Expression of the phosphoenolpyruvate carboxykinase gene in 3T3-F442A adipose cells: effects of retinoic acid and differentiation

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3T3-F442A adipocytes express the gene encoding cytosolic phosphoenolpyruvate carboxykinase (GTP) (PEPCK). Retinoic acid (RA) caused a 5-fold induction of PEPCK mRNA within 6 h in these cells with a half-maximal effective concentration of $\sim 75 \mu\text{M}$. This effect was independent of cycloheximide and inhibited by actinomycin D. *In vitro* run-on experiments using isolated nuclei confirmed that the RA-induced increase was mainly due to an increased rate of transcription of the gene. Stable transfectants bearing either the region of the PEPCK promoter from -2100 to $+69$ fused to the chloramphenicol acetyltransferase (CAT) gene (pPL1-CAT) or -600 to $+69$ fused to CAT (pPL9-CAT) were used to study PEPCK gene

regulation during differentiation. The same transfected cells were used to analyse the RA effect. Preadipocytes containing pPL1-CAT expressed a much lower level of CAT activity than did adipocytes. pPL9-CAT was not expressed in either preadipocytes or adipocytes. RA induced the expression of CAT activity in preadipocytes and adipocytes transfected with pPL1-CAT, but had no effect in cells transfected with pPL9-CAT. These results suggest that one or more DNA sequences located between -2100 and -600 bp of the PEPCK promoter is required for adipocyte-specific expression of this gene. RA action is independent of the state of differentiation and appears to require different elements in fat cells from those required in liver.

INTRODUCTION

Cytosolic phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32; PEPCK) catalyses a critical step in hepatic gluconeogenesis, the conversion of oxaloacetate into phosphoenolpyruvate. This enzyme is also expressed in white and brown adipose tissues, kidney cortex, small intestine and mammary gland [1]. The role of PEPCK in adipose tissue is to maintain glyceroneogenesis when the glucose supply is limited [2]. PEPCK activity is not known to be regulated by post-translational modification of the enzyme in any of these tissues, so modulations of enzyme activity are a reflection of the PEPCK mRNA level which, in turn, is due to the balance between mRNA synthesis and degradation.

The hormonal regulation of PEPCK gene expression has been extensively studied in liver and H4IIE rat hepatoma cells. Cyclic AMP, glucocorticoids and tri-iodothyronine stimulate PEPCK gene transcription whereas insulin inhibits it; all act through *cis*-acting elements present in the promoter region of the gene [3–7]. The vitamin A derivative, retinoic acid (RA), also stimulates PEPCK gene transcription [8]. Fusion gene constructs consisting of PEPCK promoter sequences ligated to the chloramphenicol acetyltransferase (CAT) reporter gene (PEPCK–CAT) have been used to locate an RA receptor element (RARE) [8–10]. The functional boundaries of the RARE coincide with the functional boundaries of the accessory factor 1 element which is an integral part of the complex PEPCK glucocorticoid-response unit (GRU) [4,11].

The hormonal control of PEPCK gene expression in adipose tissue is less well documented but is known to be affected by nutritional and hormonal manipulations. Starvation increases PEPCK protein and mRNA whereas refeeding and gluco-

corticoids decrease them [12,13]. In 3T3-L1 adipocytes, PEPCK mRNA is rapidly increased by cyclic AMP and is decreased by dexamethasone [13,14]. Moreover, insulin and dexamethasone inhibit the cyclic AMP-induced increase in PEPCK mRNA [14]. Similar results have been obtained with the 3T3-F442A adipocytes (S. Franckhauser, J. Antras-Ferry, P. Robin, D. Robin, D. K. Granner and C. Forest, unpublished work). Thus the PEPCK gene provides an excellent model for studying the multihormonal regulation of tissue-specific gene expression in adipose tissue.

