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A retinoic acid response element is part of a pleiotropic domain in the phospho*enol*pyruvate carboxykinase gene

(retinoic acid receptor α/complex response units/glucocorticoid response unit/tissue-specific gene expression)

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ABSTRACT **Several hormones, including insulin, glucagon, and glucocorticoids, regulate the expression of the rate-limiting gluconeogenic enzyme, phospho*enol*pyruvate carboxykinase [GTP:oxaloacetate carboxy-lyase (transphosphorylating); EC 4.1.1.32; PEPCK] in liver. In this report we demonstrate that retinoic acid (RA) also regulates PEPCK expression by inducing a 3-fold increase in the rate of transcription of the PEPCK gene. A RA response element located between −468 and −431 in the PEPCK promoter mediates a 7-fold increase in expression of a chimeric construct containing the basal PEPCK promoter ligated to the chloramphenicol acetyltransferase reporter gene. This element confers RA responsiveness through the heterologous thymidine kinase promoter and functions relatively independent of position and orientation. An 18-base-pair core sequence (−451 to −434) (*i*) mediates an effect of RA on PEPCK gene expression and contains motifs found in two other RA response elements; (*ii*) corresponds to AF1, an accessory factor element that is an integral component of the complex glucocorticoid response unit in the PEPCK gene promoter; (*iii*) is in a region involved in the developmental expression of the PEPCK gene; and (*iv*) shows homology to elements involved in the tissue-specific regulation of genes, including the hepatic apolipoprotein genes and the α_1-antitrypsin gene.**

Recent studies indicate that retinoic acid (RA), a vitamin A derivative, influences a variety of physiological processes including cell growth and differentiation (1–3), tissue development (4, 5), and tissue-specific gene expression (6–11). Three related RA receptors (RAR-α, -β, and -γ) have been identified in human and murine tissues (12–16). Recently, a fourth, distinct receptor (hRXR-α) has been found in human liver and kidney (17). All receptors show structural similarity to members of the steroid/thyroid hormone superfamily of receptors. It is thought that RA exerts its effects through regulating transcription of specific genes and that these receptors function as ligand-activated transcription factors. RA is capable of regulating gene expression through thyroid hormone response elements (18); however, because certain genes are regulated by RA but not by 3,5,3′-triiodothyronine (T_3), distinct RA and T_3 hormone response elements must exist. Selective RA response elements (RAREs) have been described in the laminin B1 (19) and RA receptor β genes (20, 21), but the determination of what constitutes a consensus RARE and how this differs from a thyroid hormone response element requires the characterization of several more such elements in other genes.

Phospho*enol*pyruvate carboxykinase [GTP:oxaloacetate carboxy-lyase (transphosphorylating); EC 4.1.1.32; PEPCK] catalyzes a rate-controlling step in hepatic gluconeogenesis. The PEPCK gene is expressed at a distinct time in development (22, 23) and is subsequently regulated by hormones in a complex, tissue-specific manner (24, 25). In this report we show that RA stimulates PEPCK gene transcription in cultured H4IIE rat hepatoma cells. This effect is largely mediated by a RARE that spans an 18-base-pair (bp) region in the PEPCK promoter from −451 to −434. This element contains motifs found in two recently characterized RAREs (19, 20) and is located in a region of the promoter involved in pleiotropic regulation of the PEPCK gene.

