The orphan receptors COUP-TF and HNF-4 serve as accessory factors required for induction of phospho*enol*pyruvate carboxykinase gene transcription by glucocorticoids

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ABSTRACT Glucocorticoids stimulate hepatic phospho*enol*pyruvate carboxykinase (PEPCK; EC 4.1.1.32) gene expression, thereby increasing the rate of gluconeogenesis. The effect of glucocorticoids on PEPCK gene expression is mediated by a set of promoter elements collectively referred to as the glucocorticoid response unit. The response unit spans a 100-bp segment and includes two glucocorticoid receptor binding sites (GR1 and GR2) and two accessory factor binding sites (AF1 and AF2), all of which are required for a maximal glucocorticoid response. The AF1 element also serves as a retinoic acid response element and may be involved in developmental and tissue-specific expression of the gene. In this study we report that COUP-TF and HNF-4, two orphan members of the nuclear receptor superfamily, bind to the AF1 element and function as accessory factors for the glucocorticoid response of the PEPCK gene.

Phospho*enol*pyruvate carboxykinase [PEPCK; GTP: oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32] catalyzes the rate-limiting step in gluconeogenesis and the amount of the mRNA encoding this protein is regulated both transcriptionally and posttranscriptionally by a variety of hormones (1–3). Transcription of the PEPCK gene in liver is positively regulated by glucocorticoids, retinoic acid, and cAMP and is negatively regulated by insulin and phorbol esters (3–7). The negative effectors are dominant. A maximal response to each of these hormones requires several elements within the PEPCK gene promoter (3, 8, 9).

The glucocorticoid response requires the presence of two glucocorticoid receptor (GR) binding sites called GR1 and GR2 and two accessory-factor elements termed AF1 and AF2. AF1 and AF2 are located just 5′ of the receptor binding sites, and the whole complex spans ≈100 bp (from −451 to −351 relative to the transcription start site at +1) (8, 10). This complex has been termed a glucocorticoid response unit (GRU) (8). The GRU itself is not sufficient for full activation of PEPCK gene expression by glucocorticoids. Such activation requires the interaction of the GRU with the cAMP response element (CRE) that is located ≈300 bp nearer the transcription start site (11). The objective of the work described in this paper was to understand how AF1, one component of this complex array, functions. We demonstrate here that the transcription factors COUP-TF and HNF-4, two members of the nuclear receptor superfamily whose ligands have not been identified ("orphan" receptors), bind the AF1 element and show that each of these proteins can act as accessory factor 1 in the PEPCK glucocorticoid response.

MATERIALS AND METHODS

Plasmids and Transfections. The construction of plasmids pPL32, pB450, and pC443 and the transfection of H4IIE rat

hepatoma cells and measurment of chloramphenicol acetyltransferase (CAT) activity have been described (12–14).

Gel Mobility-Shift Assays. The binding of nuclear factors to selected oligonucleotide probes was accomplished by adding 5 μg of crude rat liver nuclear extract (15) to each gel shift reaction mixture. A typical reaction mixture (20 μl) contained 2×10^4 cpm of the appropriate end-labeled double-stranded oligonucleotide, 10 mM Tris·HCl (pH 7.5), 50 mM NaCl, 10 mM EDTA, 1 μg of poly(dI-dC)·poly(dI-dC), 5% (vol/vol) glycerol, and 1 mM dithiothreitol. After incubation for 10 min at room temperature, the samples were loaded on an 8% polyacrylamide gel. The electrophoresis buffer was composed of 36.4 mM boric acid, 32.5 mM Tris base, and 0.25 mM EDTA. The gels were run at room temperature at a constant voltage of 180 V (≈20 mA), dried, and analyzed by autoradiography. The HNF-4 antiserum was described previously (16), and COUP-TF and retinoid X receptor α (RXRα) antisera were kindly provided by Ming-Jer Tsai (Baylor College of Medicine) and Jackie Dyke (Salk Institute), respectively. RXRα was expressed in bacteria and purified by the method of Forman *et al.* (17).

