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Activation of the Phosphoenolpyruvate Carboxykinase Gene Retinoic Acid Response Element Is Dependent on a Retinoic Acid Receptor/Coregulator Complex

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The accessory factor 1 (AF1) element is an upstream transcriptional control region that plays a role in the response of the phosphoenolpyruvate carboxykinase (PEPCK) gene to both glucocorticoids and retinoic acid. We demonstrate here that retinoic acid receptor alpha (RARα) binds to a sequence within the AF1 element, TGACCT (site B), that is a consensus retinoic acid response element (RARE) half-site. A similar DNA sequence, TGGCCG (site C), located 1 bp downstream of site B, is not involved in the binding of RARα monomers or dimers but is required for the constitution of a functional RARE. Site C is also required for the formation of a complex involving RARα and a liver nuclear factor designated CR, for coregulator. Mutational analysis of the AF1 element shows that the RARα/CR complex is the *trans*-acting unit that mediates the retinoic acid response of the PEPCK gene. Another member of the retinoid receptor family, retinoid X receptor alpha (RXRα), can also form a complex with RARα and the AF1 element. Several observations, including the observation that RXRα antibody interacts with CR, indicate that RXRα and CR are identical or closely related proteins. Though RXRα forms a complex with RARα and the AF1 element, we demonstrate that the AF1 element is functionally distinguishable from a retinoid X response element. Taken together, our results show that the AF1 element contains an RARE that mediates a retinoic acid response by binding an RARα/coregulator complex; this coregulator is presumably RXRα.

The effects of the steroid/thyroid/retinoid family of hormones on gene expression are mediated by intracellular receptors that bind to their cognate hormone response elements in target genes (2, 11, 14, 45, 47). The half-sites that compose these hormone response elements differ in their primary sequence and arrangement (33). Response elements for estrogen, vitamin D, thyroid hormone, and retinoic acid are composed of two or more similar copies of the consensus half-site sequence TGACCT arranged as either palindromic or direct repeats (33). The spacing of the half-sites relative to each other is a critical parameter that is, at least in part, responsible for determining which receptor binds to a given response element and mediates a hormone response (35, 46). In general, elements consisting of direct repeats separated by 1, 3, 4, or 5 bp function through the binding of retinoid X receptor (RXR [32]), vitamin D receptor (VDR [46]), thyroid hormone receptor (T3R [46]), or retinoic acid receptor (RAR [46]), respectively. Retinoic acid response elements (RAREs) have been found in a variety of genes (for a review, see reference 33), including the phosphoenolpyruvate carboxykinase (PEPCK) gene, wherein a RARE was initially identified between positions −468 and −431 relative to the transcription initiation site. Further analysis, with 4- and 6-bp block mutations, delineated the RARE to an 18-bp sequence located between positions −451 and −433 (28). This core sequence mediates a retinoic acid response when inserted upstream of a heterologous promoter. The functional boundaries of the RARE coincide with the functional boundaries of the accessory factor 1 (AF1) element which is an integral part of the PEPCK glucocorticoid response unit (GRU [19]; for a review, see reference 16). Deletion of the AF1 element results in a 50% reduction in the PEPCK glucocorticoid response (19) and an even greater reduction in the retinoic acid response (28). Thus, the AF1 element plays a critical role in two distinct hormone responses.

RARα binds to the AF1 element and forms two retarded complexes in a gel shift assay (28). These two complexes represent the interaction of monomeric and dimeric forms of RARα with the AF1 element (27). Mutational analysis, combined with gel mobility shift assays, showed that a consensus RARE half-site (TGACCT, site B [the nucleotide boundaries of site B in this work correspond to those of site B in reference 28]) within the AF1 element is critical for binding of RARα (28). Site B and a similar sequence located 1 bp downstream (TGGCCG, site C [the nucleotide boundaries of site C in this work are −444 and −439, inclusively, whereas those of site C in reference 28 are −445 and −441, inclusively]) are both required for the retinoic acid response (28).

The similarity between the B and C sites suggested that site C was a second half-site. However, the 1-bp spacing between sites B and C is atypical for a RARE and is suggestive of an element that functions through the binding of RXR. Such an element has been described for the cellular retinol-binding protein type II gene (CRBPII) and has been termed a retinoid X response element (RXRE) (32). The CRBPII RXRE mediates a retinoic acid response by productively binding RXR. RAR binds to this element but does not form a competent *trans*-acting complex. In fact, RAR impairs the ability of RXR to mediate a retinoic acid response through this element, (32), which may be a distinguishing characteristic of RAREs and the CRBPII RXRE.

The binding of RAR (13), VDR (26, 42, 43), T3R (3, 5, 7,

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34, 41), and the orphan receptor COUP-TF (44) to their cognate response elements is greatly enhanced by accessory proteins called coregulators. Recently, members of the RXR family (α, β, and γ) have been shown to possess coregulator activity in that they interact with and stabilize the specific DNA binding of RAR (22, 24, 51, 52), VDR (22, 51), and T3R (22, 23, 51, 52) to their respective elements. A recent report suggests that RXR can also interact with COUP-TF (21).

In addition to its coregulator activity and its ability to mediate a retinoic acid response through the CRBPII RXRE, RXR is activated by 9-*cis* retinoic acid, a natural metabolite of retinoic acid (18, 25, 31). In fact, 9-*cis* retinoic acid is about 40 times more potent than all-*trans* retinoic acid at activating RXR (18). In contrast, RAR responds equally well to all-*trans* retinoic acid and 9-*cis* retinoic acid (18).

