

# Exogenous expression of a dominant negative ROR $\alpha$ 1 vector in muscle cells impairs differentiation: ROR $\alpha$ 1 directly interacts with p300 and MyoD

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## ABSTRACT

ROR/RZR is an orphan nuclear receptor that has no known ligand in the 'classical sense'. In the present study we demonstrate that ROR $\alpha$  is constitutively expressed during the differentiation of proliferating myoblasts to post-mitotic multinucleated myotubes, that have acquired a contractile phenotype. Exogenous expression of dominant negative ROR $\alpha$ 1 $\Delta$ E mRNA in myogenic cells significantly reduces the endogenous expression of ROR $\alpha$ 1 mRNA, represses the accumulation and delays the activation of mRNAs encoding MyoD and myogenin [the muscle-specific basic helix–loop–helix (bHLH) proteins] and p21<sup>Waf-1/Cip-1</sup> (a cdk inhibitor). Immunohistochemistry demonstrates that morphological differentiation is delayed in cells expressing the ROR $\Delta$ E transcript. Furthermore, the size and development of multinucleated myotubes is impaired. The E region of ROR $\alpha$ 1 interacts with p300, a cofactor that functions as a coactivator in nuclear receptor and MyoD-mediated transactivation. Consistent with the functional role of ROR $\alpha$ 1 in myogenesis, we observed that ROR $\alpha$ 1 directly interacts with the bHLH protein MyoD. This interaction was mediated by the N-terminal activation domain of the bHLH protein, MyoD, and the ROR $\alpha$ 1 DNA binding domain/C region. Furthermore, we demonstrated that p300, ROR $\alpha$ 1 and MyoD interact in a non-competitive manner. In conclusion, this study provides evidence for a biological role and positive influence of ROR $\alpha$ 1 in the cascade of events involved in the activation of myogenic-specific markers and cell cycle regulators and suggests that crosstalk between the retinoid-related orphan (ROR) nuclear receptors and the myogenic bHLH proteins has functional consequences for differentiation.

## INTRODUCTION

Members of the nuclear receptor superfamily bind specific DNA elements and function as transcriptional regulators (1,2). This group includes the 'orphan receptors' which have no known

ligands in the 'classical sense'. The orphan receptor ROR/RZR $\alpha$  (retinoic acid receptor-related orphan receptor) is closely related to Rev-erbA $\alpha$ , RVR/Rev-erb $\beta$ /BD73 and *Drosophila* orphan receptor E75A, particularly in the DNA binding domain (DBD) and the putative ligand binding domain (LBD). ROR, Rev-erbA $\alpha$  and RVR bind as monomers to an asymmetric (<sup>A</sup>/<sub>T</sub>)<sub>6</sub>RGGTCA motif. ROR functions as a constitutive transactivator of gene expression; in contrast, Rev-erbA $\alpha$  and RVR do not activate transcription, mediate transcriptional repression and can repress constitutive transactivation from this motif by ROR $\alpha$  (3–9).

Three ROR/RZR genes have been identified. ROR $\alpha$  encodes four ROR $\alpha$  isoforms,  $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 3 and RZR $\alpha$ , which are alternatively spliced products of the ROR $\alpha$  gene and are predominantly expressed in blood, brain, skeletal muscle and fat cells (8,10). ROR $\beta$ /RZR $\beta$  is expressed specifically in the brain (11) and ROR $\gamma$  is found at high levels in skeletal muscle (12–14).

Although, ROR $\alpha$  is expressed in skeletal muscle and myogenic cells, its functional role has not been established. However, evidence for a physiological/biological role of this group of related but oppositely acting nuclear orphan receptors has come from cell culture studies. Exogenous expression of Rev-erbA $\alpha$  and RVR in muscle cells antagonistically regulates differentiation and inhibits expression of the hierarchical *myoD* gene family and the cdk inhibitor p21<sup>Waf-1/Cip-1</sup> (15–17), which control muscle-specific gene expression and cell cycle exit during myogenesis, respectively. Furthermore, during myogenic differentiation, the expression of Rev-erbA $\alpha$  and RVR mRNAs is repressed, hence, one of our objectives was to elucidate the functional role of ROR $\alpha$  in muscle differentiation and to identify putative targets of ROR $\alpha$  action in muscle.

Muscle differentiation is the process whereby proliferating myoblasts permanently exit the cell cycle and fuse to become post-mitotic, multinucleated myotubes with a contractile phenotype, that express myogenic markers (reviewed in 18,19). Insights into this process have been provided by the identification of a group of basic helix–loop–helix (bHLH) proteins encoded by the *myoD* gene family (*myoD*, *myf-5*, *myogenin* and *MRF-4/myf-6/herculin*). The products of the *myoD* gene family are muscle-specific transactivators that can direct cell fate, repress proliferation and activate differentiation and the contractile phenotype. The muscle-specific bHLH proteins function at the nexus of command circuits

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that control the mutually exclusive events of division and differentiation (reviewed in 18–20). Gene targeting studies have suggested that while *myoD* and *myf-5* are required for determination (21), *myogenin* is specifically required for differentiation (22).

