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RESEARCH ARTICLE

Nuclear receptor binding to the retinoic acid response elements of the phosphoenolpyruvate carboxykinase gene in vivo: effects of vitamin A deficiency☆

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Abstract

Vitamin A deficiency decreases hepatic phosphoenolpyruvate carboxykinase (PEPCK) gene expression in mice and expression is restored with retinoic acid treatment in vivo. This report examines further the mechanism of retinoid regulation of the PEPCK gene in vivo. We have identified nuclear receptors that bind to retinoic acid response elements (RAREs) in the PEPCK promoter by electrophoretic mobility shift assay and have verified these in vivo using chromatin immunoprecipitation (ChIP) in mouse liver. Based on the results of our ChIP assay, hepatic nuclear factor (HNF)-4α, retinoid X receptor (RXR) α, retinoic acid receptor (RAR) α, peroxisome proliferator-activated receptor (PPAR) α and chicken ovalbumin upstream promoter transcription factor (COUP-TF) II bind to the downstream retinoic acid response unit RARE1/RARE2, and PPARα and RXRα bind to the upstream RARE3 of the PEPCK gene. HNF-4α, RXRα, RARα, PPARα and COUP-TFII bind PEPCK RAREs in a specific pattern that, with the exception of PPARα, does not change significantly with vitamin A deficiency. PPARα binding to the upstream retinoic acid response element is decreased in the vitamin A-deficient liver, when compared to the vitamin A-sufficient state. These results provide the first in vivo measures of nuclear receptor binding to the upstream and downstream RAREs of the PEPCK gene under conditions where the nucleosomal structure of the chromatin is maintained and the nuclear receptors are physically cross-linked in situ to the PEPCK DNA in intact liver.

Keywords: Nuclear receptors; Phosphoenolpyruvate carboxykinase gene; Retinoids; Liver

1. Introduction

Cytosolic phosphoenolpyruvate carboxykinase [PEPCK (EC 4.1.1.32)] catalyzes the conversion of oxaloacetate to phosphoenolpyruvate in the first committed step of hepatic gluconeogenesis. PEPCK mRNA levels are acutely elevated by glucocorticoids and glucagon and epinephrine via cAMP when blood glucose levels are low due to fasting, and inhibited by insulin postprandially when blood glucose is elevated, especially following a high carbohydrate meal [1]. Ex vivo and in vivo studies have shown that hepatic PEPCK gene expression is also regulated by vitamin A [2–6]. However, because sudden fluctuations in retinoid levels do not occur physiologically, it is hypothesized that retinoids play a role in maintaining basal PEPCK gene transcription [7] or in enhancing hormonal influences [4,5] that contribute to its coordinated regulation. Vitamin A depletion in fasted mice decreases not only the retinoid-mediated PEPCK response in liver but camp induction as well [8].

The PEPCK promoter is a multifaceted regulatory domain wherein there lies a multitude of overlapping response elements for its different regulators [1]. Vitamin A

Abbreviations: ChIP, chromatin immunoprecipitation; COUP-TF, chicken ovalbumin upstream promoter transcription factor; HNF, hepatic nuclear factor; KO, knockout; PEPCK, phosphoenolpyruvate carboxykinase; PGC, peroxisome proliferator-activated receptor-γ coactivator-1; PPAR, peroxisome proliferator-activated receptor; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid response element; RXR, retinoid X receptor; SRC-1, steroid receptor coactivator-1; VAD, vitamin A-deficient; VAS, vitamin A-sufficient.

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and its downstream metabolites all-*trans* and 9-*cis* retinoic acid (RA) exert their influence on PEPCK gene transcription through the binding of nuclear receptors to three identified retinoic acid response elements (RAREs) in the PEPCK promoter. RARE1 and RARE2 were originally characterized in transfected rat hepatoma cells [3,4,9] and correspond to −451/−439 and −337/−321 of the mouse PEPCK promoter, respectively. RARE3 was first characterized in rat adipose tissue [10] and maps to −1018/−1006 of the PEPCK promoter in the mouse. Nuclear receptors that bind the PEPCK RAREs have been identified using cell extracts and overexpressed nuclear receptors [2,9–13], as well as mouse liver nuclear extracts [14].

In this report, we explore nuclear receptor binding to the mouse PEPCK gene in vivo with native chromatin structure intact, as well as the effect of vitamin A deficiency on this binding pattern. We find that hepatic nuclear factor (HNF)-4α, retinoid X receptor (RXR) α, retinoic acid receptor (RAR) α, chicken ovalbumin upstream promoter transcription factor (COUP-TF) II and peroxisome proliferator-activated receptor (PPAR) α bind to the upstream and downstream retinoic acid response units in a specific pattern in vivo. Of these nuclear receptors, PPARα binding to the upstream response element, RARE3, is decreased with vitamin A deficiency. These measures of nuclear receptor binding to the PEPCK RAREs are consistent with our earlier report on coactivator docking and associated histone acetylation of the PEPCK RAREs in mouse liver [15]. The data reported here provide a more comprehensive foundation for further exploration of nuclear receptor-induced chromatin events, especially as they relate to changes in nutrient status of the whole animal and in nutrient concentrations in individual organs such as liver.

2. Methods and materials

2.1. Animal treatments

Briefly, pregnant C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME, USA) were fed the AIN93G diet [16] without vitamin A (Dyets, Bethlehem, PA, USA) from the 10th day of gestation until the pups were weaned. Pups were continued on the vitamin A deficient (VAD) diet until they were killed at 9–10 weeks of age. Vitamin A-sufficient (VAS) mice were fed the same diet with 500,000 IU/g of retinyl palmitate (Dyets).

Three hours before sacrifice for extraction of nuclear proteins for electrophoretic mobility shift assay (EMSA), VAD mice undergoing retinoid treatment were supplemented with a total of 10 mg/kg body weight of all-*trans* RA (Sigma-Aldrich, St. Louis, MO, USA), 9-*cis* RA (Toronto Research Chemicals, North York, Canada) or both, delivered in peanut oil by gavage, with control counterparts administered peanut oil alone. Total volumes did not exceed 180 μl. All mice (VAS, VAD and treated) were food-deprived for 15 h before they were sacrificed.

The above protocols were approved by the Institutional Animal Care and Use Committee at the University of Connecticut (Animal Care Protocol #E1200501).

2.2. Measurement of hepatic retinol

Total lipid was extracted from finely minced VAS and VAD liver tissue [17]. Lipid extract was dried under nitrogen, weighed for total lipid, resolubilized in chloroform/methanol (2:1, v/v) with 450 μM of butylated hydroxytoluene and used for total retinol analysis. An aliquot of lipid extract was saponified in 10 volumes of 95% ethanol/5% potassium hydroxide (Fisher Scientific, Pittsburgh, PA, USA) and 1% pyrogallol (99%, Acros Organics, Pittsburg, PA) [18]. The cooled sample was mixed vigorously with 20 volumes hexane and then 10 volumes of water. After brief centrifugation, the upper phase was dried under nitrogen and resuspended in chloroform/methanol (1:4, v/v). All-*trans*-retinyl acetate was added as an internal standard to monitor extraction efficiency. Retinol separation was performed using a Beckman high-performance liquid chromatography (Beckman Instruments, Fullerton, CA, USA) equipped with a C-18 reversed-phase column (Alltima C18, 5 μm, 4.6×150 mm; Alltech Associates, Deerfield, IL, USA) and System Gold software (Beckman). Methanol/water (99:1, v/v) was used as the mobile phase and propelled at 1 ml/min [19]. Detection was monitored at 325 nm (UV detector, Module 168, Beckman). Retinol and retinyl acetate were eluted at 3.4 and 4.6 min, respectively. Standard curves were constructed using pure retinol and retinyl acetate.

