when co-transfected together with HNF-4α (Fig. 5, a and b). In agreement with these results, De Martino et al. (53) recently reported that COUP-TF either cooperates or inhibits GR-mediated induction of transcription from the PEPCK-C gene promoter (among others) in HepG2 cells.

The data described above suggest that the dAF1 and dAF2 sites in the extended GRU of the PEPCK-C gene promoter duplicate the AF1 and AF2 sites. Therefore, we next assessed whether all four accessory sites are required for the glucocorticoid regulation of transcription from the PEPCK-C gene promoter (−2000 to +73). To this end each of the four accessory sites was individually mutated as were the two GRE sites (GRE 1 and 2) (13). A combined mutation of both GRE 1 and GRE 2 sites abolished not only the response of pck-2000-CAT to GR alone but also the synergistic response of GR together with either PPAR/RXR (Fig. 6a)
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**FIGURE 3. Electrophoresis mobility shift assays.** In panel a the 32P-labeled dAF1 and in panel b the 32P-labeled dAF2 site probes were radioactively labeled by PCR amplification containing [32P]dCTP. The mobility shift assay included 7500 cpm of 32P-labeled probe and 10 μg of nuclear protein extract from rat liver. a, free probe; Fp, no nuclear protein extract added; *, nuclear protein extract bound to 32P-labeled dAF1. Competitors added: dAF1 × 50 μM excess (dAF1), AF1 × 50 μM excess (AF1), AF2 × 100 μM excess (AF2), and dAF2 × 100 μM excess (dAF2). Nuclear protein extract was incubated with HNF-4α antiserum (1 μl) (HNF-4α), HNF-3β antiserum (1 μl) (HNF-3β), GR antiserum (1 μl) (GR), and PPAr antiserum (1 μl) (PPARα), b, free probe; Fp, no nuclear protein extract added; *, nuclear protein extract bound to 32P-labeled dAF2. Competitors added: dAF2 × 50 μM excess (dAF2), AF1 × 100 μM excess (AF1), dAF1 × 100 μM excess (dAF1), and AF2 × 50 μM excess (AF2). Nuclear protein extract was incubated with HNF-3β antiserum (1 μl) (HNF-3β), HNF-4α antiserum (1 μl) (HNF-4α), phosphorylated FOXO1 (FKHR) antiserum (1 μl) (p-FOXO1), and FOXO1 antiserum (1 μl) (FKHR).

**FIGURE 4. ChiP assay of AF2, dAF2, and dAF1 accessory sites in the extended GRU of the rat PEPCK-C gene promoter in rat liver in vivo.** The association in vivo of hepatic nuclear proteins with AF2, dAF2, and dAF1 sites of the PEPCK-C gene promoter was carried out using ChiP analysis of DNA isolated from hepatocytes of rats fasted overnight. Isolated hepatocytes were incubated either overnight with dexamethasone (Dex) and/or with insulin for the last 2 h and, additionally, 5 min before fixation as indicated above the figures followed by cross-linking the DNA and associated proteins with formaldehyde. The specific binding of transcription factors to the AF2, dAF2, and dAF1 sites of the PEPCK-C gene promoter was identified using antibodies as indicated on the left side of the figures. The amplified DNA was separated by electrophoresis on 2% agarose gels and visualized using ethidium bromide staining. Panel a, the AF2 site comprises positions 437 to 384. Panel b, the dAF2 site comprises positions 1394 to 1335 of the PEPCK-C gene promoter. Panel c, the dAF1 site comprises positions 1088 to 948 of the PEPCK-C gene promoter. Starting input chromatin DNA is shown below as indicated (Input).

