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Identification of a regulatory function for an orphan receptor in muscle: COUP-TF II affects the expression of the *myoD* gene family during myogenesis

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ABSTRACT

COUP-TF II is an 'orphan steroid receptor' that binds a wide variety of AGGTCA repeats and represses thyroid hormone (T_3) and retinoid dependent *trans*-activation; however, very little is known of its functional and/or developmental role during mammalian cell differentiation. T_3 and retinoids have been demonstrated to promote terminal muscle differentiation via activation of the muscle specific *myoD* gene family (*myoD, myogenin, myf-5* and *MRF-4*). The *myoD* gene family can direct the fate of mesodermal cell lineages, repress proliferation, activate differentiation and the contractile phenotype. Hence, we investigated the expression and functional role of COUP-TF II during muscle differentiation. Proliferating C2C12 myoblasts expressed COUP-TF II mRNA which was repressed when cells were induced to differentiate into post-mitotic multinucleated myotubes by serum withdrawal. Concomitant with the decrease of COUP-TF II mRNA was the appearance of muscle specific mRNAs (e.g. myogenin, α-actin). We show that *Escherichia coli* expressed full length and truncated COUP-TF II bound in a sequence specific manner to the T_3 response elements (TREs) in the *myoD* and *myogenin* regulatory HLH genes [Olson (1992) *Dev. Biol.* 154, 261–272]; and the TRE in the skeletal α-actin contractile protein gene. COUP-TF II diminished the homodimeric binding of the thyroid hormone receptor and the heterodimeric binding of thyroid hormone and retinoid X receptor complexes to these TREs. Constitutive over-expression of COUP-TF II cDNA in mouse C2C12 myogenic cells suppressed the levels of myoD mRNA and blocked the induction of myogenin mRNA, whereas constitutive expression of anti-sense COUP-TF II cDNA significantly increased the steady state levels of myoD mRNA and hyper-induced myogenin mRNA. These studies demonstrate for the first time (i) that COUP-TF II, functions as a physiologically relevant antagonistic regulator of myogenesis via direct effects on the *myoD* gene family and (ii) direct evidence for the developmental role of COUP-TF II during mammalian cell differentiation.

INTRODUCTION

The process of myogenesis involves two events: determination and differentiation. Determination is the process by which pluripotential embryonic cells become committed to the myogenic lineage. Skeletal myoblasts have their origin early in embryogenesis within specific somites. Differentiation is the mechanism by which these committed cells (myoblasts) acquire and express a muscle specific phenotype. The process of muscle differentiation involves the fusion of proliferating myoblasts to form post-mitotic multinucleated myofibres that have a myogenic phenotype. This transition is accompanied by muscle specific gene activation that encodes contractile proteins and enzymes involved in energy metabolism. This structurally diverse group of proteins encode a functional sarcomere responsible for the major activity of this specialised cell, i.e. contraction. The myofibres are in a state of dynamic adaptation in response to hormones, mechanical activity and motor innervation that modulate differential gene expression and splicing during this functional acclimatisation (reviewed in 1,2 and references therein).

In tissue culture, the differentiation of myoblasts is tightly regulated through a suppression mechanism mediated by a gamut of oncogene products and growth factors/receptors that prevent cell cycle arrest and repress *trans*-activation of myogenic gene expression. Hormonal stimulation [(thyroid hormone (T3), retinoic acid (RA) and insulin-like growth factors (IGFs)] and growth factor deprivation induce proliferating mono nucleated myoblasts to exit the cell cycle and differentiate.

Insight into the molecular mechanisms that control these events was provided by the recent characterisation of the *myoD* gene family (*myoD, myf-5, myogenin* and *MRF-4/myf-6/herculin*) that encode helix–loop–helix (HLH) proteins, that are *trans*-activators of muscle specific gene expression (reviewed in 1,2 and references therein). The *myoD* gene family are regulatory myogenic factors that can direct the fate of mesodermal cell lineages, repress proliferation, activate differentiation and the

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contractile phenotype. Analyses of the MyoD gene family has provided perceptions into myogenesis because these factors operate at the nexus of regulatory networks that control the mutually exclusive events of division and differentiation and serve as targets for the variety of physiological stimuli transmitted from the cell surface to the nucleus that control myogenesis (reviewed in 1,2 and references therein).

Recent gene targeting studies indicated that myoD and myf-5 are required for determination (3), while myogenin (4) is specifically required for differentiation. In cell culture, myoD/myf-5 are expressed in proliferating myoblasts and are markers for the committed myoblast state; in contrast myogenin expression strictly coincides with the terminal differentiation of myoblasts into myotubes and expression of contractile proteins. MyoD and myogenin function is negatively regulated by a ubiquitously expressed HLH protein, Id (inhibitor of differentiation) (1,2).

