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The orphan nuclear receptor, COUP-TF II, inhibits myogenesis by post-transcriptional regulation of MyoD function: COUP-TF II directly interacts with p300 and MyoD

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ABSTRACT

COUP-TF II is an orphan nuclear receptor that has no known ligand in the 'classical sense'. COUP-TF interacts with the corepressors N-CoR, SMRT and RIP13, and silences transcription by active repression and trans-repression. Forced expression of the orphan nuclear receptor COUP-TF II in mouse C2 myogenic cells has been demonstrated to inhibit morphological differentiation, and to repress the expression of: (i) the *myoD* gene family which encodes myogenic basic helix–loop–helix (bHLH) proteins; and (ii) the cell cycle regulator, p21$^{Waf-1/Cip-1}$. In the present study, we show that COUP-TF II efficiently inhibits the *myoD*-mediated myogenic conversion of pluripotential C3H10T1/2 cells by post-transcriptional mechanisms. Furthermore, repression of MyoD-dependent transcription by COUP-TF II occurs in the absence of the nuclear receptor cognate binding motif. The inhibition of MyoD-mediated trans-activation involves the direct binding of the DNA binding domain/C-region and hinge/D-regions [i.e. amino acid (aa) residues 78–213] of COUP-TF II to the N-terminal activation domain of MyoD. Over-expression of the cofactor p300, which functions as a coactivator of myoD-mediated transcription, alleviated repression by COUP-TF II. Further binding analysis demonstrated that COUP-TF II interacted with the N-terminal 149 aa residues of p300 which encoded the receptor interaction domain of the coactivator. Finally we observed that COUP-TF II, MyoD and p300 interact in a competitive manner, and that increasing amounts of COUP-TF II have the ability to reduce the interaction between myoD and p300 *in vitro*. The experiments presented herein suggest that COUP-TF II post-transcriptionally regulates myoD activity/function, and that crosstalk between orphan nuclear receptors and the myogenic bHLH proteins has functional consequences for differentiation.

INTRODUCTION

The process of skeletal muscle differentiation involves the activation and induction of a large array of muscle-specific genes, and exit from the cell cycle. The *myoD* gene family encodes myogenic specific basic helix–loop–helix (bHLH) proteins that activate and control this process (reviewed in 1–3). MyoD plays a dual role during myogenesis, activating both muscle specific gene transcription and promoting cell cycle exit by inducing the expression of p21$^{Cip-1/Waf-1}$, an inhibitor of cyclin-dependent kinases and cellular proliferation (4–6). Transactivation by MyoD involves: (i) the bHLH domain, that is involved in both DNA binding and dimerisation; (ii) the heterodimerisation of MyoD with the ubiquitously expressed E2A gene products, E12 and E47 (7); (iii) the binding of MyoD-E2A heterodimers to specific E-box motifs (CANNTG) in muscle-specific enhancers (reviewed in 1–3); and (iv) the recruitment of the cofactors p300 and PCAF (8–10). The cofactors p300 and PCAF are critical coactivators for MyoD during myogenic commitment and differentiation. The N-terminal acid-rich activation domain of MyoD directly interacts with p300 and recruits PCAF to form a ternary multimeric complex on promoter elements (8,11). These events lead to hyper-acetylated and transcriptionally-permissive chromatin. Moreover, p300 and PCAF coactivate myoD-mediated trans-activation of the p21 gene, and are necessary for MyoD-mediated cell cyle arrest (11).

The transcriptional activity of MyoD is modulated by environmental cues related to the concentration of growth factors, receptors and oncogene products which promote cell division (reviewed in 12). These agents inhibit the transcriptional activity of MyoD by promoting: (i) the direct phosphorylation of the bHLH region and/or the interaction with c-jun, which prevent DNA binding; (ii) the activation of inhibitor of differentiation expression (Id), a bHLH protein that lacks DNA binding ability, and functions as a dominant negative; and (iii) the suppression/sequestering of myogenic specific transcription factors and cofactors.

The orphan nuclear receptors COUP-TF II, Rev-erbA and RVR are members of the nuclear receptor superfamily, and are

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abundantly expressed in proliferating myoblasts (12–15). However, the expression of this group of receptors is repressed during differentiation. Constitutive expression of COUP-TF II cDNA in mouse C2 myogenic cells blocks morphological differentiation by suppressing the levels of myoD mRNA, increasing cyclin D1 protein levels, and blocking the induction of myogenin and p21 mRNAs after serum withdrawal (14,16). The molecular basis for these effects has not been elucidated.

COUP-TF II is ubiquitously expressed in adult tissues, and regulated by the morphogen, sonic hedgehog (17). During embryogenesis, COUP-TF II is expressed in the developing CNS in a spatio-temporal manner (18,19), and in the dermatomial region of the somite at 10 days post coitum, which will give rise to the axial skeletal and tongue muscle (20). COUP-TF proteins are highly expressed in many other developing organs and the expression level decreases with completion of differentiation, suggesting that COUP-TF is involved in organogenesis (21,22). COUP-TF binds to direct and inverted repeats of the RGGTCA motif spaced by 0–12 nt (23–26).

COUP-TF isoforms I and II have been demonstrated to: (i) repress hormonal trans-activation of target genes by the nuclear receptor family (23–26); (ii) repress trans-activation by the potent GAL4VP16 chimera (16); (iii) interact with TFIIB; and (iv) negatively regulate the transcription of many other genes (e.g. Apolipoprotein AI gene) (21,27–29). Inhibition of transcription by COUP-TF II has been shown to be mediated by two main mechanisms, active repression and transrepression (30). Active repression by COUP-TF involves direct binding and competition for the occupancy of a common response element (31). Transrepression involves inhibition of transcription by COUP-TF II in the absence of its cognate binding motif, or independent of nucleic acid binding (30). Interestingly, we (16) and Achatz *et al.* (30) have demonstrated that the DNA binding domain (DBD)/C-region of COUP-TF II is necessary for transrepression. The molecular mechanism underlying COUP-TF II transrepression is unclear; however, it has been suggested that direct protein–protein interactions between COUP-TF and essential cofactors may be involved. Whether COUP-TF II directly regulates the transcription of the bHLH regulators is unclear. Furthermore, the fact that COUP-TF II represses transcription in the presence and absence of its cognate binding motif raises the scenario that COUP-TF II may control MyoD activity or the formation of the functional MyoD ternary complex.

Active repression by COUP-TF II is mediated by the nuclear corepressors (i.e. N-CoR/RIP13 and SMRT/TRAC-2) (16,32–35). Corepressors actively repress transcription by: (i) interacting with Sin3A/B and recruiting histone deacetylases (i.e. HDAc-1 or Rpd3) which actively promote chromatin condensation via hypo-acetylation of the chromatin (36–39); and (ii) binding key components of the basal transcriptional machinery (i.e. TFIIB, $TAF_{II}32$ and $TAF_{II}70$) (40). In the present study, we demonstrate that that repression of MyoD-mediated transactivation by COUP-TF II involves direct binding to MyoD and p300.