MyoD plays a dual role during myogenesis, activating both muscle-specific gene transcription and promoting cell cycle exit by inducing the expression of p21<sup>Waf-1/Cip-1</sup>, an inhibitor of cyclin-dependent kinases and cellular proliferation (23–28). Transactivation by MyoD involves: (i) the bHLH domain, that is involved in both DNA binding and dimerization; (ii) the heterodimerization of MyoD with the ubiquitously expressed E2A gene products, E12 and E47 (27); (iii) the binding of MyoD–E2A heterodimers to specific E-box motifs (CANNTG) in muscle-specific enhancers (reviewed in 23–25); (iv) the recruitment of the cofactors p300 and PCAF (29–31). The cofactors, p300 and PCAF, are critical co-activators for MyoD during myogenic commitment and differentiation. The N-terminal activation domain of MyoD directly interacts with p300 and recruits PCAF to form a ternary multimeric complex on promoter elements (29,32). These events lead to hyperacetylated and transcriptionally permissive chromatin. Moreover, p300 and PCAF co-activate myoD-mediated transactivation of the *p21* gene and are necessary for MyoD-mediated cell cycle arrest (32).

The transcriptional activity of MyoD is modulated by environmental cues related to the concentration of growth factors, receptors and oncogene products that promote cell division (reviewed in 20). 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 Id (inhibitor of differentiation) expression, an HLH protein that lacks DNA binding ability and functions as a dominant negative; (iii) the suppression/sequestering of myogenic-specific transcription factors and cofactors.

Co-activators that mediate transactivation by nuclear receptors include TIF1, ERAP160, RIP140 and p300/CBP. CBP (CREB binding protein) and the parahomologue p300 are integrators of multiple signal transactivation pathways. p300/CBP has been shown to interact with both nuclear receptors and the transcriptional machinery through multiple dimerization interfaces (33–35). The involvement of the p300 family with orphan receptors, in particular ROR $\alpha$ , is an unexplored area of nuclear receptor research.

To gain insight into the function of ROR $\alpha$ 1 in myogenesis we used loss of function studies in myogenic cells as a tool to elucidate the biological role of ROR in muscle. Ablation of ROR $\alpha$  expression in muscle cells was achieved by the exogenous expression of a dominant negative ROR $\alpha$  $\Delta$ E. We observed that these cells no longer express endogenous ROR $\alpha$  and that the onset/accumulation and expression of myogenic-specific markers and cell cycle regulators had been altered. Furthermore, the molecular basis of these effects involves direct interactions of ROR with: (i) the myogenic-specific bHLH protein MyoD; (ii) p300, a cofactor that functions as a co-activator of MyoD- and nuclear receptor-mediated transactivation.

## MATERIALS AND METHODS

### Plasmids construction

pCMX-ROR $\alpha$ 1 was kindly provided by V.Giguere. 5'-Primer GMUQ253 (GCGGAATTCACCATGGAGTCAGCTCCGGC-AGCC) and 3'-primer GMUQ254 (GCGGAATTCCTACCCAT-

CAATTTGCATTGC), containing *EcoRI* sites, were used to synthesise the 523 amino acid full-length open reading frame of ROR $\alpha$ 1. The product was end-filled and cloned into the *SmaI* site of pBS (Stratagene). Plasmid pBS-ROR was digested with *EcoRI* and the insert was subsequently cloned into pGEX-1N and pSG5 to form pGEX-ROR and pSG5-ROR. pGAL constructs were prepared by subcloning in-frame restricted fragments of pBS-ROR, with or without end-filling with Klenow, into the multiple cloning site of vector pGAL0 (36) that contains the GAL4 DBD sequence. Likewise, VP16 constructs of ROR $\alpha$ 1 were prepared utilizing the multiple cloning site of pNLVP16. All pGAL and pVP16 and pGEX clones have been checked and found to be in the correct orientation. Double-stranded DNA sequencing shows that the inserts were in the correct reading frame.

**Construction of p300 plasmids.** The CMVbp300 plasmid was cleaved with *BamHI* between amino acids 595 and 1240 and the resulting fragment was end-filled with and cloned into *SalI*-cleaved/end-filled GAL0 plasmid. The p300 plasmid was cleaved with *ScaI* and *HindIII* between amino acids 1030 and 2414 and the resulting fragment was end-filled with and cloned into *SalI*-cleaved/end-filled GAL0 plasmid. The N-terminal end of p300 (amino acids 1–149) was produced by PCR, using primers GM320 (GCG GTC GAC ATA TGG CCG AGA ATG TGG TG) and GM322 (GCG GTC GAC TGA CTG CGT AGG ACC CTG) and cloned into *SalI*-cleaved GAL0.