The function of these HLH hierarchical regulators is modulated by environmental cues related to the concentration of growth factors (e.g. TGFβ, FGF) and oncogene products (e.g. *c-fos, c-jun*) that promote cell division (summarised and discussed in 1,2). These agents act by either (i) repression of MyoD mRNA accumulation and protein synthesis; (ii) regulation of MyoD activity/function (via phosphorylation or heterodimerization) and (iii) suppression of myogenic specific *trans*-acting factors that positively regulate the expression of myogenin and MRF-4. Thyroid hormones and retinoids regulate the transcription of the *MyoD* and *myogenin* genes and promote terminal skeletal muscle differentiation (5–7). The effects of these ligands are mediated by the thyroid hormone (TR) and retinoid X receptors (RXR) that directly interact with the thyroid hormone response elements (TREs) (8) between nucleotide positions –337/–309 and –526/–494 in the mouse *myoD* and *myogenin* promoters respectively. These TREs were comprised of direct repeats of two AGGTCA half-site motifs separated by 4 nt. TR and RXR are members of the steroid superfamily of receptors that function as ligand activated DNA binding proteins (8). This group includes the 'orphan receptors' (which have no known ligands in the 'classical sense') that appear to be the ancient progenitors of this receptor superfamily. The Chicken Ovalbumin Upstream Promoter-Transcription Factor (COUP-TF) is an 'orphan receptor' whose function is influenced by catecholamine induced phosphorylation cascades (9,10). The COUP-TF gene family is closely related to the *Drosophila seven-up (svp)* gene which is required for the development of the embryonic central nervous system and specific photopreceptors (9). COUP-TF is capable of binding to oligonucleotides containing both direct repeats and palindromes of the AGGTCA motifs with different spacing (from 0 to 12 nt); however, the orphan receptor displayed higher relative binding affinity for direct repeats (with a 1–7 nucleotide gap) (11,12). Recently, COUP-TF I and II isoforms have been demonstrated to (i) repress hormonal activation of target genes by the vitamin D, thyroid hormone and retinoid family of receptors and (ii) negatively regulate the transcription of many other genes (9,11,12). The mechanism involves direct homodimeric DNA binding competition for response element occupancy, heterodimerization with other superfamily members and subsequent formation of non-functional complexes, and squelching of general transcription factors. Although, COUP-TF is abundantly expressed in dividing skeletal myoblasts its function in muscle differentiation and mammalian development remain obscure. Hence we investigated the expression/functional role of COUP-TF II in terminal skeletal muscle differentiation and the regulation of myoD/myogenin expression with respect to the characterised link between hormonal regulation of the myoD gene family and muscle differentiation.

MATERIAL AND METHODS

Cell culture and transfection

Mouse myogenic C2C12 cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 20% foetal calf serum (FCS) in 6% CO_2 as described previously (13). This cell line was induced to biochemically and morphologically differentiate into multinucleate myotubes by mitogen withdrawal [DMEM supplemented with 2% horse serum in 6% CO_2]. Differentiation was essentially complete within 72–96 h with respect to isoform switching in the actin multigene family (14). C2C12 cells were stably transfected at ~40% confluence using the DOTAP (Boehringer Mannheim) mediated procedure by co-transfection of pCMV-NEO as described previously (15).

Plasmids

The pGEX-1-cTRα and pGEX3-mRXRγ1 plasmids were described by Muscat *et al.* (1994) (5,6). The pGEX-2T-hRXRα was described in Mangelsdorf *et al.*, (1991) (16). pGEX-1-hCOUP-TF II was constructed by the generation of an appropriate insert for cloning into *Eco*RI cleaved pGEX-1 by PCR. Two primers, GMUQ 93 5′-GCGAATTCACCATGGCAATGG-TAGTCAGG-3′ and GMUQ 94 5′-GCGAATTCTTATTTATT-GAATTGCCATATACGG-3′ were synthesised to allow PCR amplification of the entire 414 amino acid coding sequence of human COUP-TF II from the parent plasmid, pGEM 7Zf(+)-hCOUP-TF II [Cooney *et al.*, (11)] that contains ~150 bp of 5′ UTR, 1242 of coding sequence and ~200 bp of 3′ UTR. Amplification was achieved by 30 cycles of 95°C/45 s denaturation, 50°C/30 s annealing and 72°C/2 min extension. The *Taq* DNA polymerase generated 1262 bp *Eco*RI cleaved fragment was cloned into *Eco*RI digested pGEX-1. The pGEX-1-hCOUP-TF II plasmids were identified and orientation defined by *Eco*RI and *Bam*HI restriction analyses, respectively. Double stranded sequencing confirmed authenticity and if the foreign protein was being expressed as 'in frame' GST fusion. The plasmid expressing truncated COUP-TF II, pGSTag-t-hCOUP-TF II was constructed by *Sma*I/*Eco*RI cleavage of the PCR generated fragment, which created a 1060 bp fragment, lacking 69 amino acids from its amino terminal end that was subsequently cloned into pGSTag digested with *Sma*I and *Eco*RI. Cloning and orientation of the insert were determined by *Eco*RI/*Sma*I and *Bam*HI restriction analyses respectively. pSG5-hCOUP-TF II S and AS were constructed from the parent plasmid, pGEM 7Zf(+)-hCOUP-TF II [Cooney *et al.*, (11)] The *Eco*RI fragment was cloned into *Eco*RI digested pSG5. Sense and anti-sense orientations were distinguished using *Hin*dIII/*Xba*I double digests.

Expression and purification of receptors

Human RXRα, mouse RXRγ, chicken TRα and human full-length and truncated COUP-TF II were expressed as fusions with glutathione-S-transferase (GST) using the pGEX-2T, pGEX-3, pGEX-1 and pGSTag bacterial expression vectors. The GST fusion proteins were purified as described previously (5).

Oligonucleotide TRE probes

Human skeletal α-actin (HSA) TRE, –273/–249 gatcGGGCAACTGGGTCG*GGTC*AGGAGGG; mouse myogenin (MM) TRE, –526/–494 gatcGTGGTAGGTCTTTAGGGGTCTCATGGGACTGACA; mouse myoD TRE, –337/–309 gatcCTGAGGTCAGTCAAGGCTGGAGGAGTAGA; α-myosin heavy chain (MHC) TRE, –127/–159, gatcCTCTGGAGGTGACAGGAGGACAGCAGCCCTGA.

Nuclear extracts and gel mobility shift assays

Each binding mixture (25–30 μl) contained 1–2 ng of a T4 polynucleotide kinase-labelled DNA fragment, 1–30 pmol of purified receptors and 1–2 μg of poly dI-dC as a non-specific competitor (only when crude nuclear extract was used) in Dignam buffer C. The assays were incubated at room temperature for 20 min and electrophoresed through a 6% (19:1 polyacrylamide:bisacrylamide) gel in 80 mM Tris–borate and 2 mM EDTA as described.

