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The Orphan Receptor COUP-TF Binds to a Third Glucocorticoid Accessory Factor Element within the Phosphoenolpyruvate Carboxykinase Gene Promoter*

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The phosphoenolpyruvate carboxykinase (PEPCK) gene promoter contains a glucocorticoid response unit (GRU) that includes, as a linear array, two accessory factor binding sites (AF1 and AF2) and two glucocorticoid receptor binding sites. All of these elements are required for a complete glucocorticoid response. AF1 and AF2 also partially account for the response of the PEPCK gene to retinoic acid and insulin, respectively. A second retinoic acid response element was recently located just downstream of the GRU. In this study we show that mutation of the 3′ half-site of this element results in a 60% reduction of the glucocorticoid response of PEPCK promoter-chloramphenicol acetyltransferase (CAT) fusion constructs in transient transfection assays, thus the half-site is now termed AF3. A variety of assays were used to show that chicken ovalbumin upstream promoter transcription factor (COUP-TF) binds specifically to AF3 and that upstream stimulatory factor (USF) binds to an E-box motif located 2 base pairs downstream of AF3. Mutations of AF3 that diminish binding of COUP-TF reduce the glucocorticoid response, but mutation of the USF binding site has no effect. The functional roles of AF1, AF2, and AF3 in the glucocorticoid response were explored using constructs that contained combinations of mutations in all three elements. All three elements are required for a maximal glucocorticoid response, and mutation of any two abolish the response.

Phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32)[1] catalyzes the rate-limiting step of gluconeogenesis. The activity of this enzyme is therefore tightly regulated by a variety of hormonal and dietary signals that are involved in the maintenance of appropriate plasma glucose levels (for reviews, see Refs. 1 and 2). The PEPCK gene product apparently is not allosterically or post-translationally modified. Rather, the activity of PEPCK is modulated by changes in the amount of the protein, which are achieved predominantly by alterations of the rate of the transcription of the gene (3). PEPCK gene transcription is positively regulated by glucagon (via cAMP), glucocorticoids and retinoic acid (RA), while insulin inhibits PEPCK gene transcription and is dominant over the positive effectors (4–8). Thus, PEPCK is an excellent model system for studies concerning the integration of multiple metabolic signals through a single gene promoter.

The glucocorticoid response of the PEPCK gene is mediated by a complex glucocorticoid response unit (GRU). This region of DNA, located between positions −451 and −353 relative to the transcription start site, is composed of a tandem array of two accessory factor elements (AF1, −451 to −439 and AF2, −416 to −407) and two glucocorticoid receptor binding sites (GR1 and GR2, −386 to −353) (4). Mutation of either AF1 or AF2 results in a 50–60% reduction of the glucocorticoid response and deletion of both AF elements abolishes activity of the GRU (4, 9). GR1 and GR2 are essentially silent in the absence of accessory factors, which distinguishes them from simple glucocorticoid response elements (GREs) that are not dependent upon accessory factors for a glucocorticoid response (10). At least one other element is also required for a complete glucocorticoid response of the PEPCK gene. Deletion of the PEPCK cAMP response element (CRE; −93 to −86) also results in a 50–60% reduction of the glucocorticoid response (11). This effect may be mediated by protein-protein interactions, since the glucocorticoid receptor (GR) and CRE binding protein (CREB) interact in solution (11).

The accessory activity of AF1 can be attributed to the specific binding of chicken ovalbumin upstream promoter transcription factor (COUP-TF) or hepatic nuclear factor 4 (HNF-4), members of a subfamily of the steroid/thyroid/retinoid superfamily of nuclear hormone receptors (12, 13). These proteins are not known to bind ligands, hence they are called orphan receptors (14). Both HNF-4 and COUP-TF are independently capable of acting as accessory factors in the PEPCK glucocorticoid response (13). The AF2 element is bound by CCAAT enhancer-binding protein (C/EBP) family members and by hepatic nuclear factor 3 (HNF-3) (15, 16). A detailed analysis of the AF2 element has led to the conclusion that the accessory factor activity is mediated by HNF-3 and not by C/EBP family members (17).