### Cell culture and transient transfections

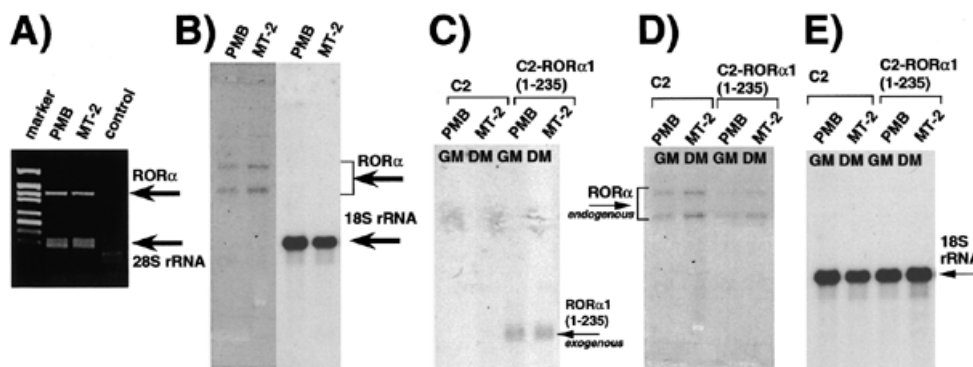
**Mammalian two hybrid assay.** Plasmids (1  $\mu$ g of G5E1bCAT reporter and 0.33  $\mu$ g of GAL-N-CoR/RIP13 or p300) were co-transfected/expressed in human choriocarcinoma JEG3 cells with either VP16 or VP16–ROR (0.33  $\mu$ g), then assayed with respect to their ability to transactivate the reporter (G5E1bCAT). Each 12-well dish of JEG3 cells (80% confluence) was transiently transfected with plasmid DNA by the DOTAP/DOSPER (Boehringer Manneheim)-mediated procedure as described previously (17).

**CAT assays.** The cells were harvested, normalised to protein concentration and the CAT activity was measured as previously described (37). Aliquots of the cell extracts were incubated at 37°C with 0.1–0.4 mCi of [<sup>14</sup>C]chloramphenicol (NEN) in the presence of 5 mM acetyl-CoA and 0.25 M Tris–HCl pH 7.8 and analysed as described previously (15–17).

**Stable transfections of C2C12.** Myogenic C2C12 cells cultured in 20% FCS in DMEM to 40% confluence were co-transfected with pCMV-NEO and either pSG5-ROR(1–235) or antisense pSG5-ROR by the DOTAP-mediated procedure. The cells were then grown for another 24 h to allow cell recovery and neomycin resistance expression before G418 selection. After 14 days selection with 400  $\mu$ g/ml G418 in culture medium, stable transfectants were cultured and maintained on 200  $\mu$ g/ml G418 medium.

### GST pulldown assay

pGEX-ROR was transformed into BL21 competent cells and grown in LB medium. The GST fusion proteins were released after IPTG induction and sonication. Lysates were bound to equilibrated glutathione beads and then extensively washed and left bound in NETN-1 buffer (0.5% NP-40, 1 mM EDTA, 20 mM



**Figure 1.** Northern analysis of endogenous and exogenous ROR $\alpha$ 1 gene expression in native C2C12 cells and stably transfected ROR $\alpha$ 1 dominant negative C2C12 cells. **(A)** ROR $\alpha$  gene expression is shown by RT-PCR with 28S rRNA as control from proliferating C2C12 (PMB) and 48 h differentiating (MT-2) C2C12 cells with negative H<sub>2</sub>O control and DNA marker (Materials and Methods). **(B)** Analysis of ROR $\alpha$  gene expression in C2C12 PMB and MT-2 cells by northern hybridization of poly(A)<sup>+</sup> RNA with mouse ROR $\alpha$  probe and 18S rRNA as control. **(C)** Proliferating C2C12 (PMB) cells (at <40% confluence) were cultured to confluence in growth medium (GM) (20% FCS in DMEM) and thereafter the medium was replaced with differentiation medium (DM) (2% horse serum in DMEM). C2C12 cells were differentiated for 2 days (MT-2) before harvesting for poly(A)<sup>+</sup> RNA extraction and analysis by northern blotting. Northern blots were probed with ROR $\alpha$ 1 showing expression of the transfected dominant negative ROR $\alpha$ 1 gene. **(D)** Mouse ROR $\alpha$  cDNA showing endogenous expression of ROR $\alpha$  in transfected and untransfected C2C12 and **(E)** end-labelled 18S rRNA probe. (Materials and Methods gives details on transfection; this data is derived from three independent hybridisations of a single blot.)