MATERIALS AND METHODS

Plasmids

Vectors used in transfection experiments and GST-pulldowns are described in detail elsewhere (8,16). The construction of new plasmids is described herein. The oligonucleotides GMUQ371 5′-GCGGTCGACATATGTGCCGCCTCAAAAAGTGCCTC-3′ and GMUQ372 5′-GCGGTCGACTTATTTATTGAATTGCCA-TATACGG-3′ were synthesised to allow PCR amplification of the COUP-TF II region spanning amino acids (aa) 134–414. The oligonucleotides GMUQ371 and GMUQ373 5′-GCGGTCGAC-CTAGGTTTTACCTACCAAACGG-3′ were used to amplify the region spanning aa 134–389. PCR amplification was performed with *Pfu* DNA polymerase (Stratagen, La Jolla, CA) according to the manufacturer's instructions. The resulting DNA fragments were digested with *Sal*I and ligated to *Sal*I-digested pNLVP16. The plasmids are described as VP16-COUP-TF II 1/2 (i.e. aa 134–414) and VP16-COUP-TF II 1/3 (i.e. aa 134–389) throughout the text. The oligonucleotides GMUQ272 and QMUQ273 (described in 16) were used to generate the COUP-TF II CD region. The resulting product was digested with *Eco*RI and end-filled with Klenow DNA polymerase according to the manufacturer's directions. The COUP-TF II CD end-filled fragment was then cloned into a pNLVP16 vector which had been cleaved with *Nde*I and end-filled with Klenow DNA polymerase. To generate the pSG5-COUP-TF II AB, ABCD and CDE expression vectors GAL4-COUP-TF II plasmids encoding the AB, ABCD and CDE domains were digested with *Eco*RI and the resulting fragments cloned into the *Eco*RI site of pSG5. pSG5-COUP-TF II CD was generated by digesting the COUP-TF II CD PCR fragment with *Eco*RI and again directly cloning into the *Eco*RI site of pSG5. To create the plasmids encoding GST-COUP-TF II AB, ABCD, CDE and DE, GAL4-COUP-TF II plasmids encoding the AB, ABCD, CDE and DE domains were digested with *Eco*RI and the resulting fragments cloned into the *Eco*RI site of pGEX-1λT. GST-COUP-TF II CD was generated by digesting the CD PCR fragment with *Eco*RI and cloning into the *Eco*RI site of pGEX-1λT. VP16, GAL and GST chimeras were sequenced to confirm the orientation and reading frame.

Cell culture and transfection

JEG-3 cells were cultured for 24 h in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% charcoal stripped fetal calf serum in 6% CO_2 at 37°C prior to transfection. JEG-3 cells grown in 6-well dishes to 60–70% confluence were transiently transfected with 3 µg of the reporter plasmid DNA G5E1b-CAT, 1 µg of either GAL4-p300-aa 1–149, GAL4-p300-aa 595–1240, or GAL4-p300-aa 1030–2414 and 1 µg of either VP16O, VP16 COUP-TF II, VP16 COUP-TF II AB, VP16 COUP-TFII ABCD, VP16 COUP-TF II CD, VP16 COUP-TF II CDE, VP16 COUP-TF II DE, VP16 COUP-TF II 1/2 or VP16 COUP-TF II 1/3 by the DOTAP/DOSPER (Boehringer Mannheim) mediated procedure as described previously (41). The DNA/DOTAP/DOSPER mixture was added to the cells in 3 ml of fresh medium. The medium was replaced 24 h after transfection. Cells were harvested and assayed for CAT enzyme activity as previously described after a further 24 h. Each transfection experiment was performed at least three times in order to overcome the variability inherent in transfections. NIH3T3 fibroblasts were grown to confluence (~5×10^6 cells) in DMEM supplemented with 10% fetal bovine serum. Cells were transfected by the calcium phosphate precipitation procedure as previously described. One µg of the 4RE-tkLUC reporter was coprecipitated with either 100 ng of EMSV, 200 ng p300 and/or 100–500 ng of the pSG5-COUP-TF II 'sense'/pSG5-COUP-TF II 'anti-sense'

plasmid. A total of 0.5 mg of the CMV-LacZ plasmid was included in each transfection. Luciferase activity was assayed as previously described (8) and corrected for with respect to the transfection efficiency by the β-galactosidase assay. The quantitative β-galactosidase assay was performed with the chlorophenol red-β-D-galactopyranoside substrate (CPRG; Boehringer Mannheim) as previously described (8). Each transfection experiment was performed at least three times in order to overcome the variability inherent in transfections.

C3H10T1/2 cell myogenic conversion

For the myogenic conversion assay, C3H10T1/2 fibroblasts were grown to 60% confluence in 12-well dishes. Cells in each well were transfected by the DOTAP/DOSPER (Boehringer Mannheim) mediated procedure, using 1 μg of EMSV-MyoD in combination with either 1 μg of pSG5-COUP-TF II, pSG5-COUP-TF II AB, pSG5-COUP-TF II ABCD, pSG5-COUP-TF II CDE or pSG5 carrier DNA so as to total 2 μg per transfection. Similarly, 0.4 μg each of EMSV-MyoD, EMSV-Myf-5, EMSV-Myogenin and EMSV-MRF4 were cotransfected with either 0.4 μg of pSG5 or pSG5-COUP-TF II. Twenty-four hours after transfection, cells were reefed on 10% DMEM until 100% confluent, whereupon the media was changed to DMEM plus 2% horse serum. Cells were grown under these conditions for 6 days, with media changes occurring every 2 days. Immunostaining was then performed using a monoclonal antibody directed towards the fast isoform of the major thick filament protein, skeletal myosin heavy chain (MHC) (Sigma; clone MY32). This procedure is described in detail elsewhere (8).

GST pulldowns

GST and GST fusion proteins were expressed in *Escherichia coli* (BL21) and purified using glutathione-agarose affinity chromatography as described previously (15). The GST fusion proteins were analysed on 10% SDS–PAGE gels for integrity and to normalise the amount of each protein. The Promega (Madison, WI) TNT-coupled transcription–translation system was used to produce [^{35}S]methionine-labelled MyoD and COUP-TF II proteins that were visualised by SDS–PAGE. *In vitro* binding assays were performed with glutathione agarose beads (Sigma, St Louis, MO) coated with ~500 ng GST fusion protein and 2–30 μl [^{35}S]methionine-labelled protein in 200 ml of binding buffer containing 100 mM NaCl, 20 mM Tris–HCl (pH 8.0), 1 mM EDTA, 0.5 % NP-40, 5 μg of ethidium bromide and 100 μg of BSA. The reaction was allowed to proceed for 1–2 h at room temperature with rocking. The affinity beads were then collected by centrifugation and washed five times with 1 ml of binding buffer without the ethidium bromide or BSA. The beads were then resuspended in 20 ml of SDS–PAGE sample buffer and boiled for 5 min. The denatured proteins were run on a 10% SDS–PAGE gel which was subsequently treated with Amersham Amplify fluor (Amersham, Arlington Heights, IL), dried and autoradiographed.