In addition to their involvement in the glucocorticoid response, AF1 and AF2 are also required for other hormone responses. The minimal functional boundaries of AF1 are co-

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[1] The abbreviations used are: PEPCK, phosphoenolpyruvate carboxykinase; RA, all-*trans*-retinoic acid; GRU, glucocorticoid response unit; AF1, accessory factor 1; AF2, accessory factor 2; GR1 and GR2, PEPCK glucocorticoid receptor binding sites 1 and 2; GRE, glucocorticoid response element; CRE, cAMP response element; GR, glucocorticoid receptor; CREB, cAMP response element-binding protein; COUP-TF, chicken upstream ovalbumin promoter transcription factor; HNF-4, hepatic nuclear factor 4; C/EBP, CAAT enhancer binding protein; HNF-3, hepatic nuclear factor 3; RARE, retinoic acid response element; RAR, retinoic acid receptor; RXR, 9-*cis*-retinoic acid receptor; RARE2, the second RARE characterized in the PEPCK promoter; CAT, chloramphenicol acetyltransferase; AF, accessory factor; AF3, accessory factor 3; USF, upstream stimulatory factor.

incident with a retinoic acid response element (RARE), and any mutation that abolishes the accessory factor function abolishes the RA response and *vice versa* (9, 12, 13, 18). Heterodimers of retinoic acid receptor (RAR) and 9-*cis*-retinoic acid receptor (RXR) bind AF1 and mediate approximately 50% of the RA effect on PEPCK gene transcription (12). AF2 mediates a portion of the dominant negative insulin or phorbol ester responses (19, 20). As mentioned above, C/EBP family members and HNF-3 bind to AF2, but these proteins apparently do not mediate the insulin or phorbol ester responses (16). The insulin and phorbol ester response factor(s) remain unknown.

A recently identified second RARE in the PEPCK gene also binds RAR/RXR and mediates approximately half of the PEPCK retinoic acid response (21). This element (termed RARE2) is located between −337 and −321. RARE2 is composed of three potential binding sites for RAR-type nuclear receptors, the α, β, and γ half-sites. RAR/RXR binds to (and *trans*-activates through) the α and γ half-sites, arranged as direct repeats positioned 5 base pairs apart.

In this paper we demonstrate that the transcription factor COUP-TF binds to a sequence coincident with the γ half-site of RARE2. Mutations that disrupt the binding of COUP-TF also diminish the glucocorticoid response of PEPCK promoter-chloramphenicol acetyltransferase (CAT) fusion genes, thus defining a third accessory factor element (AF3) in the PEPCK promoter. The functional roles of AF1, AF2, and AF3 were explored using combinations of mutations of all three AF elements. A mutation of any one of the AF elements results in a 60% reduction of the glucocorticoid response, while mutations of any two AF elements reduce the glucocorticoid response to near basal levels.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The construction of pPL32 has been described (22). Site-directed mutations of pPL32 were constructed by either the oligonucleotide-directed mutagenesis method or by the polymerase chain reaction megaprimer method as described previously (23–25). The AF3αm, AF3γm, and AF3Em constructs were made using the polymerase chain reaction megaprimer method as described previously (23, 25). The AF2 double point mutation was also made by the megaprimer method and had the sequence 5′-TGGGCTTTTG-3′ instead of the core AF2 wild type sequence of 5′-TGGTGTTTTG-3′. The construction of the AF1 double point mutation was described previously (pB450 in Ref. 13). The sequence of all constructs was verified by dideoxy sequencing. All oligonucleotides were produced on a Perceptive Biosystems Expedite 8909 DNA synthesizer located in the Vanderbilt University Diabetes Research and Training Center.

Transient Transfections—The maintenance and transfection of H4IIE cells and the measurement of CAT activity have been described previously (12, 22, 26). The mammalian expression vectors pRShRARα and pSV2GR were provided by Ronald Evans (Salk Institute, San Diego, CA) and Keith Yamamoto (University of California, San Francisco), respectively.

Gel Mobility Shift Assays—Electrophoretic gel shift assays were performed as described previously (13). The HNF-4 antiserum was provided by Frances Sladek (University of California, Riverside, CA), and the COUP-TF and RXR antisera were provided by Ming-Jer Tsai (Baylor College of Medicine) and Jackie Dyke (Salk Institute), respectively. The USF antiserum was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rat liver nuclear extract was prepared by the method of Gorski *et al.* (27). RAR and RXR were expressed in bacteria and purified according to the method of Forman *et al.* (28). The consensus USF oligonucleotide had the sequence 5′-GATCTCCGGTCACGTGACCGGA-3′ (29).

Methylation Interference—Methylation interference was performed as described previously (12, 21).