The PEPCK gene is not expressed in preadipocytes but it is expressed in terminally differentiated cells [15]. The molecular basis for this change probably relies, at least in part, on the presence of adipose-specific DNA sequence(s) in the PEPCK gene. Using transgenic mice, a region conferring the expression of the PEPCK gene promoter in adipose tissue has been recently discovered [16]. This region lies between -2086 and -888 bp relative to the transcription-start site of the gene and is located far upstream from the region responsible for hepatic expression [17] and from the region involved in hormone-regulated expression in the liver [11]. This observation implies that the hormone-response elements active in liver may be different, or controlled differently, from those in adipose cells. 3T3 adipose cell lines can also be used to study the molecular events leading to induction or repression of specific genes during the process of differentiation. For example, the identification of ARE6, an adipose-specific enhancer element located in the promoter region of the *ap2* gene, has recently been identified through the use of these cells [18].

In 3T3-F442A cells, RA prevents adipose differentiation at an early stage [19,20]. However, once converted into adipocytes, these cells respond to RA with a rapid increase in S14 gene

Abbreviations used: PEPCK, phosphoenolpyruvate carboxykinase; RA, retinoic acid; CAT, chloramphenicol acetyltransferase; RARE, retinoic acid receptor element; GRU, glucocorticoid-response unit; DMEM, Dulbecco's modified Eagle's medium; $1 \times \text{SSC}$, $0.15 \text{ M NaCl} + 0.015 \text{ M sodium citrate}$; $1 \times \text{SSPE}$, $0.18 \text{ M NaCl} + 1 \text{ mM EDTA} + 10 \text{ mM sodium phosphate}$; RAR, retinoic acid receptor; RXR, retinoid X receptor.

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transcription [21] and a specific decrease in the half-life of adipsin mRNA [22]. In the present study, we examined the role RA plays in the expression of the PEPCK gene in 3T3-F442A cells. We demonstrate that RA is a potent activator of PEPCK gene transcription in these cells, and that it acts through the 5' flanking region of the gene by a mechanism independent of the state of differentiation. We show also that the region between -2100 and +69 bp of the PEPCK gene promoter is sufficient to confer differentiation-dependent expression of a CAT reporter gene in stably transfected 3T3-F442A cells.

MATERIALS AND METHODS

Cell culture

3T3-F442A preadipose cells were grown and differentiated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, penicillin (200 units/ml), streptomycin (50 mg/l), biotin (8 mg/l) and pantothenate (4 mg/l). They were grown at 37 °C in a humidified atmosphere of 10% CO₂/90% air. After reaching confluence, insulin (20 nM) was added to the medium to promote triacylglycerol accumulation. All experiments were performed on mature adipose cells grown for 8 days after confluence. Cells were maintained in serum-free insulin-free medium during the 24 h of culture immediately before experimentation.

RNA extraction and analysis

Total RNA was extracted from cell monolayers by the method of Chomczynski and Sacchi [23]. Total RNA (20 µg) was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde [24] and blotted on to Hybond-N membranes (Amersham Corp.). The integrity and relative amounts of RNA were assessed by Methylene Blue staining as described [24]. Membranes were then hybridized in Quick Hyb (Stratagene) using cDNA labelled with [α -³²P]dCTP by random priming (Stratagene) according to the manufacturer's recommendations. Membranes were washed twice for 15 min at room temperature in 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7)/0.1% SDS and then for 30 min at 60 °C with 0.1 × SSC/0.1% SDS. Specific cDNA probes used were PC116, a rat PEPCK cDNA fragment [25], pA21, a fragment of mouse actin cDNA [26], and PT76, a fragment of the S14 gene from between -289 and +493 bp relative to the transcription start site [27]. The PEPCK mRNA signal was quantified by densitometric scanning and was corrected for differences in RNA loading by a comparison with the signals generated by either the actin mRNA or 18S ribosomal RNA probes [28].

Nuclear transcription assay

The nuclear run-on transcription assay was performed essentially as described by Antras et al. [22]. Briefly, about 2 × 10⁷ nuclei were incubated for 30 min at 30 °C with 250 µCi of [α -³²P]UTP (400 Ci/mmol) (Amersham). Then 7 × 10⁶ c.p.m. of the ³²P-labelled reaction products were hybridized for 72 h at 42 °C to nitrocellulose filters previously bound with 5 µg of each of the linearized cDNA of genomic probes. The β -actin and S14 probes were the same as described above. The PEPCK probe was a DNA fragment (.R3) of the PEPCK gene [25]. Filters were washed twice in 2 × SSPE (1 × SSPE is 0.18 M NaCl, 1 mM EDTA, 10 mM sodium phosphate, pH 7.7) at 37 °C for 15 min,

and treated for 30 min at 37 °C in 2 × SSPE containing 10 µg/ml DNAase-free RNAase A. Final washing was carried out in 2 × SSPE/0.5% SDS for 30 min at 42 °C. Filters were then exposed to Kodak XAR5 film for 48 h at -80 °C with intensifying screens. Quantification of the signals was performed by densitometric scanning of the spots and signals were standardized using the β -actin signal.