or with HNF-4α (Fig. 6b). This result corroborates earlier findings by Granner and coworkers (13, 14, 54) who noted that a mutation in any single element of the GRU only marginally affected the response of the gene promoter to glucocorticoids, and a double mutation of any two elements of the GRU completely abolished the response to glucocorticoids. Likewise, we report that a single mutation of each accessory site only moderately affected the response of the PEPCK-C gene promoter to GR alone. In contrast, the synergistic response was markedly affected by a single mutation of any one of the accessory sites. Thus, mutation of the AF1 site alone abolished the synergistic response to GR with either PPAR/RXR (Fig. 6a) or HNF-4α (Fig. 6b). Similar results were obtained when the AF2 site in the PEPCK-C gene promoter was mutated (Fig. 6, a and b). Mutation of the dAF1 site had a minimal effect on the stimulation of the PEPCK-C gene promoter by GR alone, whereas it markedly reduced the cooperative stimulation by GR together with either PPAR/RXR or HNF-4α (Fig. 6, a and b). Mutating the dAF2 site in the PEPCK-C gene promoter completely abolished the synergistic response of the promoter to the GR and PPAR/RXR (Fig. 6a), but it only partially reduced the synergistic response of GR and HNF-4α (Fig. 6b). Finally, a mutation of either the dAF1 or dAF2 sites in the extended GRU reduced the trans-activation of the PEPCK-C gene promoter by HNF-4α alone (Fig. 6b) but did not affect the response to PPAR/RXR alone (Fig. 6a).
These results establish the requirement of each of the four accessory sites despite their duplication for the synergistic response of PEPCK-C gene promoter by a combination of GR with either of the two non-steroidal nuclear receptors.

The Role of the dAF1 Site in Vivo in the Response of Hepatic PEPCK-C Gene to Diabetes—The findings using HepG2 cells demonstrating the requirement of all four accessory sites for the synergistic response of the PEPCK-C gene promoter to nuclear receptors might be specific to this cell line. An implication of their physiological significance should come from studies in vivo. This was especially important in view of the bulk of evidence on the role of the GRU that has been derived from the use of transformed cell lines (13). Using mice, which contain a targeted block mutation of the dAF1 (PPARE) site in the PEPCK-C gene promoter (34), we determined the effect of a loss of this site on the hepatic expression of the gene in response to streptozotocin-induced diabetes. Mice that were homozygous for a mutation in the dAF1 site in the PEPCK-C gene promoter and a mixed population of heterozygous and wild type mice were made diabetic by the injection of streptozotocin. The concentration of blood glucose was determined 3 days after streptozotocin injection. The mean concentration of blood glucose was determined for 13 diabetic mice lacking the dAF1 site in the PEPCK-C gene promoter (−/−) had a lower level of blood glucose as compared with 18 control littersmates comprising a mixture of 8 wild type and 10 heterozygous mutants (+/−) (414 ± 28.1 mg/dl versus 547 ± 28.7 mg/dl). When wild-type mice were made diabetic in a parallel experiment, the level of blood glucose was reduced by about 25% after adrenalectomy (data not shown); this is about the same difference that of the endogenous gene, suggesting that sequences outside of the transgene help to moderate the response (Fig. 8). Alternatively, the more rapid de-induction of transcription from the transgene might be related to its rat origin (although the rat and mouse PEPCK-C genes are highly similar (32)). These results establish that the transgene contains sufficient information to elicit the highly specific hepatic regulation of the PEPCK-C gene expression.

**DISCUSSION**

The factors that control the hepatic transcription of the gene for PEPCK-C have been extensively studied over 25 years (1, 2), making its gene promoter one of the most thoroughly studied of any eukaryote gene promoters. However, much of this research has focused on a region that is ~500 bp 5′ of the start site of gene transcription. This region of the gene promoter contains many critical elements that regulate the response of the gene to diet and hormones (1, 2). However, a
number of observations over the years have suggested that a segment of the gene promoter that extends considerably up-stream from the better-characterized down-stream region is involved in both the hormonal and tissue-specific control of PEPCK-C gene transcription. For example, the expression of the gene for PEPCK-C in adipose tissue requires a PPAR binding site that is present at 1000 in the gene promoter (34, 49). In addition, there is hepatic-specific HSS that maps at approximately 1400 of the gene promoter, the function of which has not been studied in any detail. Finally, the elegant studies by Granner and co-workers (13, 14, 22, 51, 54, 56–60) delineating the GRU in H4IIEC3 rat hepatoma cells using a construct that contained the putative GRU but extended only to 500 had the unexplained problem in HepG2 cells, which lacked a response to glucocorticoids using the same gene promoter (29). In this report we provide evidence for an extended GRU in the PEPCK-C gene promoter (from 1400 to 385), which involves four individual regulatory elements that share a common sequence identity. This extended GRU responds as predicted to nuclear receptors, and its function is consistent with the known regulation of PEPCK-C gene transcription by these hormones. The extended GRU is strongly supported by the altered response of the PEPCK-C gene to a mutated dAF1 site in mice (Fig. 7) and by recent evidence from targeted PPARα null mice (61).

