The Synergistic Effect of Dexamethasone and All-trans-retinoic Acid on Hepatic Phosphoenolpyruvate Carboxykinase Gene Expression Involves the Coactivator p300*

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Activation of phosphoenolpyruvate carboxykinase (PEPCK) gene transcription in response to all-trans-retinoic acid (RA) or a glucocorticoid such as dexamethasone (Dex) requires a distinct arrangement of DNA-response elements and their cognate transcription activators on the gene promoter. Two of the accessory factor-binding elements involved in the Dex response (gAF1 and gAF3) coincide with the DNA-response elements involved in the RA response. We demonstrate here that the combination of Dex/RA has a synergistic effect on endogenous PEPCK gene expression in rat hepatocytes and H4IIE hepatoma cells. Reporter gene studies show that the gAF3 element and one of the two glucocorticoid receptor-binding elements (GR1) are most important for this effect. Chromatin immunoprecipitation assays revealed that when H4IIE cells were treated with Dex/RA, ligand-activated retinoic acid receptors (retinoic acid receptor/retnoid X receptor) and glucocorticoid receptors are recruited to this gene promoter, as are the transcription coregulators p300, CREB-binding protein, p/CIP, and SRC-1. Notably, the recruitment of p300 and RNA polymerase II to the PEPCK promoter is increased by the combined Dex/RA treatment compared with Dex or RA treatment alone. The functional importance of p300 in the Dex/RA response is illustrated by the observation that selective reduction of this coactivator, but not that of CREB-binding protein, abolishes the synergistic effect in H4IIE cells.

Extracellular signals received by eucaryotic cells are transferred to the nucleus where the transcription of specific sets of genes is modulated (1). The final targets of these signaling pathways are different combinations of transcription factors that bind to DNA-response elements and form various protein-protein interactions on gene promoters and thereby either activate or repress gene transcription. DNA-response elements in gene promoters often overlap or are adjacent to one another (2). This provides various possibilities for the recruitment of transcription factors and their cognate coregulators in response to distinct signals and creates the potential for considerable flexibility in gene regulation. Regulation of the phosphoenolpyruvate carboxykinase (PEPCK) gene provides an excellent model system for exploring the mechanisms of signal integration in the context of gluconeogenesis, an important physiologic process (2). The expression of hepatic PEPCK is primarily accomplished at the transcriptional level (3). Glucagon (acting through cAMP), glucocorticoids, all-trans-retinoic acid (RA), and thyroid hormone activate PEPCK gene expression, whereas insulin and glucose are inhibitory (3–6).

Activation of PEPCK gene transcription by RA occurs through a multicomponent retinoic acid-response unit (RARU) that consists of two separate retinoic acid-response elements, RARE1 and RARE2, each of which binds an RAR/RXR heterodimer (4, 7). Activation of PEPCK gene transcription by Dex also requires a multicomponent glucocorticoid-response unit (GRU), which consists of two nonconsensus glucocorticoid receptor-binding sites (GR1 and GR2) (8), and four accessory factor-binding sites (gAF1, gAF2, gAF3, and the cAMP-response element (CRE)), which bind COUP-TF/HNF4, HNF3β, COUP-TF, and C/EBPβ, respectively (9–12). A mutation of any one of the accessory elements results in a 50–60% reduction of Dex-induced PEPCK gene transcription in H4IIE hepatoma cells (11). Any combination of the mutation of two elements nearly abolishes this response (11).

Most interesting, RARE1 and RARE2 in the RARU are coincident with gAF1 and gAF3 in the GRU (4, 7, 9, 13). The respective transcription factors are present in about the same abundance in the H4IIE cell nucleus, and each binds with approximately the same affinity to these elements. Thus, it is of interest to understand how a cell responds to either of these inducers, whether different constellations of accessory factors and coregulators are used for each response and whether there is any functional interaction between the two units in the presence of both ligands.

In this study, we demonstrate that the Dex/RA combination has a synergistic effect on endogenous PEPCK gene expression in both primary hepatocytes and H4IIE hepatoma cells. This response is most dependent on the gAF3 and GR1 elements. Upon treatment with Dex/RA, ligand-activated RAR/RXR heterodimers, GR, HNF3β, HNF4α, p/CIP, SRC-1, CBP, p300, and

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1 The abbreviations used are: PEPCK, phosphoenolpyruvate carboxykinase; RA, all-trans-retinoic acid; Dex, dexamethasone; GR, glucocorticoid receptor; CBP, CREB-binding protein; CREB, cAMP-response element-binding protein; ChIP, chromatin immunoprecipitation assays; RAR, retinoic acid receptors; RARE, retinoic acid-response element; RARU, retinoic acid-response unit; cRARE, consensus RARE; RXR, retinoid X receptor; siRNA, small interfering RNA; DMEM, Dulbecco’s modified Eagle’s medium; MCD, metabolic control domain; pol, polymerase.
RNA polymerase II (pol II) are present on the PEPCK gene promoter. Notably, the recruitment of p300 and pol II on the PEPCK promoter is increased upon Dex/RA treatment. The important role of p300 is illustrated by the fact that its reduction results in a selective loss of the synergistic effect of Dex/RA, whereas a similar reduction of CBP has no effect.

