Chicken ovalbumin upstream-promoter transcription factor (COUP-TF) could act as a transcriptional activator or repressor of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene

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INTRODUCTION

The nuclear hormone receptor superfamily is formed by a growing number of receptors that are ligand-activated transcription factors. This superfamily includes receptors for different kinds of hormones, retinoids and vitamin Dα. In addition to these receptors with a known ligand, many other proteins have been included in this family on the basis of homology in characteristic domains. No ligand has yet been identified for several of them, which have therefore been grouped as orphan members of the superfamily [1].

The chicken ovalbumin upstream-promoter transcription factor (COUP-TF) is an orphan member of this superfamily that was originally identified by binding to and activation of the chicken ovalbumin gene [2–4], with the aid of the non-DNA binding factor TF-IIB [5]. Since then it has been shown that COUP-TF or COUP-TF analogues are involved in the regulation of several genes [6]. COUP-TFs bind as homodimers to diverse response elements consisting of perfect or imperfect GGTCA repeats, with dramatic differences in the number of nucleotides separating the half-sites and in their orientation [7]. Although COUP-TF can stimulate the expression of a few genes [3,8–10], it has mainly been identified as a negative regulator. It inhibits ligand-induced transactivation by other nuclear receptors (retinoic acid receptor, thyroid hormone receptor, vitamin D receptor [7], retinoid X receptor (RXR) [11], peroxisome-proliferator-activated receptor (PPAR) [12,13], hepatic nuclear factor 4 [14]) via three possible mechanisms. The first is direct binding competition to common responsive elements, the second is heterodimer formation between COUP-TF and RXR, thyroid hormone receptor and retinoic acid receptor, and the third is the active silencing of the transcriptional machinery [15,16]. It has recently been shown that the cholesteryl ester transfer protein gene is either repressed or activated by COUP-TF depending on the promoter context [17].

We have recently shown that the gene for the mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase, the enzyme that catalyses the condensation of acetyl-CoA with acetoacetyl-CoA to form HMG-CoA and CoA in mitochondria, is a target for PPAR, and that this receptor mediates the induction of the gene by fatty acids [18]. With the aim of gaining further insight into the function and regulation of this gene, which codes for an enzyme representing a major control point in hepatic production of ketone bodies [19], we examined the effect of COUP-TF on its expression.

Here we show that COUP-TF has a dual regulatory role in the mitochondrial HMG-CoA synthase gene. We show that in HepG2 cells COUP-TF activates transcription from the mitochondrial HMG-CoA synthase gene promoter; interestingly this activation is independent of the peroxisome proliferator responsive element (PPRE) and is promoted by the fragment from coordinates –62 to +28 of the gene. On the other hand we show that COUP-TF competes with PPAR for the binding to the same DNA sequence (–104 to –92) and that this competition leads to the inhibition of the transcriptional activation produced by PPAR in co-transfection experiments. This effect is manifest both with a construct containing the mitochondrial synthase PPRE upstream of the thymidine kinase promoter in HepG2 cells, and in the context of the natural promoter, in a Leydig tumour cell line (R2C), where this gene is also specifically expressed.

In general, a particular regulator of gene transcription acts either as an activator or as a repressor. Nevertheless, several transcriptional regulators activate in some circumstances and repress in others. The present study adds COUP-TF to this list of regulators and points to an important role of this transcription factor in the regulation of the mitochondrial HMG-CoA synthase gene.
factor in the tissue-specific expression of the mitochondrial HMG-CoA synthase gene.

**MATERIALS AND METHODS**

**Cells**

Human hepatoma HepG2 cells were cultured in minimal essential media supplemented with non-essential amino acids and 10% (v/v) fetal-calf serum. Rat Leydig tumour R2C cells were maintained in Waymouth MB752/1 medium plus 15% (v/v) horse serum.