RESULTS

COUP-TF II inhibits myoD-mediated myogenic conversion of pluripotential C3H10T1/2 cells

We have previously demonstrated that proliferating C2C12 myoblasts express COUP-TF II mRNA and that constitutive expression of COUP-TF II cDNA in mouse C2 myogenic cells blocks morphological differentiation by suppressing the expression levels of the *myoD* gene family and critical cell cycle regulators (14,15). Moreover, preliminary evidence suggests that COUP-TF II directly regulates the transcription of the bHLH/contractile gene families and/or the cell cycle regulators (P.B. and G.M., unpublished data). However, the fact that COUP-TF II represses transcription in the presence and absence of its cognate binding motif, and interacts with the corepressors N-CoR and SMRT, raises the scenario that COUP-TF II may control MyoD activity or function by post-transcriptional mechanisms, which include transrepression.

We tested this hypothesis by investigating the impact of SV40-driven COUP-TF II expression on the MyoD-mediated myogenic conversion of pluripotential C3H10T1/2 cells. In this system, transient transfection of an EMSV-MyoD expression vector under the control of a moloney sarcoma virus promoter leads to the phenotypic/myogenic conversion of these cells as scored by immunostaining with a monoclonal antibody directed against a muscle-specific marker. The antibody is against the fast isoform of the major thick filament protein, skeletal MHC. MHC is associated with the contractile phenotype and is expressed during the process of muscle differentiation. Utilisation of an EMSV-driven MyoD expression vector, which is essentially refactory to repression by COUP-TF II, assured that any observed affects of COUP-TF II on phenotypic conversion were independent of myoD down-regulation, and mediated by post-transcriptional events. We demonstrated that the identical moloney sarcoma virus promoter linked to the CAT reporter in the same vector was not affected/regulated by COUP-TF II expression. We cotransfected increasing amounts of either pSG5, the SV40 expression vector alone, or pSG5-COUP-TF II with EMSV-CAT. We observed that at the concentrations of DNA transfected in this experiment, neither the vector alone nor pSG5-COUP-TF II affected the activity of the EMSV promoter (Fig. 1A).

In the initial experiment, C3H10T1/2 cells were transiently transfected with EMSV-MyoD in combination with either pSG5 (vector/vehicle only) or pSG5-COUP-TF II. As expected, cells transfected with EMSV-MyoD and pSG5 were found to be positively stained with the MHC antibody. (Fig. 1B, panel b). In stark contrast, cells that were transfected with both MyoD and wild type COUP-TF II did not undergo myogenic conversion (Fig. 1B, panel c).

To delimit the region within COUP-TF II which is involved in the ablation of the MyoD-mediated acquisition of the myogenic phenotype, we cotransfected 10T1/2 cells with EMSV-MyoD and expression vectors encoding the AB, ABCD and CDE regions of COUP-TF II (Fig. 1C). After immunostaining, it was found that the ABCD and CDE regions were sufficient to inhibit myogenic conversion (Fig. 1B, panels e and f). In contrast, the AB region of COUP-TF II failed to ablate the MyoD-mediated acquisition of the contractile phenotype (Fig. 1B, panel d). This is in agreement with previous studies that show that the CD region of COUP-TF II (encoding the DBD and hinge domain) blocks the

trans-activation of GAL4-dependent reporter genes by the potent chimeric trans-activator GAL4VP16 (16).

We then examined whether forced expression of the myogenic factors myoD, myogenin, myf-5 and MRF-4 could overcome the block mediated by COUP-TF II. C3H10T1/2 cells were transiently transfected with expression vectors for the myogenic factors myoD, myogenin, myf-5 and MRF-4 in the presence and absence of COUP-TF II. It was found that although cotransfection of C3H10T1/2 cells with the complete repertoire of myogenic factors results in very efficient myogenic conversion, as scored by positive αMHC immunostaining, COUP-TF II still blocks the acquistion of the contractile phenotype (Fig. 1D, panel b versus panel c).

In summary, the experiments presented above suggest that the CDE region of COUP-TF II is involved in the inhibition of myoD-mediated myogenic conversion of pluripotential C3H10T1/2 cells. Furthermore, the orphan nuclear receptor abrogates 10T1/2 myogenic conversion in the absence of its cognate binding motif. The ability of COUP-TF II to over-ride the entire repertoire of myogenic factors also suggests that it may sequester or bind essential cofactors.

COUP-TF II inhibits myoD-mediated trans-activation of a reporter gene downstream from a MyoD-dependent heterologous promoter

The immunohistochemical analysis suggested that COUP-TF II acts to repress 10T1/2 myogenic conversion by a mechanism which is independent of MyoD gene expression. Hence, it was of interest to determine whether COUP-TF II could repress MyoD-dependent transcription in the absence of its cognate binding site. To achieve this we utilised a 4RE-tkLUC reporter construct, in which four copies of the right E box (CANNTG motif) of the muscle specific MCK enhancer are placed upstream of a minimal thymidine kinase promoter linked to the LUC reporter gene. This reporter construct does not contain a cognate binding site for COUP-TF II, and has been demonstrated to facilitate MyoD-dependent trans-activation.

C3H10T1/2 cells were cotransfected with the 4RE-tkLUC reporter in combination with either EMSV-MyoD and/or COUP-TF II sense and anti-sense expression vectors. As expected, co-transfection of the reporter with EMSV-MyoD alone activated the expression of the LUC reporter gene very efficiently (Fig. 2A). However, cotransfection of 10T1/2 cells with EMSV-MyoD and increasing quantities of the pSG5 COUP-TF II expression vector (i.e. 100, 300 and 500 ng) significantly repressed MyoD-mediated trans-activation in a dose-dependent fashion (Fig. 2A). In contrast, cotransfection of EMSV-MyoD with the anti-sense COUP-TF II construct did not have any affect on MyoD trans-activation. This result clearly shows that COUP-TF II is capable of blocking MyoD-mediated trans-activation in a dose-