We report here that the binding of the monomeric and dimeric forms of RARα to the AF1 element is inhibited by methylated G residues in site B, but not by those in site C. We also show that site C binds a liver nuclear factor, designated CR for coregulator. Both sites are required for the function of the PEPCK RARE, which suggests that the RARα/CR complex is the *trans*-acting unit responsible for mediating the retinoic acid response of the PEPCK gene. RXRα mimics the effects of CR in gel mobility shift assays, and RXRα antibody interacts with CR, which indicates that the two factors are identical or closely related. The response of a PEPCK/chloramphenicol acetyltransferase (CAT) reporter gene to coexpression of RARα and RXRα and to 9-*cis* retinoic acid suggests that the PEPCK promoter contains a RARE and not a RXRE.

MATERIALS AND METHODS

Plasmids and oligonucleotides. The parent plasmid, pPL32, was used to construct plasmids containing mutations in the AF1 region. pPL32 contains the PEPCK gene sequence from −467 to +69 fused to the coding region of the CAT gene (38). pC443-CAT and pB450-CAT were constructed by site-directed mutagenesis with a kit purchased from Bio-Rad. The template used for mutagenesis (pPL32-18) contains the PEPCK gene sequence from pPL32 inserted into pEMBL18. The oligonucleotide primers used were 5′-GATCCCTTCTC ATGACCTTTTTCCGTGGGAGTGACACCT-3′ and 5′-GAT CCCTTCTCATTCCCTTTGGCCGTGGGAGTGACACCT-3′ for the construction of pC443-CAT and pB450-CAT, respectively. Double-stranded oligonucleotides for use in gel mobility shift experiments were made by annealing gel-purified, complementary oligonucleotides to those described above. The double-stranded oligonucleotide representing the wild-type AF1 element has the sense-strand sequence 5′-GAT CCCTTCTCATGACCTTTGGCCGTGGGAGTGACACT-3′.

Preparation of RARα and RXRα. RARα was expressed in *Escherichia coli* and purified by the method of Foreman et al. (10, 12). The bacterial expression vector, pET8CRARα, and the *BL21, DE3 pLYSs* host cells were gifts from Herbert Samuels (New York University Medical Center, New York, N.Y.). RXRα cDNA was inserted into pGEM 7 (Promega), and sense RNA was produced by using an Ambion in vitro transcription kit. This RNA was translated into protein by using the Promega in vitro translation kit.

Gel mobility shift assays. Two types of gel mobility shift assays were used in this study. A low-stringency assay (10) was used to detect RARα monomer and dimer binding. The probe used in these assays was either a wild-type AF1 double-stranded oligonucleotide, end labeled with [γ-^{32}P] ATP and polynucleotide kinase, or a *BglI-NdeI* restriction fragment, derived from pPL1 (38), that contained PEPCK sequence from −364 to −509. A typical binding mixture consisted of 2 × 10^4 cpm of probe, 25 mM Tris-HCl (pH 7.8), 0.5 mM EDTA, 88 mM KCl, 10 mM 2-mercaptoethanol, 0.1 μg of aprotinin, 0.05% Triton X-100, 10% glycerol, and 0.1 μg of poly(dI-dC) · poly(dI-dC). Approximately 100 fmol of purified RARα was included in the binding mix where indicated and incubated at room temperature for 30 min and then on ice for 10 min. Electrophoresis was performed in a 6% polyacrylamide gel with a buffer composed of 10 mM Tris-HCl (pH 7.8), 7.5 mM acetic acid, and 40 μM EDTA. The gels were dried and analyzed by autoradiography.

A high-stringency assay (37) was employed when nuclear extract was used in the binding reaction. A typical binding reaction consisted of 2 × 10^4 cpm of probe, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.8), 50 mM NaCl, 1 mM spermidine, 10 mM dithiothreitol, 10% glycerol, 0.1% Nonidet P-40, and 2 μg of poly(dI-dC) · poly(dI-dC). Approximately 5 μg of crude rat liver nuclear extract prepared by the method of Gorski et al. (15) was incubated with the binding mix at room temperature for 10 min. Electrophoresis was performed in a 6% polyacrylamide gel with a buffer containing 25 mM Tris-HCl, 190 mM glycine, and 1 mM EDTA. In experiments using HNF-4 antibody (anti-445 antiserum from Frances M. Sladek, University of California at Riverside, Riverside), 1 μl of a 1:10 dilution was added to the binding reaction. RXRα antiserum (containing antibody specific for amino acids 214 to 229 of human RXRα) and preimmune serum (gifts of Jacqueline Dyck and Ron Evans, Salk Institute for Biological Studies, La Jolla, Calif.) were used undiluted.

Methylation interference assay. A *BglI-NdeI* restriction fragment containing PEPCK sequence from −364 to −509 was labeled with [γ-^{32}P]ATP at either end by using polynucleotide kinase. Approximately 5 × 10^6 cpm of the labeled fragment was partially methylated with dimethyl sulfate. About 10^5 cpm of methylated fragment was used in 10 separate gel shift binding reactions, and all were loaded onto a single gel. After electrophoresis, the gel was exposed to film overnight at 4°C. DNA from the bound and free bands was electroeluted onto DE81 paper, purified, and precipitated with ethanol. The pellets were resuspended in cold 1 M piperidine, and strand cleavage was carried out at 90°C for 30 min and was followed by three cycles of washing and vacuum drying. The final pellets were resuspended in loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and an equal number of counts per minute of DNA from the free and bound bands was subjected to electrophoresis in a 10% denaturing polyacrylamide gel.