MATERIALS AND METHODS

Plasmid Construction. The construction of a series of reporter constructs containing 5′ deletion mutations of the PEPCK promoter ligated to the chloramphenicol acetyltransferase (CAT) gene has been described (26). Plasmid pTK-CAT contains the herpes simplex virus thymidine kinase (TK) promoter sequence from −105 to +51 ligated to the CAT gene (26, 27). An expression vector encoding the human RA receptor α (pRShRARα; ref. 13), for use in transfection experiments, was provided by R. M. Evans (Salk Institute for Biological Studies, La Jolla, CA). Oligonucleotides were synthesized with an Applied Biosystems 380A DNA synthesizer. A double-stranded oligonucleotide spanning the region of the PEPCK promoter from −468 to −431 (relative to the transcription start site) and containing *Hin*dIII compatible ends was inserted into the *Hin*dIII site located immediately upstream of position −306 in pPL33, a PEPCK–CAT 5′ deletion mutant containing the promoter sequence between −306 and +69 (26). Double-stranded oligonucleotides corresponding to the sequence −460 to −425, or mutants thereof, and containing *Bam*HI compatible ends were inserted into the polylinker located immediately upstream of the −105 position in pTK-CAT. All plasmid DNA constructs were sequenced and double-banded in cesium chloride gradients.

Receptor Purification. The human RA receptor α was expressed in *Escherichia coli* and purified to apparent homogeneity. The purified receptor migrated as a single band on a silver-stained SDS gel with the expected molecular mass of 51 kDa. Bacterial expression and purification of the RA receptor will be described in detail elsewhere.

Cell Culture and Transient Transfection. H4IIE rat hepatoma cells, grown as described (28), were transfected with either 15 μg of PEPCK–CAT or 20 μg of TK–CAT plasmid

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Abbreviations: RA, all-*trans*-retinoic acid; PEPCK, phospho*enol*pyruvate carboxykinase; CAT, chloramphenicol acetyltransferase; RARE, retinoic acid response element; TK, thymidine kinase; apo, apolipoprotein.

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DNA by using a calcium phosphate transfection procedure (28). Cotransfections included 5 μg of pRShRARα in the calcium phosphate–DNA coprecipitate. After transfection, cells were incubated for 24 hr in serum-free medium without or with various concentrations of RA (Sigma).

CAT Assay. Transiently transfected cells were harvested by trypsin digestion and sonicated in 250 μl of 250 mM Tris (pH 7.8). Samples were then heated to 60°C for 10 min and centrifuged briefly to remove cellular debris. CAT activity in the supernatant was assayed by a modification of the method of Nordeen *et al.* (29, 30). Background activity from a lysate-free blank was subtracted from all assays. CAT activity was corrected for the protein concentration in the lysate, as measured by the Pierce bicinchoninic (BCA) assay (31).

Nuclear Run-On Transcription Assay. H4IIE cells were grown to 50% confluency in Dulbecco's modified Eagle's medium containing (vol/vol) 5% calf, 3% newborn calf, and 2% fetal bovine serum. Twenty to 24 hr before the experiment, cells were switched to serum-free medium. Hormone treatments were for 60 min at a final concentration of 5 μM RA. The run-on transcription assay was then performed with a procedure described in detail (25) except that the T1 RNase concentration in the washing buffer was 434 units/ml and washes were done at 60°C.

Gel Retardation Assay. A double-stranded oligonucleotide corresponding to PEPCK promoter sequence from −460 to −425 was labeled with [α-^{32}P]dATP, using the Klenow fragment of *E. coli* DNA polymerase I. Twenty-five thousand counts per minute of the resulting probe (specific activity, 3170 cpm/fmol) was incubated with ≈100 fmol of purified human RA receptor α for 30 min at room temperature, followed by 10 min at 4°C. The incubation was done in a 30-μl volume in 25 mM Tris, pH 7.8/500 μM EDTA/88 mM KCl/10 mM 2-mercaptoethanol/0.1 μg of aprotinin/0.1 μg of poly(dI-dC)·poly(dI-dC)/0.05% Triton X-100 (vol/vol)/10% glycerol (vol/vol). Unlabeled DNA fragments (at 100-fold molar excess) were added for competition analyses. Samples were loaded onto a 6% polyacrylamide gel and separated by electrophoresis (20–25 mA) at 4°C for 2 hr with a buffer containing 10 mM Tris, 7.5 mM acetic acid, and 40 μM EDTA (pH 7.8). Gels were dried, and binding was analyzed by autoradiography.