At the time of sacrifice, VAD mice showed no overt differences in physical characteristics as compared to VAS mice (Table 1).

2.3. Chromatin Immunoprecipitation Assay

Chromatin isolation was carried out according to the methods of Eberhardy et al. [20] and Bennett and Osborne [21] with modifications. Dissected livers were minced and cross-linked with 1% formaldehyde for 30 min at room temperature. Phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (both from Sigma-Aldrich) were used as protease inhibitors. The chromatin was sonicated on ice in 0.5- ml aliquots and pulsed four times for 5-s intervals on

Table 1
Hepatic retinol levels in VAS and VAD mice

Weight (g)	
VAS (n=10)	23.36 (1.18)
VAD (n=24)	22.14 (0.78)
Liver weight (g)	
VAS (n=10)	0.91 (0.10)
VAD (n=24)	0.98 (0.04)
Hepatic retinol (nmol/g)	
VAS (n=4)	2263 (201)
VAD (n=4)	5.47 (2.15)*

All values given as mean (S.E.M).
* P=.00003: VAS vs. VAD mice.

setting #5 of a Fisher Model 60 sonicator (Fisher Scientific). Samples were frozen at -80°C until the immunoprecipitation (IP) step.

Before IP, chromatin was precleared twice for 1 h at 4°C with rotation using either 70 μL of protein A or G Sepharose (1.5 g/L; Zymed Laboratories, S. San Francisco, CA, USA) that had been previously treated with 0.2 g/L salmon sperm DNA and 0.5 g/L bovine serum albumin.

Antibodies specific to HNF-4α, RXRα, RARα, PPARα and COUP-TFII (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) were used for IP. The equivalent of 0.03 g of liver, corresponding to approximately 12 μg of input chromatin, was used in each chromatin immunoprecipitation (ChIP) assay for RXRα and HNF-4α. In order to obtain more visible signals by polymerase chain reaction (PCR), the equivalent of 0.04 g of liver (approximately 16 μg of input chromatin) was used in ChIP assays conducted for proteins that were present in lower abundance in liver (RARα, PPARα and COUP-TFII), whereas the ratio of input chromatin to Protein A and/or G Sepharose beads (Zymed Laboratories) was kept constant. Optimal amounts of antibody used in each ChIP assay were determined empirically. IP was done overnight (14–16 h) with rotations at 4°C.

Equal amounts of IP and control samples were added to pretreated protein A or G Sepharose beads in a microspin chromatography column (BioRad Laboratories, Hercules, CA, USA) and incubated 1–2 h at 4°C with rotation. The columns were centrifuged at 1000*g* for 2 min at 4°C, and the beads in the columns were washed twice with two volumes of ice-cold wash buffer (1 ml/L Triton X-100/20 mmol/L Tris-Cl, pH 8.0/150 mmol/L NaCl/2 mmol/L EDTA), high-salt wash buffer (1 g/L sodium dodecyl sulfate (SDS)/10 ml/L Triton X-100/2 mmol EDTA/20 mmol Tris-Cl, pH 8.0/500 mmol NaCl), LiCl wash buffer (0.25 mmol/L LiCl/10 ml/L Igepal/10 g/L deoxycholate/1 mmol/L EDTA/10 mmol/L Tris-Cl, pH 8.1) and once with TRIS-EDTA (TE), followed by 2 min of centrifugation at 1000*g*.

The immune complexes were eluted by two successive 3-min incubations with 150-μL elution buffer (10 g/L SDS/50 mmol/L $NaHCO_3$) at 65°C. After each elution, the samples were centrifuged at 1000*g* for 2 min and the eluates pooled for the respective conditions.

To reverse the formaldehyde cross-links, the NaCl concentration was adjusted to 0.3 mol/L, and 1 μL RNase A (10 g/L) was added per 200 μL of original diluted chromatin. The samples were incubated at 65°C for 3–4 h. DNA was purified using the Qiaquick Purification Kit (Qiagen, Valencia, CA, USA) and subjected to PCR.

2.4. Polymerase chain reaction

PCR (50 μL) contained 4 μL of purified DNA from immunoprecipitated or control samples, along with 0.6 μM of each primer; 200 μM each dATP, dCTP, dGTP and dTTP; 1× PCR buffer containing 1.5 mM of $MgCl_2$ (Qiagen) and 1.25 U HotStarTaq DNA Polymerase (Qiagen). After 29 cycles of PCR amplification with an annealing temperature of 57°C, PCR products were run on a 1.5% agarose gel, visualized and quantified by ethidium bromide staining using Quantity One v.4.1 software (BioRad Laboratories). Primers used for the region encompassing mouse PEPCK RARE1/RARE2 were 5′-AGGTAACACACCCCAGCTAAC-3′ and 5′-GGCTCTTGCCTTAATTGTCAG-3′, and for mouse PEPCK RARE3, 5′-GGCATGAAGGTCTGTGGCTAC-3′ and 5′-TAGACACCATCACCCTTGGAG-3′ (Fig. 1A).

2.5. Extraction of nuclear proteins from mouse liver

Nuclear proteins were extracted from mouse liver according to the method of Gorski et al. [22]. Briefly, each mouse liver (~1.0-1.5 g) was minced and homogenized to lyse the cells. The homogenate was diluted, layered over homogenization buffer and centrifuged at 29,000 rpm for 30 min in an SW50.1 rotor. The pellets were resuspended in homogenization buffer, and the centrifugation step was repeated. The nuclear pellet was resuspended in nuclear lysis buffer using a dounce homogenizer. The lysate was precipitated with 1/10 volume of 4 M $(NH_4)_2SO_4$ for 30 min, and the mixture was centrifuged at 36,000 rpm for 25 min in a Ti70 rotor to pellet chromatin. Nuclear proteins were precipitated using 0.3 g/ml $(NH_4)_2SO_4$ on ice, and the latter centrifugation step was repeated. The pellet was reserved, resuspended in 1-ml dialysis buffer, and the nuclear lysate was dialyzed twice for 2 h. The protein concentration was determined by the method of Kalb and Bernlohr [23].