RESULTS

Mutations Within the AF1 Element Attenuate the PEPCK Glucocorticoid Response. A reporter construct that contains the PEPCK promoter sequence from −467 to +69 linked to the CAT reporter gene (pPL32), when transfected into H4IIE rat hepatoma cells, has been shown to mimic the endogenous PEPCK gene in its response to a variety of hormones (9). This segment of the promoter also directs proper hormonal regulation of the PEPCK gene in the livers of transgenic mice (18–20). All studies described in this work used pPL32, or reporter constructs produced by site-directed mutagenesis of specific sequences within pPL32, so as to maintain the natural spacing of the elements under study.

Double point mutations were introduced into the upstream half-site (the B box) or downstream half-site (the C box) of the AF1 element (stars in Fig. 1*A*). The resulting constructs, designated pB450 and pC443, respectively, were compared with pPL32 with regard to how expression of the CAT reporter gene was affected by dexamethasone (Fig. 1*B*). H4IIE rat liver hepatoma cells were cotransfected with either pPL32, pC443, or pB450 and the indicated receptor expression vector, and CAT activity was measured. A 9-fold increase in CAT expression in response to dexamethasone was noted in H4IIE cells transfected with pPL32. Cells transfected with either pC443 or

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A

B

FIG. 1. The AF1 element is required for the glucocorticoid response. (*A*) Site-directed mutagenesis was used to create double point mutations in the AF1 element contained within pPL32. The B box and C box are the upstream and downstream half-sites, respectively. The positions of the B450 and C443 mutations in the AF1 element are shown by stars. The plasmids containing these mutations—pB450 and pC443, respectively—are otherwise identical to pPL32. (*B*) H4IIE cells were transfected with either pPL32, pC443, or pB450 reporter plasmids along with vectors using the Rous sarcoma virus (RSV) promoter to express the neomycin-resistance gene (RSVneo), the GR gene (RSVGR), or the retinoic acid receptor (RAR) gene (RSVRAR). After transfection the cells were either left untreated or treated with 0.5 μM dexamethasone. The cells were harvested after 18 hr and CAT assays were performed. The results are expressed as fold induction, which represents the ratio of CAT activity of dexamethasone-treated cells over that of untreated cells. The vertical bars represent the means ± SEM of at least three independent experiments.

pB450 had responses 50–75% lower than that noted in cells containing pPL32 (Fig. 1*B*). This reduction is equivalent to the dexamethasone response seen when the entire AF1 element is deleted (8). Thus, either of these sets of mutations completely disables AF1. Cotransfection of the GR expression vector RSVGR with pPL32 increased the induction of CAT activity by dexamethasone but did not affect the relative decrease caused by mutations in either the B or the C box. Thus, the requirement for an intact AF1 element cannot be overridden by overexpression of GR.

The AF1 element contains retinoic acid response element (RARE) that binds an RARα/RXRα heterodimer in a functional complex (13). This binding is abolished by the double point mutations contained in either pC443 or pB450 (13). In H4IIE cells the overexpression of RARα dramatically increases the retinoic acid response mediated by the AF1 element in pPL32 (5, 13). However, overexpression of RARα, which presumably results in increased formation of RARα/RXRα complexes, does not enhance dexamethasone-induced CAT gene expression from pPL32 (Fig. 1*B*). Thus, it appears from this transfection study that the RARα/RXRα complex does not serve as an accessory factor in the glucocorticoid response.

Liver Nuclear Factors Bind the AF1 Element. To define the protein(s) that bind to AF1 and mediate the function of this element in the glucocorticoid response, a labeled double-stranded oligonucleotide containing the wild-type AF1 ele-

ment was used as a probe in a gel mobility-shift assay. Two major DNA/protein complexes (*a* and *b*) were observed when the wild-type AF1 probe was incubated with rat liver nuclear extract (Fig. 2). An identical gel mobility-shift pattern has been observed with extracts isolated from H4IIE cells (13). A 100-fold molar excess of unlabeled wild-type AF1 oligonucleotide abolished formation of the two complexes, but a nonspecific oligonucleotide (containing the PEPCK CRE) had no effect. A 100-fold molar excess of the oligonucleotides containing the B450 or C443 mutations also did not compete for complex formation. There is, therefore, a direct correlation between the specific formation of these two complexes and the functional activity of the AF1 element.