RESULTS

The RARE2 (AF3) Element Is Required for a Complete Glucocorticoid Response—The PEPCK RARE1 (AF1) element is required for complete glucocorticoid and retinoic acid responses (4, 9, 12, 13, 18, 21). Therefore, we tested whether a mutation

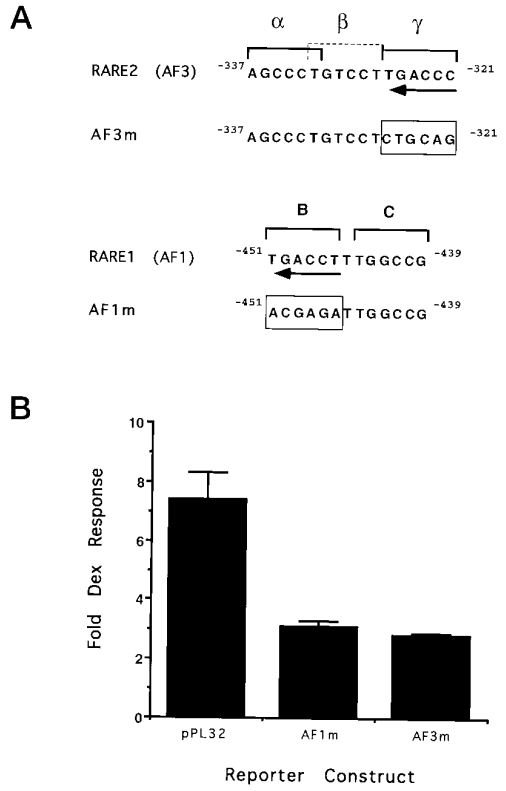

FIG. 1. **Colocalization of an RARE and a glucocorticoid accessory factor element.** *A*, the sequence of RARE2 (*AF3*) contains two overlapping degenerate RAR/RXR half-sites (α and β) and a consensus half site (γ). The γ half-site is *underlined* by an *arrow* to indicate the conventional orientation of nuclear hormone receptor half-sites (43, 44). The AF3m construct contains a block mutation of the γ half-site which is *outlined* with a *box*. Also shown is the sequence of RARE1 (*AF1*) and the block mutation in the B half site, *AF1m*, which is also *highlighted* with a *box*. *B*, constructs that contain the wild type PEPCK promoter ligated to CAT (pPL32) or constructs that contain block mutations of either AF1 (*AF1m*) or AF3 (*AF3m*), were cotransfected (10 μg each) with pRShRARα (5 μg) into H4IIE rat hepatoma cells. CAT activity was measured in cell lysates 18 h after treatment with or without 0.5 μM dexamethasone. The data are expressed as an average fold induction of CAT activity (with dexamethasone/without dexamethasone) ± S.E. of ≥4 separate experiments.

in RARE2 (AF3), previously shown to disrupt the RA response (21), would also decrease the glucocorticoid response (Fig. 1). These experiments were initially performed in parallel with the RA experiments, so that H4IIE rat hepatoma cells were cotransfected with PEPCK promoter-CAT constructs and a vector expressing RAR (21). A block mutation of either RARE2 (AF3) or RARE1 (AF1) resulted in about a 50% reduction of the glucocorticoid response (compare *AF1m* and *AF3m* with wild type *pPL32* in Fig. 1*B*). The same effect on the glucocorticoid response was seen when constructs containing RARE2 (AF3) mutations were either transfected in the absence of an expression vector or cotransfected with a construct expressing GR (see below). For clarity, RARE2 (AF3) will be henceforth referred to as simply AF3, since this element exhibits glucocorticoid accessory factor activity.

COUP-TF Binds to AF3—Mobility gel shift assays were utilized to examine whether AF1 and AF3 bind common rat liver nuclear proteins. Two shifted bands, I and II, were evident when AF3 was used as the probe (Fig. 2*B* and Ref. 21). A faint third band that migrates between bands I and II has previously been identified as RAR/RXR (21). A 100-fold molar excess of an oligonucleotide containing AF1 effectively competed for the formation of band II and for formation of the RAR/RXR·DNA complex. The AF1 element binds HNF-4 and COUP-TF (13), so