**MATERIALS AND METHODS**

**Plasmids**—The PEPCKLuciferase (PEPCK-LUC) reporter gene construct was made by cloning the PEPCK gene promoter (−467 to +69) into pGL3 basic (Promega), as described previously (14). The gAF1-Gal4 PEPCK-LUC, gAF2-Gal4 PEPCK-LUC, or gAF3-Gal4 PEPCK-LUC reporter constructs were constructed by replacing gAF1, gAF2, or gAF3 with a Gal4 DNA-binding element, as described previously (15). gAF1-gAF3-Gal4 PEPCK-LUC was constructed by further mutating the gAF1 element of gAF3-Gal4 PEPCK-LUC plasmid to a Gal4 DNA-binding element using the QuickChange site-directed mutagenesis kit (Stratagene). The sequence of the Gal4 DNA-binding element is 5'-C-GGAGGACTGTCCTCCGGACCA-3'. gAF3m PEPCK-LUC, gAF3-m PEPCK-LUC, gAF1-FOU-TP-F PEPCK-LUC, and gAF1-HNF4a PEPCK-LUC were made as described previously (15). mGR1 PEPCK-LUC and palGR1 PEPCK-LUC were made by converting the GR1 element to a block mutation or palindromic GR-binding element, respectively. mGR2 PEPCK-LUC was constructed by replacing the GR2 site with a block mutation. mGR1/2 PEPCK-LUC was made by replacing the GR1 site in mGR2 PEPCK-LUC with a block mutation. The oligonucleotides used to construct mGR1, mGR2, and palGR1 were described previously (8). gAF1-eRARE PEPCK-LUC or gAF3-eRARE PEPCK-LUC was made by replacing gAF1 or gAF3 element individually with a consensus retinoic acid receptor-binding element (cRARE) (16). The oligonucleotides used to generate gAF1-cRARE PEPCK-LUC and gAF3-cRARE PEPCK-LUC are as follows: gAF1-eRARE, 5'-ATTCCCTCTGTTGTCACCGAAGATGCAGGTGATCACCC-3', and gAF3-eRARE, 5'-CGTCCTCCGGCGTCTCCACAGGATTCGATACCGAACA-3'. The sequence of all of the constructs was verified by DNA sequence analysis.

**Cell culture and Transfection**—H4IIE hepatoma cells were grown in stationary culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum at 37 °C in 5% CO₂. Transfection was performed using the calcium phosphate precipitation method as described previously (17) using 10 μg of a reporter plasmid (wild-type or mutations of PEPCK-LUC). The cells were subsequently washed and incubated for 20 h in DMEM containing 500 nM dexamethasone (Dex) and/or 5 μM all-trans-retinoic acid (RA). Sigmas. Cells were harvested by trypsin digestion and resuspended in 200 μl of lysis buffer (Promega). Firefly luciferase activity was measured and corrected for transfection efficiency with Renilla luciferase activity using the dual-luciferase reporter assay system (Promega). The PEPCK promoter is increased upon Dex/RA treatment. The sequence of the forward cyclophilin primer was 5'-GGAATCAGCCAGCTGTAATCCCG-3', and the sequence of the reverse PEPCK primer was 5'-CATTGTGTTCTGCTGGTTGTCGG-3'. The sequence of the forward cyclophilin primer was 5'-AAGGTGAAAGAAGCATGAGA-3', and the sequence of the reverse cyclophilin primer was 5'-GGATGTCACACAGGGATGGG-3'. The following real time PCR protocol was used: denaturation and enzyme activation at 95 °C for 10 min followed by a two-step amplification and quantification program consisting of 40 cycles of denaturation for 15 s at 95 °C and annealing/extension for 1 min at 60 °C. Data were analyzed using the SDS version 1.7a Instiller software package. Results represent the means ± S.E. of four to six independent experiments. The horizontal bar in the Dex/RA column in this and other figures indicates the value that would have been achieved if the result was additive.