RNA extraction and Northern hybridisation

Cytoplasmic RNA was extracted using Nonidet P-40 and urea by the method of Schreiber *et al.*(17). Northern blots, random primings and hybridisations were performed as described in Sambrook *et al.* All the actin probes used in the hybridisations were described by Bains *et al.* (14). The mouse myogenin (18), myoD (19) and Id (20) cDNAs were excised from the pEMSV-scribe (Moloney Sarcoma Virus) based expression vectors that have been previously described.

RESULTS

COUP-TF II mRNA is repressed during myogenic differentiation

COUP-TFs are ubiquitously expressed, however very abundant levels of COUP-TF II have been observed in skeletal muscle and cardiac tissue (21). Since thyroid hormones and retinoids are major regulators of muscle development (7) and orphan receptors modulate hormonal responses (9), we investigated the expression of COUP-TF II during myogenic differentiation in culture.

To examine whether COUP-TF II functions in myogenesis, we used the mouse C2C12 myoblast cell line as a model. Proliferating C2C12 myoblasts can be induced to biochemically and morphologically differentiate into post-mitotic multinucleated myotubes by serum withdrawal in culture over a 48–96 h period. This transition from a non-muscle phenotype to contractile phenotype is associated with the repression of non-muscle proteins and the activation of contractile apparatus. Total cytoplasmic RNA was isolated from dividing myoblasts and post-mitotic myotubes and examined by 'Northern Blot' analysis (see Fig. 1). COUP-TF II mRNA was abundantly expressed in myoblasts, however, this transcript is suppressed as myoblasts exit the cell cycle and fuse to form differentiated multinucleated cells that have acquired a contractile phenotype. The induction of myogenin mRNA and repression of cytoskeletal non-muscle β-actin mRNA, relative to the equivalent levels of 18S rRNA confirmed that these cells had terminally differentiated. The expression of myoD mRNA in myoblasts and myotubes confirmed the myogenic nature of these cells. The differential expression of COUP-TF II suggested that this orphan receptor had a regulatory function in myogenesis.

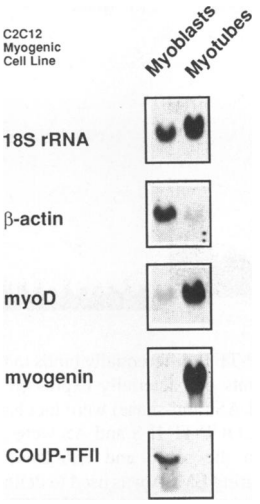

Figure 1. Expression of 18S rRNA, β-actin, myoD, myogenin and COUP-TF II mRNAs in normal C2C12 cells. Total cytoplasmic RNA was isolated from native proliferating myoblasts and myotubes (after 72 h of serum withdrawal). These RNAs were Northern blotted and probed with 18S rRNA, β-actin, myoD, myogenin and COUP-TF II random primed cDNA probes.

COUP-TF II interacts with the TREs in the muscle specific skeletal α-actin, myogenin, myoD and α-myosin heavy chain genes

COUP-TFs are capable of binding to oligonucleotides containing both direct repeats and palindromes and with different spacing (from 0 to 12 nt) of the $^{A}/_{G}$GGTCA motifs. However, COUP-TFs displayed higher relative binding affinity for direct repeats (with a 2–7 nt gap) compared to palindromes (10). Since recent investigations (i) revealed that COUP-TFs are able to repress hormonal induction of target genes by thyroid hormone (11,12) and (ii) identified TREs in the mouse *myogenin* (MM) (5), mouse *MyoD* (6) and human skeletal α-actin (HSA) (22) muscle specific genes, we determined whether bacterially expressed COUP-TF II interacted with these sequences. These TREs (MM, myoD and HSA) were comprised of direct repeats of two AGGTCA half site motifs separated by 4 nucleotides and interacted functionally with heterodimeric TR–RXR complexes (see Materials and Methods for precise sequences). We used the well characterised rodent α-myosin heavy chain (MHC) DR-4 TRE as a control (22); COUP-TF II has been demonstrated to repress *trans*-activation of this response element by TR and interact strongly with DR-4 motifs (12). Electrophoretic Mobility Shift Assays (EMSA) were utilised to evaluate COUP-TF II binding to these TRE elements from myogenic genes. We cloned both full length COUP-TF II and an amino-terminal truncated COUP-TF II (t-COUP-TF II (ΔAB), first 69 amino acids deleted) into pGEX; these two proteins had been previously demonstrated to have DNA binding characteristics and antigenic properties akin to those of native COUP proteins 21,11). We incubated increasing amounts (0.15–3.5 pmol) of purified COUP-TF II to fixed quantities of ^{32}P end-labelled MM, HSA and α-MHC TREs (Fig. 2). The orphan receptor bound as monomers and dimers to all these TREs. It is apparent though, that a COUP-TF II dimeric species preferentially forms with increasing

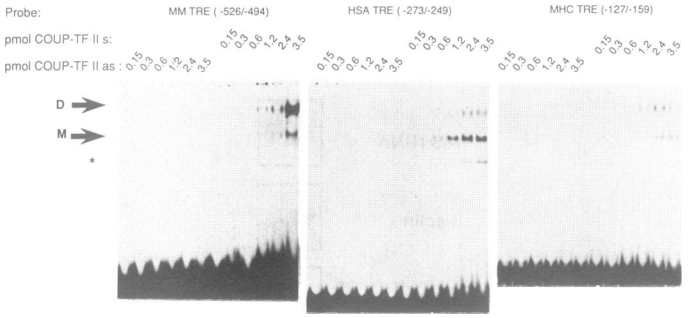

Figure 2. Human COUP-TF II differentially binds to the MM, HSA and MHC TREs. Increasing amounts of bacterially expressed human COUP-TF II S (sense) and COUP-TF II AS (anti-sense) were incubated with fixed quantities of ^{32}-P labelled TREs. COUP-TF II S and AS were produced by cloning the COUP-TF II cDNA in the sense and anti-sense orientations in pGEX respectively. Non-denaturing EMSA was used to delineate binding complexes. M and D, represent the positions of the COUP-TF II monomer and dimer respectively. The * represents non-specific DNA binding.