Tris pH 8.0, 100 mM NaCl) with Complete protease inhibitor cocktail (Boehringer Manneheim). GST-MyoD, GST-MyoD N-terminus, GST-MyoD HLH and GST-MyoD C-terminus have been described by Sartorelli *et al.* (29). pBluescript-p300, pSG5-MyoD, EMSV-MyoD and pSG5-ROR $\alpha$ 1 were transcribed and translated using the rabbit reticulolysate TnT kit according to the manufacturer's instructions (Promega). pGEX-ROR or pGEX-MyoD beads or control pGSTag were incubated for 40 min at room temperature with <sup>35</sup>S-labelled proteins in NETN-1 buffer with protease inhibitors and blocking agents (BSA and EtBr). The beads were extensively washed three times with NETN-1. Bound proteins were released by adding SDS-PAGE sample buffer and heated to 95°C for 10 min. The samples were run on miniprotein 10 or 12% SDS-PAGE gels (Bio-Rad) as described by Sartorelli *et al.* (29).

### Northern analysis

Total RNA was extracted by the acid guanidinium thiocyanate phenol/chloroform method. Poly(A)<sup>+</sup> RNA was further extracted from total RNA using a mRNA isolation kit (Boehringer Manneheim). Total RNA and poly(A)<sup>+</sup> RNA were electrophoresed in a formaldehyde gel and vacuum blotted onto Hybond N membranes. The membranes were subsequently UV crosslinked and dried in an incubator. Prehybridized membranes were hybridized at 42°C in 4× Denhardt's solution, 45% formamide, 0.2% SDS, 0.1 mg/ml herring sperm DNA with random primed DNA probes. After 2 days, the membranes were washed twice in 0.5–2× SSC, 0.1% SDS at 65°C for 30 min. Autoradiographs were scanned using a computer scanner and Adobe Photoshop.

### Multiplex RT-PCR

C2C12 total RNA was reverse transcribed by Superscript reverse transcriptase (Gibco) using random hexamers as primers. The first strand product was subsequently amplified by touchdown PCR using Taq polymerase and mouse ROR $\alpha$  primers (L-primer, GMUQ305, GCGAGATCTTTCATCAGCTTTGTGTTTGAATTT; R-primer, GMUQ306, GCGAGATCTGGCGGACATT-

TACCCATCGATTT) and 28S rRNA primers (L-primer, primer A, ATCTAGTAGCTGGTTCCTC; primer B, CCTCTAATCATT-CGCTTTAC).

## RESULTS

### ROR $\alpha$ is constitutively expressed in myogenic C2C12 cells

ROR $\alpha$  has been shown to be expressed in skeletal muscle tissue. This observation suggested that this protein may play a role during mammalian myogenesis. Furthermore, the closely related but oppositely acting Rev-erbA/RVR orphan nuclear receptors are abundantly expressed in proliferating myogenic cells; however, they are repressed during myogenic differentiation. To elucidate the functional role of ROR $\alpha$  in mammalian differentiation we investigated the expression of ROR $\alpha$  mRNA relative to 18S/28S rRNA during myogenic differentiation in the mouse C2C12 myoblast cell line. Using the Genbank sequence of ROR $\alpha$  to design specific primers for mouse ROR $\alpha$  amplification, we were able to amplify the mouse ROR $\alpha$  mRNA transcript from total RNA isolated from proliferating and differentiated C2C12 cells by multiplex RT-PCR (Fig. 1A). Subcloning and DNA sequencing identified the expressed amplified product as ROR $\alpha$ . We further substantiated that ROR $\alpha$  was constitutively expressed during mouse myogenesis in culture by northern hybridisation analysis of RNA isolated from proliferating and differentiated C2C12 cells (after 48 h serum withdrawal) (Fig. 1B). ROR $\alpha$  is seen transcribed as a double band in C2C12 as described in differentiated P19 cells (9). This data demonstrated that the ROR $\alpha$  mRNA transcript was constitutively expressed during myogenic differentiation in culture, relative to the 18S and 28S rRNAs (Fig. 1A and B).

### Exogenous dominant negative ROR $\alpha$ expression delays the activation and significantly reduces the expression of the MyoD, myogenin and p21<sup>Waf-1/Cip-1</sup> mRNAs

Proliferating C2C12 cells can be induced to biochemically and morphologically differentiate into post-mitotic, multinucleated myotubes by serum withdrawal in culture over a 48–96 h period.