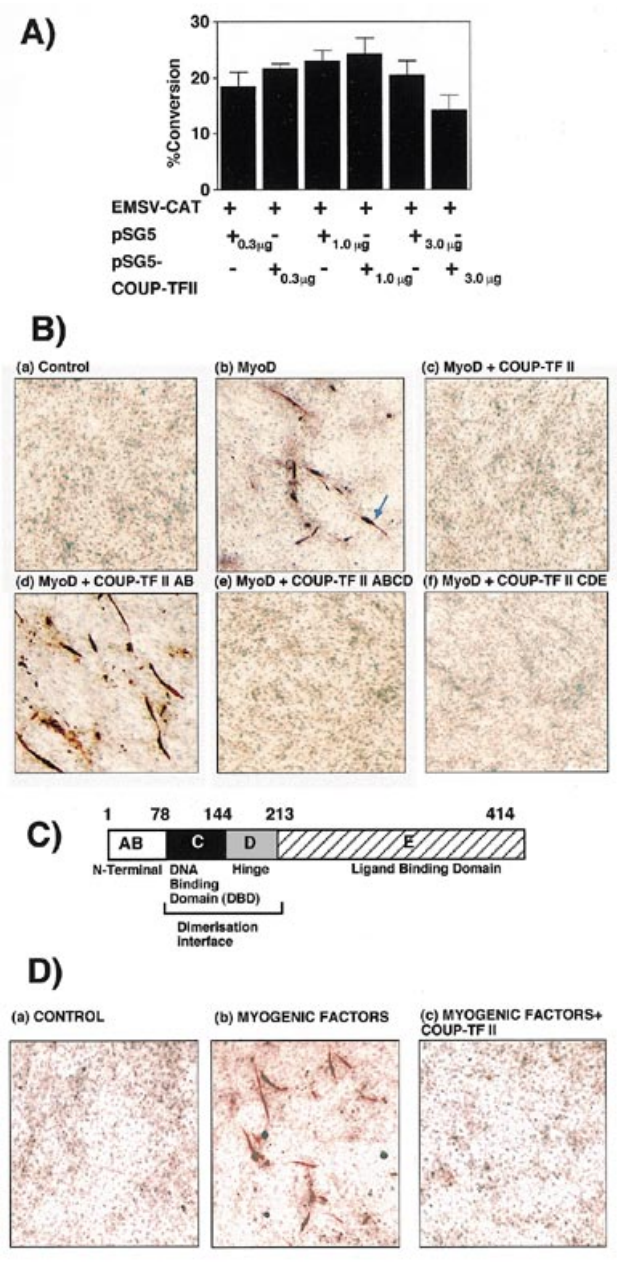

Figure 1. (**A**) COUP-TF II expression does not affect the activity of the EMSV promoter. C3H10T1/2 cells were transiently co-transfected with EMSV-CAT in combination with either increasing quantities (0.3–3.0 μg) of pSG5 alone or pSG5-COUP-TF II. They were subsequently assayed for CAT activity; results shown are mean ± standard deviation and were derived from three independent transfections. (**B**) COUP-TF II inhibits the myoD-mediated myogenic conversion of pluripotential C3H10T1/2 cells. The ABCD and CDE regions of COUP-TF II are sufficient to inhibit myogenic conversion. C3H10T1/2 cells were transiently transfected with EMSV-MyoD in combination with either pSG5 alone (panel b), pSG5-COUP-TF II (panel c), pSG5-COUP-TF II AB (panel d), pSG5-COUP-TF II ABCD (panel e) or pSG5-COUP-TF II CDE. Myogenic conversion was then scored with respect to the untransfected control cells (panel a) by immunostaining with a monoclonal antibody directed towards the fast isoform of the major thick filament protein, skeletal αMHC after 4 days in 2% horse serum. MHC-positive cells stain red. (**C**) Diagrammatic representation of COUP-TF II, highlighting the key domains: AB domain (i.e. aa 1–78); C domain or DBD (i.e. aa 79–144); D domain or Hinge (i.e. aa 145–213); and E domain of putative LBD (i.e. aa 214–414). (**D**) Inhibition of 10T1/2 myogenic conversion by COUP-TF II is not overcome by the forced expression of the myogenic factors myoD, myogenin, myf-5 and MRF-4. C3H10T1/2 cells were transiently transfected with expression vectors for the myogenic factors MyoD, myogenin, myf-5 and MRF-4 in the absence (panel b) and presence (panel c) of COUP-TF II. As before, myogenic conversion was scored with respect to the untransfected control cells (panel a) by immunostaining with an MHC antibody. MHC-positive cells are stained red and highlighted by an arrow.

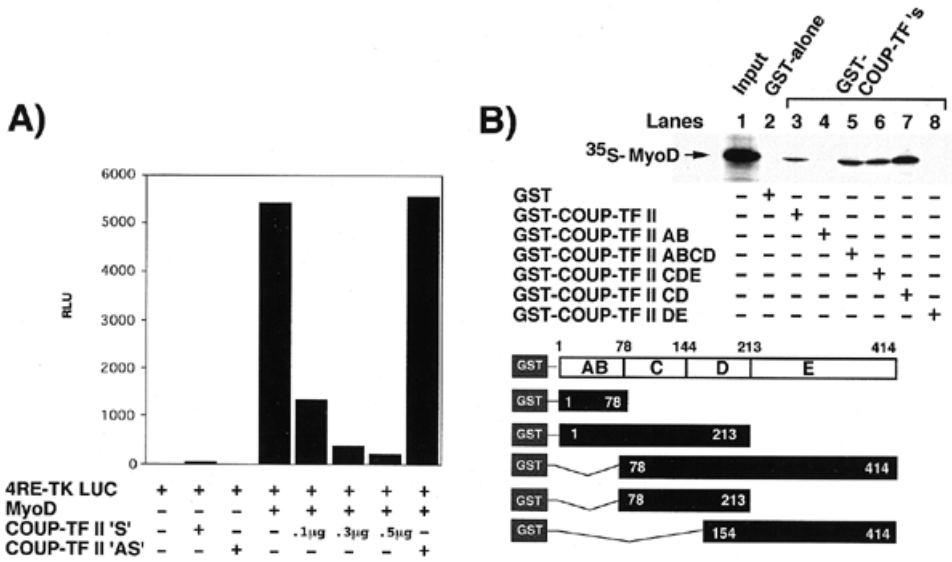

Figure 2. (**A**) COUP-TF II inhibits the MyoD-dependent transactivation of a 4RE reporter construct via a mechanism that is independent of DNA binding. NIH 3T3 cells were transiently transfected with the 4RE-tkLUC reporter (1 µg) in combination with either MyoD (100 ng), COUP-TF II sense 'S' (100, 300 or 500 ng) or COUP-TF II anti-sense 'AS' (500 ng) as indicated (+). After 48 h, cells were harvested and assayed for luciferase activity as described in the Methods and Materials. (**B**) COUP-TF II directly interacts with MyoD *in vitro*; the CD region of COUP-TF II alone is capable of mediating this interaction. MyoD was radiolabelled with [^{35}S]methionine by *in vitro* transcription/translation [the input (lane 1) represents ~10% of the total protein] and was assayed for its ability to interact with glutathione-immobilised GST alone (lane 2), GST-COUP-TF II (lane 3), GST-COUP-TF II AB (lane 4), GST-COUP-TF II ABCD (lane 5), GST-COUP-TF II CD (lane 6), GST-COUP-TF II CDE (lane 7) and GST-COUP-TF II DE (lane 8). The retained proteins are shown in lanes 3–8.

dependent manner via a trans-repression mechanism which is independent of COUP-TF II DNA binding.