Transfection and CAT assays. H4IIE rat hepatoma cells were grown under conditions previously described (19). The cells were transfected, by using the calcium phosphate procedure (38), with 10 μg of reporter plasmid and, where indicated, 5 μg of pRShRARα (kindly provided by Herbert Samuels) or 5 μg of pMT2-RXRα (kindly provided by Sotirios Karathanasis, Lederle Laboratories, Pearl River, N.Y.). The amount of DNA transfected was kept constant by the addition of RSVneo. Five hours later the cells were subjected to a 4-min treatment with 20% dimethyl sulfoxide. Fresh serum-free Dulbecco modified Eagle medium, containing either no addition, 2 μM retinoic acid, or the specified amount of 9-*cis* retinoic acid (synthesized and provided by David Ong, Vanderbilt University, Nashville, Tenn.), was then added. The cells were harvested 18 h later. CAT

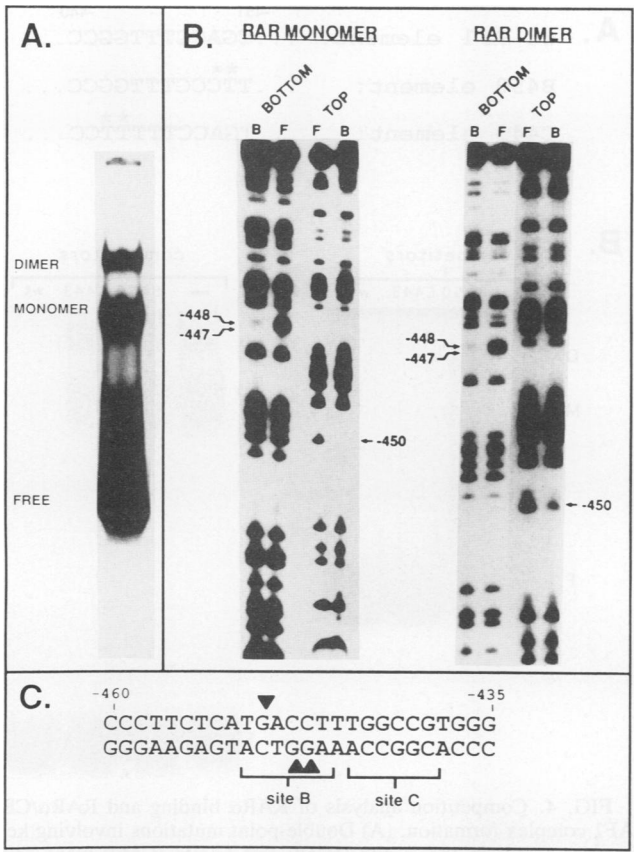

FIG. 1. The monomeric and dimeric forms of RARα bind to a single half-site. (A) Approximately 100 fmol of RARα and an end-labeled, partially methylated *BglI-NdeI* restriction fragment were incubated together under low-stringency binding conditions. The bound and free probes were separated in a low-stringency gel shift assay, as described in Materials and Methods. (B) DNA isolated from the RARα monomer, dimer, and free bands was subjected to methylation interference analysis. Both the top and bottom DNA strands were analyzed. B and F indicate the bound (monomer and dimer) and free DNA fractions, respectively, isolated from the gel depicted in panel A. (C) The G residues that interfere with binding of the RARα monomer and dimer to the AF1 element are indicated by the closed triangles. The RARE consensus half-site (site B) and a similar sequence (site C) are indicated by the brackets.

assays were performed according to the method of Nordeen et al. (36) with slight modifications (19).

RESULTS

Monomeric and dimeric forms of RARα bind to an RARE half-site. The AF1 element contains a consensus RARE half-site, TGACCT (site B), and a similar sequence, TGG CCG (site C), located 1 bp downstream (28) (Fig. 1C). A methylation interference analysis was performed to determine which G residues are important for RARα binding to the AF1 element. Both the monomeric and dimeric forms of the receptor were detected in a binding reaction that contained end-labeled *BglI-NdeI* restriction fragment and approximately 100 fmol of RARα (Fig. 1A). This finding corroborated a previous observation obtained when a labeled double-stranded oligonucleotide was employed as a probe (28). The bands representing free probe and either bound monomer or bound dimer were excised from the gel,

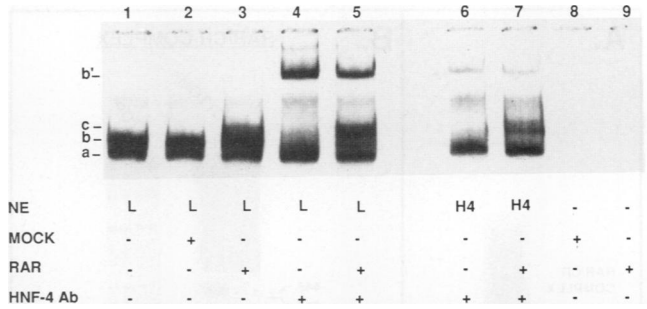

FIG. 2. RARα and a factor from nuclear extract form a complex with the AF1 element. A double-stranded, end-labeled AF1 oligonucleotide was incubated with nuclear extract (NE) from liver (L) or H4IIE (H4) cells, under high-stringency conditions, as described in Materials and Methods. Additional components of the various reactions included: extract from *BL21 DE3 pLYSs* cells that had not been transformed with pET8CRARα (MOCK), 1 μl (~100 fmol) of RARα, and 1 μl of a 1:10 dilution of anti-445 HNF-4 antiserum.

and the DNA was subjected to methylation interference analysis as described in Materials and Methods. As illustrated in Fig. 1B, methylation of G residues at −448 and −447 on the bottom strand and −450 on the top strand interfered with RARα monomer binding. There was no evidence of involvement of G residues in site C. An identical methylation interference pattern was seen on both strands when DNA from either the RARα monomer or dimer complex was analyzed. Therefore, the monomeric and dimeric forms of RARα specifically recognize only the G residues in site B. A summary of the methylation interference data with RARα is shown in Fig. 1C.