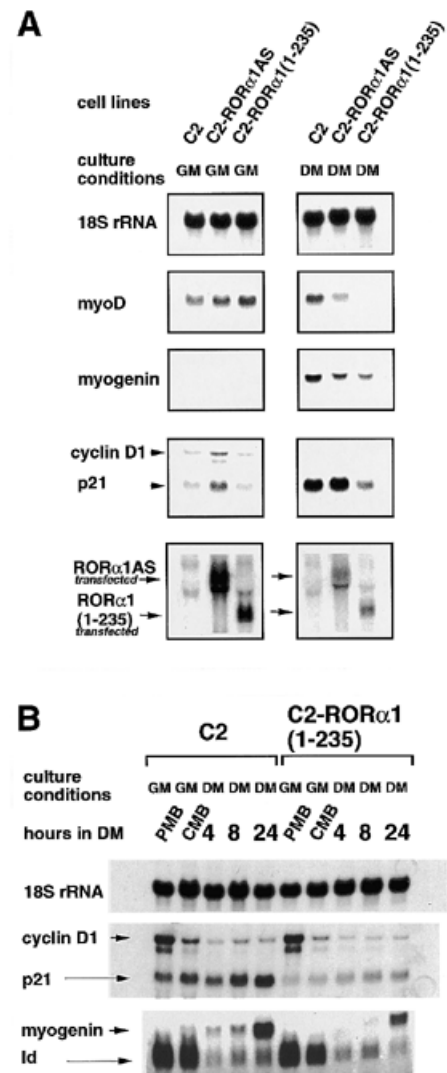
This transition from a non-muscle phenotype to a contractile phenotype is associated with repression of non-muscle proteins and activation of a structurally diverse group of genes. This gene activation encodes a functional sarcomere responsible for the major activity of this specialised cell type, i.e. contraction. The events are characterised by the transition of the actin multigene family. Non-muscle  $\beta$ - and  $\gamma$ -actins are down-regulated; in contrast, the sarcomeric cardiac and skeletal  $\alpha$ -actins are induced. These isoform transitions correlate with the repression of cyclin D1 (that is involved in the maintenance of the proliferative state), induction of the muscle-specific bHLH gene *myogenin* and the cdk inhibitor p21, that are involved in activation of muscle-specific gene expression and permanent cell cycle arrest, respectively.

To understand the biological role of ROR $\alpha$ 1 during myogenesis and to identify the putative target(s) of this orphan receptor in muscle cells, we proceeded to examine the effect of knocking out/ablating ROR $\alpha$  function in myogenic C2C12 cells. We stably (and independently) transfected C2C12 cells with dominant negative and antisense ROR $\alpha$  expression vectors with pCMV-NEO. Stable transfectants were isolated as a polyclonal pool of G418-resistant colonies (comprised of >20 individually resistant colonies). The C2-ROR $\alpha$ 1(1–235) (also denoted as C2-ROR $\Delta$ E) cells were transfected with pSG5-ROR(1–235), which contained a cDNA that only encoded amino acids 1–235. This dominant negative construct lacked the entire E region and part of the hinge/D region. This was chosen because the ‘staggerer’ phenotype in mice is due to a frameshift at amino acid 273 (38), and McBroom *et al.* (39) reported that deletion of this region preserved DNA binding but destroyed transactivation. Furthermore, we noted that ROR $\alpha$ 1(1–235) was not sufficient to transactivate gene expression from an optimal and single monomeric response element linked to the basal tk-CAT reporter (data not shown). In contrast, C2-ROR $\alpha$ 1 AS cells were transfected with the construct pSG5-ROR $\alpha$ 1 AS, which contained the full-length ROR $\alpha$  transcript cloned in the antisense orientation.

The C2-ROR $\alpha$ 1(1–235) cells abundantly expressed the transfected/exogenous transcript relative to the endogenous full-length transcript (Fig. 1C and D, respectively). Interestingly, we observed that the endogenous levels of the ROR $\alpha$  mRNA transcripts were significantly reduced in the C2-ROR $\alpha$ 1(1–235) cells (Fig. 1D) relative to the equivalent levels of 18S rRNA (Fig. 1E). Similarly, we detected the expression of the ROR $\alpha$  antisense transcript in the C2-ROR $\alpha$ 1 AS cells (Fig. 2A).

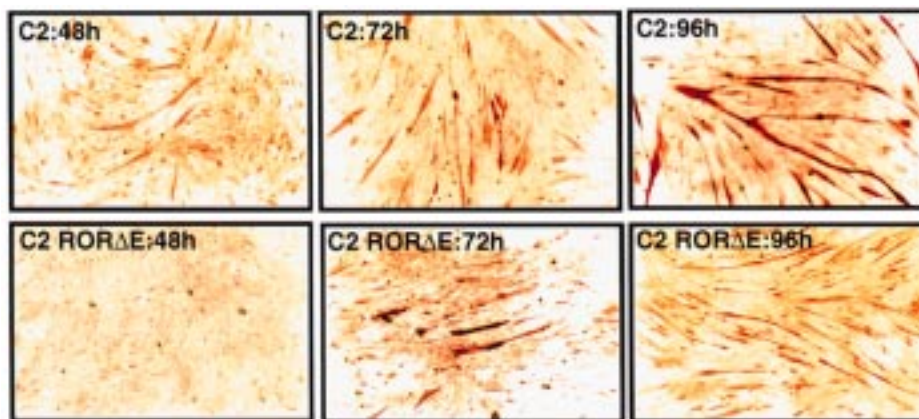
Down-regulation of the endogenous ROR $\alpha$  transcripts is not unexpected; mRNA pool sizes during myogenesis and in muscle tissue are under strict control (40), a mechanism exists that senses total output from exogenous and endogenous genes (37). Furthermore, exogenous expression of a number of different contractile protein transgenes in the mouse (e.g. myosin light chain 2, troponin I fast and skeletal and cardiac actin) results in a decline in the expression of the corresponding endogenous gene (37,41–44).