**Regulation of PEPCK gene expression by Dex/RA in rat primary hepatocytes and H4IIE hepatoma cells.** A, H4IIE cells were serum-deprived overnight, and then were either left untreated or were treated with 500 nM Dex and/or 5 μM RA for 4 h. Total RNA was extracted from these cells, and 0.5 μg of RNA was subjected to reverse-transcribed RNA using primers encoding cDNA sequences for PEPCK and cyclophilin, as described under **Materials and Methods.** B, the experiment described in A was performed using isolated rat hepatocytes. Data were analyzed using the SDS version 1.7a Instiller software package. Results represent the means ± S.E. of four to six independent experiments. The horizontal bar in the Dex/RA column in this and other figures indicates the value that would have been achieved if the result was additive.
Immune complexes were collected using 40 μl of a 1:1 protein-A/G-agarose slurry plus 100 μg/ml salmon sperm DNA, with constant rotation for 3 h at 4°C. The agarose beads were pelleted at 1000 × g for 2 min and washed for 10 min at room temperature with 1 ml of each of the following buffers in succession: low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8, 20 mM Tris-HCl, pH 8, 500 mM NaCl, pH 8), LiCl wash buffer (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 10 mM Tris-HCl, 1 mM EDTA, pH 8), and 2× with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). Chromatin complexes were eluted from the beads by two 1-h incubations with 160 μl of elution buffer (1% SDS, 0.1 M NaHCO3) at room temperature. The DNA-protein cross-links were reversed by incubating the samples at 65°C for 12 h. The RNA and protein in the samples were digested using an RNase mixture (Ambion) and 50 μg/ml proteinase K, respectively. The DNA was purified using the QIAquick PCR purification kit (Qiagen) and resuspended in 50 μl of nuclease-free water. Five microliter aliquots were used for each PCR or real time PCR utilizing primers specific for PEPCK or β-actin promoters. The forward primer used for the PEPCK gene promoter was 5'-GTTTCACGTCTGAGCTGA-3'; the reverse primer was 5'-ACCGTGACTGTTGCAGTGC-3'. The forward and reverse primers used for the β-actin gene promoter were described previously (18). PCR products were run on a 2% agarose gel and visualized by ethidium bromide staining. Real time PCR was performed as described above. The antibodies used for GR, RAR, RXR, HNF3α, HNF4α, PCAF, p300, CBP, SRC-1, and p/CIP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-RNA polymerase II was from Berkeley Antibody Co. (Richmond, CA).

Small Interfering RNA—The mammalian vector pSUPER was used for the expression of siRNAs specifically targeted against rat p300 and CBP mRNAs. The pSUPER vector consists of an H1-RNA promoter, which directs the synthesis of siRNA transcripts (19). The target sequence of the p300 mRNA is a 21-nucleotide sequence corresponding to nucleotide positions 88–108 downstream from the transcription start site of this gene, which is separated by a 9-nucleotide noncomplementary spacer sequence followed by the reverse complement of the same 21-nucleotide sequence. The cloning of the sequence was facilitated by BamHI and HindIII flanking sites. This vector is referred to as p300 siRNA. The siRNA specific for CBP was made in a similar way by using a 21-nucleotide sequence corresponding to nucleotide positions 122–142 of the CBP mRNA. The resulting vector is referred to as CBP siRNA.

Western Blot Analysis—siRNA-treated KNRK cells were washed twice with ice-cold PBS. These cells were disrupted in a detergent lysis buffer (20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, 20 mM Na4P2O7, 0.2% Triton X-100, 10 mM microcystin, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8) and the protein content was determined using the Bradford assay.
β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml apro- tinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin). A 40-μg aliquot of protein from each treatment was loaded on a 6% Tris-glycine gel (Invitrogen). After electrophoresis, the separated proteins were trans- ferred to a nitrocellulose membrane (Invitrogen). The membrane was incubated for 2 h with the appropriate antibody, followed by incubation with an anti-rabbit or anti-mouse IgG conjugated to horseradish per- oxidase. Immunoreactive proteins were detected using the ECL immu- nodetection system (Amersham Biosciences). To assess the specificity of siRNA, the membrane probed with p300 antibody was re-probed with CBP antibody and vice versa.

Statistical Analysis—Statistical analyses were performed using Student’s t test, with the significance level set at p < 0.05.

RESULTS

Dexamethasone and Retinoic Acid Have a Synergistic Effect on PEPCK Gene Expression—Dexamethasone (Dex) and reti- noic acid (RA) each induce transcription of the PEPCK gene in H4IIE hepatoma cells through some shared DNA elements (2, 4, 7, 9, 13). This is not a clonal cell line (20), so it is possible that different populations of cells in a culture respond to each in-ducer. If so, the summed responses to the two ligands should be additive. To determine the effect of the Dex/RA combination on PEPCK gene expression, real time RT-PCR assays were per- formed to quantify PEPCK mRNA upon Dex and/or RA treat- ment. Cyclophilin mRNA was also measured as a normaliza- tion control. RA and Dex caused a 4- and 2.7-fold induction, respectively, and the combination resulted in a synergistic 13.3-fold effect in H4IIE cells (Fig. 1A). In rat primary hepatocytes, PEPCK mRNA is induced 15- and 7.4-fold upon treat- ment with RA or Dex, respectively. When both ligands are present, a much greater than additive 39-fold induction was observed (Fig. 1B). This indication of an effect on mRNA induc- tion within a single population of cells was confirmed at the protein level in an immunofluorescent experiment performed on primary hepatocytes. PEPCK expression is induced in 71, 67, or 70% of hepatocytes upon treatment with RA, Dex, or Dex/RA, respectively (data not shown). Because more than 50% of the cells respond to treatment with either or both ligands, we con- clude that there are no discrete populations of hepatocytes that respond to either Dex or RA or to the combination. Also, in keeping with the observations noted in Fig. 1B, the intensity of staining was greater in the cells treated with the Dex/RA combination (data not shown).