RARα and a liver nuclear factor bind the AF1 element. Since site C is required for the activity of the PEPCK RARE (28) but not for the binding of RARα we reasoned that another essential factor might bind to this sequence. However, no specific protein binding to site C was observed when nuclear extracts were mixed with an oligonucleotide in which the RARα binding site was mutated (data not shown). Thus, factor binding to site C could require the presence of an intact RARα binding site. We then performed gel mobility shift experiments in which RARα was added to rat liver nuclear extract to investigate whether a factor bound to site C in the presence of RARα. As shown in Fig. 2, two retarded complexes, *a* and *b*, were observed when the AF1 probe was incubated with liver nuclear extract either alone (lane 1) or with extract from *BL21 DE31 pLYSs E. coli* that had not been transformed with pET8CRARα (lane 2). We have shown, using antibody supershift experiments, that the *b* band represents HNF-4 bound to AF1 (16a). The identity of the *a* band is presently unknown. No shifted complexes were observed when only RARα was used in the binding reaction, probably owing to the high concentration of poly(dI-dC) · poly(dI-dC) and the high ionic strength of the gel system (lane 9). A unique complex did form when approximately 100 fmol of RARα was added to a binding reaction containing liver nuclear extract (the *c* band in lane 3). The *c* band most often appeared as a poorly resolved doublet in gel shift assays (lanes 3, 5, and 7); however, some preparations of RARα resulted in the apparent formation of a single shifted complex. The significance of this observation is not clear. Since the *c* band was so close to the *b* band, HNF-4 antibody was added to the binding reaction in an effort to separate the two. This resulted in the diminution of the *b*

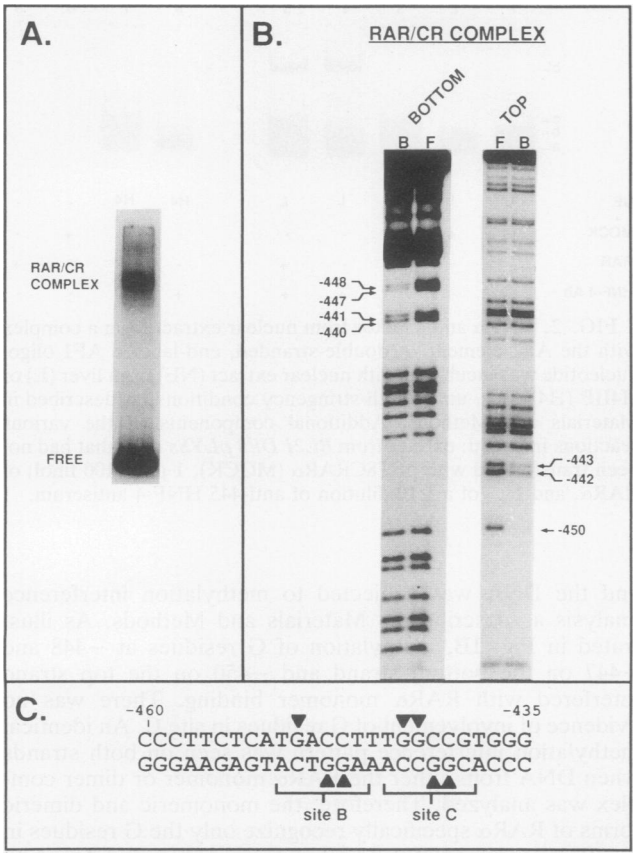

FIG. 3. The binding site for the RARα/CR complex includes site C. (A) An end-labeled, partially methylated *BglI-NdeI* restriction fragment was incubated with approximately 100 fmol of RARα, 5 μg of rat liver nuclear extract, and 30 mM $MgCl_2$, and then a high-stringency gel mobility shift assay was performed. Under these conditions the *a* and *b* bands defined for the experiment shown in Fig. 2 do not appear. (B) DNA isolated from the bound (B) and free (F) bands was subjected to methylation interference analysis. The interference pattern of both the bottom and top strands are shown. (C) The G residues that interfere with binding of the RARα/CR complex are indicated by the closed triangles. The consensus RARE half-site (site B) and the adjacent sequence (site C) are indicated by the brackets.

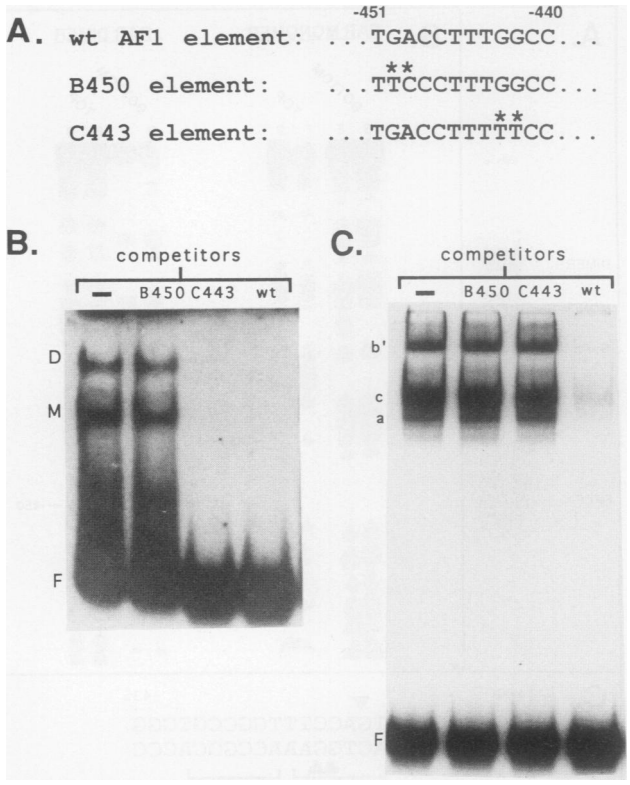

FIG. 4. Competition analysis of RARα binding and RARα/CR/AF1 complex formation. (A) Double-point mutations involving key contact points in the B site (B450) and C site (C443) were constructed as described in Materials and Methods. Positions of the double-point mutations relative to the wild-type AF1 element are shown by asterisks. (B) A low-stringency gel mobility shift assay in which the binding reactions contained end-labeled wild-type AF1 oligonucleotide probe, approximately 100 fmol of RARα, and a 100-fold molar excess of the oligonucleotide competitor (B450 or C443) (indicated above each lane) was performed. The free (F), monomer (M), and dimer (D) bands are indicated. (C) A high-stringency gel mobility shift assay in which the binding reactions contained the same amount of probe, RARα, and oligonucleotide competitor as in the experiment illustrated in panel B, in addition to 5 μg of rat liver nuclear extract, was performed. The *a*, *b'*, and *c* bands are defined in the text.