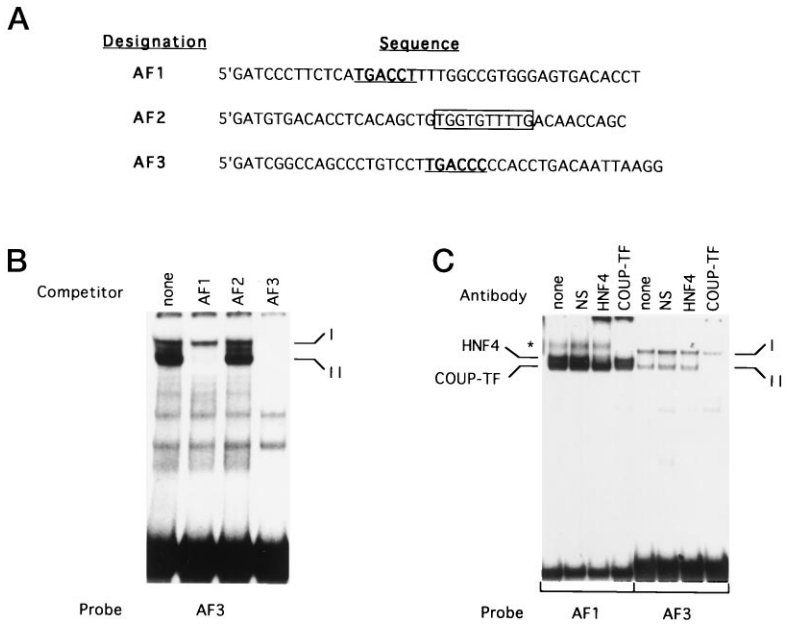

FIG. 2. **COUP-TF binds specifically to AF3.** *A*, the sequences of the oligonucleotides used as radiolabeled probes and unlabeled competitors are shown. The consensus nuclear hormone receptor binding sites are *underlined* and shown in *bold type* (43, 44). The 10-base pair core sequence of AF2 is *outlined* with a *box* (16, 20). *B*, this panel illustrates a mobility gel shift assay when AF3 was used as the probe. The indicated double-stranded oligonucleotide competitors were added in a 100-fold molar excess prior to the addition of 5 μg of rat liver nuclear extract. *Labels I* and *II* denote protein-DNA interactions detected by the AF3 probe. The faint band that migrates between band I and II is RAR/RXR (21). *C*, radiolabeled AF1 or AF3 oligonucleotides were incubated for 15 min with 5 μg rat liver nuclear extract and 1 μl of antiserum directed against COUP-TF, HNF-4, or a nonspecific protein. The *asterisk* indicates a COUP-TF/RXR heterodimer that is sometimes visible when AF1 is used as the probe. The autoradiographs shown are representative of at least two experiments.

an antibody supershift analysis was used to determine whether proteins antigenically related to either COUP-TF or HNF-4 bind to AF3 (Fig. 2*C*). For simplicity, we refer to COUP-TFI and the very closely related gene products COUP-TFII and EAR-2 collectively as COUP-TF. When AF1 was used as the probe the HNF-4 antiserum supershifted the HNF-4 band and the COUP-TF antiserum diminished the COUP-TF complex (13) and also supershifted a band demonstrated to be a COUP-TF/RXR heterodimer (data not shown). Formation of band II was disrupted by incubation with an antiserum directed against COUP-TF when AF3 was used as the probe. In contrast, the HNF-4 antiserum did not affect the formation of either band I or II (Fig. 2*C*). Thus, band II contains COUP-TF, but not HNF-4. COUP-TF binds as a homodimer, and it can heterodimerize with RXR (14). In other experiments, anti-RXR antiserum failed to supershift the COUP-TF-containing complex on AF3 (data not shown).

Methylation interference was performed in order to define the contact sites made by COUP-TF and the protein component of band I. The methylated guanine residues that interfered with the binding of both COUP-TF and band I proteins are shown in Fig. 3. The γ half-site was required for efficient binding of COUP-TF as well as for a complete glucocorticoid response (Figs. 1 and 3 and see also Fig. 5). However, we have not been able to ascertain the location of a second half-site for COUP-TF binding. COUP-TF could make nonspecific contacts along the phosphate backbone or could bind to a single half-site. Fig. 3 also demonstrates that the protein component of band I binds to an E-box motif (CANNTG) located 2 base pairs downstream from the γ half-site. E-box motifs are bound by members of the basic helix-loop-helix family of transcription factors, including Myc, MyoD, and USF (30).

The closely related transcription factors USF-I (43 kDa) and USF-II (44 kDa) bind with high affinity to the consensus sequence CACGTG, but can also bind to the sequence CACCTG, which is present in the PEPCK gene promoter E-box motif (Refs. 29 and 31 and Fig. 3). A 100-fold molar excess of an oligonucleotide containing the CACGTG motif effectively competed for the formation of band I in a competition mobility gel shift assay, which indicated that band I proteins interact with a consensus USF binding site (Fig. 4*A*). Antibody supershift analysis, utilizing an antiserum that detects both USF-I and USF-II, demonstrated that band I is antigenically related to the USF family of transcription factors (Fig. 4*B*). For simplicity, the protein component of band I will henceforth be referred to as USF, although we recognize that we did not distinguish between USF-I and USF-II.