Transient transfections

3T3-F442A adipocytes (3–4 days after confluence) were transfected in suspension using the calcium phosphate/DNA co-precipitation method. Each 100 mm dish was transfected with 20 µg of either pPL1-CAT (-2100 to +69 bp of the PEPCK gene promoter fused to the CAT gene) or 5 µg of pSV2-CAT (simian virus 40 promoter fused to the CAT gene) [29]. The amount of transfected DNA (20 µg) was maintained constant using pUC18. Precipitates were prepared exactly as described previously [6]. Cells were detached with trypsin, spun down at 800 g for 5 min in serum-containing medium and resuspended in the calcium phosphate/DNA co-precipitate. After 15 min of contact at room temperature, serum-containing medium was added to the cells and the suspension was seeded on to new plates in order to obtain a confluent monolayer (about 1.5 original plates were used for each new plate).

The precipitate was allowed to remain on the cells for 6 h. After a 20% dimethyl sulphoxide shock for 3 min, the cell monolayer was washed three times with DMEM, then fresh differentiation medium was added. Medium was replaced the next day and cells were further incubated for 2 days, after which hormonal treatments were performed for 15 h in serum-free medium.

Stable transfections

First 2 µg of pSV2-NEO [30], the expression of which confers resistance to the antibiotic geneticin, G418 (Gibco laboratories), was co-transfected with 20 µg of either pPL1-CAT or pPL9-CAT (-600 to +69 of the PEPCK gene promoter fused to the CAT gene) by calcium phosphate/DNA co-precipitation on to exponentially growing 3T3-F442A preadipocytes (10⁵ cells per 100 mm plate). Cells were then treated essentially as described previously [6]. Under these conditions the transfection efficiency was about 5 × 10⁻⁵. After two weeks exposure to G418 (0.4 mg/ml), several colonies of cells resistant to the antibiotic were either isolated or pooled (about 20 colonies per pool) and analysed. Analysis revealed that cells from 90% of the isolated colonies were still able to differentiate. About 90% of these expressed CAT and about 70% of CAT-expressing cell colonies showed a stimulation of CAT activity in response to hormones.

CAT assay and primer-extension analysis

Preparation of cell homogenates and CAT assays were performed exactly as detailed previously [6]. One unit of CAT converts 1 nmol of chloramphenicol, with [³H]acetate (ICN Pharmaceuticals) as substrate, to chloramphenicol 3-acetate/min at pH 7.8 and 37 °C.

For primer extension, an oligonucleotide (CAT30; 5'-TAG-CTTCCTTAGCTCCTGAAAATCTCGCCA-3') was designed to be complementary to the portion of the CAT construct located between +81 and +111 bp relative to the transcription start site of the pPL1 plasmid [29]. This was 5' end-labelled with [γ -³²P]ATP (> 5000 Ci/mmol; Amersham) to a specific radioactivity of about 10⁹ c.p.m./µg [24]. Primer-extension analysis

was performed essentially as described [6], except that 20 μ g of total RNA was used instead of polyadenylated RNA.