band and the appearance of a supershifted HNF-4 complex, which was designated *b'* (lane 4). Under these conditions, the *c* band, which forms when RARα is added to the binding reaction, is clearly visible (lane 5). The binding conditions for lanes 6 and 7 were identical to those in lanes 4 and 5, respectively, except that H4IIE cell nuclear extract was used instead of rat liver nuclear extract. Therefore, the cell line in which the functional studies are performed also contains a nuclear factor that influences RARα binding, and this factor appears to be identical to that found in liver nuclear extracts. For the purposes of discussion, this factor is referred to as the coregulator (CR).

The RARα/CR complex forms on sites B and C. The gel shift experiments showed that CR and RARα form a complex with the AF1 element. To determine whether site C was involved in formation of this complex, we performed a methylation interference analysis on the RARα/CR/AF1 complex (Fig. 3). In order to resolve the RARα/CR/AF1 complex from the HNF-4/AF1 complex in a preparative mobility shift assay, 30 mM $MgCl_2$ was included in the binding reaction. This effectively prevented the formation of the HNF-4 complex and the *a* band complex (data not shown) but had little effect on the formation of the RARα/CR/AF1 complex (Fig. 3A). The methylation interference pattern for the RARα/CR/AF1 complex extended beyond the RARα binding site and included the G residues at positions −450, −443, and −442 on the top strand and −448, −447, −441, and −440 on the bottom strand (Fig. 3B). This pattern, summarized in Fig. 3C, indicates that the G residues in site B and all but the last G residue in site C are important for formation of the RARα/CR/AF1 complex.

A mutation within site C prevents formation of the RARα/CR/AF1 complex. Two double-point mutations were introduced into the AF1 element in order to assess the contributions that site B and site C make to the binding of RARα and the formation of the RARα/CR/AF1 complex (Fig. 4A). The sites of the double-point mutations were selected on the presumption that each set of mutations would prevent the

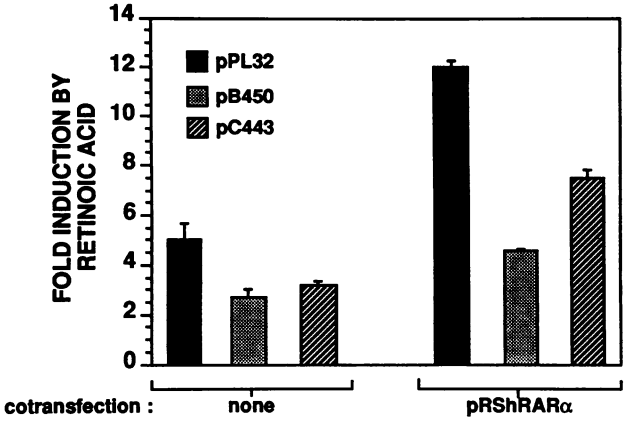

FIG. 5. Mutations in the AF1 element reduce the retinoic acid response of a PEPCK/CAT fusion gene. pPL32 contains the wild-type PEPCK promoter from −467 to +69 fused to the CAT reporter gene. pB450 is identical to pPL32 except that it contains a double-point mutation at positions −450 and −449. pC443 is identical to pPL32 except that it contains a double-point mutation at positions −443 and −442. The construction of these plasmids is described in Materials and Methods. Each of the indicated reporter constructs was transfected either alone or with pRShRARα into H4IIE cells. The amount of DNA transfected was kept constant with the addition of RSVneo. CAT activity was measured 18 h after the addition of 2 μM retinoic acid. Vertical bars represent the means ± standard errors of the mean of five independent experiments.

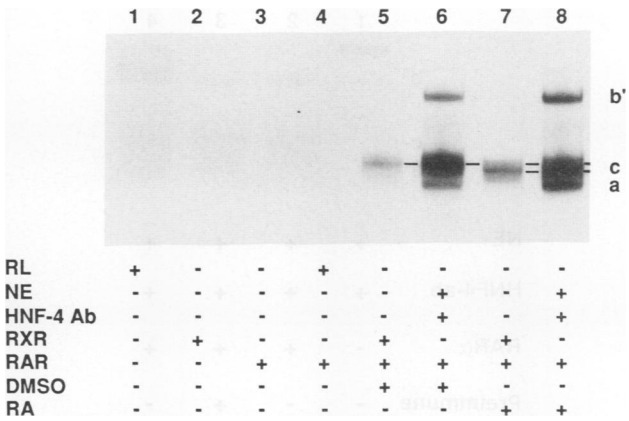

FIG. 6. RXRα mimics CR in a gel mobility shift assay. A high-stringency gel shift assay in which the binding reactions contained the indicated components was performed. The amount of each component added is the same as in Fig. 2. Other components used in this experiment included 4 μl of reticulocyte-produced RXRα; 1 μl dimethyl sulfoxide (DMSO), the solvent for retinoic acid; and retinoic acid (RA) at a final concentration of 2 μM. The *c* denotes both the RARα/RXRα/AF1 and RARα/CR/AF1 complexes. The *a* and *b'* bands are defined in the text.

binding of one factor but not the other. To test the effect of the mutations on RARα binding, double-stranded oligonucleotides containing either wild-type or mutated AF1 elements were used as competitors against a labeled wild-type probe in a low-stringency gel mobility shift assay (Fig. 4B). Oligonucleotides containing either the wild-type PEPCK RARE sequence or the C443 mutation competed for binding of RARα. An oligonucleotide containing the B450 mutation did not compete for binding of RARα. This again illustrates the point that RARα binding requires an intact site B but not an intact site C.