2.6. Electrophoretic mobility shift assay

EMSA was performed as previously described [14]. Probes for EMSA were prepared by end-labeling a double-stranded oligonucleotide containing PEPCK RARE1, RARE2 or RARE3 (Fig. 2A) with [γ-^{32}P-ATP] and T4 polynucleotide kinase (Invitrogen, Carlsbad, CA, USA) and purified over a G-25 spun column (Amersham Biosciences, Piscataway, NJ, USA). Liver nuclear extracts (1 μg protein/binding reaction) were incubated with 50,000 cpm of [^{32}P]-labeled probe and 1 μg poly (dI-dC) [24].

For supershift experiments, 3 μg of antibody specific to RXRα or HNF-4α (sc-553× and sc-6556×, respectively; Santa Cruz Biotechnologies) were added to the binding reaction after the addition of probe. In competition studies, unlabeled PEPCK RARE1, RARE2 or RARE3 were added at concentrations up to 100× to the concentration of labeled oligonucleotide in the binding reaction.

Samples from the binding reactions were subjected to electrophoresis on 6% (29:1) gels in 1× TRIS-Borate-EDTA (TBE) buffer at 180 V. The gels were dried under vacuum and autoradiographed using Kodak Biomax film (Eastman Kodak, Rochester, NY, USA). For the gel shift Western, following EMSA, the resolved protein–DNA complexes were transferred to nitrocellulose. The nitrocellulose was cut into strips, and each strip hybridized independently with the above antibodies to HNF-4α or

RXRα-specific binding was determined using the VECTASTAIN ABC kit (Vector Labs, Burlingame, CA, USA).

2.7. Statistical analysis

For animal characteristics, results are presented as mean ±S.E.M. For ChIP assays, results are reported as mean image densities± S.E.M., with the negative control (No Ab) for each ChIP assay set to a value of 1. Differences between group means were analyzed by Student's *t* test and were considered significant at $P<.05$. All data were evaluated using MS Excel 2002 (Microsoft, Redmond, WA, USA).

3. Results

3.1. Hepatic retinol levels are reduced in VAD mice

VAD mice that were used for chromatin isolation and nuclear protein extraction showed no overt differences in physical characteristics, as compared to VAS mice at the age of sacrifice. Body weight and liver weight did not differ despite the dramatic reduction in hepatic retinol in VAD mice (Table 1). Hepatic retinol levels, measured from lipids extracted from VAS and VAD livers, decreased from 2263±201 to 5.47±2.15 nmol/g liver, respectively ($P=.00003$). In mice placed on the same dietary protocol in our laboratory, plasma retinol levels were reduced in VAD mice to 20% of retinol in VAS mice [8], confirming that with vitamin A deficiency, hepatic retinol stores are depleted before circulating retinol in the blood.

3.2. Hepatic HNF-4α, RXRα, RARα, PPARα and COUP-TFII bind to RARE1/RARE2 of the mouse PEPCK promoter in vivo

An assessment of in vivo binding of nuclear receptors to the PEPCK RAREs in intact liver has not been conducted before. We used the ChIP assay to characterize nuclear receptor binding to the PEPCK RAREs in intact mouse liver. Under conditions of a moderate fast, when PEPCK gene expression is induced, we measured in vivo binding of the liver-specific transcription factor HNF-4α and the 9-*cis* RA receptor RXRα to the DNA sequence encompassing RARE1/RARE2 of the PEPCK promoter (Fig. 1A) in VAS mouse liver (Fig. 1B). In addition, mice were made VAD, and the binding of nuclear receptors was tested under the same conditions of a moderate fast, conditions under which we have previously measured a decrease in induction of PEPCK mRNA levels in the VAD liver [6,8]. A comparison of in vivo binding of HNF-4α and RXRα in VAS and VAD mouse liver showed that HNF-4α and RXRα bound RARE1/RARE2 to a similar extent (Fig. 1B). In order to assess the specificity of the ChIP assay conducted, the PCR products generated with the RARE1/RARE2 primers were measured using input chromatin and HNF-4α-immunoprecipitated chromatin and compared to the no-antibody control (Fig. 1C). In addition, HNF-4α bound to RARE1/RARE2 but not RARE3 (Fig. 1D). The results in Fig. 1D also indicate that the DNA fragments immunoprecipitated with HNF-4α antibody generated a product with the RARE1/RARE2 primers but were within the 500-bp range (as indicated in Methods and materials) and, therefore, not long enough to include the upstream RARE3 sequence and generate a nonspecific RARE3 PCR product.

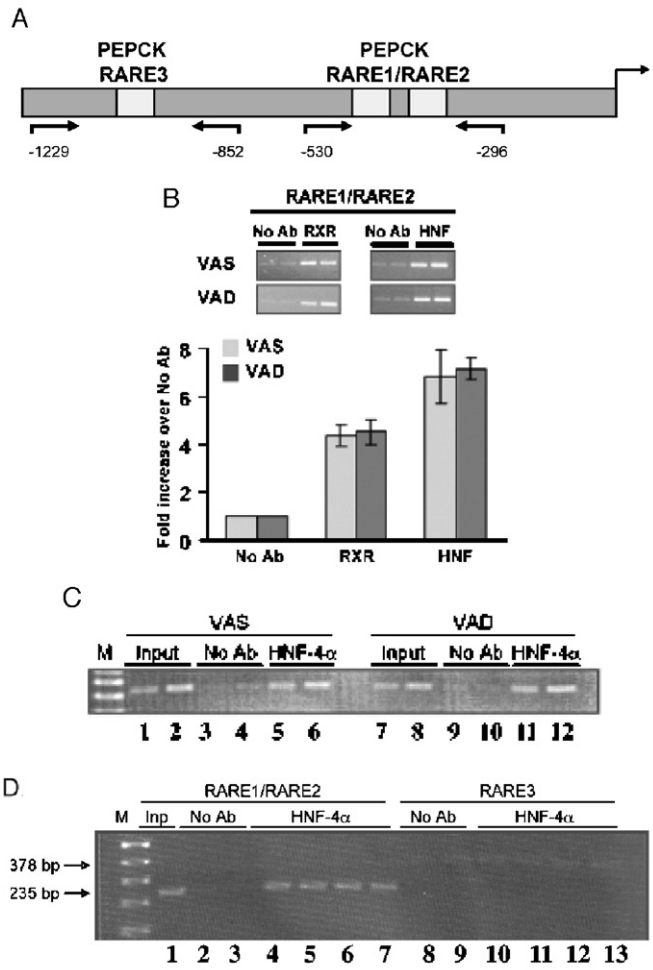

Fig. 1. HNF-4α and RXRα bind RARE1/RARE2 of the PEPCK promoter in vivo in VAS and VAD livers. Chromatin was isolated from VAS and VAD mouse liver for the ChIP assay, and antibodies specific to HNF-4α and RXRα were used as described in Methods and materials. (A) primers specific to the regions encompassing RARE1/RARE2 and RARE3 of the mouse PEPCK regulatory domain were used as indicated. (B) RXRα and HNF-4α bind at PEPCK RARE1/RARE2 in VAS and VAD mice. The negative control lanes (No Ab) show ChIP assays performed without antibody. The relative intensity of the positive signal is measured as an increase over that of the No Ab signal, which is set to 1 for each experiment. (C) input chromatin (5 and 10 μl of 1:16 dilution) from VAS (Lanes 1 and 2) and VAD (Lanes 7 and 8) livers; No Ab control for VAS (Lanes 3 and 4) and VAD (Lanes 9 and 10) livers; and 5 and 10 μl of immunoprecipitated DNA with HNF-4α antibody from VAS (Lanes 5 and 6) and VAD (Lanes 11 and 12) livers. (D) specificity of HNF-4α binding to RARE1/RARE2 versus RARE3. Input chromatin is shown in Lane 1 and the No Ab controls in Lanes 2 and 3 and Lanes 8 and 9. Immunoprecipitation with HNF-4α antibody and PCR with RARE1/RARE2 primers (Lanes 4–7) vs. RARE3 primers (Lanes 10–13). The results shown are representative of 4–6 individual experiments, and the fold differences are given as mean±S.E.M.