COUP-TF and HNF-4 Form the Two AF1/Protein Complexes. The sequence of the AF1 element matches the HNF-4 binding site in the apolipoprotein (Apo) CIII gene promoter at 11 of 12 positions (16, 21). This element also binds the ApoAI gene-regulatory protein ARP-I (22). ARP-1 and COUP-TF share 98.5% identity in their DNA-binding domains and 95% identity in their carboxyl-terminal domains (22). Therefore, we used antisera raised against COUP-TF or HNF-4 in an attempt to define the components of the AF1/protein complexes. The COUP-TF and HNF-4 antisera caused a specific loss of the *a* and *b* DNA/protein complexes, respectively, in a gel mobility-shift assay (Fig. 3). This identifies HNF-4 and COUP-TF, or antigenically related proteins, as the factors responsible for the formation of these bands. We have shown previously that neither complex contains RXRα (13, 23). For simplicity, we will continue to refer to the proteins that form the *a* and *b* bands as COUP-TF and HNF-4 even though we recognize that our antibody and DNA-binding studies cannot distinguish between proteins that are closely related to COUP-TF and HNF-4.

COUP-TF and HNF-4 Bind to Unique DNA Elements. Although many DNA elements bind both COUP-TF and HNF-4, there are sequences that can bind only one or the other of these proteins (24). We reasoned that substitution of the AF1 element for a DNA sequence that binds only COUP-TF,
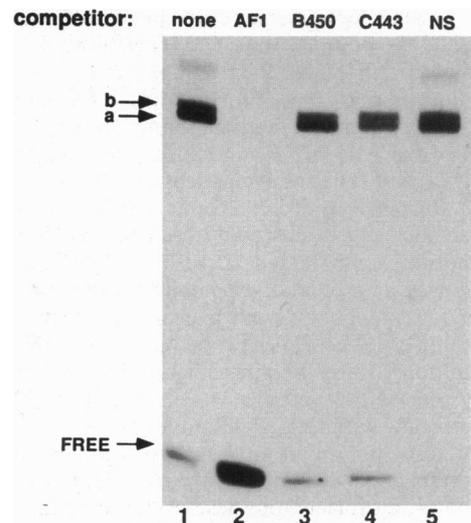
FIG. 2. Two major DNA/protein complexes form on the AF1 element. End-labeled, wild-type AF1 oligonucleotide was incubated with 5 μg of rat liver nuclear extract in the presence or absence of a 100-fold molar excess of the indicated competitor oligonucleotide, which was added to the reaction mixtures before the addition of extract. The resulting DNA/protein complexes were separated from free AF1 oligonucleotide in a gel mobility-shift assay. The sequences of the oligonucleotides containing the B450 and C443 mutations are identical to the AF1 oligonucleotide except for the changes shown by the stars in Fig. 1*A*. The nonspecific (NS) oligonucleotide contained the PEPCK CRE and surrounding sequence.
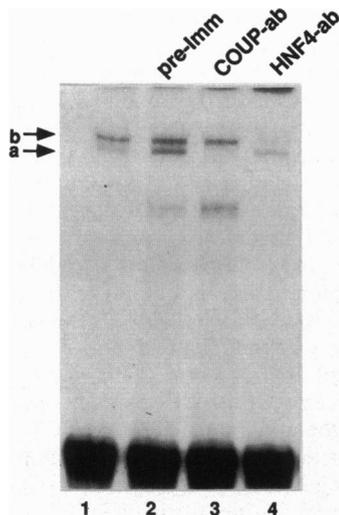
FIG. 3. COUP-TF and HNF-4 interact with the AF1 element. End-labeled wild-type AF1 oligonucleotide was incubated with 1 μg of crude rat liver nuclear extract. Preimmune serum, COUP-TF antiserum, and HNF-4 antiserum were added to the reaction mixtures that were loaded in lanes 2–4, respectively. The resulting DNA/protein complexes were separated from free AF1 oligonucleotide in a gel mobility-shift assay.