**Plasmid constructions**

Reporter chloramphenicol acetyltransferase (CAT) construct pSMPCAT1 contains 1148 bp of the 5’,flanking region and the first 28 bp of exon 1 of the rat HMG-CoA synthase gene in pCAT-Basic vector (Promega), and has been described previously [20]. pSMPCAT6D contains 62 bp of the 5’ regulatory region and the same 3’ deletion end point in exon 1 of the gene. In order to prepare it, a 149 bp fragment was isolated from the digestion of pSMPCAT6C [18] with HindIII and was digested with DdeI to yield a 93 bp fragment that was cloned into the Sacl site of pCAT-Basic. 5’,Overhangs were filled in with the Klenow fragment of DNA polymerase I where necessary. Reporter plasmids pSMPCAT6A, pSMPCAT1M, pSMPCAT6AM and pTKCAT(3)(x)SM116-69, and receptor expression plasmids pSG5-mPPARα (mPPARα is mouse peroxisome-proliferator-activated receptor α) and pJCXR8 have been described [18]. pFLCOUP-TF I and pRSShCOUP-TF I (generously provided by Dr. Ming-Jer Tsai, Baylor College of Medicine, Houston, TX, U.S.A.) contain the full-length COUP-TF I cDNA in the pGEM7zl(+) (Promega) vector and the Rous sarcoma virus eukaryotic expression vector, respectively [7]. A plasmid encoding a truncated form of hCOUP-TF I (tCOUP-TF I) was constructed as described by Miyata et al. [12].

**Transfections and measurement of CAT activity**

HepG2 cells were transfected by the calcium phosphate co-precipitation procedure, essentially as already described [21]. For R2C cells, transfection assays were carried out similarly except that 4 h after the addition of the precipitates, the cells were treated with a 15% (v/v) glycerol solution for 2 min. The cells received 5 µg of a reporter gene construct and, when indicated, 0–2 µg of pSG5-mPPARα and/or pJCXR8, and 0–2.5 µg of pRSiCOUP-TF I expression plasmids. Effector plasmid dosage was kept constant by the addition of appropriate amounts of the empty expression vector pSG5 (Stratagene). Preparation of plasmids and CAT assays were performed as described [18]. Cell extracts were normalized to the total amount of protein, as determined by the method of Bradford [22] because COUP-TF expression vectors were shown to repress expression of β-galactosidase expression vectors [7]; moreover, we have confirmed, by dot-blot analysis of the intracellular plasmid DNA, that the DNA uptake by both cell lines is the same (results not shown).

**Transcription/translation in vitro**

*In vitro* transcription of cDNAs encoding receptors and subsequent translation in rabbit reticulocyte lysate were performed using a commercially available kit according to the instructions of the manufacturer (Promega); we used 1 µl of TNT™ T7 RNA polymerase for mPPARα (pSG5-mPPARα) and human 9-cis-retinoic acid receptor α (pJCXR8), 1 µl of TNT™ SP6 RNA polymerase for hCOUP-TF I (pFLCOUP-TF I) and tCOUP-TF I and 0.5 µl of each polymerase for co-translations. Plasmid (1 µg) was used for separate translations and 0.75 µg of each plasmid was used for co-translations. Translations of proteins for use in electrophoretic mobility shift analysis (EMSA) were performed with unlabelled methionine.

**EMSA**

Proteins synthesized *in vitro* were preincubated on ice for 10 min in 10 mM Tris/HCl (pH 8.0)/40 mM KCl/0.05% (v/v) Nonidet P40/6% glycerol/1 mM dithiothreitol/poly(dI-dC) (1 µg). Then 0.8 ng of 32P-labelled MSPPRE oligonucleotide (see below) was added and the incubation was continued for 15 min at room temperature. The final volume for all reactions was 20 µl. Binding reactions were electrophoresed at 4 °C on a 4.5% (w/v) polyacrylamide gel in 0.5 x TBE buffer (45 mM Tris/45 mM boric acid/1 mM EDTA, pH 8.0). MSPPRE is the fragment corresponding to coordinates −116 to −69 of the mitochondrial HMG-CoA synthase gene and contains the PPAR response element of this gene (−104 to −92) (5’-TGACTTTGTTCAGAGCTTTTGCCAGGTCATTAGTTTTTCTGGAGACCTTTGAGGCAGGCAGAGG-3’). For competition experiments MSPPRE, MSA or MB double-stranded probes (see below) were included during preincubation. MSA is a synthetic oligonucleotide corresponding to coordinates −116 to −96 (5’-tccAAAGGTCAGACTTTGACGAGGGG-3’); MB is an unrelated synthetic oligonucleotide (5’-AGCTAGTTTTTATGAAGCCCA-3’) including the TATA box of the mitochondrial HMG-CoA synthase gene.

**PAGE**

[35S]Methionine-labelled co-translation products from pCOUP-TF I and pJCXR8, pSG5-mPPARα or pSG5 plasmids were analysed as follows. Receptor proteins synthesized *in vitro* (5 µl) were boiled with 5 µl of 2 x SDS loading buffer [0.125 M Tris/HCl (pH 6.8)/6% (w/v) SDS, 10% (v/v) β-mercaptoethanol/20% glycerol/0.025% (v/v) Bromophenol Blue] for 5 min. Then samples were electrophoresed on an SDS/10% polyacrylamide gel according to Laemmli [23]. The gel was treated sequentially with DMSO, DMSO/20% (v/v) 2,5-diphenyloxazole and water before drying between cellophane sheets and placing in contact with X-ray film at −70 °C.