COUP-TF, but Not USF, Is Required for a Complete Glucocorticoid Response—Constructs were made wherein selected guanine residues were changed based on the contacts made by either COUP-TF or USF (see Fig. 3). In addition, the α half-site mutant previously shown to decrease both RAR/RXR binding and the RA response (21), and a block mutation of the β half-site, were examined to determine whether these elements had any effect on the glucocorticoid response (Fig. 5*A*). Mutation of the α half-site, the β half-site, or the E-box motif did not affect the glucocorticoid response (Fig. 5*A*). In contrast, mutation of the γ half-site decreased the glucocorticoid response by 60%, an effect similar to that noted when the entire γ half-site was mutated (Fig. 1).

Mobility gel shift experiments were performed using rat liver nuclear extract and AF3 as the probe to determine whether COUP-TF and USF binding correlated with the functional data presented in Fig. 5*A*. Double-stranded oligonucleotides that contain the mutations employed in the study described in Fig. 5*A* were used as competitors. Oligonucleotides with the wild type AF3 sequence, or the α or β half-site mutations, competed effectively for the formation of both the COUP-TF·DNA and USF·DNA complexes (Fig. 5*B*). However, the γ half-site mutant did not compete for the binding of COUP-TF, and the E-box mutant did not compete for the binding of USF. These obser-

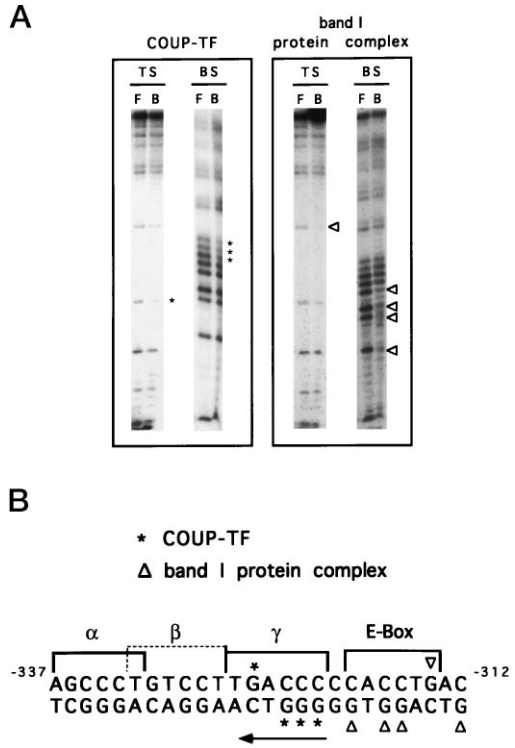

FIG. 3. **COUP-TF makes contact with a single consensus nuclear receptor half-site.** *A*, mobility gel shift assays were performed using 10 μg of rat liver nuclear extract and the end-labeled and partially methylated AF3 element as the probe. DNA was isolated from the free and protein-bound bands and subjected to piperidine cleavage and separation on a 6% denaturing polyacrylamide gel. The methylated guanine residues that interfered with COUP-TF binding are indicated with an *asterisk*. The guanine residues that interfered with the formation of band I are indicated with *arrowheads*. *TS*, top strand; *BS*, bottom strand; *F*, free DNA; *B*, protein-bound DNA. *B*, the sequence of AF3 is shown and the methylated guanine residues that interfere with COUP-TF binding or the formation of band I are indicated with *asterisks*. The *arrow* designates the conventional orientation of the COUP-TF half-site (43, 44). The protein component of band I makes contacts with guanine residues within and adjacent to the apparent E-box motif, as indicated with *arrowheads*.

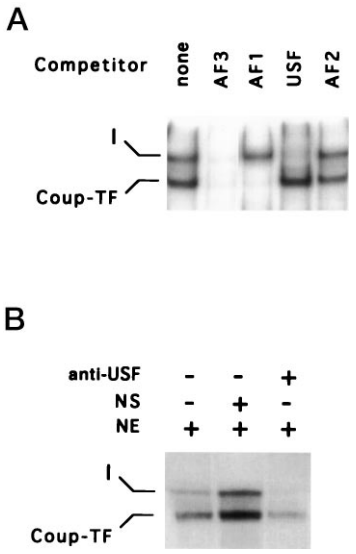

FIG. 4. **USF binds specifically to the E-box motif.** This figure illustrates mobility gel shift assays in which AF3 was used as the probe. *A*, the indicated competitor oligonucleotides were added in a 100-fold molar excess prior to the addition of 5 μg of rat liver nuclear extract. The positions of band I and the COUP-TF·DNA complex are shown. The lane marked USF represents the competitor oligonucleotide that contains the consensus sequence for USF binding (29). The sequences of the AF1, AF2, and AF3 competitor oligonucleotides are shown in Fig. 1. *B*, this panel represents a mobility gel shift experiment in which the binding reaction incubation included 5 μg of rat liver nuclear extract and 2 μl of antiserum directed against USF or a nonspecific antiserum. For simplicity, only the portion of the gel containing bound complexes is shown; no other complexes were evident on the gel. The autoradiographs shown are representative of three separate experiments.