RESULTS

Induction of PEPCK mRNA by RA in 3T3-F442A adipocytes

RNA isolated from RA-treated 3T3-F442A adipocytes was analysed by Northern-blot hybridization. PEPCK mRNA increased rapidly for 4 h after the addition of 1 μ M RA (Figure 1). The new steady-state level, which represented a 5-fold induction over the basal value, was maintained for at least 24 h. A treatment time of 4 h was chosen to determine the concentration-response relationship of this RA action. As shown in Figure 2, the increase in PEPCK mRNA in response to RA was concentration-dependent, with an EC_{50} reached at about

Figure 1 Time course of RA action on PEPCK mRNA in 3T3-F442A adipocytes

Differentiated cells (day 8) were maintained in serum-free medium throughout the 24 h experiment. RA (1 μ M) was added to the medium either at time 0 or for 24, 8, 6, 4, 2, 1 and 0.5 h before the cells were harvested. Cells from two 60 mm dishes were pooled and RNA was extracted and analysed as described in the Materials and methods section. The data, obtained by scanning densitometry, were normalized for differences in RNA loading by using the actin mRNA signal. Data are expressed as the percentage of the maximum PEPCK signal (6 h value). Each value is the mean $\pm$ S.E.M. of data collected from three independent experiments.

Figure 2 Effect of RA concentration on PEPCK mRNA in 3T3-F442A adipocytes

Differentiated cells (day 8) were maintained in serum-free hormone-free DMEM for 20 h and then treated with the indicated concentration of RA for 4 h. RNA was extracted from two 60 mm dishes and analysed as described in the Materials and methods section. The data, obtained by scanning densitometry, were normalized for differences in RNA loading by using the actin mRNA signal. Data are expressed as the percentage of the maximum PEPCK signal, which was obtained with 1 μ M RA. Each value represents the mean $\pm$ S.E.M. of results obtained from three independent experiments.

Figure 3 Influence of cycloheximide and actinomycin D on RA effects on PEPCK gene expression in 3T3-F442A adipocytes

Differentiated cells (day 8) maintained in serum and hormone-free DMEM for 20 h were then preincubated or not with cycloheximide (10 μ M) (a) or actinomycin D (4 μ g/ml) (b) for 30 min. Cells were then incubated for an additional 4 h with RA (1 μ M) in the presence or absence of the inhibitors. Total RNA (20 μ g per lane) was subjected to blot transfer analysis. Autoradiograms obtained with the same blot hybridized sequentially with the PEPCK and 18S rRNA probes are shown.

70–80 nM. This EC_{50} is in good agreement with that observed for the RA-mediated decrease in adipsin mRNA half-life [22].

Direct stimulation of PEPCK gene transcription in 3T3-F442A adipocytes by RA

To study the mechanism of RA action, 3T3-F442A adipocytes were first treated with the protein-synthesis inhibitor cycloheximide. RA induced PEPCK mRNA in cells preincubated for 30 min with 10 μ M cycloheximide before a 4 h treatment with 1 μ M RA (Figure 3a). This concentration of cycloheximide is effective in preventing the dexamethasone-induced decrease in PEPCK mRNA in these cells (S. Franckhauser, J. Antras-Ferry, P. Robin, D. Robin, D. K. Granner and C. Forest, unpublished work). Thus this action of RA does not require protein synthesis.

The observed changes in PEPCK mRNA may be the consequence of an increased rate of gene transcription or a change in mRNA stability. Two different approaches were used to distinguish between these alternatives. In the first approach cells were preincubated for 30 min with 4 μ g/ml actinomycin D then treated with 1 μ M RA. Whereas actinomycin D did not affect PEPCK mRNA in control cells, it totally prevented the RA-induced increase in PEPCK gene expression (Figure 3b). The second approach employed is more direct in that the rate of transcription of the PEPCK gene was measured. A representative experiment is shown in Figure 4. A 2 h treatment of the cells with 1 μ M RA specifically induced a large increase in labelled transcripts representing PEPCK (4–5-fold) and S14 (5–6-fold). These results strongly suggest that the effect of RA on the PEPCK gene is mainly exerted at the level of transcription.

RA and differentiation-dependent DNA elements are present in the PEPCK gene promoter

In H4IIE hepatoma cells, RA stimulates PEPCK gene transcription through a *cis*-acting element (RARE) located in the 5'-flanking region of the gene. We performed transient and stable transfections in 3T3-F442A cells, using the pPL1-CAT construct,

Figure 4 Hormonal regulation of PEPCK and S14 gene transcription in 3T3-F442A adipocytes

Differentiated cells (day 8) were serum-deprived for 23 h then treated or not (control) for 2 h with RA (1 μ M). Nuclei were prepared and used for an *in vitro* transcription assay as described in the Materials and methods section. Autoradiograms of labelled transcripts representing PEPCK, S14, actin and pUC18 are shown. Exposure to X-ray film was for 4 days.