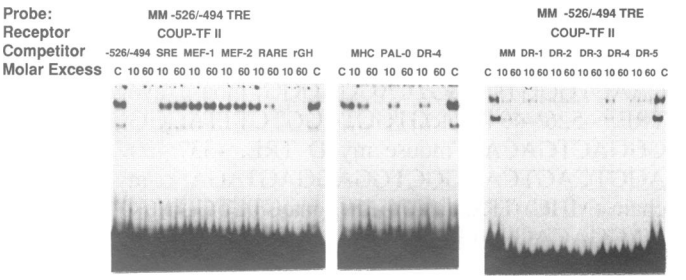

Figure 3. The mouse myogenin MM TRE interacts with full length hCOUP-TF II in a sequence-specific fashion. The effect of competition (10–60-fold molar excess), by self; non-hormone response elements [SRE (GAAGGGGACCAAA-TAAGGCAAGGTG), E-box/MEF-1 (CCCCCCCAACACCTGCTGCCTGA-GCC), MEF-2 (GCCCCATATATCAGTGATATAAATAGAACCTGCAG)]; the characterised hormone; response elements [CRBP I RARE (–1016/–989 TTAG-TAGGTCAAAAGGTCAGACACTGAA), rGH (–190/–166 gatcAAGG-TAAGATCAGGGACGTGACCGC), MHC (–127/–159 gatcCTCTGGAGGT-GACAGGAGGACAGCAGCCCTGA), PAL-0 (gatcTCAGGTCATGAC-CTGA) and DR-4 (gatcAGGTCACAGGAGGTCA)] and the synthetic Direct Repeat response elements based on the 3-4-5 rule [DR-1(gatcAGGTCAgAGGT-CA), DR-2 (gatcAGGTCAggAGGTCA), DR-3 (gatcAGGTCAaggAGGTCA), DR-4 (gatcAGGTCAcaggAGGTCA) and DR-5 (gatcAGGTCAccaggAGGT-CA)] on the complex formed between the probe MM TRE (–526/–494 gatc GTGGTAGGTCTTTAGGGGTCTCATGGGACTGACA) and the COUP-TF dimer. The molar excess of each DNA competitor is indicated. C, denotes the control binding reaction in the absence of any unlabelled competitor.

receptor concentration on the MM and MHC TREs, whereas a monomeric species seems to predominate on the skeletal α-actin TRE (Fig. 2). Monomeric binding to this TRE may be favoured over dimeric interaction due to the presence of a perfect AGGTCA core half-site between the imperfect DR-4 sequence. COUP-TF II cloned in the anti-sense orientation in pGEX (COUP-TF II AS), expressed and affinity purified from *E.coli* showed no interaction with the MM, HSA or MHC TREs. Similar results were observed with the myoD TRE (data not shown).

The binding of COUP-TF II to these TREs suggests that this orphan receptor is likely to perform a key regulatory role by modulating hormonal control of these TREs during skeletal myogenesis. Finally, that monomeric and dimeric species differentially bind to the MM, HSA and MHC TREs may suggest that COUP-TF II has distinct regulatory effects on these TREs.

The interaction of COUP-TF II with the myogenic TREs is specifically competed by classical TREs and 'AGGTCA' sequence motifs

We conducted electrophoretic mobility shift analysis (EMSA) competition studies to confirm that the binding of COUP-TF II to these TREs was sequence specific. We characterised the specificity of this interaction with a large bank of unlabelled oligonucleotides that represent a wide variety of binding sites. Competition probes used, included the classical and well characterised natural rodent growth hormone (rGH) and MHC TREs, and the synthetic PAL-0 TRE; a wild type CRBP I RARE, (cellular retinol binding protein I, retinoic acid response element) MEF-1/E-box [Myocyte enhancer factor 1 is an E-box, (CANNTG) and interacts with helix–loop–helix proteins], MEF-2 [an integral component of muscle specific enhancers that interacts with the MADS box transcription factor family (named for the yeast mating type transcription factor, MCM1, two plant homeotic genes agamous and deficiens, and human serum response factor)] and SRE (Serum Response Element that interacts with serum response factor). The sequence of the oligonucleotides used in this study are listed in the Figure 3 legend with the motifs underlined. The competition studies were carried out at 10- and 60-fold molar excess of oligonucleotide with respect to the MM TRE probe (Fig. 3). These studies demonstrated that the complex formed between the MM TRE sequences and the COUP-TF II dimer could be specifically competed by the established wild-type and synthetic TREs (that have been demonstrated to interact with heterodimeric TR–RXR receptor complexes) and the wild type RARE. These results indicated that the MM TRE–COUP-TF II interaction was highly specific. To further examine and characterise this specific binding of COUP-TF II we also conducted EMSA competition with an array of synthetic hormone response elements as predicted by the 3-4-5 rule, arranged as direct repeats of the AGGTCA motif with spacings of 1, 2, 3, 4 and 5 nt (designated DR-1, DR-2, DR-3, DR-4 and DR-5, respectively) as described in Kliewer *et al.* (32) (Fig. 3 legend). A functional relationship among the RXR, VDR, TR and RAR has recently been described in which these receptors bind and activate through tandem direct repeats AGGTCA N_x AGGTCA with spacing of 1, 3, 4 and 5 nt respectively. We tested these synthetic hormone response elements in EMSA competition assays to assess the specificity and gap/spacing preference of the COUP-TF II dimeric binding to the MM TRE sequence (Fig. 3). DR-1, DR-2, DR-3, DR-4 and DR-5 were used in the binding reactions at 10- and 60-fold molar excess with respect to the MM TRE probe (Fig. 3). This data indicated that DR-4/DR-5 > DR-3 > DR-2 > DR-1 all competed for the formation of the COUP-TF II dimer on the MM TRE. Identical analyses on the myoD TRE was performed with the same array of competitors with identical results (data not shown).