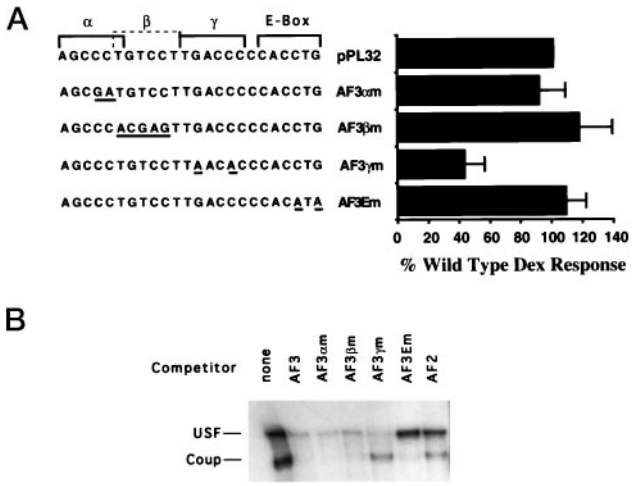

FIG. 5. **Correlation of COUP-TF binding with the glucocorticoid response.** *A*, the sequences of specific AF3 element mutations are shown on the *left*. Results from experiments in which H4IIE rat hepatoma cells were transfected with PEPCK-CAT fusion genes that contain the indicated mutations of the AF3 element are shown on the *right*. CAT activity was measured in cell lysates 18 h after treatment with or without 0.5 μM dexamethasone. Results are expressed as the average percentage of wild type CAT activity ± S.E. of ≥4 separate determinations. *B*, mobility gel shift assays were performed with a double-stranded, end-labeled AF3 oligonucleotide as the probe. The indicated competitor oligonucleotides were added in a 100-fold molar excess prior to the addition of 5 μg of rat liver nuclear extract. Only the portion of the gel containing bound complexes is shown, since no other bands were noted. The autoradiograph shown is representative of two independent experiments.

vations are consistent with the methylation interference data and, together with the functional data in Fig. 5A, suggest that COUP-TF mediates the accessory factor activity of AF3.

Two of the Three Accessory Factors Are Required for Partial GRU Function—Constructs were made wherein combinations of mutations were introduced in AF1, AF2, and AF3 in the context of the intact PEPCK promoter in an effort to understand the functional role of AF3 in relation to AF1 and AF2. Two base pairs were altered in each element so that the factor known to mediate glucocorticoid accessory factor activity from that element could not bind (Refs. 13 and 17 and Fig. 5). A 60% decrease of the glucocorticoid response was evident when any one of the AF elements was mutated (Fig. 6). This result confirms previous observations made with deletion mutations and block mutations of AF1 and AF2 (4, 9, 17). When any two AF elements were mutated, or all three AF elements were mutated, the glucocorticoid response dropped to the level exhibited by pPL33, a PEPCK promoter-CAT construct with a 5′ end point of −306, that removes all three AF elements as well as both GR binding sites (4). Thus, mutation of any two AF elements essentially eliminates the glucocorticoid response mediated by the GRU, and, conversely, any combination of two intact AF elements confers a partial (3-fold) glucocorticoid response.

DISCUSSION

We have identified a third glucocorticoid response accessory factor element (AF3) in the PEPCK gene promoter. AF3 binds

the orphan receptor COUP-TF, and the binding of this factor correlates closely with the functional activity of AF3. In addition, AF3 exhibits accessory factor activity equivalent to that of AF1 and AF2, since a mutation in any of the AF elements results in a similar reduction of the glucocorticoid response. Furthermore, none of the AF elements alone is sufficient to confer accessory factor activity to the glucocorticoid response since mutations in any two AF elements abolish the glucocorticoid response. The fact that any combination of two AF elements allows the same partial glucocorticoid response implies that the accessory factor activities of AF1, AF2, and AF3 have quantitatively similar functional roles.