Shown in Fig. 4C are the results of the competition in a high-stringency gel shift assay with RARα in the presence of nuclear extract. Again, to aid in visualization of the RARα/CR/AF1 complex (the *c* band), HNF-4 antibody was added to the binding reaction so that the majority of the HNF-4 band was supershifted (the *b'* band). An oligonucleotide containing the C443 mutation, which effectively competed for RARα binding, did not compete with the wild-type AF1 element for the formation of the RARα/CR/AF1 complex. An oligonucleotide containing the B450 mutation failed to compete for the formation of the RARα/CR/AF1 complex, just as it failed to compete for the binding of RARα alone. Thus, the B450 mutation prevents the binding of RARα and the formation of the RARα/CR/AF1 complex, whereas the C443 mutation has no effect on RARα binding but prevents the formation of an RARα/CR/AF1 complex.

Activity of the PEPCK RARE is abrogated by the B450 and C443 mutations. The same double-point mutations used in the binding studies were introduced into the AF1 element of pPL32, which contains PEPCK sequence from −467 to +69 fused to the CAT reporter gene, to give pB450 and pC443. These constructs were compared with the wild-type pPL32 for PEPCK RARE function. The reporter constructs pPL32, pB450, and pC443 were transfected into H4IIE rat hepatoma cells, either alone or with a RARα expression vector, pRShRARα. The results are shown in Fig. 5. The construct pPL32, which contains the wild-type AF1 element, exhibited a fivefold induction with 2 μM retinoic acid in the absence of cotransfected pRShRARα. The retinoic acid response of the mutant constructs was only 50 to 60% that of pPL32. Cotransfection with pRShRARα increased the retinoic acid response of all the constructs approximately twofold, but the effect of the mutations was qualitatively similar under both conditions. The degree of reduction in the retinoic acid response caused by the mutations is identical to that reported previously for a construct in which the entire AF1 element was removed by 5′ deletion (28). The residual retinoic acid responsiveness is due to the presence of a second, distinct RARE located closer to the transcription start site (unpublished observation).

RXRα mimics CR. The finding that site C is required for CR binding and function of the PEPCK RARE suggested that CR is a coregulator. We therefore asked whether RXRα, a known coregulator (22, 24, 42, 52), forms a complex with RARα on the AF1 element. RXRα and RARα were examined for binding to AF1 under the high-stringency gel shift assay conditions. As shown in Fig. 6, virtually no binding to the AF1 oligonucleotide was observed when reticulocyte lysate control, RXRα, or RARα was added alone (lanes 1 to 3). Adding the reticulocyte lysate control with RARα did not result in the binding of RARα to the AF1 probe; however, an RARα/RXRα/AF1 complex was detected when RARα and RXRα were added to the binding reaction (lane 5). The RARα/RXRα/AF1 complex comigrated with the RARα/CR/AF1 complex (compare lanes 5 and 6).

Retinoic acid affected the mobility of both the RARα/RXRα/AF1 and the RARα/CR/AF1 complexes, as shown in Fig. 6, lanes 7 and 8. In fact, the mobility of the two complexes changed similarly. The complex believed to be the functional *trans*-acting unit in the cell may undergo a conformational change in the presence of retinoic acid.

RXRα antibody interacts with CR. The similar behavior of

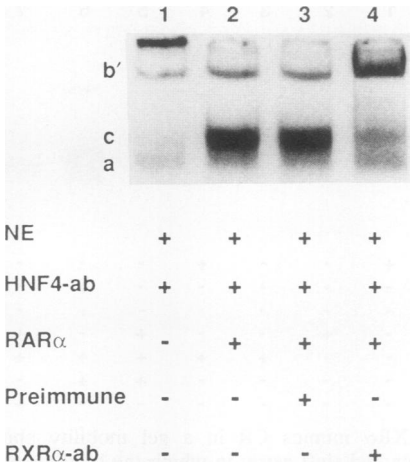

FIG. 7. RXRα antibody interacts with CR. An end-labeled, double-stranded AF1 oligonucleotide was incubated with liver nuclear extract (NE) and HNF-4 antiserum in a set of high-stringency binding reactions. 1 μl (~100 fmol) of RARα, 4 μl of RXRα reticulocyte lysate, 1 μl of preimmune serum, or 1 μl RXRα antiserum was added to the reactions as indicated below each lane. Electrophoresis was performed under high-stringency gel mobility shift conditions. The *a*, *c*, and *b'* bands are defined in the text. The dark band above the *b* band in lane 1 is an artifact that appears occasionally when HNF-4 antiserum is used.

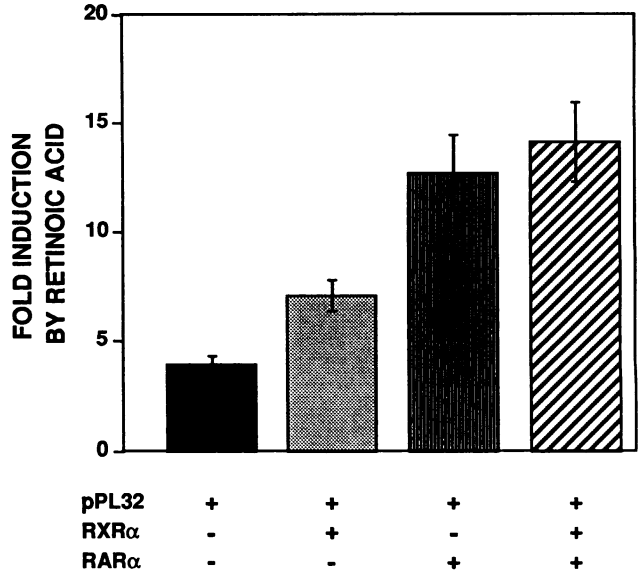

FIG. 8. RXRα and RARα are not functionally counteractive. H4IIE cells were transfected with 10 μg of pPL32 alone, with 5 μg of pMT2-RXRα or pRShRARα, or with both. RSVneo was added as needed to bring the total amount of DNA per transfection to 20 μg. The cells were exposed to 2 μM retinoic acid for 18 h, after which CAT activity was determined. Vertical bars represent the means ± standard errors of the means of five independent experiments.