RAR/RXR Heterodimers at the gAF3 Element Are Primarily Inolved in the Synergistic Effect—The involvement of specific cis-elements and their cognate transcription factors in this synergistic effect was defined by using a number of variations of the PEPCK-LUC reporter plasmid, which contains the wild type PEPCK promoter from −467 to +69 ligated to the lucif- erase reporter gene. Upon treatment of H4IIE cells with 5 μM
RA, 500 nM Dex, or a combination of the two, PEPCK promoter-driven luciferase reporter gene expression is induced 3.4-, 8.6-, and 16.3-fold, respectively (see Fig. 2). This greater than additive response is qualitatively similar to that noted in expression of the endogenous gene (see Fig. 1A); hence, this transient transcription approach can be used to define the DNA elements and associated transcription factors involved in this response.

Mutations of PEPCK-LUC that replace γAF1, γAF2, or γAF3 with a Gal4 DNA-binding element(s) individually, or γAF1 and γAF3 in combination, were made to identify the involvement of these accessory elements in the synergistic effect. Results consistent with previously published data from our laboratory show that any single mutation of these three γAFs reduces the Dex-induced response by about 50%, and the mutation of both γAF1 and γAF3 abolishes the response (Fig. 2) (11). By contrast, the major effect of RA is facilitated through γAF3, although a small effect is mediated through γAF1. γAF2 is not involved in the RA effect, as noted earlier (13). Correspondingly, γAF2 has no qualitative effect on the synergistic response (the total effect is reduced because of the decreased response to Dex), whereas both γAF1, and especially γAF3, have major effects (Fig. 2).

The γAF1 element was converted to a consensus binding element for either HNF4α or COUP-TF which do not bind RXR (9). In accordance with a previous observation, the presence of consensus HNF4α- or COUP-TF-binding elements at γAF1 decreases the RA-induced response by about 50% (Fig. 3) (7). A significant synergistic effect of Dex/RA was not achieved from these constructs. However, the presence of a consensus RAR/RXR-binding element (cRARE) at γAF1, which excludes HNF4α or COUP-TF binding, promotes a significant increase of the RA response but reduces the Dex response by about 50% (Fig. 3).

Nonetheless, the synergistic effect is fully supported when γAF1 is replaced by a cRARE (γAF1-cRARE PEPCK-LUC).

The γAF3 element includes two RAR/RXR sites, α and γ, separated by a 5-bp β linker sequence (see Fig. 4). An E box, which binds the transcription factor USF, is located immediately downstream (Fig. 4). The α and γ elements, and the E box, were mutated in the context of PEPCK-LUC to determine which component of γAF3 is involved in the synergistic effect. As expected from previous studies (11), the E box mutation does not affect either of the Dex or RA responses nor does it have an effect on the Dex/RA response. A mutation of the α site slightly reduces the RA-induced response but has no significant effect on the Dex-induced response or on the Dex/RA-induced synergistic effect. By contrast, a mutation of the γ site of γAF3 significantly reduces both the RA and Dex effects and abolishes the synergistic effect of Dex/RA on PEPCK gene expression (Fig. 4). These data indicate that transcription factors binding to the γ site of γAF3 mediate the synergistic effect of Dex/RA on PEPCK gene expression.

The approach employed in the experiment described in Fig. 3 was used to infer the binding of transcription factors to the γAF3 element upon Dex/RA treatment (Fig. 5). The full synergistic effect was supported only when γAF3 was converted to a cRARE (γAF3-cRARE PEPCK-LUC). The cRARE binds RAR/RXR heterodimers with a higher affinity than either HNF4 or COUP-TF (16), thus the RA response in a construct in which γAF3 was changed to a consensus RAR/RXR-binding element is greater than that obtained from the wild type PEPCK-LUC (12-fold versus 3.4-fold; Fig. 5). The response of constructs in which both γAF1 and γAF3 were converted to cRAREs (γAF1/γAF3-cRARE PEPCK-LUC) was similar to that of γAF3-cRARE PEPCK-LUC (data not shown). This
implies that gAF3 is more important than gAF1 in promoting the synergistic effect of Dex/RA on PEPCK gene expression and that ligand-bound RAR/RXR heterodimers play a critical role.

The GR1 Glucocorticoid Receptor-binding Site Is Involved in the Synergistic Effect—Block mutations of GR1 and/or GR2, which prevent the binding of GR, were made in the context of the PEPCK-LUC construct. The GR1 mutant (mGR1 in Fig. 6) reduces Dex-induced PEPCK gene expression by 60%, whereas mGR2 has little effect, as expected (Fig. 6). The synergistic effect of Dex/RA on PEPCK gene expression is totally abolished in mGR1 but is not affected by mGR2 (Fig. 6). These results indicate that GR1, but not GR2, is involved in the synergistic effect of Dex/RA on PEPCK gene expression, which is consistent with our previous report (8) that GR1 is more important for Dex-induced PEPCK gene expression.