COUP-TF was originally identified as a positive transcription factor as it was required for the expression of the chicken ovalbumin gene (32). More recently, COUP-TF has been described as a repressive factor in that it counteracts the positive transcriptional effects mediated by RAR/RXR, thyroid hormone receptor/RXR, 1,25-dihydroxyvitamin D_3 receptor/RXR, peroxisome proliferator-activated receptor/RXR, HNF-4, and estrogen receptor (33–36). In the context of the PEPCK gene, COUP-TF is apparently required as an accessory factor for a complete glucocorticoid response, and this activity is mediated through both AF1 and AF3 (Ref. 13 and this work). This requirement is not unique as a number of different transcription factors provide accessory factor activity to GREs in a variety of promoter contexts. For example, Sp1, NF1, OTF, CP1, and the CACCC-box factor all potentiate the glucocorticoid response of constructs that contain a mouse mammary tumor virus GRE and cognate binding sites for these transcription factors linked to a CAT reporter gene (37). In addition, our previous studies have shown that HNF-4, COUP-TF, HNF-3, and CREB have accessory factor activity in the PEPCK GRU (Fig. 7 and see below). In spite of the large number of accessory factors, there seems to be some specificity in this requirement. For example, Allan *et al.* (38) demonstrated that COUP-TF could not substitute for NF1 accessory factor activity when a synthetic promoter was tested in a GR-dependent *in vitro* transcription system. Promoter context must be important, since COUP-TF is an accessory factor in the context of the PEPCK promoter (Ref. 13 and this study). In addition, Wang *et al.* (17) have recently demonstrated that Sp1 cannot substitute for AF2 in the context of the PEPCK promoter.

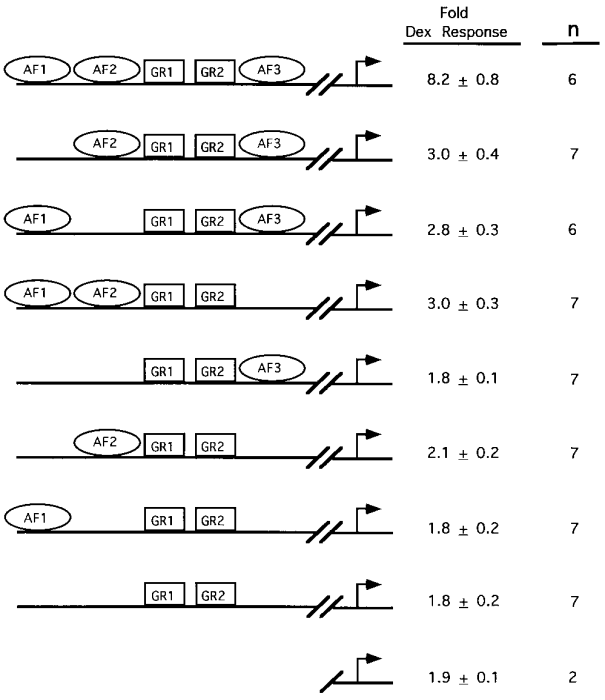

FIG. 6. **Functional roles of the accessory factor elements of the PEPCK gene GRU.** Mutations were introduced into the accessory factor elements of the PEPCK promoter, either separately or in combination. All of the mutations were made in the context of PEPCK promoter-CAT fusion gene constructs and were double point mutations that prevent the binding of the cognate accessory factor. The function of these constructs was compared with that of the full-length wild type PEPCK promoter-CAT reporter (pPL32; −467 to +69) and to a PEPCK promoter-CAT construct that lacks AF1, AF2, and AF3 (pPL33; −306 to +69). Ten μg of reporter plasmid and 5 μg of pSVGR were cotransfected into H4IIE cells, and CAT activity was measured in cell extracts 18 h after treatment of the cells with or without 0.5 μM dexamethasone (*Dex*). The data are expressed as an average fold induction of CAT activity (with dexamethasone/without dexamethasone) ± S.E. The number of independent transfections performed is indicated (*n*).

Since AF1 and AF3 both bind COUP-TF and RAR/RXR (Refs. 12, 13, and 21 and this study), and both elements function as RAREs, it is reasonable to postulate that RAR/RXR could be an accessory factor. The assignment of COUP-TF (and HNF-4 for AF1) as a glucocorticoid accessory factor is based on the following observations. COUP-TF and HNF-4 are both present in high abundance in H4IIE cells and bind AF1 with approximately equal high affinity (13). By contrast, RAR is much less abundant in H4IIE cells and, while there is enough RAR present in these cells to allow a 3–4-fold induction of the endogenous gene by RA, cotransfection of RAR is necessary for a robust RA response in transient transfection assays (12, 18, 21). Furthermore, RAR must be added to rat liver nuclear extracts in order to observe appreciable amounts of RAR/RXR heterodimer formation in mobility gel shift assays when either AF1 or AF3 are used as the probe (12, 18, 21). Importantly, cotransfection of RAR with PEPCK promoter-CAT constructs does not increase the glucocorticoid response (13). Thus, in the absence of ligand, RAR/RXR does not appear to have accessory factor function. However, we cannot rule out the possibility that ligand-bound RAR/RXR could act as an accessory factor (from either AF1 or AF3 or both) under certain metabolic circumstances *in vivo*.