Table 1 RA modulation of CAT expression in transiently transfected 3T3-F442 adipocytes

Cells were transfected with either pPL1-CAT (20 μ g/dish) or a mixture of pSV2-CAT (5 μ g/dish) and pUC18 (15 μ g/dish) as described in the Materials and methods section. They were then treated with RA (1 μ M) for 15 h in serum-free medium. CAT activities from untreated cells and fold inductions by RA are presented. Values represent the mean $\pm$ S.E.M. of data obtained from five independent experiments. * $P < 0.04$ (Student's *t* test for unpaired data) when compared with pSV2-CAT stimulated by RA.

Plasmid construct	CAT activity (munits/mg of protein)	Fold induction by RA
pPL1-CAT	0.25 $\pm$ 0.06	2.75 $\pm$ 0.58*
pSV2-CAT	2.94 $\pm$ 0.70	1.20 $\pm$ 0.23

in an attempt to delineate such an element in adipocytes. This plasmid contains the region from -2100 to +69 bp of the PEPCK promoter ligated to the CAT gene. Transiently transfected adipocytes responded to a 15 h RA exposure with a 2.8-fold increase in CAT activity (Table 1). This effect was specific, as the level of CAT gene expression from cells transfected with the pSV2-CAT control plasmid remained unchanged (Table 1).

To confirm the presence of an RA-response sequence in the 5'-flanking region of the PEPCK gene, 3T3-F442A preadipocytes were co-transfected with pPL1-CAT and pSV2-NEO, and several

Figure 5 Effect of various RA concentrations on CAT activity in the FL18 stable transfectant

Differentiated cells (day 8) from the FL18 stable transfectant were treated with RA for 15 h in serum-free medium. Cells were then harvested and CAT activity was determined. The results are expressed as the percentage of the maximum stimulation at 1 μ M. Values represent the mean $\pm$ S.E.M. of data obtained from three independent experiments.

G418-resistant independent colonies were selected (see the Materials and methods section). Three of these, FL15, FL18 and FL1J, were analysed in detail. Cells containing these stable transfectants differentiated at a frequency similar to that of the parental cell line (not shown). Primer-extension analyses using RNA isolated from these differentiated cells confirmed that transcription of the pPL1-CAT transgene was initiated at the correct site (not shown). This result corroborates earlier observations made using stable PEPCK-CAT/H4IIE cell transfectants [6]. FL15, FL18 and FL1J preadipocytes cultured for 15 h in serum-free medium expressed very low levels of CAT activity (Table 2). Expression of CAT activity increased markedly, although to different extents in the various lines, upon differentiation into adipocytes (Table 2). The increase in CAT expression consequent on differentiation was about 10-, 64- and 11-fold for FL15, FL18 and FL1J cells respectively (Table 2). This result is consistent with the existence of an adipose-specific sequence in the regulatory region of the PEPCK gene. Although CAT expression was quite different between preadipocytes and adipocytes, both cell types responded to a 15 h treatment with RA with an increase in CAT expression (Table 2). The stimulations noted were 4.8-, 2.9- and 1.4-fold for FL15, FL18 and FL1J preadipocytes respectively and 2.1-, 3.5- and 2.5-fold for FL15, FL18 and FL1J adipocytes respectively. We chose one of these transfectants, FL18, to determine the concentration-response relationship of this action of RA. As shown in Figure 5, FL18 adipocytes responded to RA with a concentration-dependent increase in CAT activity. The EC_{50} was about 100 nM,

Table 2 Effect of RA and adipose conversion on CAT expression in pPL1-CAT/3T3-F442A stable transfectants

Cell extracts from FL15, FL18 and FL1J stable transfectants were obtained from 100 mm dishes as described in the Materials and methods section. Preadipocytes (day 0) and adipocytes (day 8) were cultured for 15 h in serum-free medium with or without RA (1 μ M), then harvested for measurement of CAT activity. CAT activities from untreated cells and fold inductions by RA are presented. Values represent the mean $\pm$ S.E.M. of data obtained from four to five independent experiments.