GR1 is a weak, nonconsensus GR-binding site which, by itself, cannot induce a Dex response on PEPCK gene expression (8). The accessory factor elements gAF1–gAF3, with associated transcription factors, increase the affinity of binding of liganded GR to GR1/GR2 and stabilize this binding (21). The gAF1 and gAF3 accessory elements are not necessary when GR1/GR2 are replaced with high affinity, palindromic GR-binding elements (21). To test whether ligand-activated RAR/RXR heterodimers are necessary in the presence of a high affinity GR-binding element, GR1 was converted to a palindromic glucocorticoid-response element (palGRE) in the context of PEPCK-LUC. PEPCK luciferase activity is induced 80-fold by Dex in the context of palGR1 PEPCK-LUC, but when both Dex and RA are present, PEPCK gene expression is not further augmented (Fig. 6). Most interesting, the synergistic effect of Dex/RA is not seen when GR1 is converted to a palindromic high affinity GR-binding site, which is independent of gAF1 and gAF3 (8). By contrast, when either gAF1 or gAF3 is converted to a consensus RAR/RXR-binding element, in the context of the wild type GR1/GR2 elements, the synergistic effect of Dex/RA on PEPCK gene expression is actually augmented (see Figs. 3 and 5). Taken together, we conclude that the synergistic effect of Dex/RA involves enhanced GR binding to the nonconsensus GR1 element and that this may be facilitated by the binding of RAR/RXR heterodimers.

In some genes RA is thought to bind to DNA-bound RAR/RXR heterodimers, and the RA-RAR interaction results in the release of a corepressor complex and the subsequent association of a coactivator complex (22, 23). There is a significant association of RAR and RXR to the PEPCK gene promoter in the absence of ligand, but there is a substantial increase of binding of both (~2-fold increase) when RA is added to the cells (Fig. 7). This association is not increased by the concomitant administration of Dex.

HNF3β binds to the PEPCK gene promoter constitutively, presumably to gAF2 (Fig. 7) (10, 18), which is consistent with functional data in which we have shown that this element is not involved in either the RA or Dex/RA-induced synergistic responses on PEPCK gene expression (Fig. 2). HNF4α is also
bound to the PEPCK gene promoter in untreated cells and in cells treated with Dex and/or RA (Fig. 7).

SRC-1 and p/CIP, members of the p160 nuclear receptor family, are recruited to the PEPCK gene promoter upon treatment with Dex and/or RA (Fig. 8). Although Dex causes a somewhat greater recruitment of SRC-1 and p/CIP to the PEPCK gene promoter than RA (3.5- versus 2-fold and 2.8- versus 2-fold, respectively, Table I), the recruitment of these proteins is not further increased when both ligands are present. PCAF, a HAT family protein, is present in H4IIE cells but is not located on the PEPCK gene promoter under any of these treatment conditions. This is probably not due to epitope masking, as antibodies specific for different epitopes of PCAF were used. CBP and p300 are both recruited to the PEPCK gene promoter upon Dex and/or RA treatment but, despite their structural homology, the binding of CBP and p300 differs. The recruitment of CBP is induced 3.7- and 6.9-fold upon treatment with Dex or RA, respectively (Fig. 8 and Table I). The recruitment of CBP is not further increased (6.5-fold) by combined treatment with Dex/RA. By contrast, recruitment of p300 is increased 4.7-fold upon treatment with Dex/RA combination as compared with a 2.0-fold increase upon treatment with either RA or Dex alone, respectively. Antibodies specific for different epitopes of CBP and p300 were also used in this experiment, and similar recruitment profiles of CBP and p300 were observed in each case (data not shown). The increased recruitment of RNA pol II (2-, 2-, and 5-fold upon treatment with RA, Dex, and RA/Dex, respectively) parallels the change in p300 binding (Fig. 8). Similar changes in the binding patterns of these nuclear proteins were noted in primary hepatocytes (data not shown).

**p300 Is Specifically Involved in the Synergistic Effect**—The difference in association of p300 and CBP when H4IIE cells are exposed to Dex/RA is interesting in view of the observation that these proteins interact with many transcription factors and nuclear receptors in the regulation of gene transcription and are often considered to be interchangeable. Selective reduction of p300 or CBP was used to establish the role of these proteins in the synergistic effect of Dex/RA on PEPCK gene expression. siRNAs directed against p300 or CBP mRNA were cloned into the pSUPER mammalian expression vector (designated as p300 siRNA and CBP siRNA, respectively). A Western blot analysis, performed using cell lysates prepared 72 h after transfection of these siRNAs into KNRK cells, shows that the siRNA directed against either p300 or CBP mRNA inhibited the endogenous expression of each of these proteins (Fig. 9, A and B). To validate the specificity of the siRNAs, the nitrocellulose membrane used in the p300 analysis was re-blotted with the CBP antibody and vice versa. The p300 siRNA does not affect CBP gene expression, nor does the CBP siRNA affect p300 gene expression. α-Actin expression is not affected by either treatment. Thus, the siRNAs cause a major and selective reduction of the cognate proteins.