RESULTS

Regulation of PEPCK Gene Transcription by RA. A 60-min exposure of H4IIE rat hepatoma cells to RA increased PEPCK gene transcription 3-fold (Fig. 1*A*). In other experiments a corresponding increase of PEPCK mRNA was noted by primer-extension analysis (data not shown).

The transient expression of a reporter construct containing 2100 bp of PEPCK promoter DNA ligated to the CAT gene (pPL1; ref. 32) was analyzed to determine whether cis-acting elements in the PEPCK promoter mediated this RA enhancement of gene transcription. In the absence of cotransfected receptor expression vector, RA induced CAT expression in a concentration-dependent fashion; a half-maximal response occurred at ≈2×10^{-8} M (Fig. 1*B*). Numerous studies have used cotransfection of expression vectors for receptors of the steroid/thyroid hormone superfamily to elevate transcriptional responses to corresponding agonists (17–21, 30, 33, 34). Cotransfection with an expression vector for the α subtype of the RA receptor had no effect on basal expression of the reporter gene (data not shown), but it allowed for greater maximal induction of CAT activity in response to RA (6.7-fold compared with 3.0-fold; Fig. 1*B*). Thus, all subsequent studies used cotransfection with the RA receptor expression vector pRShRARα. These results indicate that RA induces transcription of the PEPCK gene through one or more

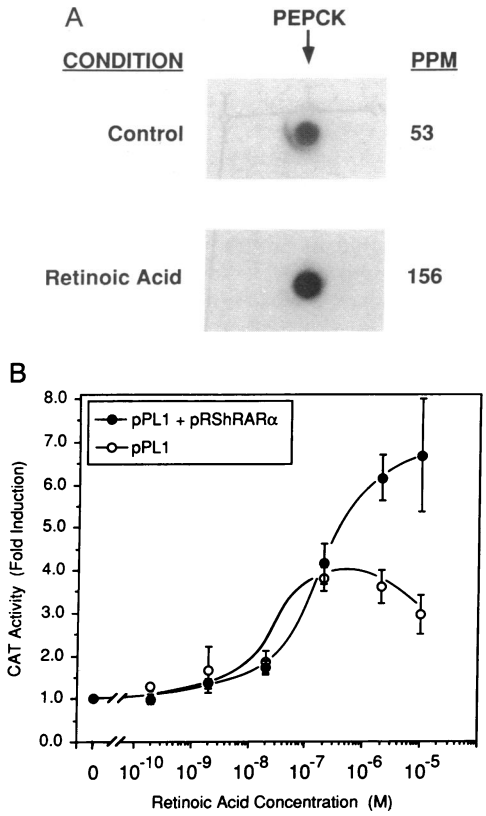

FIG. 1. RA regulation of PEPCK gene expression. (*A*) H4IIE rat hepatoma cells were incubated with or without 5 μM RA for 60 min. Nuclei were then prepared; and nascent RNA transcripts were labeled, isolated, and measured by hybridization to a PEPCK-specific probe as described. The autoradiograph is from a representative experiment in which data are expressed as parts PEPCK RNA transcribed per million parts total RNA synthesized (ppm). (*B*) H4IIE cells were transfected with 15 μg of a reporter construct containing 2100 bp of PEPCK 5′-flanking DNA ligated to the CAT gene (pPL1; ref. 32). Cotransfections were performed as indicated by adding 5 μg of expression vector encoding the human RA receptor α (pRShRARα; ref. 13). After transfection, cells were incubated for 24 hr without or with various concentrations of RA. Cells were then harvested, extracts were prepared, and CAT activity was measured. Results are expressed as the fold induction of CAT activity (+RA/−RA) and represent the average ± SEM of two to nine separate determinations at each hormone concentration.

cis-acting elements located within 2100 bp of the transcription start site.