Although HNF-4α association with PEPCK RARE1/RARE2 did not change with alterations in vitamin A status, we also tested HNF-4α binding under fed conditions. In the fed state, PEPCK mRNA levels are low [1], so we used alteration to the fed state to test changes in HNF-4α binding to PEPCK RARE1/RARE2. In the fed state, HNF-4α binding to PEPCK RARE1/RARE2 decreased by approximately 50%, from 6.9±0.45- to 3.4±0.3-fold in the fasted and fed state, respectively ($P<.05$).

As shown in Fig. 2, COUP-TFII, PPARα and RARα also bound to PEPCK RARE1/RARE2 in liver in vivo. In the VAD liver, there was a decrease in PPARα and RARα binding to RARE1/RARE2 of 40% and 45%, respectively, although this was not statistically significant. These results may indicate a decrease in PPARα and RARα affinity for the PEPCK RARE or a decrease in the amount of PPARα and RARα protein available to bind PEPCK RARE1/RARE2.

3.3. Hepatic PPARα and RXRα bind to RARE3 of the mouse PEPCK promoter in vivo

The upstream Direct Repeat (DR)1 element (GTGGTAAAGGTCT) of the PEPCK promoter between −1002/−1014 in the mouse gene (Fig. 1A) has not been examined in liver. This element is considered an adipocyte-specific enhancer required for PEPCK gene expression during adipogenesis [25]. In adipose tissue, PPARγ binds to this element [25]. Fig. 3 shows that the liver-enriched PPARα binds to the RARE3 in liver in vivo. As assessed by the ChIP assay, PPARα and RXRα both bind to RARE3. There was no detection of HNF-4α, RARα or COUP-TFII binding to this upstream element. PPARα binding to RARE3 was significantly decreased in VAD liver ($P<.05$), and there was a 20% decrease in RXRα binding to RARE3 in VAD liver, although this was not statistically significant. Previous studies in our laboratory have shown no difference in RARα and RXRα mRNA or protein levels in VAS and VAD livers [6,14]. HNF-4α mRNA and protein levels are also unchanged in VAD liver.[2] However, both RNA and protein levels for PPARα are decreased in VAD liver,[2] and this may contribute to the significant decrease in PPARα binding at PEPCK RARE3, as well as the trend to decreased binding at PEPCK RARE1/RARE2.

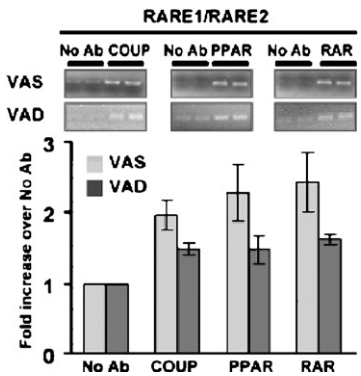

Fig. 2. COUP-TFII, PPARα and RARα bind RARE1/RARE2 of the PEPCK promoter in vivo in VAS and VAD livers. Chromatin was isolated from VAS and VAD mouse liver for the ChIP assay and antibodies specific to COUP-TFII; PPARα and RARα were used as described in Methods and materials. The results shown are representative of 3–6 individual experiments, and the fold differences are given as mean±S.E.M.

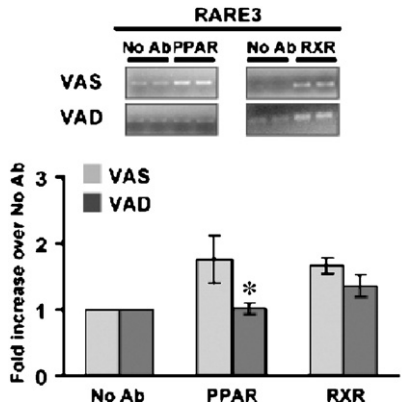

Fig. 3. RXRα and PPARα bind RARE3 of the PEPCK promoter in vivo in VAS and VAD livers. Chromatin was isolated from VAS and VAD mouse liver for the ChIP assay, and antibodies specific to RXRα and PPARα were used as described in Methods and materials. PPARα and RXRα bind at PEPCK RARE3 in VAS and VAD mice. The results shown are representative of 3–4 individual experiments, and the fold differences are given as mean±S.E.M. *Different from VAS, $P<.05$.

3.4. Distinguishing nuclear receptors that bind PEPCK RARE1, RARE2 and RARE3 in vitro

In order to determine the specific binding of nuclear receptors to PEPCK RARE1 vs. RARE2, which could not be distinguished by ChIP assay due to the close proximity of these elements, EMSA was conducted with the designated oligonucleotides for the mouse PEPCK gene (Fig. 4A). As shown in Fig. 4B, both HNF-4α and RXRα bound to PEPCK RARE1. This confirms our in vivo results and indicates the specific RARE with which both HNF-4α and RXRα are associated. Antibody to HNF-4α and RXRα each shifted a protein–DNA complex of approximately similar molecular weight, although much less with the RXRα antibody. The addition of retinoids, all-*trans* and 9-*cis* RA, did not change the gel shift pattern. EMSA competition experiments showed that the RXRα supershift was almost completely eliminated with 25× cold RARE1 competitor, and the HNF-4α supershift was also diminished by 25× cold RARE1 competitor (Fig. 4C). There was no difference in the competition pattern when nuclear extracts from either VAS or VAD liver were used in the binding reaction. Resolution of the protein–DNA complexes at RARE1 that were supershifted with HNF-4α and RXRα antibodies, individually and in combination, showed that the same molecular weight band was supershifted by the individual antibodies, and the addition of both antibodies to

[2] Kang H, Reddy GB, Odom DP, Fernandez M-L, and McGrane MM. Altered macronutrient metabolism in the vitamin A deficient liver. J. Lipid Res, submitted.