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**Fig. 6.** GR1 is involved in the synergistic effect. PEPCK-LUC constructs were made that contain a mutated GR1 (mGR1), a mutated GR2 (mGR2), a combined mutation of GR1 and GR2 (mGR1/2), or a palindromic GR-binding element (palGR1). H4IIE cells were transiently transfected with 10 μg of PEPCK-LUC or mutant forms of PEPCK-LUC. The results illustrated are normalized to the basal activity of each PEPCK-LUC reporter gene construct. Data represent the mean ± S.E. of five to eight independent experiments.

**Table 1.**

| Designation | Reporter Constructs |
|-------------|---------------------|
| PEPCK-LUC   |                     |
| mGR1        | gAF1                 |
| PEPCK-LUC   | gAF2                 |
| mGR2        | gAF1                 |
| PEPCK-LUC   | gAF2                 |
| mGR1/2      | gAF1                 |
| PEPCK-LUC   | gAF2                 |
| palGR1      | gAF1                 |
| PEPCK-LUC   | gAF2                 |
or 500 nM Dex/5 M RA.

3 h under the following conditions: no hormone, 5 nM RA, 500 nM Dex, or 500 nM Dex/5 M RA. No Ab lanes show the results of immunoprecipitations performed in parallel without the application of primary antibodies. Input lanes show the results from samples not subjected to immunoprecipitation. B, the association of GR, RAR, RXR, HNF3β, and HNF4α with the endogenous β-actin gene promoter was measured by ChIP with the indicated antibody after ChIP. The data are expressed as the fold change relative to the control (untreated) samples from four to six independent experiments.

### Table I

The occupancy of the PEPCK gene promoter by transcription factors and coregulators changes upon treatment with Dex and/or RA

| Control | RA | Dex | Dex/RA |
|---------|----|-----|--------|
| GR      | 1.00 | 1.2 ± 0.2 | 13.0 ± 3.8 | 21.2 ± 6.2 |
| RAR     | 1.00 | 2.2 ± 0.2 | 1.3 ± 0.1 | 2.4 ± 0.4 |
| RXR     | 1.00 | 1.3 ± 0.1 | 0.8 ± 0.1 | 1.4 ± 0.2 |
| HNF3β   | 1.00 | 1.0 ± 0.1 | 1.1 ± 0.1 | 1.1 ± 0.1 |
| HNF4α   | 1.00 | 0.9 ± 0.1 | 1.1 ± 0.2 | 1.1 ± 0.1 |
| SRC-1   | 1.00 | 2.0 ± 0.2 | 3.5 ± 0.1 | 3.5 ± 0.2 |
| pCIP    | 1.00 | 2.1 ± 0.1 | 2.8 ± 0.1 | 2.8 ± 0.3 |
| CBP     | 1.00 | 6.9 ± 0.1 | 3.9 ± 0.5 | 6.5 ± 0.6 |
| p300    | 1.00 | 2.0 ± 0.4 | 2.1 ± 0.2 | 4.7 ± 1.1 |
| pol II  | 1.00 | 2.0 ± 0.2 | 2.2 ± 0.1 | 4.9 ± 0.7 |

H4IIE cells were transfected with PEPCK-LUC, RSV-GR, CMX-RAR, and CMX-RXR expression vectors with either pSUPER alone (control), p300 siRNA, or CBP siRNA. 5 μM RA, 500 nM Dex, or a combination of the two, was added to the cells 18 h before harvest. By using PEPCK-LUC reporter gene expression with Dex/RA treatment as 100%, the induction by RA or Dex is 10 and 65%, respectively (Fig. 9C). This is quantitatively similar to the pattern of endogenous PEPCK gene expression. The Dex-induced response was reduced to 35% of the maximal response in p300 siRNA-transfected H4IIE cells. By contrast, the RA-induced response was not changed. The Dex/RA-induced response is, however, reduced to 37%, which is similar to the reduction of the Dex-induced response. This result indicates that the synergy achieved with Dex/RA is abolished when p300 expression is reduced. The Dex-induced response was also reduced to 29% in CBP siRNA-transfected H4IIE cells, and the RA-induced response was not affected. The Dex/RA-induced response was only reduced to 53%, which is mainly because of the decreased response to Dex; however, the

![Fig. 7.](http://www.jbc.org/)

**DISCUSSION**

The GRU and RARU in the PEPCK gene promoter consist of several overlapping DNA elements, as described in the Introduction. Although any of these DNA elements and associated factors could be involved in the synergistic action of Dex/RA, it appears that gAF3, and to a lesser extent gAF1, are of most importance, and gAF2 is not involved (Fig. 2). In particular, the γ site of gAF3, which binds a RAR/RXR heterodimer, is critical (Fig. 4). The role of RAR/RXR binding is supported by the observation that substitution of an element at gAF1, which selectively binds RAR/RXR,-promotes synergism, but elements that selectively bind either HNF4 or COUP-TF, which bind to the wild type gAF1, do not (Fig. 3). Finally, the substitution of a consensus RARE at gAF1 (Fig. 3), and especially at gAF3 (Fig. 5), markedly enhances the synergistic effect; the cRARE at gAF3 results in a 4-fold greater synergistic response than that obtained from the wild type promoter (Fig. 5).