The addition of AF3, and the inclusion of the CRE, expands the PEPCK gene GRU to include, 5′ to 3′, AF1 (−451 to −439), AF2 (−416 to −407), two glucocorticoid receptor binding sites, GR1 and GR2 (−386 to −353), AF3 (−326 to −321), and the CRE (−93 to −86) (Refs. 4 and 11 and this study and see Fig. 7). There are a number of similarities between AF3 and the other AF elements. AF3 is located adjacent to the GRs, as are AF1 and AF2, and all these elements mediate the same mag-

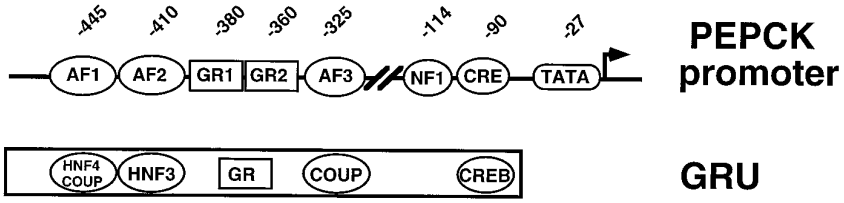

FIG. 7. **Components of the PEPCK gene GRU.** This diagram of the PEPCK gene promoter illustrates the characterized *cis* elements and associated *trans*-acting factors that are required for a complete glucocorticoid response. The location of each element with respect to the transcription start site is indicated above the schematic of the PEPCK promoter.

nitude of accessory factory activity (Fig. 6). AF3 participates in more than one hormonal or dietary response, as do AF1 and AF2 (Refs. 4, 9, 18, 20, and 21 and this study). In addition, several protein complexes bind to AF3, a characteristic shared by AF1 and AF2 (Refs. 12, 13, 15, 16, and 21 and Fig. 2). We have now identified the accessory factors that bind to each of the AF elements: AF1 activity is mediated by either HNF-4 or COUP-TF; AF2 activity is mediated by HNF-3; and AF3 activity is mediated by COUP-TF (Refs. 13 and 17 and this study). These observations have been summarized in Fig. 7.

As noted above, all three AF elements, as well as the CRE, participate in more than one effector response. Thus, AF1 and AF3 bind RAR/RXR and mediate the RA response (12, 21). Similarly, AF2 binds HNF-3 and C/EBP family members, and other, as yet unidentified factors, and this element mediates a significant portion of the dominant negative insulin and phorbol ester responses (15, 16, 19, 20). In addition, the CRE binds CREB and C/EBP family members, the former being integral to PEPCK basal transcription as well as the cAMP and glucocorticoid responses (11, 15, 39–41). Importantly, each individual element requires functional interactions with at least one other element to mediate a complete effector response. Thus, each hormonal response is mediated by a response unit, comprised of multiple, functionally interdependent elements and associated factors. Accordingly, the RA response requires both AF1 and AF3 (retinoic acid response unit), the cAMP response requires the CRE and a C/EBP binding site located between −233 and −225 (P3(I), Ref. 5; together with the CRE, the CRU), the glucocorticoid response requires AF1, AF2, AF3, and the CRE (together, the functionally complete GRU, see Fig. 7), and the insulin response requires AF2 and at least one other element that remains to be identified (the IRU).

The use of multiple elements to mediate a complete effector response results in functional redundancy, thus a mutation in any one element (or binding factor) would not result in an ineffective response to a particular metabolic signal. In the case of PEPCK gene expression such a single defect would presumably not greatly alter gluconeogenesis and glucose homeostasis. Additionally, with multiple protein complexes capable of binding to each element, this multicomponent (and overlapping) structure for each hormonal or dietary response allows a finely tuned, functionally integrated response to a wide variety of environmental conditions. Thus, the amount (and/or affinity) of the various protein complexes that bind to these elements must determine the capacity of the promoter to respond to particular components of multiple simultaneous environmental signals. These concepts have led us to describe the PEPCK promoter as a metabolic control domain wherein multiple environmental signals are integrated (21, 42). It is not unreasonable to predict that metabolic control domains will be defined in the promoters of genes subject to complex regulation.

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