The Influence of Glucocorticoids and Insulin on the Binding of Transcription Factors to the Extended GRU—The presence of an extended GRU in the PEPCK-C gene promoter requires a re-analysis of the significance of the binding pattern of transcription factors to this larger regulatory unit in response to hormones. For example, the proximal AF1 and AF2 accessory sites are duplicated in the extended GRU, yielding the corresponding distal AF1 and AF2 counterpart sites, thus forming two couples, with each couple exhibiting a high degree of sequence identity between the proximal and distal partners. It would, thus, be reasonable to assume that these sites would share a common pattern of transcription factor binding. This is especially important in considering the mechanism by which diabetes alters transcription of the gene for PEPCK-C in the liver. The increase in hepatic PEPCK-C gene transcription noted in diabetes is due not only to the removal of the negative
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It is important to note that a ChIP assay does not necessarily provide evidence of DNA binding but, rather, documents complexes of proteins ultimately associated with the transcription factor(s) that binds to an amplified DNA fragment. Therefore, this assay determines not only factors that directly bind to a specific site on the PEPCK-C gene promoter but also those associated with the site via protein-protein interactions that occur off the promoter. Such is the case with the ChIP analysis, which indicates intensified binding of PPARα to complexes formed at both AF2 and dAF2 sites upon the addition of dexamethasone (Fig. 4, a and b). The similarity of AF2 and dAF2 sites is even more striking when compared with the different glucocorticoid-induced pattern of binding factors to the dAF1 site. Thus, the hormones induced the binding to dAF1 of HNF4α but not PPARα or FOXO1 (Fig. 4c).

The ChIP analysis shown in Fig. 4 suggests that insulin treatment of isolated hepatocytes causes an association of P-FOXO1 with the AF2, dAF2, and dAF1 sites of the PEPCK-C gene promoter (Fig. 4). It is thus, likely that the phosphorylation of FOXO1 stimulated by the addition of insulin disrupts a complex formed between FOXL1 and other transcription factors such as PPARα or HNF-4α, which occurs at the AF2, dAF2, and dAF1 sites, respectively, of the extended GRU. It is widely accepted, however, that insulin-induced phosphorylation of P-FOXO1 (via its activation of protein kinase B) results in a rapid exodus of the transcription factor from the nucleus and its subsequent degradation in the cytoplasm (62). By this model the phosphorylation of FOXO1 in the presence of insulin would cause its removal from the complex of transcription factors, resulting in an inhibition of PEPCK-C gene transcription. However, Tsai et al. (63) have shown that ~25% of the FOXO1 remains in the nucleus one h after insulin treatment of hepatocytes. Thus, although insulin does cause a major redistribution of transcription factors within the cell, a significant fraction of the P-FOXO1 remains in the nucleus after insulin addition. The authors also noted that a mutation replacing leucine at position 375 in FOXO1, which is likely that the phosphorylation of FOXO1 stimulated by the addition of insulin disrupts a complex formed between FOXO1 and other transcription factors, resulting in an inhibition of PEPCK-C gene transcription. However, Tsai et al. (63) have shown that ~25% of the P-FOXO1 remains in the nucleus one h after insulin treatment of hepatocytes.

regulation exerted by insulin but also to the chronically increased level of circulating glucocorticoids and, in turn, the strong stimulation of gene transcription.

Previous studies have shown that the AF1 site binds the hepatic-enriched orphan receptors HNF-4 (16), COUP-TFII (17), PPARγ2 (18), the RARα (19), and RXRα (20). The AF2 site binds members of the Forkhead family including HNF-3β (Foxa2) (50, 51, 54), FOXO1, and its phosphorylated form (21). Recently, Wolfrum et al. (23), using a ChIP assay, reported that the AF2 site primarily binds FOXO1. Our findings essentially corroborate theirs (Fig. 4).