The Dex response was reduced by 50–60% in the experiment in which a cRARE was substituted for gAF3, which normally binds COUP-TF as an accessory factor in the Dex response (11, 13). Thus, unliganded RAR/RXR, if bound to the cRARE, cannot serve as an accessory factor for the Dex response. However, when RA is added, a robust synergistic effect is obtained, as noted above (and Fig. 5). Although indirect, this suggests that ligand-bound RAR/RXR can serve as an accessory factor in the Dex response.

The role of the accessory factors at gAF1, gAF2, and gAF3 (HNF4/COUPTF, HNF3, and COUP-TF, respectively) is to increase the affinity of binding of GR to the GR1 element (21). Thus, when GR1 is converted to a high affinity site, gAF1 and
of the accessory factors HNF4 and HNF3 is not affected by any treatment, as noted previously (18).

The promoter occupancy of PCAF, SRC-1, p/CIP, p300, and CBP was analyzed under the various hormone treatments. There was no binding of PCAF under any of the treatment conditions (Fig. 8). This was somewhat surprising in view of the observations that RA-activated RAR/RXR heterodimers recruit PCAF to DNA segments that contain the typical DR5 RARE in vitro (26). RA and Dex each recruit p/CIP and SRC-1; however, there is no additional binding when the Dex/RAR combination is employed. Indeed, the binding of these p160 family members is the same in Dex/RAR as in Dex-only treated cells (Fig. 8). A different pattern was noted with CBP. RA treatment resulted in more recruitment of CBP than did Dex treatment, and the Dex/RAR level was equivalent to that of RA alone. The most interesting observation concerns the binding of p300 to the PEPCK gene promoter. RA and Dex each increase the binding of p300 by ~2-fold; however, the combination results in about a 5-fold increase (Fig. 8 and Table I). A similar quantitative change occurs with the binding of pol II to the promoter (Table I). In concert with this observation, McGrane and co-workers (27) recently showed that pol II association with the PEPCK gene promoter is reduced in vitamin A-deficient mice.

A key role for p300 in the synergistic action of Dex/RA was established by selectively reducing the cellular levels of p300 and CBP by siRNA. Elimination of either had no effect on the RA response, whereas both reduced the Dex effect by about 50%. The absence of CBP had no effect on the synergistic effect of the Dex/RA combination, but removal of p300 abolished the response (Fig. 9). CBP and p300 are closely related proteins. Although encoded by different genes, they have several functional domains in common. The designation CBP/p300 implies a common overall function; however, recent reports (28) suggest each may govern unique cellular events. Mice with inactivation of a single allele of CBP develop a variety of hematological abnormalities that were not seen in mice that had a similar inactivating mutation in one of the p300 alleles. By contrast, the selective reduction of p300 makes F9 cells resistant to RA-induced differentiation, whereas a similar reduction of CBP has no effect (29).

The linear increase of the recruitment of p300 and pol II in conjunction with a nonlinear increase of the transcription response is not unexpected. Veitia (30) has explained this in the context of a series of cooperative interactions between promoter elements/enhancers and transcription factors with a succession of coregulator proteins and ultimately the basal transcription apparatus. The activation of the interferon-β gene in response to viral infection is a case in point. The synergistic transcription response of this gene is thought to be the result of the formation of a unique activating surface composed of a domain in the p65 subunit of NFκB and the activation domains of IRF1 and ATF-2/c-Jun with the architectural protein HMGI(Y). This enhancesome recruits CBP/p300 to the basal transcription complex (31). The likely participation of other components unique to the complex assembled on the PEPCK gene promoter in response to Dex/RA awaits further investigation.

In addition to the PEPCK gene, a number of other genes that encode enzymes involved in metabolic processes have promoters that allow for a range of gene expression. Examples include the tyrosine aminotransferase, fatty-acid synthase, L-type pyruvate kinase, insulin-like growth factor-binding proteins, and the glucose 6-phosphate catalytic subunit genes (32–37). We have used the phrase “metabolic control domain” (MCD) to describe an array of DNA elements and associated transcription factors that provide an integrated response to a variety of signals (2, 15). As the PEPCK gene MCD is elucidated, the
central importance of one element, gAF3, has become apparent. This DNA sequence (see Fig. 4) is involved as an accessory factor in the glucocorticoid response (8), as an important element in the retinoic acid response (7), and as a component of the cyclic AMP response unit (15), in the amplifying effect of PGC-1 (38), and now in the synergistic effect of Dex/RA (Figs. 2, 4, and 5). The proteins (15) and in the amplifying effect of PGC-1 (38), and now in the synergistic effect of Dex/RA (Figs. 2, 4, and 5). The proteins