In summary we provide evidence that COUP-TF II complex on the MM and myoD TREs is specifically competed by response elements containing RGGTCA core motifs arranged as direct repeats separated by 1–5 nt, or as a palindromic sequence. In contrast SRE, MEF-1 and MEF-2 show no apparent affinity for COUP-TF II.

Mutagenesis of the myogenin TRE identifies the RGGTCA motifs as essential COUP-TF II binding sites

We characterised the nucleotides in the MM TRE that interacted with the COUP-TF II dimer by mutating the MM TRE sequentially

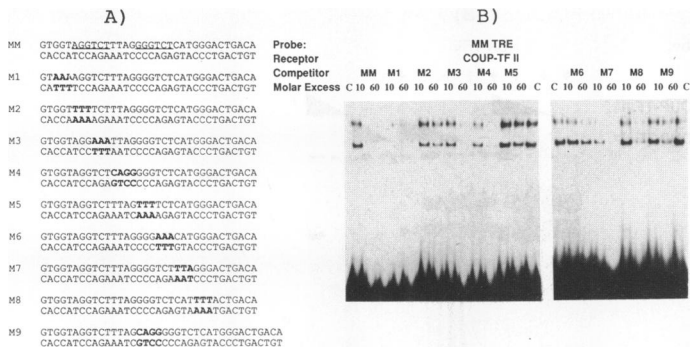

Figure 4. (**A**) Pictorial representation of the various site specific mutations in the mouse myogenin TRE. The wild type TRE sequence is depicted and the mutations in the M1, M2, M3, M4, M5, M6, M7, M8 and M9 TREs are denoted by bold text. (**B**) Mutational analyses of the mouse myogenin TRE. The core receptor binding motifs in the MM –526/–494 TRE are important for binding. The effects of competition by a battery of mutations in the MM TRE, designated M1, M2, M3, M4, M5, M6, M7 and M8, on the complex formed between the MM TRE probe and full length COUP-TF II. The molar excess of each DNA competitor is indicated. C, denotes the control binding reaction in the absence of any unlabelled competitor.

from the 5′ to 3′ direction by nine triplet changes that spanned the half site motifs. These mutant MM TREs were designated, MM M1–M9 (see Fig. 4A) and used in EMSA competition analyses to ascertain their ability to disrupt the complex formed between the MM TRE and COUP-TF II. We independently incubated wild type MM TRE (–526/–494) probe with COUP-TF II (Fig. 4B). and competed with 10- and 60-fold molar excesses of the MM M1, M2, M3, M4, M5, M6, M7, M8 and M9 mutant TREs. Figure 4B depicts the ability of the mutant MM TRE oligos, M1–M9, to compete for binding to COUP-TF II relative to the wild type sequence. The mutant MM TREs M1, M3, M4, M7 and M8 competed efficiently for binding to COUP-TF II. This demonstrated that the sequences flanking the direct repeats and the sequence of the 4 nt gap were not important for binding to the COUP-TF II. In contrast, M2, M5 and M6 TREs did not compete for binding to COUP-TF II, indicating that two functional AGGTCA motifs were required for efficient binding. This data strongly reinforced the role of the direct repeats (i.e. the two half-sites) and the importance of the half-site sequence (especially the AGG's) in the efficient formation of a COUP TF II complex. The mutant M9 that had an 8 nt gap between the wild type direct repeats did not compete for binding demonstrating that the 8 nt gap spacing hindered efficient complex formation (Fig. 4B).

COUP-TF II binding represses the formation of TR monomers and homodimers on the skeletal α-actin and α-myosin heavy chain TREs

We have previously characterised the efficient binding of bacterially expressed TRα to the HSA TREs and we amongst others have characterised the strong interaction of TR to the MHC TRE (22), hence we examined the effect of COUP-TF II on the binding of TRα to the HSA and MHC TREs.

In our previous analysis of TRα binding to the HSA and MHC TREs, we observed that TR formed monomeric and dimeric complexes. A similar result is evident in Figure 5A. It is clear that in the presence of COUP-TF II S, the formation of TRα homodimers on these TREs is inhibited (Fig. 5A). This is a specific result, since COUP-TF II AS (anti-sense) co-incubation with TR, has no effect on TR monomer–homodimer complex formation. Whether, the formation of TR monomers on these TREs is prevented in the presence of COUP-TF II, is uncertain when full length COUP-TF II is utilised, as COUP-TF and TR monomeric binding on the HSA TRE runs with similar mobility on EMSA gels. We addressed this issue by incubating increasing amounts of truncated COUP-TF II with a fixed quantity of TRα with the HSA TRE. This was utilised as it has been previously demonstrated that truncated COUP-TF II migrates faster than full length COUP-TF II on EMSA gels (Fig. 5B). The truncated COUP-TF II receptor prevents the formation of TR monomers and homodimers on the HSA TRE (Fig. 5B). Hence, we can conclude that COUP-TF II prevented the formation of TR monomers and homodimers in a dose-dependent manner on the HSA and MHC TRE. This inhibition of TR binding is due to direct binding and competition for TRE occupancy.

COUP-TF II represses the heterodimeric binding of RXR and TR on the myoD and myogenin TREs

We have previously identified and investigated the functional formation of TR and RXR heterodimeric complexes on the myoD, MM, α-MHC and HSA TREs that mediated T3 dependent *trans*-activation of gene expression. Since COUP-TF II has been demonstrated to repress TR, RXR, RAR and VDR dependent hormonal *trans*-activation we examined the effect of full length and truncated COUP-TF II on the formation of TR–RXR heterodimeric complexes on these myogenic TREs (Fig. 6). These experiments indicate that COUP-TF II represses the formation of functional TR–RXR heterodimeric complexes on the myoD, MM and α-MHC TREs in a dose dependent manner (Fig. 6). A similar effect was observed on the HSA TRE (data not shown). Curiously, although heterodimer formation was inhibited, we did not observe the binding of COUP-TF II to these elements in the presence of TR and RXR. This data may suggest that non-functional/binding complexes were being formed.