A

PEPCK Oligonucleotides used in EMSA		
Name	**Region**	**Sequence (5' → 3')**
Mouse RARE1	-459/-433	CCTTCTCA**TGACCT**T**TGGCCG**TGGGAG
		CTCCCACGGCCAAAGGTCATGAGAAGG
Mouse RARE2	-342/-316	CGGCT**AGGCCT**GCCCT**TGACCC**CCACC
		GGTGGGGGTCAAGGGCAGGCCTAGCCG
Mouse RARE3	-1020/-1007	ACAACT**GTGGTA**A**AGGTCT**TG
		CAAGACCTTTACCACAGTTGT

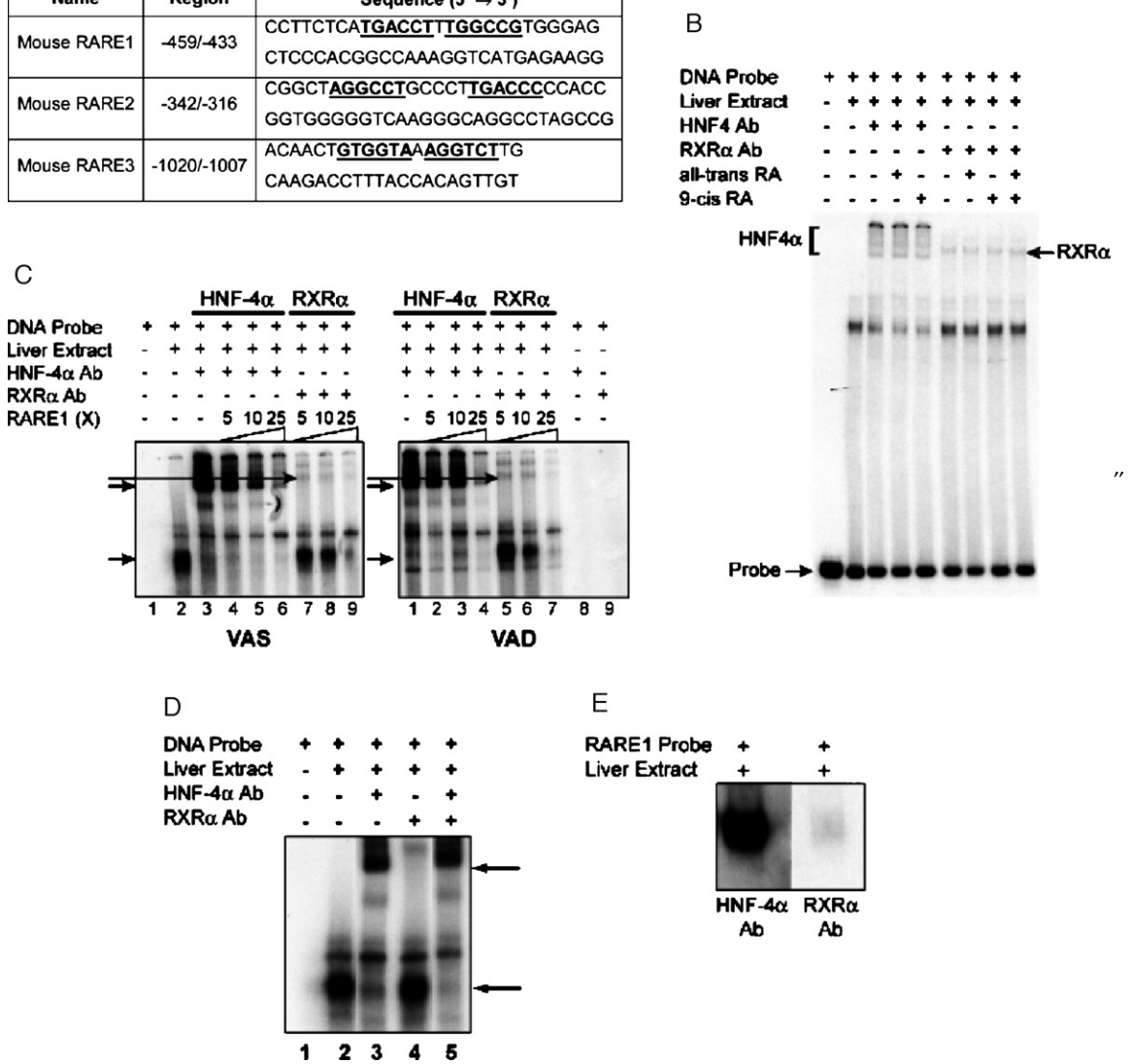

Fig. 4. RXRα and HNF-4α specifically bind PEPCK RAREs in vitro. (A) oligonucleotide sequences of the mouse PEPCK RARE1, RARE2 and RARE3 used for EMSA. Nuclear proteins (1 μg per reaction) were extracted from mouse liver as described and incubated with 50,000 cpm of double-stranded [^{32}P]-labeled probe for mouse PEPCK RARE1 or RARE3. Where indicated, antibodies specific to HNF-4α or RXRα were added to the binding reaction for antibody supershifts. (B) EMSA was performed using [^{32}P]-labeled probe for mouse PEPCK RARE1 and antibodies specific to HNF-4α or RXRα, ±ligand all-*trans* RA (9 μM), 9-*cis* RA (9 μM) or both. (C) EMSA competition with unlabeled mouse PEPCK RARE1. EMSA was performed using [^{32}P]-labeled probe for mouse PEPCK RARE1, nuclear extract from VAS and VAD livers, and antibodies specific to HNF-4α (Lanes 3–6 VAS and 1–4 VAD) or RXRα (Lanes 7–9 VAS and 5–7 VAD). In Lanes 4–6 VAS and 2–4 VAD and 7–9 VAS and 5–7 VAD, unlabeled mouse PEPCK RARE1 was added to the binding reaction at 5×, 10× and 25× the concentration of labeled probe. Lanes 8 and 9 in the VAD autoradiogram represent DNA probe with either HNF-4α or RXR α antibody, in the absence of liver nuclear extract. (D) Resolution of the protein–DNA complexes at RARE1 that were supershifted with HNF-4α and RXRα antibodies, individually and in combination. (E) The EMSA was conducted as in (B) and (C), but with unlabeled DNA probe. After completion of the EMSA, the resolved protein–DNA complexes were transferred to nitrocellulose, then individual strips of nitrocellulose with the shifted DNA–protein complex were used for Western blot with antibodies to HNF-4α or RXRα.

the binding reaction caused an increase in the amount of supershifted band (Fig. 4D). A gel shift-Western was conducted with the DNA–protein complexes formed on RARE1; these were transferred to nitrocellulose and hybridized with antibodies to either HNF-4α or RXRα and detected using the VECTASTAIN ABC kit. The results shown in Fig. 4E indicate that HNF-4α and RXRα were bound in a molecular complex of the same relative mobility in the gel. It is most likely that this represents both HNF-4α and RXRα homodimers bound to RARE1, resulting in the same relative mobility of the gel shift band, as the two nuclear receptors are known to bind as homodimers and have an almost identical amino acid sequence and molecular weight.