To investigate the effect of exogenous dominant negative and antisense expression on factors involved in the regulation of myogenesis (e.g. MyoD and myogenin) and the control of the cell cycle (e.g. cyclin D1 and p21). We isolated total RNA from normal, C2-ROR $\alpha$ 1 AS and C2-ROR $\alpha$ 1(1–235) C2C12 proliferating myoblasts (cultured in growth medium) and myotubes (after 96 h serum withdrawal). RNA samples were northern blotted and probed for 18S rRNA, MyoD, myogenin, cyclin D1 and p21 mRNA expression. Northern analysis revealed important differences in the



**Figure 2.** Analyses of the functional role of ROR $\alpha$ 1 in muscle differentiation. Proliferating C2C12 cells (at <40% confluence) are cultured to confluence in growth medium (GM) (20% FCS DMEM) thereafter medium is replaced with differentiation medium (DM) (2% horse serum DMEM). C2C12s were differentiated for 1–4 days before being harvested for total RNA extraction and northern hybridization. Multinucleated myotubes are formed after 4 days. (A) Changes in gene expression observed in native C2C12 cells, and cells that exogenously express ROR $\alpha$ 1 anti-sense and dominant negative transcripts, before and after 96 h of differentiation. (B) Changes in gene expression observed in native C2C12 cells, and cells that exogenously express the ROR $\alpha$ 1 dominant negative transcripts during the first 24 h of differentiation. (See Materials and Methods for details on transfection.)

biochemical profile during myogenesis. In contrast to normal C2C12 cells, the cells stably transfected with antisense ROR $\alpha$ 1 and dominant negative ROR $\alpha$ 1(1–235) had reduced steady-state levels of MyoD mRNA after the induction of differentiation by serum withdrawal (Fig. 2A). Furthermore, the level of myogenin activation/induction was reduced in the stably transfected cell lines. Interestingly, dominant negative ROR $\alpha$ 1 expression had a stronger impact than expression of the antisense construct on MyoD and myogenin expression (Fig. 2A). The induction/activation of p21, which induces cell cycle exit, is also blocked in the



**Figure 3.** Exogenous ROR $\Delta$ E expression delays myotube development. Native C2C12 cells and C2-ROR $\Delta$ E cells were harvested after 2–4 days in 2% horse serum and were immunostained with a monoclonal antibody directed towards the fast isoform of the major thick filament protein, skeletal myosin heavy chain (MHC). MHC-positive cells are stained red.

C2-ROR $\alpha$ 1(1–235) cell line (Fig. 2A). The altered biochemical profile of mRNA expression in these stably transfected cells correlated with the reduced ability of the C2-ROR $\alpha$ 1(1–235) cells to differentiate in culture. This stable transfection analysis suggested that ROR $\alpha$ 1 expression affected the expression of two members of the muscle-specific bHLH gene family, *myoD* and *myogenin*, and the cdk inhibitor, p21<sup>Waf-1/Cip-1</sup>, that are involved in the activation of myogenic transcription and cell cycle exit, respectively.

To determine whether the reduced steady-state levels of MyoD, myogenin and p21 in the C2-ROR $\alpha$ 1(1–235) cell line were due to a slower rate of differentiation, we conducted a time course study. We isolated total RNA from C2 and C2-ROR $\alpha$ 1(1–235) cells as proliferating myoblasts (PMB), confluent myoblasts (CMB, harvested 24 h after harvesting of PMB cells), and 4, 8 and 24 h after serum withdrawal in DM (i.e. 4, 8 and 24 h after the harvesting of the CMB sample in GM). These RNAs were northern blotted and probed with 18S rRNA, myogenin, Id, cyclin D1 and p21 (Fig. 2B). Northern analysis demonstrated that the rate of terminal differentiation in the C2-ROR $\alpha$ 1(1–235) cell line is delayed. The time of myogenin and p21 mRNA induction is delayed by 8 and 24 h, respectively. Furthermore, the extent of myogenin and p21 mRNA accumulation is reduced. The expression of these genes that are responsible for initiating the differentiation process and arresting the cell cycle, respectively, has been altered by dominant negative expression of the ROR $\alpha$ 1 variant, suggesting that the ROR $\alpha$ 1 orphan nuclear receptor has a positive influence on the differentiation programme of myogenic cells.