COUP-TF II directly interacts with MyoD: the CD region of the nuclear receptor mediates the interaction

We postulated that COUP-TF II could be functioning to repress MyoD activity/function by directly interacting with MyoD. To determine this we utilised the *in vitro* GST pulldown assay, in which glutathione agarose-immobilised GST-COUP-TF II was incubated with *in vitro* ^{35}S-radiolabelled full-length MyoD. This assay clearly showed that COUP-TF II and MyoD were indeed capable of a direct interaction *in vitro* (Fig. 2B).

To map the region within COUP-TF II that was involved in mediating this interaction, a variety of COUP-TF II domains were sub-cloned into the GEX-1 expression vector, producing the fusion protiens GST-COUP-TF II AB, GST-COUP-TF II ABCD, GST-COUP-TF II CD, GST-COUP-TF II CDE and GST-COUP-TF II DE. The full-length and deleted GST-COUP-TF II chimeric proteins were then incubated with *in vitro* translated MyoD. Interestingly, the COUP-TF II domains ABCD, CD and CDE interacted strongly with full-length MyoD (Fig. 2B). In contrast, neither the AB region nor the DE region retained the ability to interact with full-length MyoD. This demonstrated that the CD region of COUP-TF II, which spans aa 78–213 and encodes the DBD and hinge region, is required for MyoD binding. This observation was also confirmed by examining the ability of *in vitro* translated full-length COUP-TF II to interact with GST-MyoD (Fig. 3A). As expected, we observed specific binding between ^{35}S-radiolabelled COUP-TF II and GST-MyoD *in vitro*. These experiments suggested protein–protein interactions were involved in the transrepression of myoD-mediated transcription.

The N-terminal acid-rich activation domain of MyoD mediates the interaction with COUP-TF II

To identify the domain within MyoD (Fig. 3B) which interacts with COUP-TF II, immobilised GST-COUP-TF II was incubated in combination with either ^{35}S-radiolabelled N-terminal MyoD (aa 1–100) or C-terminal MyoD (aa 162–318). It was found that the N-terminal acid-rich activation domain of MyoD, but not the C-terminal region, interacted with COUP-TF II (Fig. 3C). It should be noted that the N-terminal activation domain of MyoD has also been demonstrated to interact with p300.

To rigorously confirm that the N-terminal of MyoD interacts with COUP-TF II, we examined the ability of a number of GST-MyoD fusion chimeras containing functional subdomains of MyoD and immobilised on glutathione agrose beads [i.e. GST-MyoD (aa 1–318), GST-N terminal MyoD (aa 1–100), GST-bHLH (aa 102–161) and GST-C-terminal MyoD (aa 162–318)] to interact with the ^{35}S-radiolabelled CDE region of COUP-TF II (Fig. 3B). As expected, MyoD and the N-terminal of MyoD linked to GST interacted strongly with *in vitro* translated COUP-TF II-CDE (Fig. 3D). In contrast, the GST-MyoD-C-terminal and -bHLH regions did not support any significant interaction with the CDE domain of COUP-TF II. These experiments suggest that the N-terminal acid-rich activation domain of MyoD, which is represented by the first 100 aa of the protein, is required for COUP-TF II binding.

The coactivator p300 alleviates COUP-TF II-mediated repression of MyoD-dependent trans-activation

Nuclear receptors have been demonstrated to inhibit AP-1 function, in the absence of any observable affects on DNA recognition, by competing for limiting amounts of p300/CBP

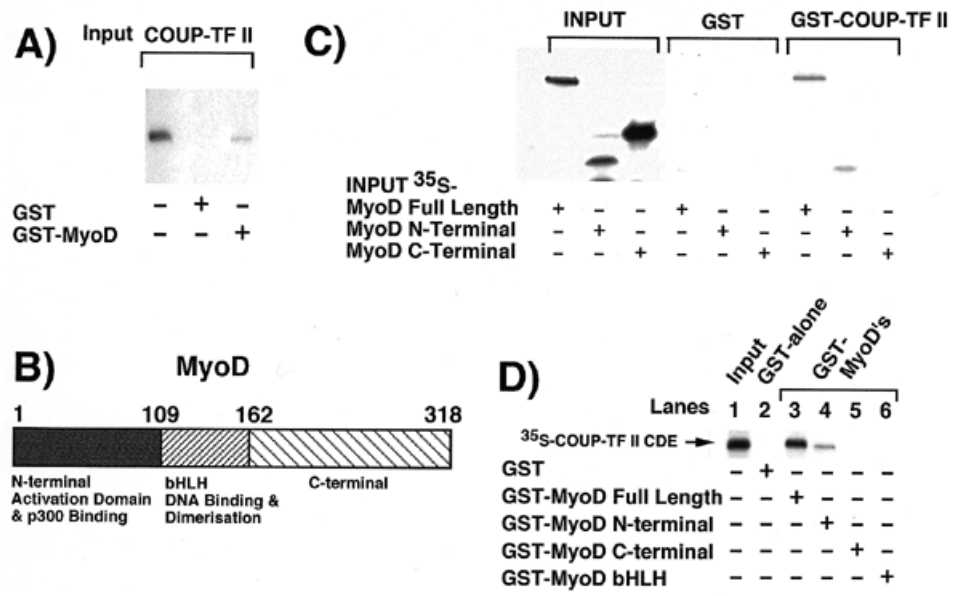

Figure 3. The N-terminal acid-rich activation domain of MyoD mediates the interaction with COUP-TF II *in vitro*. (**A**) GST pulldown showing an interaction between ^{35}S-radiolabelled COUP-TF II and glutathione agarose-immobilised GST-MyoD. The input lane represents ~10% of the total protein. (**B**) Diagrammatic representation of the MyoD protein, highlighting key regulatory domains. (**C**) Glutathione agarose-immobilised GST and GST-COUP-TF II proteins were incubated with either ^{35}S-radiolabelled full-length MyoD, N-terminal MyoD (aa 1–100) or C-terminal MyoD (aa 162–318). The input lanes represent ~10% of the total protein. (**D**) COUP-TF II directly interacts with the N-terminal of MyoD *in vitro*. The CDE domain of COUP-TF II was radiolabelled with [^{35}S]methionine by *in vitro* transcription/translation [the input (lane 1) represents ~10% of the total protein] and assayed for its ability to interact with glutathione-immobilised GST alone (lane 2), GST-MyoD full-length (lane 3), GST-MyoD N-terminal (lane 4), GST-MyoD C-terminal (lane 5) and GST-MyoD bHLH (lane 6). The retained proteins are shown in lanes 3–6.