To determine the relative affinity of HNF-4α and RXRα for PEPCK RARE1, RARE2 and RARE3, EMSA competition experiments were conducted. Binding of HNF-4α to RARE1 was specific, and the HNF-4α supershift was eliminated with 25× cold competitor (Fig. 5A). HNF-4α had the highest affinity for RARE1. Excess unlabelled RARE2 did not compete off HNF-4α (Fig. 5A), except at high concentrations (100×). However, direct HNF-4α binding to mouse PEPCK RARE2 (a DR5 element) was not detected by EMSA with HNF-4α antibodies (data not shown). Even at 100× concentration of unlabeled RARE3 competitor, there was still HNF-4α bound to RARE1, suggesting that, despite the similar configurations of RARE1 and RARE3 (both are DR1 elements), HNF-4α has a very low affinity for the nucleotide sequence at RARE3. This is consistent with the ChIP analysis that did not show HNF-4α binding to RARE3 in vivo. It should be noted that RARE1 is a DR1 with only one imperfect repeat (in the 5′ position), whereas RARE3 has two imperfect repeats. Our initial studies conducted with the rat RARE3 sequence, which differs from the mouse RARE3 sequence by only two nucleotides, did show HNF-4α binding at this element (data not shown), illustrating the specificity that is required for DNA binding by this protein when nuclear extracts are used. At RARE3, competition studies using unlabeled RARE1, RARE2 or RARE3 showed RXRα binding was similar for all three RAREs (Fig. 5B). This is consistent with our ChIP results that indicate RXRα binds to RARE1/RARE2 and RARE3 in vivo.

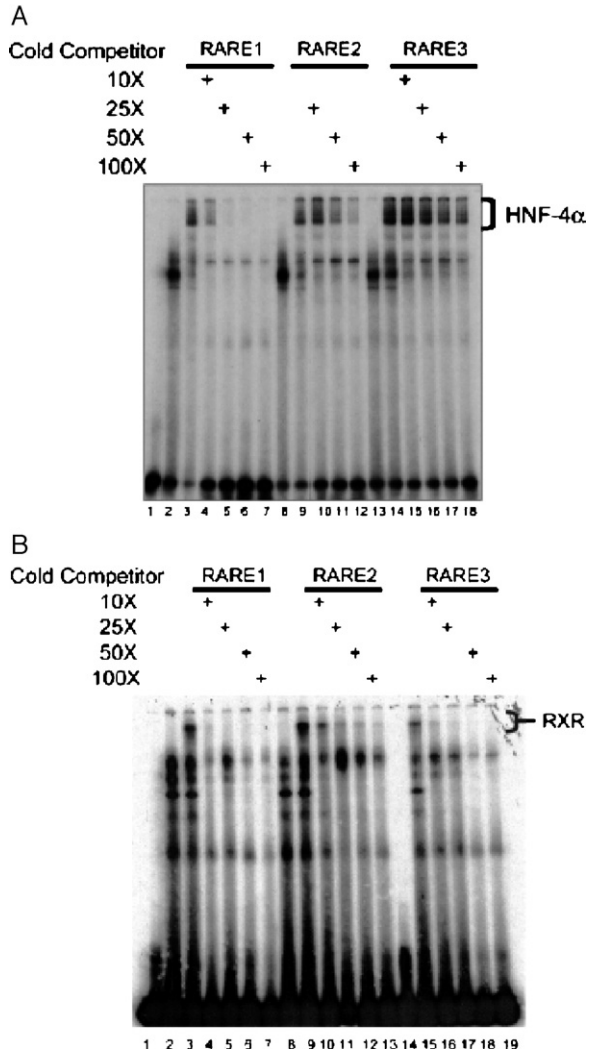

Fig. 5. HNF-4α and RXRα affinity for PEPCK RARE1, RARE2 and RARE3. (A) competition studies were conducted to examine the affinity of HNF-4α binding (Lanes 3–7, 9–12 and 14–18) to PEPCK RARE1, RARE2 and RARE3. Shown are [^{32}P]-labeled PEPCK RARE1 probe alone (Lane 1), with liver nuclear extract alone (Lane 2, 8 and 13), with NE plus antibody to HNF-4α (Lanes 3, 9 and 14), and the addition of unlabeled mouse PEPCK RARE1 (Lanes 4–7), RARE2 (Lanes 10–12) or RARE3 (Lanes 15–18) to the binding reactions at 10×, 25×, 50× and 100× the concentration of labeled PEPCK RARE1 probe. (B) shown are [^{32}P]-labeled PEPCK RARE3 probe alone (Lane 1), with liver nuclear extract alone (Lanes 2 and 8), with NE plus antibody to RXRα (Lanes 3, 9 and 15) and the addition of unlabeled mouse PEPCK RARE1 (Lanes 4–7), RARE2 (Lanes 10–13) or RARE3 (Lanes 16–19) to the binding reactions at 10×, 25×, 50× and 100× the concentration of labeled PEPCK RARE1 probe.

We have also shown that COUP-TFII and RARα bind to both RARE1 and RARE2 in vitro, although with much lower abundance than for RXRα and HNF-4α [14], consistent with the ChIP data presented here. Although PPARα was measured as binding to RARE1/RARE2 and RARE3 by ChIP, it was not detected by EMSA (data not shown), indicating the importance of native chromatin structure for measuring the binding of certain nuclear receptors to their cognate DNA response elements.

4. Discussion

The results presented here identify the nuclear receptors that bind to PEPCK RAREs in liver with the chromatin structure of the hepatocyte nuclei intact, as well as the differential binding of these nuclear receptors under conditions of altered vitamin A status. Of the PEPCK RAREs, RARE1 fits most closely the common motif for a DR element, i.e., a DR1 with a 5′ imperfect and 3′ perfect AGGTCA motif, with an A in the spacer position [26]. Consistent with this, we measure HNF-4α, RXRα, COUP-TFII, PPARα and RARα association with this promiscuous element, as has been determined for other genes with DR1 elements in vitro [26,27]. Because multiple nuclear receptors bind the PEPCK RARE1, as determined by both EMSA and ChIP assay, further insight into the potential specificity of nuclear receptor binding to this element can be determined by comparison of the nucleotide sequence of the PEPCK RARE1 to the consensus DR1 element. Our results are consistent with reports on the C3P element of the *apoCIII* gene, which has a high affinity for HNF-4, COUP-TFII, RAR/RXR and PPAR/RXR and contains an imperfect 5′ repeat which diverges at the 1 and 4 nucleotide, as does PEPCK RARE1. More specifically, according to Nakshatri and Nakshatri [26], a change to a pyrimidine at Position 1 in

C3P decreases the affinity of COUP-TFII, RAR/RXR and PPAR/RXR, but not HNF-4, for this element. The imperfect 5′ repeat of the PEPCK RARE1 has a C in Position 1 and, therefore, is similar to the described mutation in C3P. Consistent with this, we measure HNF-4α as the nuclear receptor that binds RARE1 in greatest abundance, with much lower levels of RXRα, RARα, COUP-TFII and PPARα binding, when mouse liver nuclear extract is used and there is no overexpression of an individual receptor. HNF-4α also has a greater affinity for RARE1 than RARE2 or RARE3. RXRα, on the other hand, has an equal affinity for the three PEPCK RAREs, as determined by EMSA in this report.