Constitutive expression of COUP-TF II in myogenic cells blocks the induction of myogenin mRNA and suppresses myoD mRNA levels

Our studies demonstrated that (i) COUP-TF II mRNA repression correlates with the biochemical and morphological differentiation of myogenic cells that results in the transition from a non-muscle phenotype to contractile phenotype and (ii) COUP-TF II protein bound to the myogenic specific TREs in a sequence specific manner, and prevented the formation of TR monomers and homodimers, and TR–RXR heterodimers on these TREs. Hence to confirm these conclusions *in vivo* and rigorously identify the target(s) of this orphan receptor in differentiation and myogenesis we proceeded to examine the effect of constitutive COUP-TF II S expression in the C2C12 cell line. The construct pSG5-COUP-TF II S (an expression vector that contained COUP-TF II cDNA in the correct orientation under the control of the SV40 promoter) was co-transfected with pCMVNEO. Stable transfectants were isolated as a polyclonal pool of G418 resistant colonies (comprised of >20 individual resistant colonies). This cell line was denoted as C2: COUP-TF II S.

The process of myogenesis involves two events: determination (the process by which a pluripotential embryonic cell becomes committed to the myogenic lineage) and differentiation (the

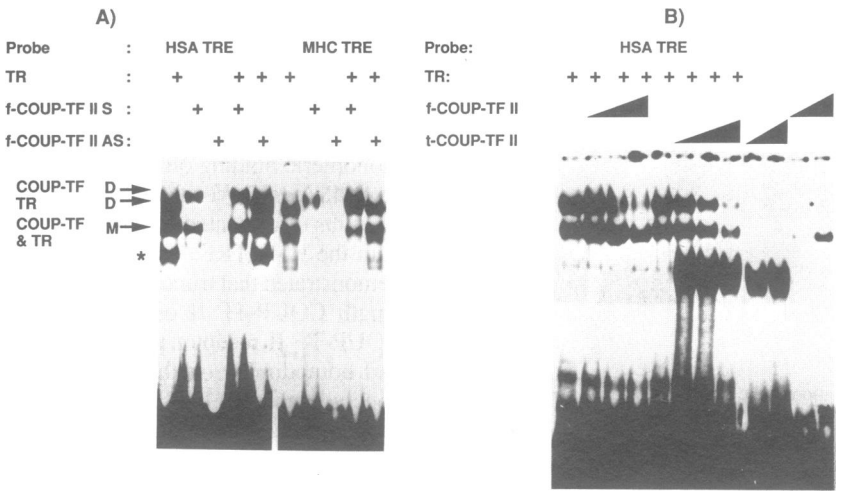

Figure 5. (**A**) Effects of full length (f)-COUP-TF II sense (S) and anti-sense (AS) on the formation of TRα monomers and homodimers on the HSA and MHC TREs. *E.coli* expressed and affinity purified TRα (1.5 pmol) and COUP-TF II (3 pmol) were incubated as indicated by the + signs with a fixed quantity of end-labelled TREs. M and D, represent monomeric and dimeric complexes respectively. The * represents non-specific DNA binding. (**B**) Effects of full length (f) and truncated (t) COUP-TF II on the formation of TRα monomers and homodimers on the HSA TRE. *E.coli* expressed and affinity purified TRα and f- and t-COUP-TF II were incubated as indicated by the + signs with a fixed quantity of end-labelled HSA TRE.

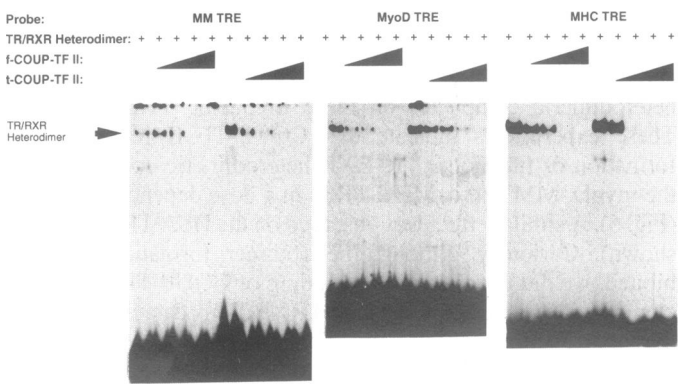

Figure 6. The effect of truncated (t) and full length (f) -hCOUP-TF II on the formation of TR–RXR heterodimers on the MM, myoD and MHC TREs. *E.coli* expressed and affinity purified TRα, RXRγ and f- and t-COUP-TF II were incubated as indicated by the + signs with a fixed quantity of end-labelled MM, MyoD and MHC TREs.

process by which committed myoblasts fuse to form multi-nucleated myofibres that express a muscle specific phenotype). Recent studies have indicated that the muscle specific, HLH proteins, myoD and myogenin, are required for determination and differentiation respectively (3,4). In cell culture, myoD is expressed in proliferating myoblasts and is a marker for the committed myoblast state; in contrast myogenin expression strictly coincides with the terminal differentiation of myoblasts into myotubes and expression of contractile proteins. MyoD and myogenin function is negatively regulated by a ubiquitously expressed HLH protein, Id (inhibitor of differentiation).