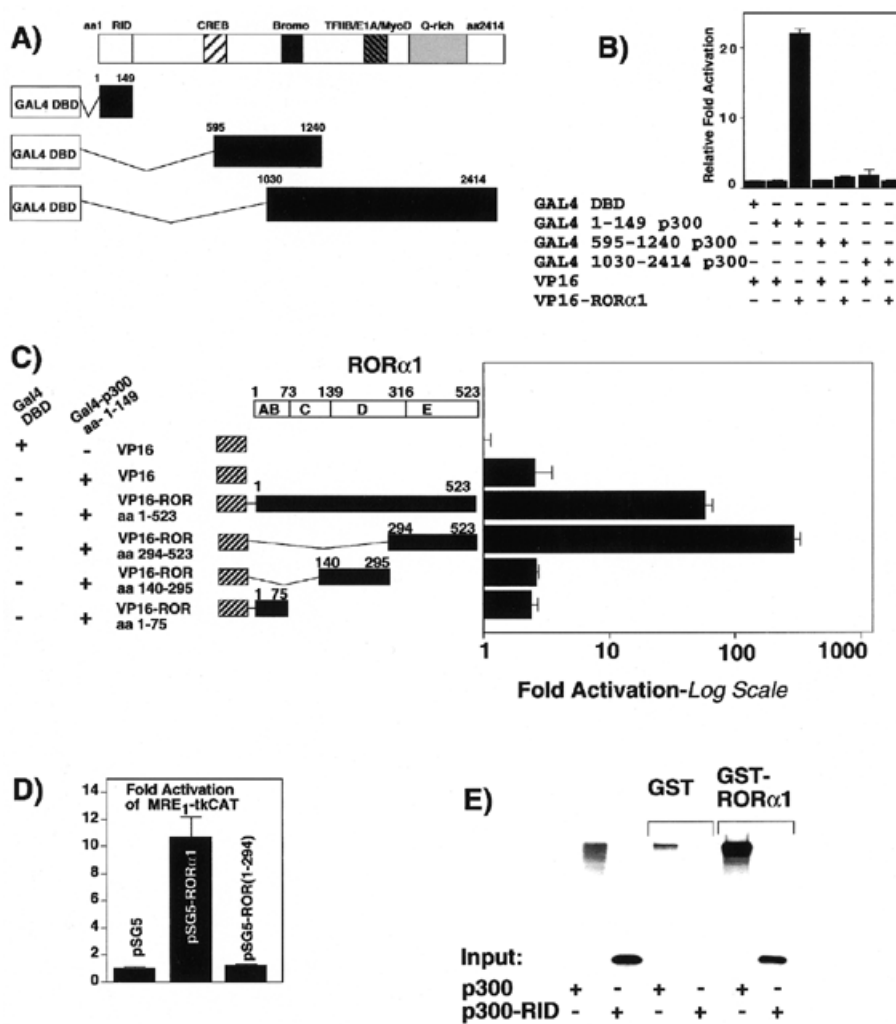
To put these results into a morphological context we examined the extent of myogenic conversion in the C2:ROR(1–235) cells (denoted C2-ROR $\Delta$ E cells in these experiments) relative to native C2C12 cells; as measured by immunostaining with a monoclonal antibody directed against a muscle-specific contractile protein marker. The antibody is against the fast isoform of the major thick filament contractile protein, skeletal myosin heavy chain (MHC). MHC is associated with the contractile phenotype and is expressed during the process of muscle differentiation. Immunostaining of native C2C12 cells after 48 h serum withdrawal (to induce differentiation) in native C2C12 cells detected the appearance of multinucleated myotubes (Fig. 3). In contrast,

ROR $\Delta$ E cells after 48 h serum withdrawal lacked any significant cellular immunostaining of MHC or myotube structures. After 96 h serum withdrawal significant immunostaining was observed in both cell types; however, the native myotubes consistently appeared larger, longer and more developed (Fig. 3). These experiments are consistent with a delay in the time of and extent of induction of the mRNAs regulating differentiation (e.g. myogenin) and cell cycle exit (e.g. p21) observed in the northern analysis above.

### ROR $\alpha$ 1 directly interacts with the co-activator p300

Exogenous expression of the dominant negative ROR construct in myogenic cells suggested that this member of the orphan nuclear receptor family plays a positive role in myogenesis. We then examined whether the constitutive transactivator, ROR $\alpha$ 1, interacts with the cofactor, p300. This coactivator of nuclear receptor- and MyoD-mediated transactivation is an important regulator of cell cycle control (and p21 mRNA expression) during muscle differentiation. CBP/p300 is a cofactor that interacts with a host of transcription factors and mediates transcriptional signalling, either through its histone acetylase activity or by functioning as a bridge to recruit another coactivator(s) with histone acetyltransferase activity, e.g. P/CAF (45,46). Regions of p300 that have been demonstrated to interact with a variety of transcription factors are schematically depicted in Figure 4A. In particular, the N-terminal region of p300, between amino acids 1 and 149, has been shown to function as a receptor interaction domain (RID) and to interact with a number of nuclear receptors, including RAR, TR, RXR (33–35) and COUP-TF II (47).