HNF-4α bound the mouse PEPCK RARE1 equally in VAS and VAD mice (Fig. 2C). HNF-4α is required for PEPCK gene activation in the fasted state and may be necessary as a docking site for the coactivators steroid receptor coactivator (SRC)-1 [28] and peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α [29]. Stafford et al. [29] have shown that SRC-1 associates with HNF-4α in liver in vivo and that this requires the AF2 domain of HNF-4α. SRC-1 enhances the transactivation capacity of HNF-4α, as tested in HepG2 cells. HNF-4α is typically associated with gene activation, and its expression is induced in adipose cells by retinoids [30]. However, we do not measure a difference in the amount of HNF-4α bound from either VAS or VAD nuclear extracts, nor do we measure a difference in liver HNF-4α protein by Western analysis (data not shown). A recent study suggests that HNF-4α is bound constitutively to RARE1, and inhibition of PEPCK gene transcription is due, in part, to displacement of PGC-1α from its docking site on HNF-4α by the inhibitory steroid response element binding protein 1 [31]. Hypothetically, the lack of coactivator binding to an HNF-4α dimer at PEPCK RARE1 would diminish the transactivation capacity of HNF-4α because of the lack of histone acetylation and chromatin remodeling that would normally accrue to the PEPCK gene due to docking of coactivators such as SRC-1 and PGC-1α to this site. As reported previously, we measure a decrease in SRC-1 association with PEPCK RARE1/RARE2 in VAD liver in vivo [15]. So it is likely coactivator docking to HNF-4α, rather than HNF-4α binding itself, that is decreased in VAD liver at this downstream retinoic acid response unit of the PEPCK gene.

RARα also bound RARE1/RARE2 as determined by ChIP in vivo (Fig. 2). The binding of RARα to RARE1/RARE2 is consistent with our previous report that RARα binds RARE2, as shown by EMSA [14]. We have determined in transgenic mice that RARE1 is a 9-*cis* RA response element, whereas RARE2 (DR5) is an all-*trans* RA response element [14]. Therefore, it is likely that RARE1 binds RXRα as a homodimer, whereas RARE2 binds RARα as a heterodimer with RXRα. Ijpenberg et al. have recently shown that RXR homodimer formation at a DR1 element is stabilized by both 9-*cis* RA and the coactivator SRC-1 [32]. As indicated, we measure SRC-1 association with the PEPCK RARE1/RARE2 in vivo and a decrease in this association in VAD liver [15]. Therefore, SRC-1 potentially interacts with both HNF-4α and RXRα homodimers at PEPCK RARE1, and when SRC-1 association is decreased with VAD, this contributes to decreased expression of the PEPCK gene proportionate to the HNF-4α and RXRα homodimers bound.

PPARα binds both PEPCK RARE1/RARE2 and RARE3; there is a significant reduction in PPARα binding at RARE3, as well as a 40% decrease in binding to RARE1/RARE2 in VAD liver (Figs. 2 and 3). It should be noted that this report provides the first evidence of a PPAR isoform binding to the functional PEPCK promoter in liver in vivo, and the first report of the binding of any liver nuclear receptor to the PEPCK RARE3. PPAR/RXR heterodimers are capable of activating target genes, including PEPCK, in response to PPARα-specific ligands and to both 9-*cis* and all-*trans* RA [11,33]. The PPARα gene is itself regulated by retinoids in brown adipose tissue [34], and we measure a decrease in PPARα mRNA by microarray and PPARα protein by Western blot in VAD liver.[2] Of particular interest, is the fact that PPARα is required for the cellular response to fasting in liver. In PPARα knockout (KO) mice, the normal increase in fatty acid β-oxidation with fasting does not occur, and genes encoding enzymes of this pathway are not induced [35]. Our results indicate that in the absence of retinoids, PPARα binding at RARE3 is significantly diminished. As stated above, under the same conditions, we measure a decrease in hepatic PEPCK mRNA during a fast, and PEPCK gene expression is regulated almost entirely at the transcriptome level. This would predict a decrease in hepatic gluconeogenesis in the VAD liver. This may document another altered metabolic response to fasting linked to decreased PPARα availability, in this case, due to vitamin A deficiency.

Interestingly, fasted PPARα KO mice, liver-specific HNF-4α KO mice and VAD mice share a similar phenotype in that all three develop fatty livers in comparison to their respective controls[3] [35–38]. Additionally, both the liver-specific HNF-4α KO mice and VAD mice show a similar inhibition of induction of the PEPCK gene with fasting [37] and PPARα KO mice exhibit a decrease in hepatic gluconeogenesis with fasting [35]. Therefore, the metabolic outcome in PPARα and HNF-4α KO mice is similar to VAD in both lipid and carbohydrate parameters and provides further evidence that metabolic dysregulation characteristic of VAD is likely mediated by a disruption of HNF-4α and PPARα signaling in liver. Altered hepatic lipid metabolism due to VAD, PPARα KO or HNF-4α KO may also impact the relative concentrations of fatty acid metabolites, i.e., free fatty acids and fatty acyl-CoA thioesters, therefore upsetting the ratio of ligands that are agonists or antagonists for HNF-4α and PPARα [39,40].

[3] Reddy, G., Kang, H.W. and M.M. McGrane, unpublished observation.

References

[1] Hanson RW, Reshef L. Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. Annu Rev Biochem 1997;66:581–611.

[2] Hall RK, Scott DK, Noisin EL, Lucas PC, Granner DK. Activation of the phosphoenolpyruvate carboxykinase gene retinoic acid response element is dependent on a retinoic acid receptor/coregulator complex. Mol Cell Biol 1992;12:5527–35.

[3] Lucas PC, O'Brien RM, Mitchell JA, Davis CM, Imai E, Forman BM, et al. A retinoic acid response element is part of a pleiotropic domain in the phosphoenolpyruvate carboxykinase gene. Proc Natl Acad Sci U S A 1991;88:2184–8.

[4] Lucas PC, Forman BM, Samuels HH, Granner DK. Specificity of a retinoic acid response element in the phosphoenolpyruvate carboxykinase gene promoter: consequences of both retinoic acid and thyroid hormone receptor binding. Mol Cell Biol 1991;11:5164–70.

[5] Pan CJ, Hoeppner W, Chou JY. Induction of phosphoenolpyruvate carboxykinase gene expression by retinoic acid in an adult rat hepatocyte line. Biochemistry 1990;29:10883–8.

[6] Shin DJ, Tao A, McGrane MM. Effects of vitamin A deficiency and retinoic acid treatment on expression of a phosphoenolpyruvate carboxykinase-bovine growth hormone gene in transgenic mice. Biochem Biophys Res Commun 1995;213(2):706–14.

[7] Lee MY, Jung C-H, Lee K, Choi YH, Hong S, Cheong JH. Activation transcription factor-2 mediates transcriptional regulation of gluconeogenic gene PEPCK by retinoic acid. Diabetes 2002;51:3400–7.

[8] Shin DJ, McGrane MM. Vitamin A regulates genes involved hepatic gluconeogenesis in mice: phosphoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase and 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase. J Nutr 1997;127:1274–8.

[9] Scott DK, Mitchell J, Granner DK. Identification and characterization of the second retinoic acid response element in the phosphoenolpyruvate carboxykinase gene promoter. J Biol Chem 1996;271:6260–4.