**RESULTS**

**COUP-TF I activates the mitochondrial HMG-CoA synthase gene in HepG2 cells**

CAT expression from pSMPCAT1, a construct containing the CAT gene under the control of the promoter and 5’,flanking sequences of the rat mitochondrial HMG-CoA synthase gene (coordinates −1148 to +28), was increased by co-transfection with COUP-TF I in human hepatoma HepG2 cells (Figure 1). This activation was independent of the mitochondrial HMG-CoA synthase PPRE (−104 to −92), as confirmed in transfection experiments in which the PPRE was altered by mutation in pSMPCAT1 and pSMPCAT6A (coordinates −116 to +28). The scrambling of this sequence in both constructs, which obliterated the response to PPAR [16], did not eliminate the response to COUP-TF I, which even increased (Figures 1 and 2). Moreover, a deletion mutant pSMPCAT6D, extending to coordinate −62, which did not include PPRE, was still responsive to COUP-TF I (Figure 2). Interestingly, when the PPRE was altered or absent, the response was greater than in the presence of a functional PPRE.
Dual effect of chicken-ovalbumin upstream-promoter transcription factor

Figure 1 COUP-TF-I-dependent activation of the mitochondrial HMG-CoA synthase gene promoter in HepG2 cells

(a) CAT reporter constructs containing the wild-type (pSMPCAT1) or mutated (by scrambling nucleotides corresponding to PPRE, pSMPCAT1M) 5′-flanking region of the mitochondrial HMG-CoA synthase gene were co-transfected with or without 2.5 µg of pRSShCOUP-TF I into HepG2 cells. Average values of CAT activity, from at least three independent experiments with two plates each, are presented as ‘fold induction’ relative to pSMPCAT1 in the absence of COUP-TF I. The structural features of the CAT reporter constructs are shown at the bottom. The position of the 5′-flanking region end with respect to the transcription start site of mitochondrial HMG-CoA synthase gene is —1148. PPRE and the striped box refer to wild-type and mutated PPAR-responsive element respectively. 

(b) An autoradiograph showing a representative experiment.

COUP-TF I represses the mitochondrial HMG-CoA synthase gene in R2C cells

In contrast, the activity of pSMPCAT1 was inhibited by co-transfection with COUP-TF I in a rat Leydig tumour cell line (R2C). The inhibition (approx. 50%) was not observed when the PPRE had been altered (Figure 3). This result points to interaction of COUP-TF I with the PPRE as a mechanism of the inhibition.

COUP-TF I represses the induction of the mitochondrial HMG-CoA synthase gene by PPAR

As can be seen in Table 1, CAT expression from pTKCAT(3x)SM116-69, a construct containing the CAT gene under the control of the thymidine kinase gene promoter and three copies of the mitochondrial HMG-CoA synthase PPRE, is activated by co-transfection with PPAR in human hepatoma HepG2 cells[18]. This activation was repressed by co-transfection with COUP-TF I and this effect was overcome by co-transfection with increasing amounts of the PPAR expression plasmid. The same effect was also seen with pSMPCAT1 (which contains the PPRE in the context of the natural promoter) in R2C cells, although the recovery of the activity produced by increasing amounts of PPAR was not total.

COUP-TF I binds to the same DNA region in the mitochondrial HMG-CoA synthase gene as PPAR

We performed gel mobility-shift assays to analyse whether COUP-TF I binds to the PPRE of the mitochondrial HMG-CoA synthase gene. COUP-TF I, transcribed and translated in vitro, produced a prominent complex with a DNA probe containing the DNA sequence to which PPAR binds. The specificity of this complex was demonstrated by competition with an excess of unlabelled MSPPRE probe, whereas an excess of two different oligonucleotides (MSA and MSB, which do not contain the PPRE) did not lead to the disappearance of the complex (Figure 4A). COUP-TF I did not bind either to a probe containing a mutated version of the PPRE or to a probe containing the region of the gene between coordinates −62 and +28 (results not shown). This result points to competition between the PPAR–RXR heterodimer and COUP-TF I for binding to the same cis element.