FIG. 4. The α1AT and TRE oligonucleotides compete for binding of specific AF1/protein complexes. (*A*) The sequence of the PEPCK AF1 element is compared with the core sequences of the competitor oligonucleotides used in *B*. (*B*) End-labeled AF1 oligonucleotide was mixed with 5 μg of crude rat liver nuclear extract. The indicated competitor oligonucleotides were added to the reaction mixtures prior to the addition of extract. The DNA/protein complexes were separated from free probe by gel mobility-shift assay. The CRE competitor oligonucleotide, which served as the nonspecific control, was identical to that used in Fig. 2.

or for one that binds only HNF-4, would allow us to determine which, if either, of these proteins could serve as the AF1 factor required for the glucocorticoid response. The DNA elements chosen were the HNF-4 binding site in the α1-antitrypsin (α1AT) gene, which does not bind ARP-1 or COUP-TF (22, 24), and the synthetic thyroid hormone response element (TRE) palindrome, which binds ARP-1 or COUP-TF but not HNF-4 (16, 22, 24) (Fig. 4*A*). A mobility-shift assay was performed to demonstrate the specificity of these two DNA elements. A 100-fold molar excess of unlabeled AF1 oligonucleotide completely prevented binding of COUP-TF or HNF-4 to the labeled AF1 probe (Fig. 4*B*, lanes 2 and 3). In contrast, the α1AT oligonucleotide competitor prevented formation of the AF1/HNF-4 complex but had only a weak effect on formation of the AF1/COUP-TF complex (lanes 4 and 5). The TRE oligonucleotide competitor prevented formation of the AF1/COUP-TF complex and had only a weak effect on formation of the AF1/HNF-4 complex (lanes 6 and 7).

The α1AT and TRE Oligonucleotides Bind HNF-4 and COUP-TF, Respectively. To determine whether other proteins in rat liver nuclear extracts could bind the α1AT and TRE oligonucleotides, end-labeled α1AT and TRE oligonucleotides were used as probes in gel mobility-shift assays (Fig. 5). Each probe was incubated with liver nuclear extract and the indicated antisera. The α1AT probe binds only HNF-4 under these conditions, since the single band observed was supershifted by the HNF-4 antiserum (Fig. 5*A*). The TRE probe interacts strongly with COUP-TF under these conditions, but a weak interaction with an unidentified factor from rat liver nuclear extracts was also noted (Fig. 5*B*). None of the DNA/protein complexes formed on these probes contained RXRα, since no complex interacted with RXRα antiserum. An oligonucleotide that binds RXRα homodimers (DR1; ref. 25) was incubated with partially purified RXRα as a positive control for the effectiveness of the RXRα antiserum (Fig. 5*C*).

Sequences Within the α1AT and TRE Oligonucleotides Can Functionally Substitute for AF1. The AF1 element in pPL32 was replaced with either the α1AT HNF-4 binding site or the TRE by site-directed mutagenesis to determine whether these elements could function as accessory elements. The induction of CAT activity by dexamethasone in H4IIE cells transfected with the resulting constructs, pα1AT and pTRE, was com-

pared with that obtained in cells transfected with either pPL32 or pB450 (Fig. 6). Since the α1AT element (which binds HNF-4) and the TRE (which binds COUP-TF) supported the glucocorticoid response in the PEPCK promoter context, we concluded that both HNF-4 and COUP-TF can serve as the AF1 factor.