[10] Devine JH, Eubank DW, Clouthier DE, Tontonoz P, Spiegelman BM, Hammer RE, et al. Adipose expression of the phosphoenolpyruvate carboxykinase promoter requires peroxisome-proliferator-activated receptor gamma and 9-*cis* retinoic acid receptor binding to an adipocyte-specific enhancer in vivo. J Biol Chem 1999;274:13604–12.

[11] Eubank DW, Duplus E, Williams SC, Forest C, Beale EG. Peroxisome proliferators-activated receptor Y and chicken ovalbumin upstream promoter transcription factor II negatively regulate the phosphoenolpyruvate carboxykinase promoter by a common element. J Biol Chem 2001;276(32):30561–9.

[12] Hall RK, Sladek FM, Granner DK. The orphan receptors COUPTF and HNF serve as accessory factors required for induction of phosphoenolpyruvate carboxykinase gene transcription by glucocorticoids. Proc Natl Acad Sci U S A 1995;92:412–6.

[13] Scott DK, Mitchell JA, Granner DK. The orphan receptor COUP-TF binds to a third glucocorticoid accessory factor element within the phosphoenolpyruvate carboxykinase gene promoter. J Biol Chem 1996;271(50):31909–14.

[14] Shin D-J, Odom DP, Scribner KB, Ghoshal S, McGrane MM. Retinoid regulation of the phosphoenolpyruvate carboxykinase gene in liver. Mol Cell Endocrinol 2002;195:39–54.

[15] Scribner KB, Odom DP, McGrane MM. Vitamin A status in the mouse affects the histone code of the phosphoenolpyruvate carboxykinase gene in liver. J Nutr 2005;135:1161–5.

[16] Reeves PG. Components of the AIN93G diet as improvements in the AIN-76A diet. J Nutr 1997;127(5 Suppl):833S–41S.

[17] Folch J, Lees M, Sloane-Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957;226:497–509.

[18] Ross AC. Separation and quantitation of retinyl esters and retinol by high performance liquid chromotagraphy. Methods Enzymol 1986; 123:68–74.

[19] May HE, Koo SI. The reverse phase HPLC behavior of retinyl esters. J Liq Chromatogr 1989;12:1261–80.

[20] Eberhardy SR, D'Cunha CA, Farnham PJ. Direct examination of histone acetylation of Myc target genes using chromatin immunoprecipitation. J Biol Chem 2000;275:33798–805.

[21] Bennett MK, Osborne TF. Nutrient regulation of gene expression by the sterol regulatory element binding proteins. Proc Natl Acad Sci U S A 2000;97:6340–4.

[22] Gorski K, Carneiro M, Schibler U. Tissue-specific in vitro transcription from the mouse albumin promoter. Cell 1986;47:467–76.

[23] Kalb Jr VF, Bernlohr RW. A new spectrophotomteric assay for protein in cell extracts. Anal Biochem 1977;238(20):519–21.

[24] Yen PM, Sugawara A, Chin WW. Triiodothyronine (T3) differentially affects T3-receptor/retinoic acid receptor and T3-receptor/retinoid X receptor heterodimer binding to DNA. J Biol Chem 1992;267(32): 23248–52.

[25] Beale EG, Forest C, Hammer RE. Regulation of cytosolic phosphoenolpyruvate carboxykinase gene expression in adipocytes. Biochimie 2003;85(12):1207–11.

[26] Nakshatri H, Nakshatri PB. Multiple parameters determine the specificity of transcriptional response by nuclear receptors HNF-4, ARP-1, PPAR, RAR and RXT through common response elements. Nucleic Acids Res 1998;26:2491–9.

[27] Jiang G, Sladek FM. The DNA binding domain of hepatocyte nuclear factor 4 mediates cooperative, specific binding to DNA and heterodimerization with the retinoid X receptor alpha. J Biol Chem 1997;272:1218–25.

[28] Monsalve M, Wu Z, Adelmant G, Puigserver P, Fan M, Spiegelman BM. Direct coupling of transcription and mRNA processing through the thermogenic coactivator PGC-1. Mol Cell 2002;6(2):307–16.

[29] Stafford JM, Waltner-Law M, Granner DK. Role of accessory factors and steroid receptor coactivator 1 in the regulation of phosphoenolpyruvate carboxykinase gene transcription by glucocorticoids. J Biol Chem 2000;276:3811–9.

[30] Antras-Ferry J, Franckhaueser S, Robin D, Granner DK, Forest C. Expression of the phosphoenolpyruvate carboxykinase gene in 3T3-F42A adipose cells: effects of retinoic acid and differentiation. Biochem J 1994;302(Pt 3):943–8.

[31] Yamamoto T, Shimano H, Nakagawa Y, Ide T, Yahagi N, Matsuzaka T, et al. SREBP-1 interacts with HNF-4 alpha and interferes with PGC-1 recruitment to suppress hepatic gluconeogenic genes. J Biol Chem 2004;279(13):12027–35.

[32] Ijpenberg A, Tan NS, Gelman L, Kersten S, Seydoux J, Xu J, et al. Polarity and specific sequence requirements of peroxisome proliferators-activated receptor/retinoid X receptor heterodimer binding to DNA. A functional analysis of the malic enzyme gene PPAR response element. EMBO J 2004;23:2083–91.

[33] Desvergne B, Wahli W. Peroxisome proliferators-activated receptors: nuclear control of metabolism. Endocr Rev 1999;20:649–88.

[34] Valmesada A, Carmona MC, Barberá MJ, Viñas O, Mampel T, Iglesias R, et al. Opposite regulation of PPAR alpha and gamma gene expression by both their ligands and retinoic acid in brown adipocytes. Mol Cell Endocrinol 1999;154(1–2):101–9.

[35] Kersten S, Seydoux J, Peters JM, Gonzalez FJ, Desvergne B, Wahli W. Roles of PPARs in health and disease. J Clin Invest 1999;103:1489–98.

[36] Leone TC, Weinheimer CJ, Kelly DP. A critical role for PPAR in the cellular fasting response: the PPAR alpha null mouse as a model of fatty acid oxidation disorders. Proc Natl Acad Sci U S A 1999;96: 7473–7.

[37] Xu J, Xiao G, Trujillo C, Chang V, Blanco L, Joseph SB, et al. PPAR alpha influences substrate utilization for hepatic glucose production. J Biol Chem 2002;277(52):50237–44.

[38] Hayhurst GP, Lee Y-H, Lambert G, Ward JM, Gonzales JM. HNF-4 alpha is essential for maintenance of hepatic gene expression and lipid homeostasis. Mol Cell Biol 2001;21(4):1393–403.

[39] Ferre P. The biology of peroxisome proliferators-activated receptors: relationship with lipid metabolism. Diabetes 2004;53:S43–S50.

[40] Hertz R, Magenheim J, Berman I, Bar-Tana J. Fatty acyl-CoA thioesters are ligands of hepatic nuclear factor 4 alpha. Nature 1998;392:512–6.