In order to determine whether COUP-TF I can compete with PPAR–RXR for binding to the same DNA sequence, gel shift was performed using an N-terminal truncated form of COUP-TF I, PPAR and RXR transcribed and translated in vitro. The N-terminal truncated form of COUP-TF I was used to avoid
Figure 3  COUP-TF-I-dependent repression of the mitochondrial HMG-CoA synthase gene promoter in R2C cells

(a) CAT reporter constructs containing the wild-type (pSMPCAT1) or mutated (by scrambling nucleotides corresponding to PPRE, pSMPCAT1M) 5'-flanking region of the mitochondrial HMG-CoA synthase gene were co-transfected with or without 2.5 µg of pRShCOUP-TF I into R2C cells. Average values of CAT activity, from at least three independent experiments with two plates each, are presented as ‘fold induction’ relative to pSMPCAT1 in the absence of COUP-TF I. The structural feature of the CAT reporter constructs are shown at the bottom. The position of 5'-flanking region end with respect to the transcription start site of mitochondrial HMG-CoA synthase gene is -1148. PPRE and striped box refer to wild-type and mutated PPAR-responsive element respectively. (b) An autoradiograph showing a representative experiment.

Table 1  COUP-TF I represses the induction by PPAR of the transcriptional activity through the mitochondrial HMG-CoA synthase PPRE

<table>
<thead>
<tr>
<th>Cell type</th>
<th>PPARα (µg)</th>
<th>Control</th>
<th>COUP-TF I (0.5 µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>HepG2</td>
<td>7.2 ± 0.89</td>
<td>1.48 ± 0.27</td>
<td>2.49 ± 0.27</td>
</tr>
<tr>
<td>R2C</td>
<td>7.24 ± 1.79</td>
<td>1.86 ± 0.76</td>
<td>2.40 ± 0.95</td>
</tr>
</tbody>
</table>

DISCUSSION

In this paper we show that COUP-TF I has a dual role in the regulation of mitochondrial HMG-CoA synthase gene transcription; it could act either as an activator or as a repressor, depending on the promoter and the cell context. It has previously been shown that COUP-TF can function either positively or negatively through the same responsive element, depending on the promoter context. COUP-TF repressed the expression from the promoter of the gene for ornithine transcarbamylase, but it can activate the expression from a construct containing the same responsive element (DR-1) placed in front of the thymidine kinase promoter [14]. The dual transcriptional control by COUP has also been described in the case of the activation of the mouse mammary tumour virus promoter through a sequence downstream of the transcriptional start site. This activation is abolished by the addition of a DR1 or a thyroid-hormone-responsive element sequence upstream of the promoter [24]. Recently, it has been shown that overexpression of COUP-TF with cholesteryl ester transfer protein/CAT gene reporter plasmids represses confusion between the PPAR–RXR-specific and COUP-TF-I-specific complexes. Upon addition of increasing amounts of PPAR–RXR, an increase in the specific PPAR–RXR–DNA complex and a corresponding decrease in the truncated COUP-TF-I–DNA complex was observed (Figure 4B). Thus COUP-TF I and the PPAR–RXR heterodimer compete for DNA binding to the same element.

Another possible mechanism to explain the COUP-TF-I-dependent antagonism of transactivation by PPAR is the formation of functionally inactive heterodimers with PPAR and RXR. We analysed COUP-TF I, PPAR and RXR co-translated in vitro by gel shift to test for the formation of DNA-binding heterodimers on the mitochondrial synthase PPRE. If heterodimers were formed we would expect the formation of an extra complex with intermediate mobility. Upon co-translation of the different species as indicated, we observed complexes corresponding to the PPAR–RXR heterodimer or the truncated COUP-TF I homodimer. No third heterodimer complex was observed in any situation. Upon co-translation of two species (truncated COUP-TF I and PPAR or truncated COUP-TF I and RXR), the disappearance of the truncated COUP-TF-I–DNA-specific complex was observed (Figure 4C). In order to verify the presence of the protein, we performed the transcription and translation in vitro in the presence of [35S]methionine and analysed the products by SDS/PAGE. The protein was produced (Figure 4D), even though the shifted complex corresponding to COUP-TF I was not observed.
translated in two polypeptide species, is present in similar amounts in all co-translations. Competition for occupancy of DNA binding site. Labelled MSPPRE was incubated with fixed and human 9-
acid receptor α
repressor in the absence of hormone and as an activator in its presence. It has recently been shown that the thyroid hormone receptor, the DNA-bound receptor can act as a repressor in the presence of PPAR [18]. We have previously shown that mitochondrial HMG-CoA synthase gene transcription is activated in the presence of PPAR [18]. The COUP-TF-I-mediated activation in HepG2 cells is produced through a sequence other than the mitochondrial synthase PPARE (DR-1). This has been confirmed in two ways. Firstly, in transfection experiments where the PPARE (−104 to −92) was altered by mutation in both the longer and the deletion −116 promoter constructs (pSMPCAT1 and pSMPCAT6A respectively), the scrambling of this sequence in both constructs obliterated the response to PPAR, but did not eliminate the response to COUP-TF I, which even increased. Secondly, a deletion mutant pSMPCAT6D, extending up to coordinate −62, in which the PPARE is not present, was still responsive to COUP-TF I. This result localized the DNA sequence responsible for the activation by COUP-TF I between positions −62 and +28. This region contains only the TATA box and a GC box besides the transcription initiation sequence. We attempted, without success, to confirm a direct COUP-TF-I–DNA interaction by gel-shift experiments with a DNA probe containing the region from −62 to +28 and COUP-TF I produced in vitro or with HepG2-cell nuclear extracts. Recently, an indirect mechanism was suggested for the activation of the vHNF1 gene by COUP-TF [27]. Interestingly, an octamer site and not the DR-1 site is required for COUP-TF transactivation of vHNF-1 promoter. COUP-TF does not bind directly to this octamer site and evidence has been presented of an interaction between COUP-TF and the octamer binding proteins.