Hence, to examine the effect of constitutive COUP-TF II S expression on factors involved in determination and differentiation, total cytoplasmic RNA was isolated from C2C12 and C2:COUP-TF II S cells before and after 72 h of serum withdrawal. These RNAs were Northern blotted and probed with 18S rRNA, actin [cytoskeletal (non-muscle) and sarcomeric (muscle)], myoD, myogenin and Id-labelled cDNAs (Fig. 7). We noted that the C2:COUP-TF II S cell line differentiated poorly with a reduced % of multinucleated myotubes visible after serum withdrawal. The level of myoD mRNA in the C2:COUP-TF II cells in the presence and absence of serum was drastically reduced, furthermore, the induction of myogenin mRNA after serum withdrawal was not observed (Fig. 7). Interestingly, constitutive COUP-TF II did not effect the repression of cytoskeletal non-muscle β-actin mRNA and Id after serum withdrawal, relative to the equivalent levels of 18S rRNA (Fig. 7). However, the α-/β-actin ratio after serum withdrawal is reduced in C2:COUP-TF II S cells with respect to normal C2 myotubes. This data indicated that COUP-TF II, *in vivo*, specifically regulates the transcription of the muscle specific helix–loop–helix genes and functions as a physiologically important antagonistic regulator of myogenesis.

To verify the adverse effects of COUP-TF II on the *myoD* gene family and myogenesis, that we ascribed to this orphan steroid receptor from 'over-expression' studies, we examined the effects of constitutive 'anti-sense' COUP-TF II cDNA expression in C2C12 cells. The construct pSG5-COUP-TF II AS (an expression vector that contained COUP-TF II cDNA in the anti sense orientation under the control of the SV40 promoter) was co-transfected with pCMVNEO. Stable transfectants were isolated as a polyclonal pool of G418 resistant colonies (comprised of >20 individual resistant colonies). This cell line was denoted as C2: COUP-TF II AS. We noted that the C2:COUP-TF II AS cell line differentiated very efficiently with a high % of multinucleated myotubes visible after serum withdrawal and that endogenous COUP-TF II mRNA expression was notably reduced (data not shown). The level of myoD mRNA in the C2:COUP-TF II AS cells in the presence and absence of serum was significantly induced, furthermore, the myogenin mRNA was hyper-induced after serum withdrawal (Fig. 7). Constitutive COUP-TF II AS expression did not effect the repression of cytoskeletal non-muscle β-actin mRNA and Id after serum withdrawal, relative to the equivalent levels of 18S rRNA

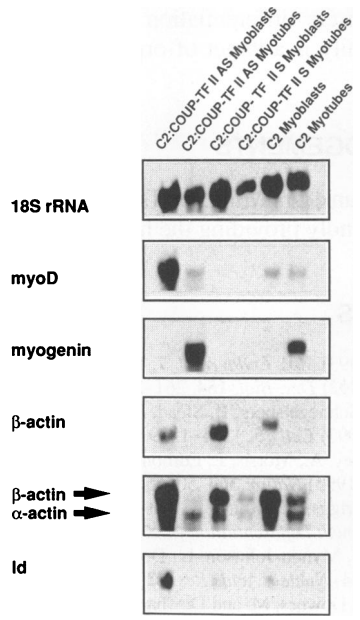

Figure 7. Expression of 18S rRNA, myoD, myogenin, β-actin, actin (cytoskeletal and sarcomeric actin, i.e. non-muscle and muscle specific actins) and Id mRNAs in normal C2C12 cells and in cells stably transfected with pSG5-COUP-TF II S (C2: COUP-TF II S) and AS (C2: COUP-TF IIAS) expression vectors. Total cytoplasmic RNA was isolated from native C2, C2: COUP-TF II S and C2: COUP-TF II AS proliferating myoblasts and myotubes (after 72 h of serum withdrawal). These RNAs were Northern blotted and probed with 18S rRNA, myoD, myogenin, β-actin, actin and Id random primed cDNA probes.

(Fig. 7). Interestingly, the α-/β-actin ratio after serum withdrawal is significantly induced in C2:COUP-TF II AS cells with respect to normal C2 myotubes, reflecting the efficient morphological differentiation of this cell line.

This sense and 'anti-sense' stable transfection analyses firmly suggested that COUP-TF II, specifically regulates the transcription of the muscle specific-helix–loop–helix genes and functions as a physiologically important modulator of differentiation.

DISCUSSION

The Chicken Ovalbumin Upstream Promoter-Transcription Factor II (COUP-TF II) is a low M_r (43–48 000) member of the COUP-TF gene family. The DNA binding domain and the 'so-called' C-terminal ligand binding domain of COUP-TF II, share ~90% amino acid sequence identity between *Drosophila* and humans, the similarity between mouse and human is between 99–100% (9,23). This striking cross species conservation is suggestive that these domains have important intracellular roles. The *Drosophila* homologue of COUP-TF, *Seven-up* (*svp*), plays a critical role in cell fate determination and differentiation of photoreceptor cells (9). Homozygous mutants of *svp* are lethal, whereas *svp* mosaic mutants have an altered defective photoreceptor phenotype. These investigations and the striking cross species conservation from 'flies' to humans indicated that COUP-TF II had an important role in development and differentiation. However, despite the studies in the *Drosophila* system and *in situ* analyses of COUP-TF expression in zebrafish, chicken and mouse development, a recent review from Ming-Jer Tsai and colleagues stated 'many questions remain to be answered. The central one concerns the physiological functions of COUP-TF...' (9). Our study showed that proliferating C2C12 myoblasts expressed COUP-TF II mRNA which was repressed when cells were induced to differentiate into post-mitotic multinucleated myotubes by serum withdrawal. The *in vitro* analyses demonstrated that COUP-TF II bound to the TREs in the promoters (1) of the *myoD* and *myogenin* regulatory HLH genes; and (2) the skeletal α-actin and α-MHC contractile protein genes in a sequence-specific manner. Furthermore, COUP-TF II diminished the homodimeric binding of the thyroid hormone receptor and the heterodimeric binding of thyroid hormone and retinoid X receptor complexes to these TREs. These *in vitro* observations correlated with (i) the significantly reduced levels of myoD mRNA and the absence of myogenin induction in mouse C2C12 myogenic cells that constitutively over-express COUP-TF II and (ii) increased levels of myoD mRNA and the hyper-induction of myogenin mRNA in C2C12 cell that constitutively express anti-sense COUP-TF II cDNA.