RXRα and CR in gel mobility shift assays suggested that RXRα and CR were the same protein or were closely related. Addition of RXRα antibody to a reaction containing liver nuclear extract and RARα resulted in a diminution of the *c* band, which contains the RARα/CR/AF1 complex (Fig. 7, lane 4). A complex of slower mobility that comigrated with the HNF-4/HNF-4 antibody/AF1 complex (band *b'*) was also detected in the presence of RXRα antibody. Preimmune serum had no effect on the RARα/CR/AF1 complex (lane 3). Thus, the RXRα antibody interacts with CR, which suggests that RXRα and CR are identical or closely related proteins.

RARα and RXRα are not counteractive. The structure of the PEPCK RARE, with 1-bp spacing between sites B and C, is reminiscent of a direct repeat with 1-bp spacing which is the optimal spacing for RXR binding (21). A naturally occurring version of this type of element is found in the CRBPII gene (32). The CRBPII element is actually composed of four direct repeats and mediates the retinoic acid response of the CRBPII gene by binding RXR. RARα is incapable of mediating a retinoic acid response from the CRBPII RXRE and, in fact, interferes with the RXRα-mediated induction of the CRBPII RXRE (32). Since this may be a distinguishing feature of RAREs and RXREs, the interaction between RARα and RXRα was analyzed in the context of the PEPCK promoter. H4IIE cells were transfected with pPL32 or were cotransfected with pPL32 and the expression vectors for RXRα and/or RARα. As shown in Fig. 8, RXRα and RARα enhanced the retinoic acid response of pPL32 approximately two- and threefold, respectively. Coexpression of RXRα and RARα resulted in a retinoic acid response similar to that observed when RARα was expressed by itself.

9-*cis* retinoic acid induces expression from pPL32. The observation that RXRα formed a complex with RARα on the AF1 element prompted us to examine the effect of 9-*cis* retinoic acid, the ligand for RXRα (18, 25, 31), on pPL32. H4IIE cells were transfected either with pPL32 alone or with pPL32 and the RXRα expression vector pMT2-RXRα. The cells were exposed to various concentrations of either retin-

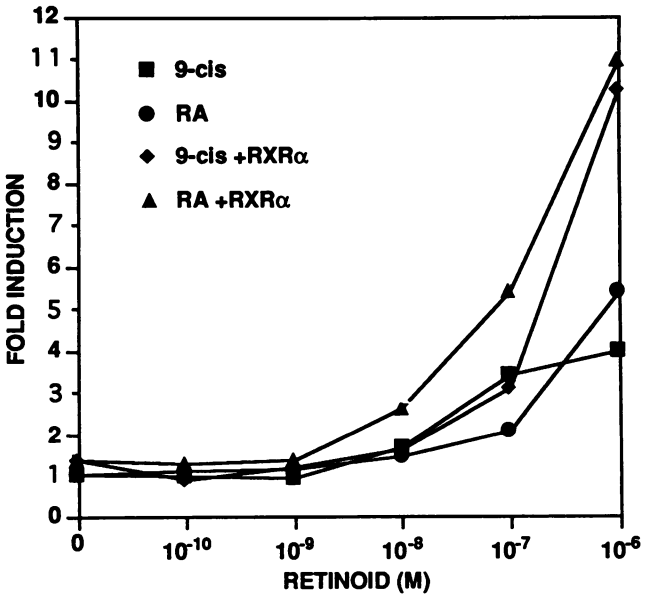

FIG. 9. 9-*cis* retinoic acid induces PEPCK promoter activity. H4IIE cells were transfected with 10 μg of pPL32 either alone or with 5 μg of pMT2-RXRα. The total amount of DNA transfected was kept at 15 μg with the addition of RSVneo as needed. The cells were exposed to the indicated concentrations of retinoid for 18 h, after which CAT activity was determined. Each point represents the average of at least three independent experiments.

oic acid or 9-*cis* retinoic acid for 18 h. The results are shown in Fig. 9. At each concentration tested, both retinoids stimulated CAT gene expression from pPL32 to a similar degree. This was true in the presence or absence of cotransfected pMT2-RXRα. Therefore, retinoic acid and 9-*cis* retinoic acid are equipotent in activating the PEPCK promoter.

DISCUSSION

The response of the PEPCK gene to retinoic acid requires a consensus RARE half site (TGACCT, site B) and an adjacent sequence (TGGCCG, site C) separated from the former by 1 bp. Site B binds monomeric and dimeric forms of RARα, but this, in the absence of site C, does not produce a competent transactivating complex. Site C binds a liver nuclear factor designated CR; this complex by itself is also not productive. Sites B and C together bind RARα and CR in a complex that supports *trans*-activation of the PEPCK promoter by retinoic acid. CR is identical to or closely related to RXRα, since RXRα mimics CR in gel mobility shift assays, and RXRα antibody interacts with CR. However, the AF1 element does not appear to function like a RXRE, since RARα, which inhibits RXRα-mediated retinoic acid induction through the CRBPII RXRE, has a stimulatory action on the AF1 element. In addition, retinoic acid and 9-*cis* retinoic acid are equipotent at activating the PEPCK/CAT fusion gene; this is more indicative of a RAR-mediated response rather than of a RXR-mediated response (18).