Beyond their similarity in sequence and binding specificity in gel shift assays, the striking similarity of AF2 and dAF2 sites of the PEPCK-C gene promoter is clearly established by the ChIP assay, where the pattern of binding proteins to these two sites markedly differs from the pattern associated with the dAF1 site. Thus, although dexamethasone stimulates the binding of FOXO1 to the AF2 and dAF2 sites but not to dAF1 site, the hormones stimulate the binding of HNF4α only to dAF1 but not to the AF2 or the dAF2 sites. These ChIP results, which clearly discriminated between the dAF1 site and AF2 and dAF2 sites, were made possible because of the relatively remote distance of dAF1 site from the other two sites. In contrast, the AF1 site is, in fact, successively adjacent to AF2 site.

FIGURE 7. A targeted mutation of the dAF1 site of the PEPCK-C gene (PPARE mice) affects the expression of the gene for PEPCK-C in the liver and the level of blood glucose in diabetic mice. Panel a, 13 homozygous (dAF1) PPARE mice (−/−) and a mixed population of 10 heterozygous and 8 wild type mice (PPARE) (+/−) were made diabetic by streptozotocin (STZ) injection. The concentration of blood glucose was determined 3 days later, and its mean level ± S.E. is shown. The difference between the two genotypes (dAF1) (PPARE) mutants (−/−) compared with (dAF1) PPARE (+/−) was 24.3% (significant at p = 0.025). Panel b, Northern hybridization assays using 10 μg of total RNA from liver and kidney of streptozotocin diabetic mice were quantified by determining the abundance of PEPCK-C mRNA relative to that of β-actin. The histograms show the means ± S.E. of the results from four mice each of wild type (+/−) (filled boxes) and mutant (−/−) (empty boxes). The 3.5-fold difference in the hepatic abundance of PEPCK-C mRNA between the wild type and mutant diabetic mice was significant (p < 0.037). The renal abundance of PEPCK-C mRNA was not significantly affected by the mutation.
FIGURE 8. The effect of glucose feeding on the levels of PEPCK-C mRNA in the livers of fasting transgenic mice containing a rat PEPCK-C transgene. Panel a, a schematic illustration of the rat transgene in transgenic mice. The **linear black boxes** indicate exons, and open boxes indicate introns and flanking regions. +1, transcription start site; A++, polyadenylation signal. The region of the RT-PCR products of the transgene and endogenous gene and its polymorphic BglII site is indicated below. Panel b, RT-PCR analysis of RNA from three fasted transgenic mice (numbered 1–3) and three fasted and re-fed with glucose (4–6). Total RNA was extracted from the liver (L), kidney (K), and white adipose tissue (A) and processed by RT-PCR. The cDNA samples were amplified using PEPCK-C-specific primers from exons 9 and 10, as specified under “Experimental Procedures.” The amplified RT-PCR products were digested with BglII, generating the transgene segments sizes 202 and 99 bp, whereas the amplified segment of the endogenous gene, which has two BglII sites, yielded three segments, sizes 148, 98, and 54 bp. Only the larger bands, 202 bp of the rat transgene and 148 bp of the endogenous gene, are shown. On the left are the markers: R, the product of the rat gene; M, the product of the endogenous mouse gene.