These studies provide the first demonstration that the orphan receptor, COUP-TF II, functions as a physiologically relevant regulator of mammalian differentiation by transcriptional repression of the *myoD* gene family. *MyoD* and *myogenin* transcription were targets of COUP-TF II action, which suggested that this orphan receptor performed a regulatory function in both myogenic determination and differentiation, similar to the *svp* gene in *Drosophila* CNS and photoreceptor differentiation (24). This work also defines a developmental role for COUP-TF II during mammalian cell differentiation.

The ability of COUP-TF II to (i) interact with the DR-4 TREs in the myoD, myogenin, skeletal α-actin and α-myosin heavy chain genes and (ii) be specifically competed by direct repeats with different spacing (1–5) of the AGGTCA motif and a variety of other steroid hormone response elements (rGH TRE, PAL-0, CRBP I RARE) is compatible with the published COUP-TF binding characteristics. COUP-TF has been previously demonstrated to interact with synthetic oligonucleotides containing both direct repeats and palindromes and with different spacing (from 0 to 12 nt) of the $^{A}/_{G}$GGTCA motifs. However, it displayed higher relative binding affinity for direct repeats (with a 1–7 nt gap) compared to palindromes (11). Mutagenesis of the myogenin TRE verified the role of the direct repeats (i.e. two half sites) and the motif sequence in complex formation. This is in agreement with Cooney *et al.* (11) that demonstrated that functional COUP-TF II binding is a dimer requiring two motifs. Furthermore, this mutagenic analyses utilised the same bank of mutants used to define the TR–RXR heterodimeric complex binding site in the MM TRE, the data indicated that the TR–RXR complex and the COUP-TF II complex bind at identical nucleotides in the MM TRE.

The dose dependent inhibitory effect of COUP-TF II on the monomeric and homodimeric binding of the thyroid hormone receptor to these myogenic TREs (HSA and MHC) correlates with (a) the negative regulation of thyroid hormone action and (b) COUP-TF being able to bind a variety of hormone response elements (HREs) as homodimers, and efficiently compete against other receptors, (including ER, VDR, RAR and TR) for HRE occupancy. Our study on natural TREs is in agreement with the work of Tran *et al.* (25) that demonstrated on the synthetic PAL-0 TRE that COUP-TF I could replace TRα on PAL-0.

Interestingly, we observed that this orphan receptor inhibited in a dose dependent manner the heterodimeric binding of thyroid

hormone and retinoid X receptor complexes to these myogenic TREs, similar to Tran *et al.* (25) that showed COUP-TF I repressed TR–RXR binding on PAL-0. However in our study, this did not involve direct COUP-TF binding in the presence of TR and RXR, but the putative formation of non-binding COUP-TF–RXR or TR complexes. Other investigations have revealed that COUP-TF is able to heterodimerize with other superfamily members and form non-functional receptor complexes. This would not only block receptors binding their specific RE's, but would also allow other receptors/factors to fill their places. Furthermore, COUP-TF's apparently form heterodimers on only subsets of response elements *in vitro*. This has potential implications in spatial and temporal regulation of hormone sensitive transcription during development. Conflicting reports exist in the literature regarding the exact nature of these heterodimers, some groups argue that the COUP-TF's specifically heterodimerize with RXR (the accessory factor for RAR, TR, VDR and PPAR); while others argue that COUP-TF's are RXR-like, and that they heterodimerize similarly with all the thyroid hormone-like receptors. Despite this, TR, RAR and RXR have all been reported to heterodimerize with COUP-TF; albeit on specific response elements. COUP-TF's are likely to play major roles in regulating gene expression by squelching of general transcription factors (COUP-TF binds TFIIB and synergistically influence transcription; and by functioning as 'activation gateways' by allowing other receptors to bind HREs they normally could not access (9,11,12,25–31).

These repression of COUP-TF II mRNA that we observed during muscle differentiation is characteristic of orphan receptors which are often temporally and spatially regulated. Furthermore, the regulation of the hierarchical *myoD* gene family that we observed after stable transfection of COUP-TF II 'sense' and 'anti-sense' expression vectors equates with the observation that orphan receptors are often major regulators of specific genes and developmental processes (e.g. *seven-up, svp in Drosophila*) (32,33). Interestingly, myogenin induction after serum withdrawal was completely blocked in C2:COUP-TF II S cells, whereas α-actin mRNA was still detectable albeit at reduced levels. This data may reflect the more efficient formation COUP-TF II dimer complexes on the MM versus HSA TREs.

Many investigations have shown that protein kinase C and c-AMP dependent protein kinase A inhibit muscle differentiation. PKC and PKA act via (i) suppression of the *trans*-activation functions of myoD/myogenin by multiple mechanisms and (ii) repression of *myoD* gene family expression (1,2). Dopamine was shown to stimulate COUP-TF dependent transcription of an appropriate target gene. Dopamine was not binding directly to COUP-TF, but to the membrane bound D1 receptor that is coupled to the adenyl cyclase and phospholipase signalling pathways. Phosphorylation via cAMP-dependent protein kinase A is likely to be involved, because 8-Br-cAMP and okadaic acid (a strong phosphatase inhibitor) also activate COUP-TF (33). Our studies and others demonstrate that the *myoD* gene family is targeted by the steroid receptor pathway, PKA, PKC and neurotransmitters.

In conclusion this investigation highlights the functional role that the orphan receptor, COUP-TF II plays in the modulation of mammalian muscle differentiation and demonstrates that the *myoD* gene family is a target of orphan receptor mediated cell signalling.

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