DISCUSSION

We have demonstrated here that HNF-4 and COUP-TF serve as accessory factor 1 in the PEPCK gene GRU. HNF-4 is found mainly in liver, kidney, and intestine (16, 26), wherein it is involved in transcriptional regulation of a number of genes. Among these are the genes encoding L-type pyruvate kinase (L-PK; ref. 27), factor IX (28), HNF-1α (29–31), medium-chain acyl-CoA dehydrogenase (32), tyrosine aminotransferase (TAT; ref. 33), ornithine transcarbamylase (OTC; ref. 34), cellular retinol-binding protein II (CRBP II; ref. 35), transferrin (36) and ApoAI (37), ApoAII (38), ApoBI (39), and ApoCIII (40). HNF-4 is also required for the liver-specific expression of the hepatitis B virus enhancer I (41) (see refs. 24 and 42 for detailed reviews). HNF-4 also interacts with an element in the L-PK gene promoter that mediates a glucose response, and it is required for repression of L-PK gene transcription by cAMP (43). In the TAT gene, an HNF-4 binding site and an adjacent CRE mediate the tissue-specific activation of the TAT gene by cAMP (33).

COUP-TF was originally defined as an activator of chicken ovalbumin gene expression (44). The negative regulatory

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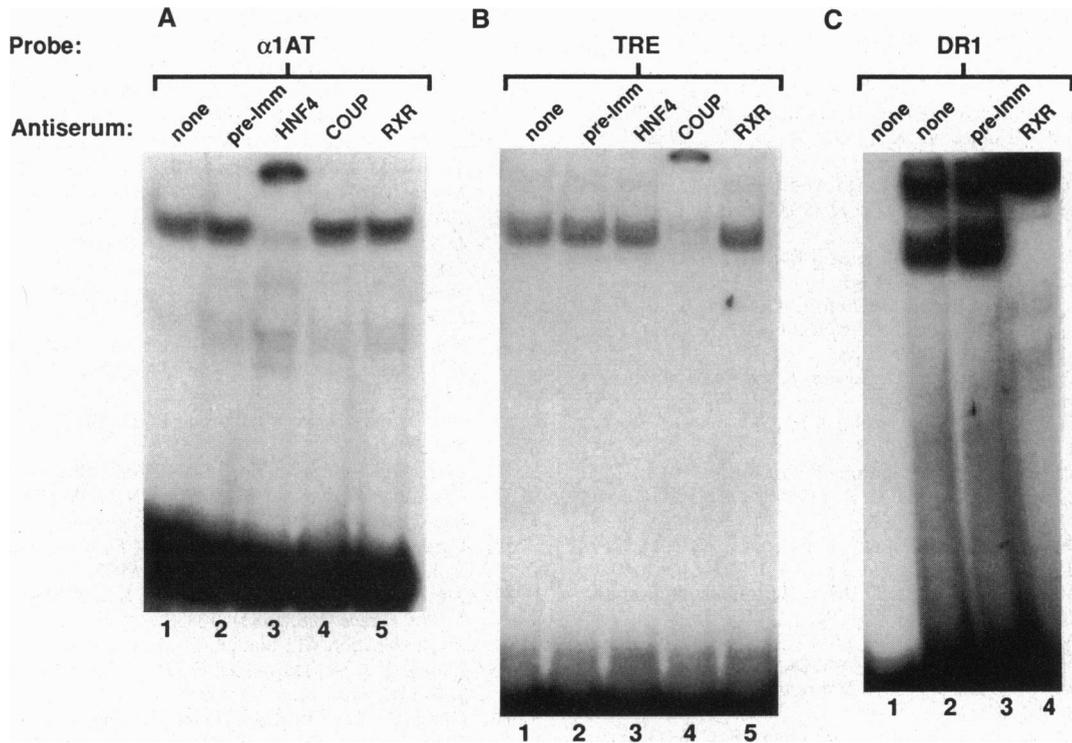
FIG. 5. HNF-4 and COUP-TF bind the α1AT and TRE oligonucleotides, respectively. (*A*) End-labeled α1AT oligonucleotide was mixed with 1 μg of crude rat liver extract. The indicated antiserum was added immediately after the addition of extract. The DNA/protein complexes were separated by mobility shift. (*B*) The conditions are the same as in *A* except that probe was an end-labeled TRE oligonucleotide. (*C*) The conditions are the same as in *A* and *B* except that an end-labeled DR1 oligonucleotide was incubated with bacterially expressed RXRα (lanes 2–4) or "mock" bacterial extract lacking RXRα (lane 1).