FIGURE 9. Model of the proposed conformational changes in the extended GRU of the PEPCK-C gene promoter in response to dexamethasone or insulin. The regulatory sites comprising the extended GRU (including the AF3 site that resides downstream of the GRE2) are shown as mapped to the PEPCK-C gene promoter in the upper portion of the figure. In the present manuscript we analyzed the GRE1 and -2 and the accessory sites residing upstream of the two GREs. The addition of dexamethasone (Dex) to primary rat hepatocytes or human hepatoma HepG2 cell line supplemented with GR causes the formation of a complex associated with a conformational bend of the DNA that juxtaposes the AF2 and dAF2 sites at the end of the bended DNA and the AF1 and dAF1 sites within the bended DNA. This alignment attracts co-activators that recruit the transcriptional machinery. The addition of insulin unites the complex by phosphorylation of the bound FOXO1 and by releasing the entire bound transcription factors, thus arresting the gene transcription. Pol II, polymerase II.
essential for the regulation of hepatic PEPCK-C gene transcription by nuclear receptors in vivo. The best example of this regulation is the enhanced glucose production characteristic of the diabetic animal since, according to our model, a mutation in any one of the four critical sites in the GRU should not only affect the abundance of hepatic PEPCK-C mRNA but potentially also alter the circulating glucose level of diabetic mice. Mice with a targeted mutation in the dAF1 provide a rigorous test for this assumption, since we previously reported that this mutation did not alter the level of PEPCK-C mRNA in their livers during fasting (34). Moreover, streptozotocin-induced diabetes raises the concentration of glucocorticoids in the blood, thereby allowing us to test the response of fed, rather than fasted mice to a targeted ablation of the dAF1 site in the PEPCK-C gene promoter. Mutating the dAF1 site markedly reduced the level of hepatic PEPCK-C mRNA (by 3.5-fold) and caused a significant decrease in the level of circulating glucose (about 25%). The large difference between the effect of the mutation on the hepatic level of PEPCK-C mRNA and its more moderate consequent effect on blood glucose noted in these mice could also be due to the induction of renal PEPCK-C gene expression via the increased metabolic acidosis that occurs during diabetes. The regulatory region in the PEPCK-C gene promoter that responds to acidosis (HNF-1) is about 600 bp downstream of the dAF1 and is independent of dAF1 because a transgene harboring only 362 bp of the 5′ flanking region of the rat PEPCK-C gene is fully responsive to acidosis in transgenic mice (7). Moreover, renal proximal tubules are not insulin-responsive. The result is that during diabetes the contribution of renal gluconeogenesis to the circulating blood glucose level is greatly enhanced (65). Because the dAF1 mutation has little effect on renal PEPCK-C gene expression, it is likely that the kidney contributes significantly to the elevated concentration of blood glucose noted in these mice. On the other hand, the marked effect of the dAF1 mutation on the hepatic expression of PEPCK-C gene compared with its marginal effect on expression of the gene in the kidney strongly supports the requirement of all four accessory sites for the synergistic response of the hepatic PEPCK-C gene promoter to nuclear receptors.

Recall that the synergistic stimulation of the PEPCK-C gene promoter by the cooperation of GR with non-steroid nuclear receptors is markedly affected by a single mutation of each of the accessory sites. A recent article by Bernal-Mizrachi et al. (61) lends strong support to the existence of such cooperation between GR and nuclear receptors in diabetes. Thus, these authors could generate diabetes by treating mice with a transgene harboring only 362 bp of the 5′ flanking region of the rat PEPCK-C gene that had been mutated in the AF2 site. The mutation of the AF2 site, one of the four accessory factors binding sites in the extended GRU of the PEPCK-C gene promoter, results in the inhibition of the diabetes-induced increase of PEPCK-C transgene transcription in the livers of transgenic mice and renders the PEPCK-C gene promoter refractory to insulin (41).

A Proposed Model for the Integrated Function of an Extended GRU—We have noted that the synergistic response of the PEPCK-C gene promoter to glucocorticoids requires the presence of each of the four accessory sites that include two duplicated sites in which the alignment of the distal accessory sites is opposite to that of the proximal sites; close inspection of the alignment of the accessory sites indicates a “macro” palindromic. One possible mechanism to explain the synergistic response of the PEPCK-C gene promoter to glucocorticoids involves a conformational bend of the DNA in the region containing the four accessory sites. This would juxtapose the occupied AF2 site with the dAF1 site at the two ends of the bent region and the AF1 and dAF1 sites within the bent loop (Fig. 9). The close juxtaposition of these sites would facilitate the recruitment of the required factors into a complex that results in the synergistic response of the gene promoter to nuclear receptors. The feasibility of the proposed model is currently being tested by introducing transgenes into mice that contain mutations in each of the four binding regions of the proposed extended GRU in the PEPCK-C gene promoter.

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