Localization of a RARE in the PEPCK Promoter. A series of 5′ deletion mutants of the PEPCK promoter ligated to CAT was used to locate the 5′ boundary of a RARE (Fig. 2*A*). Mutants with promoter endpoints of −2100, −600, and −467 all responded to RA with a 6- to 7-fold induction of CAT expression. Deletion to −437 sharply decreased RA-stimulated CAT expression (from 6.5- to 2.7-fold induction). Further 5′deletions caused a small, gradual decline in responsiveness. This allowed us to locate the 5′ boundary of a major RARE between −467 and −437 in the PEPCK promoter.

The PEPCK promoter sequence between −468 and −431 was ligated to a PEPCK–CAT 5′ deletion mutant ending at −306 (Fig. 2*B*) to further characterize this element. This sequence conferred substantial RA responsiveness to the −306 mutant which, itself, was poorly responsive. Thus, a 38-bp segment of the PEPCK promoter mediates a stimulatory effect of RA on PEPCK gene transcription.

Mutational Analysis of the PEPCK RARE. The pTK-CAT reporter construct was used to delineate the precise boundaries of the RARE. A double-stranded oligonucleotide cor-

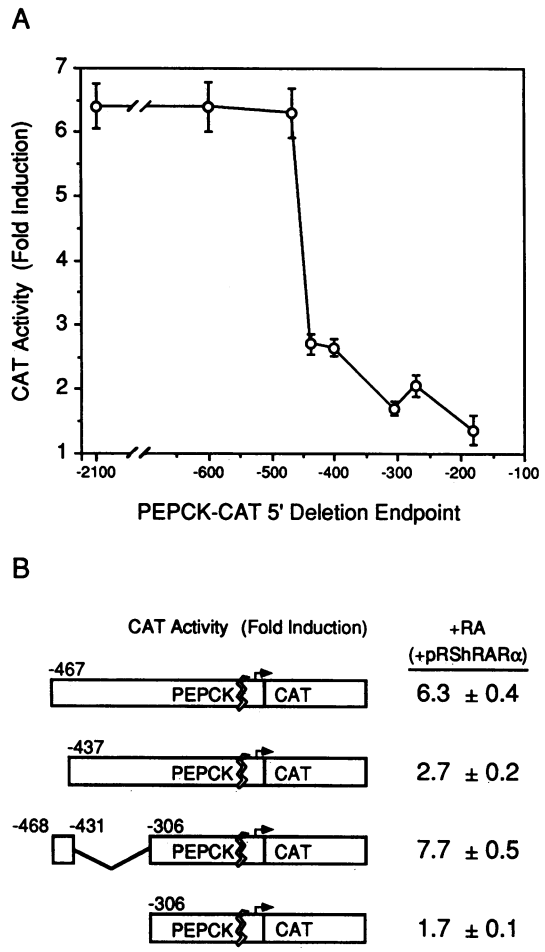

FIG. 2. RA regulation of transiently transfected 5′ deletion mutants of the PEPCK–CAT fusion gene. (*A*) A series of constructs containing 5′ deletion mutations of the PEPCK promoter fused to CAT (15 μg) were cotransfected with pRShRARα (5 μg) into H4IIE cells. Cells were incubated for 24 hr with or without 2 μM RA, and CAT activity was measured as for Fig. 1. Results are expressed as the fold induction of CAT activity (+RA/−RA) and represent the average ± SEM of four to nine separate determinations. (*B*) H4IIE cells were transiently transfected with the PEPCK–CAT reporter constructs (15 μg) diagramed and 5 μg of pRShRARα. Cells were incubated for 24 hr with or without 2 μM RA, and CAT activity was measured as for Fig. 1. Results are expressed as the fold induction of CAT activity (+RA/−RA) and represent the average ± SEM of seven to nine separate determinations.

responding to the PEPCK promoter sequence from −460 to −425 was inserted into the polylinker site immediately upstream of position −105 in the TK promoter. RA induced CAT expression nearly 3-fold from this construct (p460/425wtTK-CAT) regardless of orientation of the insert (Fig. 3). Mutational analysis of the RARE was carried out by studying RA responsiveness when block mutations of 4–6 bp were introduced in the −460 to −425 insert (Fig. 3). Mutations in regions B, C, and D completely abolished responsiveness, whereas mutations in regions A and E had little effect. These results suggest that an 18-bp region spanning the PEPCK promoter sequence from −451 to −434 comprises the core RARE.