(42). Competition for p300 in this case involves direct binding between p300 and nuclear receptors. In the context of muscle differentiation, p300 functions as a coactivator for MyoD-mediated trans-activation, and has been demonstrated to augment the trans-activation of genes which contain E-box elements in their enhancer regions. It was therefore of some interest to determine whether COUP-TF II could be functioning to repress MyoD-dependent trans-activation by competing for limiting amounts of p300. In doing so, we examined the effect of p300 over-expression on COUP-TF II-mediated repression of MyoD-dependent trans-activation. NIH 3T3 cells were cotransfected with the 4RE-tkLUC construct and COUP-TF II in the presence of either MyoD or MyoD and p300. If competition for limiting quantities of p300 also accounts for the inhibitory effect of COUP-TF II on MyoD transcriptional activity, then increased levels of p300 should restore/alleviate COUP-TF II-mediated repression. We found that cotransfection of p300 with COUP-TF II and MyoD almost completely rescued myoD transcriptional activity (Fig. 4A), suggesting that repression by COUP-TF II involves competition for limiting amounts of p300, presumably through protein–protein interactions.

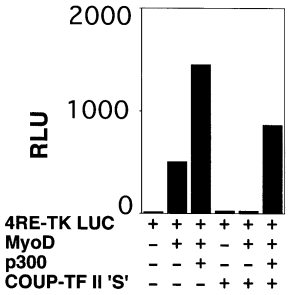

Figure 4. The coactivator p300 alleviates COUP-TF II-mediated repression of MyoD-dependent trans-activation. NIH 3T3 cells were transiently transfected with 1 µg of the 4RE-tkLUC construct, 100 ng of CMV-MyoD, 200 ng of CMV-βp300 and 300 ng of COUP-TF II as indicated (+). After 48 h, cells were harvested and assayed for luciferase activity.

COUP-TF II interacts with the N-terminal 149 aa receptor interaction domain (RID) of the cofactor p300 *in vivo*

Nuclear receptors have been shown to directly interact with the N-terminal RID encoded by aa residues 1–149 of p300 *in vivo* (Fig. 5A; 43). To determine if COUP-TF II also interacts with p300 *in vivo*, we utilised the mammalian two-hybrid assay. In this assay, the GAL4 DBD-p300 domain fusion chimeras, GAL4-p300-aa 1–149, -p300-aa 595–1240, and -p300-aa 1030–2414 were independently cotransfected in JEG-3 cells with either full-length VP16-COUP-TF II, VP16-COUP-TF II AB or VP16-COUP-TF II CDE. The capacity of the GAL-p300 and VP16-COUP-TF II fusion products to physically interact *in vivo* was then assessed by their ability to trans-activate a CAT reporter gene placed downstream of GAL4 binding sites linked to the E1b promoter. It was found that, like other members of the nuclear receptor superfamily, full-length COUP-TF II strongly interacts (~20-fold) with only the N-terminal RID of p300 (aa 1–149) (Fig. 5B). In comparison, with the nuclear receptor RXRγ (in the presence of ligand), the interaction of the N-terminal region of p300 with full-length COUP-TFII, is stronger [~5-fold; data not shown, and similar to that reported by Chakravarti *et al.* (43)]. It