Transfectant	Preadipocytes		Adipocytes	
	CAT activity (munits/mg of protein)	Fold induction by RA	CAT activity (munits/mg of protein)	Fold induction by RA
FL15	0.53 $\pm$ 0.33	4.8 $\pm$ 1.5	5.50 $\pm$ 1.04	2.1 $\pm$ 0.2
FL18	0.24 $\pm$ 0.15	2.9 $\pm$ 1.0	15.70 $\pm$ 3.80	3.5 $\pm$ 0.3
FL1J	0.23 $\pm$ 0.10	1.4 $\pm$ 0.1	2.51 $\pm$ 0.89	2.5 $\pm$ 0.2

Table 3 Expression of CAT activity in stably transfected 3T3-F442A adipocytes

Cell extracts from the various stable transfectants were obtained from 100 mm dishes as described in the Materials and methods section. Differentiated (day 8) cells were cultured for 15 h in serum-free medium then harvested for measurement of CAT activity. Values represent either a single experiment or the mean $\pm$ S.E.M. of data obtained from three to ten independent experiments (*). ND, not detected.

pPL1-CAT		pPL9-CAT	
Transfectant	CAT activity (munits/mg of protein)	Transfectant	CAT activity (munits/mg of protein)
FL1J*	2.5 $\pm$ 0.9	FL91*	0.5 $\pm$ 0.2
FL12Pool	2.8	FL92	ND
FL13Pool*	5.1 $\pm$ 1.2	FL93	ND
FL15*	5.1 $\pm$ 1.1	FL94*	ND
FL16	11	FL97*	0.13 $\pm$ 0.03
FL17	17	FL98*	ND
FL18*	15.7 $\pm$ 3.8	FL911*	ND
FL19	8.4	FL912	ND

a value close to that observed for RA induction of PEPCK mRNA (Figure 2). These results demonstrate the existence of a differentiation-independent RA-response sequence in the PEPCK-gene-regulatory region.

We performed stable transfection analyses using the pPL9-CAT construct in an initial attempt to locate an RARE. This construct contains the portion of the PEPCK-gene-regulatory region located between -600 and $+69$ bp relative to the transcription-start site of the gene [6,29], and contains the GRU [4]. Six of the eight stably transfected adipocytes analysed showed no CAT activity whether or not they were treated with RA (Table 3). Two others, FL91 and FL97, had activity that was just detectable with the CAT assay employed. RA had no effect on CAT gene expression in FL97 and stimulated CAT activity about 2-fold in FL91. In contrast, eight cell lines transfected with pPL1-CAT expressed levels of CAT activity ranging from 2.5–17 munits/mg of protein (Table 3). These results suggest that a *cis*-acting element located between -2100 and -600 bp relative to the transcription-start site is required for differentiation-dependent RA-induced PEPCK gene expression.

DISCUSSION

RA stimulates PEPCK gene transcription in 3T3-F442A adipocytes. This stimulation is rapid and does not require simultaneous protein synthesis. These results are similar to those observed in hepatocytes and hepatoma cells in which RA also stimulates PEPCK gene transcription [8,31]. The RA concentration required to affect PEPCK gene expression in these adipocytes (EC_{50} 70–80 nM; Figure 2) is relatively high compared with that determined for the induction of S14 gene transcription in the same cells (EC_{50} 5 nM) [21]. However, it is quite similar to that required to affect adipin mRNA stability in these cells (EC_{50} 50–100 nM) [22]. Such differences could reflect the involvement of a different receptor or receptor complex for each effect. Indeed, two distinct classes of receptors are known to be involved in mediating RA actions, the RARs (RAR α , β and γ) and the retinoid X receptors (RXRs). Both are members of the large superfamily of nuclear receptors [32]. RARs and RXRs differ in primary structure and in their affinity for different

retinoid ligands. It is known that heterodimers between RAR and RXR are involved in hormone responses, so the variety of these receptors and the possible association with different ligands could provide a regulatory network through which different biological effects are mediated [32].