Binding of RA Receptor α to the PEPCK RARE. Interaction of the PEPCK RARE with human RA receptor α, expressed and purified from *E. coli*, was analyzed by using the gel-retardation assay. When the PEPCK sequence from −460 to −425 was used as a probe, two bands were detected, which may indicate that both monomer and dimer forms of the receptor interact with this DNA segment (Fig. 4). Addition to the binding reaction of 100-fold molar excess of unlabeled competitor DNA, containing the wild-type −460 to −425 sequence, dramatically reduced the intensity of both retarded bands. Similar competition experiments were performed with unlabeled competitor DNAs, identical to the mutants of the −460 to −425 sequence described in Fig. 3, in an effort to correlate receptor binding with the RA response. Mutants A and E, both of which confer full RA responsiveness to pTK-CAT, competed for receptor binding as effectively as the wild-type sequence (Fig. 4). In contrast, mutant B failed to effectively compete for receptor binding, consistent with its inability to confer RA responsiveness. Analysis of mutants C and D, however, revealed a discrepancy in the correlation of binding with function. The mutant D sequence was not capable of mediating the RA response, but it could compete for receptor binding. Mutant C, also incapable of mediating a RA response, competed for the slower complex but had little effect on the more rapidly migrating complex. Gel-retardation experiments in which rat liver nuclear extract was incubated with a DNA fragment corresponding to PEPCK sequence from −441 to −426 (a fragment containing the regions altered in mutants D and E but not those regions altered in mutants A, B, or C) revealed two protein–DNA complexes (30). Therefore, it is possible that one (or more) unknown DNA-binding protein(s) may interact at site D and play a role as essential accessory factors to the RA receptor, which binds at sites B and C, in mediating the RA enhancement of gene expression.

DISCUSSION

RA treatment of H4IIE hepatoma cells results in a 3-fold enhancement of PEPCK gene transcription. An 18-bp segment, spanning nucleotides −451 to −434, serves as a minimal RARE in the PEPCK gene promoter. As seen with certain other hormone response elements, the PEPCK RARE displays enhancer-like properties; it acts in an orientation-independent fashion (Fig. 3), works through a heterologous promoter (Fig. 3), and functions equally well in at least two different locations in the PEPCK promoter (Fig. 2*B*). This element is not palindromic and bears only modest similarity to an idealized thyroid hormone response element, a sequence previously shown to mediate both RA and 3,5,3′-triiodothyronine (T_3) enhancement of gene transcription (18). In contrast, comparison of this 18-bp element to two recently described RAREs reveals considerable similarities (Fig. 5). Vasios *et al.* proposed that tandemly repeated TGACC sequences are critical components of a 46-bp RARE identified in the laminin B1 gene (19). Two such TGACC motifs also appear in tandem in the PEPCK RARE (Fig. 5); however, the region encompassed by these repeat sequences does not include site D, which is an essential component of the PEPCK gene RARE (compare Figs. 3 and 5). de The *et al.* implicated the sequence $A^{AA}_{GG}GTTCAC$ in conferring RA responsiveness to the RA receptor β gene (20), and this was subsequently confirmed (21). Interestingly, the reverse of this motif is present in the noncoding strand of the PEPCK RARE (Fig. 5), and it is separated from site D by 4 bp. Gel-retardation experiments indicate that the RA receptor α interacts specifically with sequences contained in sites B and C (Fig. 4). If the RA receptor binds the PEPCK RARE as a dimer, the TGACC motif, repeated in both sites B and C, may represent a receptor monomer-binding site. Alternatively, the dimer may bind to the $A^{AA}_{GG}GTTCAC$ motif. In either case, a second essential protein appears to bind at site D.