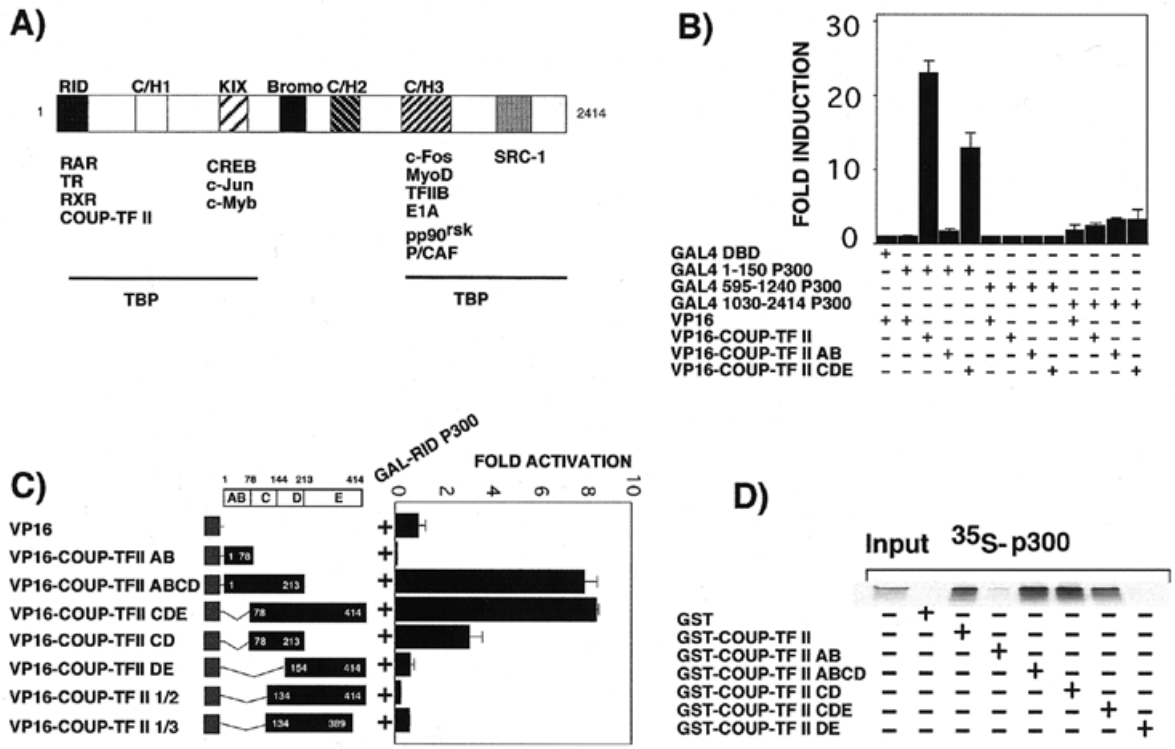

Figure 5. (**A**) Diagrammatic representation of the p300 protein, highlighting regions involved in protein–protein interactions. (**B**) COUP-TF II interacts with the RID of p300 *in vivo*. JEG-3 cells were transiently transfected with the G5e1bCAT reporter construct and expression vectors encoding either GAL4-p300-aa 1–149, GAL4-p300-aa 595–1240, GAL4-p300-aa 1030–2414, VP16-COUP-TF II full-length, VP16-COUP-TF II AB or VP16-COUP-TF II CDE as indicated (+). After 48 h on 10% charcoal stripped medium, cells were harvested and assayed for CAT activity. Results shown are mean ± standard deviation and were derived from at least three independent experiments. In each case, fold activation is relative to GAL4-p300 chimera/VP160 CAT activity, which is arbitrarily set at 1.0. (**C**) The CD region of COUP-TF II, which spans aa 78–213, interacts with the N-terminal 149 aa of p300 *in vivo*. JEG-3 cells were transiently transfected with the 3 mg G5e1bCAT reporter construct, 1 mg GAL4-p300-aa1–149 and 1 mg of the VP16-COUP-TF II chimeras as indicated (+). The results are presented as the mean ± standard deviation and were derived from at least three independent experiments. In each case, fold activation is relative to GAL4-p300-aa 1–149/VP160 CAT activity, which is arbitarily set at 1.0. (**D**) Direct binding between the CD region of COUP-TF II and full-length p300 *in vitro*. p300 was radiolabelled with [^{35}S]methionine by *in vitro* transcription/translation and assayed for its ability to interact with glutathione-immobilised GST, GST-COUP-TF II, GST-COUP-TF II AB, GST-COUP-TF II ABCD, GST-COUP-TF II CD, GST-COUP-TF II CDE and GST-COUP-TF II DE.

was also found that the RID of p300 interacted efficiently with the C-terminal region of COUP-TF II which encodes the CD and E domains [i.e. DBD, hinge and putative ligand binding domain (LBD)]. In contrast, the AB region of COUP-TF II did not interact with the RID of p300 (Fig. 5B).

The cofactor p300 directly interacts with the COUP-TF II CD region *in vivo* and *in vitro*

To delimit the region within COUP-TF II which interacts with the p300 RID *in vivo*, we again utilised the mammalian two-hybrid assay. In doing so, the VP16 chimeras VP16-COUP-TF AB, ABCD, CDE, CD, DE, 1/2 and 1/3 (depicted in Fig. 5C) were independently cotransfected with GAL RID p300 and assayed for their ability to trans-activate the G5e1bCAT reporter. It was found that the CD region of COUP-TF II, which encodes the DBD and hinge (i.e. aa 78–213), was sufficient to interact with the RID of p300. Interestingly, the VP16-COUP-TF II chimeras 1/2 and 1/3, which contain only part of the DBD (i.e. from aa 134), were not able to interact with the RID of p300, suggesting that the entire DBD is required for binding.

To confirm that COUP-TF II and p300 interact directly, we used the *in vitro* GST pulldown assay. Equal quantities of glutathione agarose-immobilised GST (negative control) and GST-COUP-TF II full-length and various sub-domains (GST-COUP-TF II AB, GST-COUP-TF II ABCD, GST-COUP-TF II CD, GST-COUP-TF II CDE and GST-COUP-TF II DE) were incubated with *in vitro* ^{35}S-radiolabelled full-length p300. The resulting pulldown clearly showed a direct interaction between full-length COUP-TF II and *in vitro* translated p300 (Fig. 5D). Furthermore, the CD region of COUP-TF II, spanning aa 78–213, is required for the interaction with p300 (Fig. 5D). Interestingly, this is the same region that interacts with MyoD. It should be noted that the ABCD and CDE regions of COUP-TF II are also sufficient to repress the MyoD-dependent conversion of C3H10T1/2 cells and that the DBD of COUP-TF II (aa 79–144) is essential for the repression of trans-activation in the absence of the cognate binding motif and nucleic acid binding.

COUP-TF II, p300 and MyoD interact in a competitive manner

We then embarked on experiments designed to analyse whether competitive binding between these interacting proteins was an issue with respect to the formation of a ternary complex. We examined the ability of increasing amounts of COUP-TF II CDE

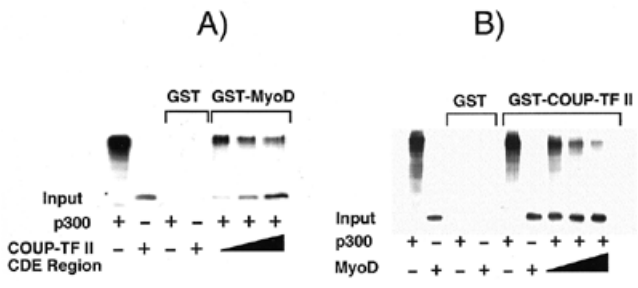

Figure 6. COUP-TF II, p300 and MyoD interact in a competitive manner *in vitro*. (**A**) Glutathione-immobilised GST-MyoD protein was incubated with 0.5 µl of ^{35}S-radiolabelled p300 and either 2, 4 or 8 µl of radiolabelled COUP-TF II CDE as indicated. In each pulldown, the input lanes represent ~10% of the total protein. (**B**) Glutathione-immobilised GST-COUP-TF II protein was incubated with 1 µl of ^{35}S-radiolabelled p300 and either 2, 4 or 8 µl of radiolabelled MyoD as indicated.

to affect the efficiency of interaction between GST-MyoD and radiolabelled p300 (Fig. 6A and B). We observed that increasing amounts of radiolabelled COUP-TF II CDE reduced the ability of a fixed amount of ^{35}S-labelled p300 to interact with GST-MyoD. Similarly, we observed that increasing amounts of myoD reduced the ability of p300 to interact with COUP-TF II (Fig. 6C).

This suggested that the interaction between COUP-TF II, p300 and myoD is competitive in nature, and that COUP-TF II may be involved in the process that regulates the interaction between MyoD and its cofactor, p300, which is essential for trans-activation of gene expression.

DISCUSSION

The regulation of myogenesis (i.e. muscle differentiation) is intimately controlled by a group of muscle specific bHLH proteins encoded by the *myoD* gene family (myoD, myogenin, myf-5, MRF-4) (reviewed in 1–3). The products of the *myoD* gene family are involved in a variety of protein–protein interactions with many factors that mediate transcription [e.g. E12 and E47, TFIIB; 7,44], control the cell cycle (e.g. RB; 45), and regulate chromatin accessibility and architecture (p300 and PCAF; 11). These protein–protein interactions regulate cellular proliferation and activate myogenic specific transcription that encodes the contractile phenotype. Insights into the process of myogenesis have been provided by the *myoD* gene family of transcription factors, because they function at the nexus of command circuits that control the mutually exclusive events of division and differentiation.

The nuclear receptor superfamily are potent regulators of development, differentiation, homeostasis and organ physiology; however, their functional role in mammalian muscle differentiation, regulation of MyoD and myogenesis has only been resolved at a descriptive level. The classical ligand activated nuclear receptors (e.g. thyroid hormone receptor) promote differentiation and muscle maturation *in vivo* (12,46). However, the orphan nuclear receptors including Rev-erbα, RVR and COUP-TF II, which are abundantly expressed in skeletal muscle and proliferating myogenic cells, antagonistically regulate muscle differentiation and appear to be involved in the maintenance of the proliferative state (13–15).