properties of COUP-TF have since become the focus of most studies on this factor. COUP-TF, or the closely related factor ARP-1 (COUP-TFII), represses transcription of the ApoAI (22, 45), ApoCIII (40, 46), ApoB (46), and ApoAII (46) genes. In addition, COUP-TF represses transcription of the murine Oct-4 and OTC (47, 48) genes. The ability of COUP-TF to repress trans-activation by vitamin D3, thyroid hormone, and RARs has been extensively analyzed in the context of the
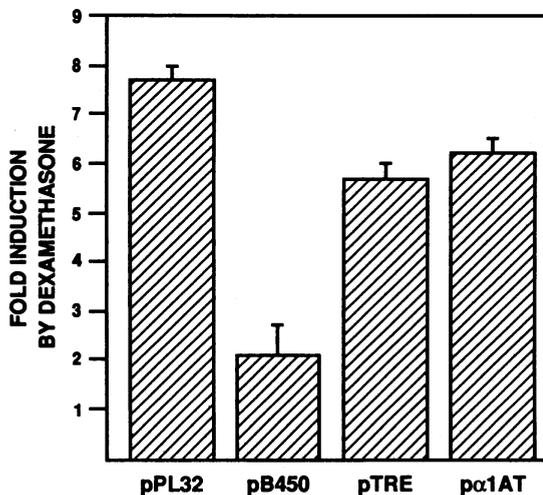
FIG. 6. The TRE and α1AT sequences can substitute for the AF1 element in the glucocorticoid response. H4IIE cells were transfected with either pPL32, pB450, pTRE, or pα1AT. The cells were treated with 0.5 μM dexamethasone, but RSVGR was not included in these transfections. The cells were harvested 18 hr later, CAT activity was measured, and the results were calculated as described in the legend to Fig. 1. The vertical bars represent the means ± SEM of at least three independent experiments.

ApoAI gene (45) and by using synthetic response elements attached to a reporter gene (35, 49–51). COUP-TF also antagonizes peroxisome proliferator-mediated activation of the enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase gene (52) and represses the expression of the mouse lactoferrin gene induced by estrogen (53).

In genes that contain an element capable of binding both COUP-TF (or ARP-1) and HNF-4, the former generally has a negative effect whereas the latter has a positive action. This is true in the genes for ApoCIII (40), OTC (48), ApoAI (37), medium-chain acyl-CoA dehydrogenase (32), ApoB (46), ApoAII (46), and CRBPII (35) and in the hepatitis B virus enhancer (41). Our results indicate that COUP-TF and HNF-4 are not antagonistic in the PEPCK glucocorticoid response but rather are functionally redundant. It is possible that COUP-TF and HNF-4 utilize a common interface to interact with other factors in the GRU or with factors that bind elsewhere in the PEPCK promoter.

The AF1 element can bind an array of proteins, including RARα monomers and dimers (5, 13), RARα/thyroid hormone receptor heterodimers (54), RARα/RXRα heterodimers (13), and COUP-TF and HNF-4 homodimers (this work). AF1-bound RARα monomers and dimers are incapable of mediating a retinoic acid response (13), and the RARα/thyroid hormone receptor/AF1 complex is nonproductive (54). The RARα/RXRα heterodimer mediates a retinoic acid response when bound to AF1 (13), but in the absence of ligand does not appear to serve as the accessory factor in the glucocorticoid response (this work). However, we cannot exclude the possibility that the RAR/RXR heterodimer may serve as an accessory factor in the presence of retinoic acid. Taken together, our studies show that the accessory function of AF1 is provided by HNF-4 or COUP-TF.

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