Several different regulatory processes appear to involve elements in or around the PEPCK RARE. This RARE

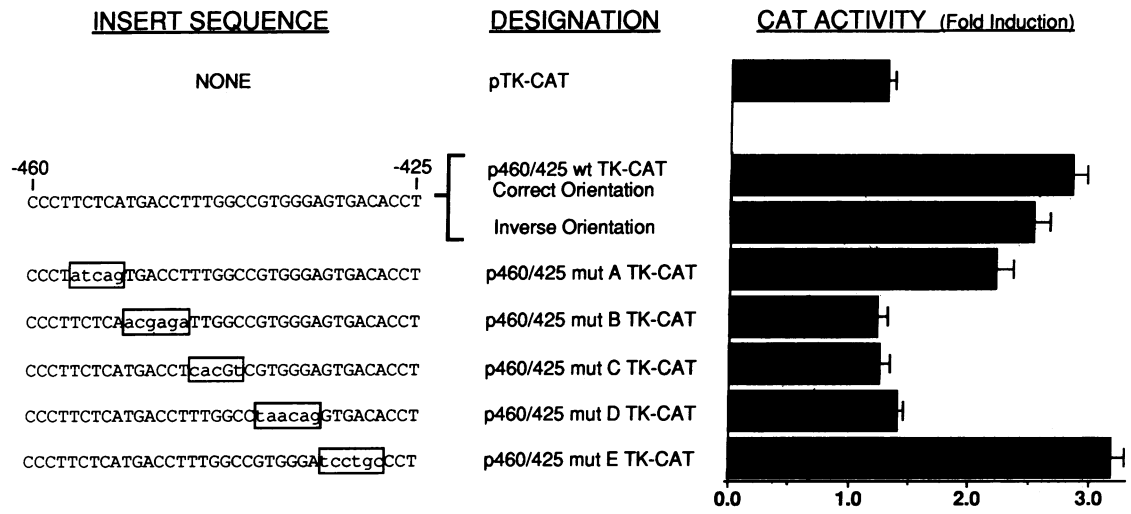

FIG. 3. Mutational analysis of the PEPCK RARE. A fragment of the PEPCK promoter spanning the region from −460 to −425 was inserted adjacent to the −105 position of pTK-CAT to create p460/425wtTK-CAT. Various mutants of the −460 to −425 oligonucleotide were also synthesized and inserted in the correct orientation into the same site. Boxed regions represent mutated segments, and lowercase letters denote the actual base changes. These constructs (20 μg) were cotransfected with pRShRARα (5 μg) into H4IIE cells. Cells were incubated for 24 hr with or without 2 μM RA before CAT activity was measured. Results are expressed as the fold induction of CAT activity (+RA/−RA) and represent the average ± SEM of 6–23 separate determinations. A pTK-CAT construct containing an insert of PEPCK sequence from −468 to −431 responded to RA with an induction of CAT expression equal to that seen with p460/425wtTK-CAT (data not shown).

coincides with an accessory factor element (AF1) that binds one or more factors essential to the function of a complex glucocorticoid response unit in the PEPCK gene (30). This glucocorticoid response unit spans the region from −455 to −349 and consists of (5′ → 3′) AF1, a second accessory factor element (AF2), and two glucocorticoid receptor-binding sites. Whether the RA receptor is the protein that binds to AF1 and mediates the function of this accessory element in the glucocorticoid response unit remains to be established; however, it is interesting that RA and dexamethasone provide synergistic induction of the endogenous PEPCK gene (unpublished work).