The observation that both monomeric and dimeric forms of RARα bind only to site B indicates that site C does not play a role in the formation of a RARα homodimer on the AF1 element. Although no direct evidence has heretofore been presented, it might be assumed, on the basis of earlier studies of other members of the steroid/thyroid/retinoid family of receptors, that an RAR dimer binds to a RARE, with a monomer binding to each half-site. For example, a T3R monomer binds a single half-site and a T3R dimer contacts both half-sites of a perfect direct repeat (23). However, glucocorticoid receptor (GR [6]), progesterone receptor (PR [8]), and estrogen receptor (ER [9]) can dimerize in the absence of DNA, so the presence of two half-sites in some specific juxtaposition is not an obligatory requirement for dimerization. This has been demonstrated most directly for GR, where crystallographic studies reveal that a dimer of the GR DNA-binding domain binds to a glucocorticoid response element half-site. One monomer makes base-specific contacts, while the other makes electrostatic contacts with the phosphate backbone (29). An interesting observation by Baniahmad et al. (1) provides further evidence (though more indirect) that a half-site can bind a receptor dimer. This group showed that a half-site derived from the silencer thyroid response element (TRE) in the chicken lysozyme promoter generates a T3R complex with an identical mobility in gel shift assays, as does the T3R complex formed on the intact palindromic binding site. The authors suggested that both the palindromic site and the half-site are bound by a single T3R dimer. Thus, the observation that a single half-site can support the binding of a receptor dimer is not unprecedented. In addition, our results are compatible with a model of monomer-dimer formation recently proposed by Samuels and coworkers (10), which suggests that receptor monomers exist in equilibrium with metastable dimers.

The structure of the AF1 element, taken with the observation that overexpression of RXRα in the presence of retinoic acid increases the expression of the PEPCK/CAT reporter gene, suggests that this element is a RXRE. However, several observations indicate that this is not the case. First, overexpression of RARα increases the retinoic acid response of the PEPCK-CAT reporter gene in pPL32. Second, coexpression of RARα with RXRα does not repress the retinoic acid response of the PEPCK-CAT reporter gene. It is important to note that RXRα is very abundant in liver (the origin of H4IIE cells) and probably not limiting for transient transfection experiments. This may be why overexpression of RXRα does not further enhance the retinoic acid response of pPL32. Third, whereas RXRα works well by itself on the CRBPII element, presumably because it binds efficiently, its binding to the PEPCK RARE is relatively weak. In fact, it binds to the PEPCK RARE 5 to 10 times less efficiently than it binds to a synthetic RXRE (data not shown). Fourth, the concentration of 9-*cis* retinoic acid required for stimulation of the PEPCK promoter is at least 100 times greater than that required for stimulation of the CRBPII element (18, 31). Since RAR is activated by 9-*cis* retinoic acid to the same degree as RXR (18), it is likely that the response of the PEPCK gene promoter to 9-*cis* retinoic acid is mediated by RAR.

The PEPCK AF1 element appears to be a composite element consisting of an RAR binding site and a binding site for another nuclear factor which fits the definition of a coregulator. A similar situation may exist in the rat growth hormone TRE (3, 7). In this system, T3R auxiliary protein (TRAP) binds to the sequence TCCCT and serves to stabilize T3R binding to a nearby half-site (7). Mutations in the TCCCT sequence abolish the TRAP-enhanced binding of T3R (7). In addition, T3R binding is required for efficient binding of TRAP to the TCCCT sequence. A mutual interaction like this was observed in the binding of RAR and CR to the PEPCK gene AF1 element. In addition, we have shown that the sequence to which CR binds is required for function of the AF1 element as an RARE.

The study of coregulators received a major boost with the discovery of the RXR family (α, β, and γ members). The RXRs enhance the binding of T3R, VDR, and RAR to their cognate elements and augment the activity of these response elements (22, 24, 51, 52). The three RXRs appear to be equally active on the various response elements but are distinctive in their tissue distribution (31). RXRα, for example, is the predominant form in liver. Since it mimics the in vitro binding properties of CR and RXRα antibody interacts with CR, we propose that CR is RXRα. Whether the RXR family constitutes the only coregulators involved in the action of the steroid/thyroid/retinoid family of receptors remains an open question.

Although the ligand is required for *trans*-activation by steroid/thyroid/retinoid hormone receptors, the mechanism by which this occurs is not known. The ability of a receptor to bind its cognate site in vitro does not appear to be affected by ligand, as free GR, PR, ER, T3R, and RAR exhibit ligand-independent DNA binding (20, 34, 40, 49, 50). This is generally true for VDR (30), but under certain conditions this receptor requires the presence of ligand to bind DNA (26). The ligand may enable the receptor to bind in vivo by promoting dissociation of an inactive complex (39), dimerization (9), or posttranslational modification (17). Such ligand-dependent changes could also result in a conformational change of the receptor (48). In support of this notion, ER, T3R, and VDR exhibit ligand-induced changes in the mobility of their respective receptor/DNA complexes in gel mobility shift assays (4, 10, 30). As illustrated above, retinoic acid alters the mobility of the RARα/CR and RARα/

RXRα complexes. The implications of this ligand-induced change of the *trans*-acting complex remain to be established.

The unique RARE described herein is found in the same region of the PEPCK gene promoter as the GRU (for a review, see reference 16). This region, which we call a metabolic response domain, encompasses elements that serve a variety of functions, including responses to glucocorticoids, retinoic acid/9 *cis*-retinoic acid, insulin, and phorbol esters, as well as tissue-specific and developmental expression of the gene. The definition of the complex interplay of factors that bind the AF1 element and the other regulatory elements in this region is of vital importance for the understanding of PEPCK gene regulation.

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