REFERENCES

1. Levine, M., and Tjian, R. (2003) Nature 424, 147–151
2. Lucas, P. C., and Granner, D. K. (1992) Annu. Rev. Biochem. 61, 1131–1173
3. 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
4. Lucas, P. C., 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
5. Park, E. A., Jerden, D. C., and Bahouth, S. W. (1995) Biochem. J. 309, 913–919
6. Scott, D. K., O'Doherty, R. M., Stafford, J. M., Newgard, C. B., and Granner, D. K. (1998) J. Biol. Chem. 273, 24145–24151
7. Scott, D. K., Mitchell, J. A., and Granner, D. K. (1996) J. Biol. Chem. 271, 6260–6264
8. Scott, D. K., Stromstedt, P. E., Wang, J. C., and Granner, D. K. (1998) Mol. Endocrinol. 12, 482–491
9. Hall, R. K., Sladek, F. M., and Granner, D. K. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 412–416
10. Wang, J. C., Stromstedt, P. E., O'Brien, R. M., and Granner, D. K. (1996) Mol. Endocrinol. 10, 794–800
11. Scott, D. K., Mitchell, J. A., and Granner, D. K. (1996) J. Biol. Chem. 271, 31909–31914
12. Yamaoka, K., Duong, D. T., Scott, D. K., Wang, J. C., and Granner, D. K. (1999) J. Biol. Chem. 274, 5880–5887
13. Sugiyama, T., Scott, D. K., Wang, J. C., and Granner, D. K. (1998) Mol. Endocrinol. 12, 3807–3811
14. Stafford, J. M., Waltner-Law, M., and Granner, D. K. (2001) J. Biol. Chem. 276, 3811–3819
15. Watanabe, M., Duong, D. T., Daniels, M. C., Herzog, B., Wang, X. L., Prasad, R., and Granner, D. K. (2003) J. Biol. Chem. 278, 10427–10435
16. Nakashatri, H., and Chambon, P. (1994) J. Biol. Chem. 269, 890–902
17. O'Brien, R. M., Noisin, E. L., Suwanichkul, A., Yamasaki, T., Lucas, P. C., Wang, J. C., Powell, D. R., and Granner, D. K. (1996) Mol. Cell. Biol. 16, 1747–1758
18. Duong, D. T., Watanabe, M., Sears, R., Sealy, L., and Granner, D. K. (2002) J. Biol. Chem. 277, 32234–32242
19. Brunsmenkamp, T. R., Bernard, R., and Agami, R. (2002) Science 296, 550–553
20. Pfitz, H. C., Perrino, C., Morse, P. A., and Potter, V. R. (1964) Nat. Cancer Inst. Monogr. 15, 229–242
21. Stafford, J. M., Wilkinson, J. C., Bechem, J. M., and Granner, D. K. (2001) J. Biol. Chem. 276, 39885–39891
22. Chen, J. D., and Evans, R. M. (1995) Nature 377, 454–457
23. Schalman, I. G., Juguilon, H., and Evans, R. M. (1996) Mol. Cell. Biol. 16, 3807–3813
24. Deleted in proof
25. Granner, D., Andreone, T., Sasaki, K., and Beale, E. (1983) Nature 305, 545–549
26. Blanco, J. C., Minucci, S., Lu, J., Yang, X. J., Walker, K. K., Chen, H., Evans, R. M., Nakatani, Y., and Ozato, K. (1998) Genes Dev. 12, 1638–1651
27. Scribner, K. B., and McGrane, M. M. (2003) J. Nutr. 133, 4112–4117
28. Kung, A. L., Rebel, V. I., Bronson, R. T., Ch’ng, L. E., Steff, C. A., Livingston, D. M., and Topp, E. (2000) Genes Dev. 14, 272–277
29. Uga, H., Uchida, K., Kawasaki, H., and Yokoyama, K. K. (1999) J. Biol. Chem. 277, 481–494
30. Veritas, R. A. (2003) Biol. Rev. Camb. Philos. Soc. 78, 149–170
31. Wie, J., Merika, M., Munch, N., Chen, G., and Thanoos, D. (1999) EMBO J. 18, 3074–3089
32. Ganes, R., Weib, F., and Schultz, G. (1994) Mol. Endocrinol. 8, 895–903
33. Schwanzer, M., Roder, K., Zhang, L., and Wolf, S. S. (2002) Biochem. Soc. Trans. 30, 1070–1072
34. Yamada, K., and Noguchi, T. (1999) Biochem. J. 337, 11–1
35. Giromini, R., Lacson, R., Yang, E., Sum, R., and Unterman, T. (1994) Endocrinology 134, 736–743
36. Suwanichkul, A., Allander, S. V., Morris, S. L., and Powell, D. R. (1994) J. Biol. Chem. 269, 58835–58841
37. Vander Kooi, B. T., Streeper, R. S., Svitak, C. A., Oeser, J. K., Powell, D. R., and O’Brien, R. M. (2003) J. Biol. Chem. 278, 11782–11793
38. Herzog, B., Hall, R. K., Wang, X., Watanabe, M., and Granner, D. K. (2004) Mol. Endocrinol. 18, 807–819
The Synergistic Effect of Dexamethasone and All-trans-retinoic Acid on Hepatic Phosphoenolpyruvate Carboxykinase Gene Expression Involves the Coactivator p300

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