However, the mechanism by which COUP-TF transactivates the mitochondrial HMG-CoA synthase gene promoter remains to be elucidated. We speculate that the activation could arise from the action of a modified form of COUP-TF resulting from: (a) the binding of COUP-TF I to a liver-specific ligand, (b) the interaction of COUP-TF I with a transcription factor present in HepG2 cells, or (c) a liver-specific COUP-TF I post-translational modification that affect its activity. In a hepatic context (HepG2 cells) this putative modified form of COUP may activate transcription in a PPARE-independent manner (downstream of position −62) and at the same time this form may have a diminished affinity for PPARE, allowing the activation of the promoter by the PPAR–RXR heterodimer. On the other hand, in R2C cells this modified form of COUP may not occur.

In co-transfection assays in R2C cells, COUP-TF repressed PPAR-dependent activation of a reporter gene linked to the mitochondrial HMG-CoA synthase promoter. Our results suggest a competition between the PPAR–RXR heterodimer and COUP-TF for DNA binding as the mechanism for the repression. We show that the repression of the PPAR-dependent activation by COUP-TF is produced through the mitochondrial HMG-CoA synthase PPARE in the context of (a) the thymidine kinase promoter in HepG2 cells or (b) the natural promoter in R2C cells. This repression can be alleviated by increasing amounts of PPAR, although not totally in R2C cells. This indicates different activation states of PPAR–RXR heterodimers [28] in those cell lines. We demonstrate here that COUP-TF binds to the PPARE of the mitochondrial HMG-CoA synthase gene, and we show that there is a competition for DNA binding sites in vitro and, presumably, formation of non-DNA-binding heterodimers between COUP-TF and RXR or PPAR, although this interaction should be confirmed by other approaches.

A similar dual regulatory role has been described for other members of the nuclear receptor superfamily. In the case of the thyroid hormone receptor, the DNA-bound receptor can act as a repressor in the absence of hormone and as an activator in its presence. It has recently been shown that the thyroid hormone

transcriptional activity of the cholesteryl ester transfer protein promoter containing sequences up to −300, but activates transcription in the context of larger constructs containing sequences up to −636 [17]. Our work represents another example of a target gene for COUP-TF I that could be either activated or repressed by the action of this receptor through different DNA sequences. Interestingly, the activation is cell-type-specific: it is seen in the hepatoma cell line HepG2 but not in the Leydig tumour cell line R2C. These are both cell lines in which the mitochondrial HMG-CoA synthase gene is expressed, mimicking the expression of the gene in liver and gonads.

PPAR is another member of the nuclear hormone receptor superfamily that has an important role at the transcriptional level in the regulation of lipid metabolism [25,26].
modulates the interaction of the receptor with the basal transcription machinery (TFIIB) [29–31]. As COUP-TF interacts with TFIIB [5,32], a similar mechanism could be proposed for the dual regulatory role of COUP-TF, if its hitherto unidentified ligand turns out to be a cell-type-specific metabolite able to modify its activity.

The present work provides a model with which to study how different cell contexts lead to opposing effects on transcription by a single transcription factor. The characterization of the mechanism by which COUP-TF activates the mitochondrial HMG-CoA synthase gene promoter in a hepatic context should provide further insight into the molecular mechanisms by which a ubiquitously expressed transcription factor has a role in the regulation of the tissue-specific gene expression.

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