This study was directed toward identifying some of the mechanistic aspects of orphan nuclear receptor-mediated repression of muscle differentiation and myogenic conversion. Our work suggested that repression of MyoD-mediated trans-activation and myogenic conversion by COUP-TF II involves direct interactions with MyoD and its coactivator, p300. Furthermore, the study provides another line of evidence in a biological context which demonstrates that COUP-TF II-mediated repression of MyoD-dependent trans-activation occurs in the absence of the orphan receptor cognate binding motif. This observation strengthens the view that trans-repression by COUP-TF II has a functional role during mammalian muscle differentiation. Finally, it demonstrates that direct crosstalk between the orphan nuclear receptor and bHLH pathways has functional consequences for the regulation of differentiation and phenotypic acquisition. This mode of action and crosstalk between two central regulatory components may turn out to be utilised in other pathways of mammalian differentiation.

Specifically, our study showed that COUP-TF II could repress MyoD-mediated myogenic conversion of pluripotential 10T1/2 cells, when myoD expression was driven by a viral promoter refractory to COUP-TF II action. This immunochemical analysis suggested that the CDE region (but not the N-terminal AB region) of COUP-TF II was necessary for the repression of myoD-mediated myogenic conversion. In particular, the C and D regions of the orphan receptor, between aa residues 78 and 213, which encode the DBD and hinge region, respectively, were specifically involved in these events. We observed that a MyoD-dependent reporter gene driven by four multimerised MyoD binding sites (from the MCK-enhancer), cloned upstream of the herpes simplex thymidine kinase promoter, was specifically inhibited by COUP-TF II expression. Our study suggested that one of the mechanisms involved in this process of repression was the direct interaction between MyoD and COUP-TF II. Additional *in vitro* experiments demonstrated that the interaction was mediated by the acid-rich N-terminal activation domain of MyoD (which also mediates p300 binding) and the CD region of COUP-TF II. Furthermore, COUP-TF II interacted with the N-terminal RID (aa 1–149) of the cofactor p300. This is perhaps not surprising, since many other classical ligand activated nuclear receptors (e.g. RAR, TR, RXR, etc.) interact with the coactivator p300 (42). However, the fact that the DBD (C-region) and hinge/D-region of COUP-TF II mediate the interaction to p300 and MyoD is novel, and perhaps suprising. With respect to the *in vitro* and *in vivo* requirement of the COUP-TF II DBD and hinge region (aa 79–213) as a dimerisation interface for myoD and p300, it should be mentioned that this domain contains the DR and T box motifs, which have been implicated as dimerisation interfaces in RXR (and other nuclear receptors). Furthermore, they are highly homologous to the similar motifs found in RXR, which is expected, since COUP-TF II and RXR belong to the same nuclear receptor sub-family (47,48). The DR box has been strongly implicated in the heterodimerisation of TR and RAR with RXR. The DR boxes in RXRγ and COUP-TF II are highly homologous. Similarly, the T-box sequence that forms a third helix in RXR, and is implicated in homo- and heterodimerisation, is very similar in COUP-TF II and RXR (49–53). Recently, COUP-TF II and SpI have been demonstrated to synergistically regulate the transcription of the HIV type I LTR (54). In support of our observations, they demonstrated that the *in vitro* and *in vivo* physical interaction with SpI is mediated by the DBD of COUP-TF. Furthermore, the

Octamer transcription factors are recruited by the C-region (DBD) of the glucocorticoid receptor (55).

COUP-TF II-mediated repression of trans-activation involves the cofactor p300, since co-expression with p300 alleviates the silencing of transcription. Our study suggested that repression may involve sequestration of this vital cofactor for myoD-mediated trans-activation, since COUP-TF II directly interacts with p300. Whether inhibition of MyoD-dependent trans-activation by COUP-TF II binding is a result of: (i) a conformational change in MyoD (which is detrimental to function); (ii) a prevention of p300/cofactor/coactivator binding; (iii) and/or sequestering of cofactors (e.g. p300) by COUP-TF II is unclear at present. However, the *in vitro* GST-pulldown data does suggest that MyoD, COUP-TF II and p300 interact in a competitive manner, and that increasing amounts of COUP-TF II have the ability to reduce the interaction between myoD and p300 *in vitro*.

Achatz and colleagues have demonstrated that repression of transcription by COUP-TF II activity is dependent on the DBD and a segment spanning aa residues 193–399 (30). Single point mutations in the DBD that ablated sequence-specific binding did not affect the silencing of transcription. Futhermore, Bailey *et al.* (16) demonstrated that the repression of GAL4VP16-mediated trans-activation involves the DBD of COUP-TF II in the absence of its cognate binding motif. Achatz suggested that the regions critical for repression of trans-activation by COUP-TF II were involved in mediating protein–protein interactions. In the present study, we show that the DBD and hinge region of COUP-TF II alone are sufficient to bind either MyoD or p300. Deletions of COUP-TF II that contain only part of the DBD (i.e. from aa 134), however, are not able to interact with the RID of p300, suggesting that the entire DBD is required for binding. Although the DBD and hinge region functions as a dimerisation interface in COUP-TF II, we also observed that the presence of the E-region strengthens the specificity of the interaction (G.E.O.M. and P.J.B., unpublished observations). While this manuscript was being reviewed, it was demonstrated by Froeschle *et al.* (56) that the DBD of the retinoic acid receptor also directly interacts with MyoD. Our data and the above data suggested that the DBD of the nuclear receptors may also encode a dimerisation interface for the bHLH family of proteins.

Forced expression of COUP-TF II in C2 myogenic cells blocks differentiation and in particular ablates the induction of p21 mRNA expression (14). Moreover, in normal proliferating C2 cells, COUP-TF II mRNA is expressed, however, during cell cycle withdrawal and differentiation, the mRNA levels are repressed (15). Transcription of the p21 gene is specifically stimulated by MyoD during myogenic differentiation, and is coactivated by a transcriptional complex that contains both p300 and PCAF (11). In the present study, we show that MyoD-mediated transcription is significantly repressed by COUP-TF II. This suggests that COUP-TF II is involved in the maintenance of the proliferative state, by controlling the trans-activation of the p21 gene.

Nuclear receptors have also been shown to interact with a growing family of PAS/bHLH domain coactivators (e.g. SRC1/N-CoA-1, TIF-2/GRIP-1, ACTR/RAC-3/pCIP) (57–59). However, the N-terminal bHLH/PAS domains in these coactivators function as activation domains, interact with other proteins or are involved in target gene specificity. There has been no documented interactions between these regions of the cofactors and the nuclear receptors.

In conclusion, the present study provides evidence that suggests that one of the mechanisms that mediates repression of muscle differentiation and myogenic conversion by COUP-TF II involves direct interactions with MyoD and its coactivator, p300. Whether the direct crosstalk between the orphan nuclear receptor and bHLH pathways has target gene specificity in a developmental context remains to be elucidated, and will be a focus of future studies in the context of mammalian differentiation.

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