In H4IIE hepatoma cells RA action is mediated through the binding of RAR α and a liver nuclear factor identical with or closely related to RXR α to a RARE located between positions -451 and -433 bp relative to the transcription-start site [9,10]. 3T3-F442A adipocytes transfected either transiently or stably with the pPL1-CAT construct respond to RA with a stimulation of CAT expression (Tables 1 and 2). This construct includes both the hormone-response domain that is effective in liver and the putative adipose-specific region [17]. However, a shorter construct, pPL9-CAT, carrying the portion of the PEPCK gene between -600 and $+69$ is not expressed in stably transfected 3T3-F442A adipocytes and does not respond to RA (Table 3). This construct contains the RARE but does not have the putative adipose-specific region. This observation is different from an earlier report [33] which showed that the region between -597 and the transcription initiation site was sufficient to confer CAT-linked expression in 3T3-F442A adipocytes. These authors used transient transfection. Indeed, we also observed a slight CAT expression after transient transfection of pPL9-CAT into 3T3-F442A adipocytes (not shown). However, our data are in total agreement with those obtained using transgenic mice in which the region between -2086 and -888 bp of the PEPCK gene promoter is required for expression in adipose tissue [17]. A discrepancy between results obtained with the same construct in transiently transfected cells and in transgenic mice has been observed for other genes such as the aP2 gene [34,35]. It would appear from the results presented in the present paper that an adipose-specific element is located between -2100 and -600 bp relative to the transcription start site of the PEPCK gene. This view is consistent with the data obtained with transgenic mice [17] and strongly suggests that this portion of the regulatory region plays a key role in the induction of the PEPCK gene linked to adipocyte differentiation.

The fact that the pPL9-CAT construct, which includes the RARE that is active in hepatoma cells, is unresponsive to RA both in transiently and in most (seven out of eight) stably transfected adipocytes deserves additional comment. First, hepatocytes and adipocytes may not respond to RA through the same DNA element. Two RARE consensus half-sites are present upstream from -888 bp. One, TGACCT, is located between -1988 and -1983 and the other, AGGTCA, is located between -1812 and -1807 . Tests of the functional significance of one or both of these sites in adipocytes should help address this point. Second, the proximal RARE may be active in both cell types but one or more additional sequences, located between -2100 and -600 bp, may be necessary for a response to RA in adipocytes. The ARE6 consensus element present in the -2086 to -888 fragment [17] could play that role. Such a sequence may bind a protein involved indirectly in the RA effect by simply allowing a sufficient level of basal expression of the gene for the RARE to function. This would be consistent with the observation that one of the pPL9-CAT stably transfected adipocytes, FL91, is slightly responsive to RA. Alternatively, a direct interaction of the factor with RAR may be necessary for RA to stimulate transcription in adipocytes. This is not unprecedented, even in this promoter. In H4IIE hepatoma cells, RAR itself does not activate the PEPCK gene RARE [10]; a heterodimer between RAR and RXR is required [10]. There is now ample evidence that the interaction of distinct regulatory pathways, i.e. cross-coupling, may exist between steroid hormones and other factors [36]. Additional

experiments, using 3T3-F442A adipocytes transfected with pPL1-CAT in which the RARE active in hepatoma cells is deleted or contains block mutations, are required to clarify these points.

The observation that the pPL1-CAT construct is responsive to RA in preadipocytes, in which the PEPCK gene is not expressed (Table 2), can be explained by the presence of RAR α in these cells [21]. Such a result implies that RA acts through the 5'-flanking region of the gene by a mechanism independent of the state of differentiation. However, RA is not sufficient for induction of PEPCK mRNA in preadipocytes (not shown) perhaps because of differences in chromatin structure between the endogenous gene and the transgene. Of interest in this regard is the fact that, in hepatoma cells, the PEPCK RARE is an integral part of the GRU. In these cells, glucocorticoids and RA both enhance PEPCK gene transcription. In adipocytes, dexamethasone reduces PEPCK gene expression whereas RA induces it. If, in adipocytes, dexamethasone also affects transcription and acts through the GRU, this hormone may then impede RA stimulation through either protein-protein or protein-DNA interactions. Such interference in actions between different nuclear receptors or between nuclear receptors and other factors has been described [36]. We are at present conducting experiments to clarify these points.

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