The PEPCK RARE also encompasses a 12-bp segment that appears in a number of promoters, including the four hepatic apolipoprotein (apoA-I, apoA-II, apoB, and apoC-III) and the α_1-antitrypsin genes (Fig. 5). This region in the α_1-antitrypsin gene is known to act as a tissue-specific basal enhancer and binds a liver nuclear factor termed LF-A1 (35, 36). LF-A1 is also capable of binding the homologous region in the apoA-I promoter (35). Similar sequences in the apoB (37, 38) and apoC-III (39, 40) genes are, likewise, critical for their tissue-specific expression. We have shown that protein(s) from liver nuclear extracts protect a region from −455 to −431 in the PEPCK promoter from digestion by DNase I (30). This region encompasses the 18-bp RARE as defined in this study. Gel-retardation assays, with probes corresponding to this region and rat liver nuclear extract, also detected several distinct protein–DNA complexes (30). It will be interesting to see whether LF-A1 is involved in expression of the PEPCK gene and, if so, how this interacts with the RA receptor. It also will be interesting to determine whether this common element confers RA responsiveness upon these other genes.

Finally, expression of PEPCK in rat liver is rigidly timed in fetal development; the enzyme is absent throughout ges-

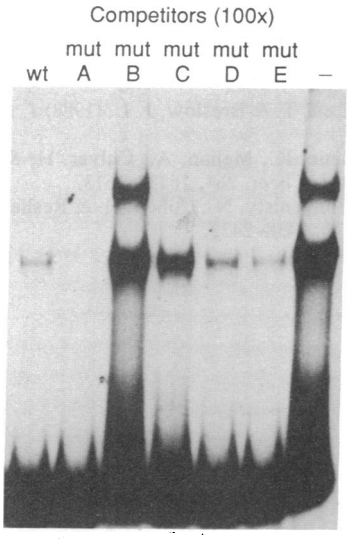

FIG. 4. Correlation of RA receptor α binding with RA response. Gel-retardation assays were done by using the PEPCK −460 to −425 sequence as labeled probe. Competitor DNAs (indicated at top of each lane) were added in 100-fold molar excess. The same quantitative results were obtained when 50-fold molar excess of competitor was used. wt, Wild type; mut, mutant.

A

Laminin B1 RARE	TGACC TGACC
PEPCK RARE	TGACCTTTGGCCGTGGGA ACTGGAAACCGGCACCCT
RA-rec. β RARE	CACTTG$^{AA}_{GG}$A

B

Gene	5'end	Sequence	Ref.
PEPCK RARE	-451	TGACCTTTGGCC	
ApoC-III	-82	·····C··	(39,40)
ApoB	-79	C·C·····A·	(37,38)
ApoA-II	-733	CA·····AC··	(38)
ApoA-I	-209	GA···C···A··	(35,38)
α_1-At	-121	··GA···A·C··	(35,36)

FIG. 5. The PEPCK RARE: Homology to other previously described RAREs. (*A*) The 18-bp core PEPCK RARE is compared with motifs demonstrated essential in two previously described RAREs. (*B*) A 12-bp segment of the PEPCK RARE is compared with sequences found in several genes expressed in liver. Dots indicate bases identical to bases in the PEPCK sequence. RA-rec. β, RA receptor β; α_1-At, α_1-antitrypsin.

tation but appears soon after birth as a consequence of the initiation of gene transcription (22). Four sequential, liver-specific protein–DNA interactions occur in the PEPCK promoter during the latest stages of gestation and in the newborn rat pup (41). The first of these occurs at gestation day 18 and involves the promoter sequence from −456 to −433, which closely corresponds to the RARE described in this report. RA may, therefore, play a role in the developmentally timed expression of PEPCK in the rat fetus.

Numerous and diverse processes, including tissue-specific and developmental expression and regulated expression through the interplay of two different hormones and their cognate receptors (RA and glucocorticoids), appear to be mediated by the interaction of several proteins with a relatively short segment of DNA in the PEPCK gene promoter.

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