To investigate whether ROR $\alpha$ 1 interacts with p300 *in vivo*, we utilised the mammalian two-hybrid assay. We tested several constructs of p300 to determine which domain/region of the cofactor interacts with ROR $\alpha$ 1. The plasmids utilised in the mammalian two-hybrid assay included p300-aa1–149, p300-aa595–1240 and p300-aa1030–2414 that include regions of the cofactor that interact with the nuclear receptors and other known transcription factors (e.g. MyoD). These regions were fused to form chimaera with the GAL4 DBD vector. The GAL4–p300 fusion proteins were then tested for interaction with



**Figure 4.** RORα1 interacts with the co-activator p300 *in vivo* and *in vitro*. RORα1 interacts with p300 *in vivo*. (A) Schematic representation of p300, highlighting domains that interact with other transcription factors (50,57) and the domains linked to the GAL 4 DBD. (B) Analysis of the interaction of RORα1 with the p300 interaction domains GAL4-p300-aa1-149, GAL4-p300-aa595-1240 and GAL4-p300-aa1030-2414. (C) Interaction of GAL4-p300-aa1-149 with VP16-RORα1 chimaera. JEG3 cells were co-transfected with 1 μg of each GAL4 and VP16 chimera as indicated, with 3 μg of the reporter pG5E1bCAT. Results shown are mean ± SD and were derived from at least three independent experiments. Relative fold activation is expressed relative to CAT activity measured after transfection with the appropriate GAL-p300 chimera and VP16 vector alone, arbitrarily set to 1.0. (D) Transactivation of MRE<sub>1</sub>-tkCAT by pSG5 RORα1 in COS-1 cells. MRE-tkCAT was constructed by ligating the double-stranded Rev-erbAα monomeric response element to tkCAT. The clone was sequenced to contain only a single monomeric binding site. pSG5-ROR(1-2294) was constructed by restriction digestion of pSG5-RORα1 with *EcoRV* + *Bam*HI and *Sac*I + *Bam*HI and then end-filled by Klenow fragment and religated. Transfection and CAT assay were as described previously. (E) GST-RORα1 on glutathione beads was incubated for 40 min with *in vitro* transcribed and translated p300 in the presence of ethidium bromide and BSA. At the end of the incubation period, the beads were extensively washed and heated at 97°C for 10 min in sample dye and analysed by SDS-PAGE. Input was 25% of the amount used in the interaction reaction. GST-RORα1 interaction with p300 and the p300 RID.

a chimeric protein consisting of the full-length RORα1 cDNA linked in-frame to the VP16 activation domain in the mammalian cell line JEG3. VP16-RORα1 strongly interacted (~20-fold) with the N-terminal domain of p300 (amino acids 1-149) (Fig. 4B). RORα1 did not interact with the other domains of p300, indicating that RORα1 interacts only with the p300 RID. In comparison, with RXRγ (in the presence of ligand), the interaction of the N-terminal region of p300 with full-length RORα1 is stronger [~5-fold, and similar to that reported by Chakravarti *et al.* (34)] (data not shown).

To identify the p300 interaction region in RORα1, we cloned different regions of RORα1 into the VP16 vector and used the

mammalian two-hybrid assay to delimit the domain that mediated the interaction with p300 (Fig. 4C). The results show that the p300 RID interacts efficiently with RORα1 in the presence of an intact E region, VP16-RORα1(295-523). The hinge/D region and N-terminal/AB region of RORα1 had a negligible or limited role in p300 binding (Fig. 4C). The importance of this region was verified by demonstrating that deletion of the E region inhibited the ability of RORα1 to transactivate a monomeric response element (Fig. 4D).

The demonstration of an interaction between p300 and RORα1 in the *in vivo* mammalian two-hybrid assay strongly suggests that these proteins may interact by a direct mechanism. However, this

does not eliminate the possibility of an indirect mechanism in which an additional factor(s) mediates this interaction. We tested this hypothesis using a biochemical approach, the *in vitro* GST pull-down assay, to confirm that p300 and ROR $\alpha$ 1 interact directly. Glutathione-agarose immobilised GST-ROR $\alpha$ 1 was tested for direct interaction with *in vitro*  $^{35}\text{S}$ -radiolabelled full-length native p300 and the RID of p300. GST-ROR $\alpha$ 1 showed a direct interaction with full-length p300 (Fig. 4E) and the p300 RID (Fig. 4E).

In conclusion, the *in vivo* and *in vitro* assays studies demonstrate that ROR directly interacts with the cofactor p300, a critical component of nuclear receptor- and MyoD-mediated transcription. The efficiency of binding by the RID of p300 relative to the native protein is weakly compromised; whether this reflects the requirements of additional regions for binding is not clear and not supported by the *in vivo* two-hybrid studies.

### ROR $\alpha$ 1 directly interacts with MyoD: p300, ROR $\alpha$ 1 and MyoD interact in a non-competitive manner

During the course of this investigation it has been reported that the positive and negative effects of RXR and COUP-TF II on myogenesis, respectively, involve novel interactions between the DNA binding domain of these nuclear receptors and MyoD (47,48). Furthermore, the observations that ROR $\alpha$ 1 interacted with the cofactor p300, a coactivator of nuclear receptor- and MyoD-mediated transactivation and an important regulator of cell cycle control/p21 mRNA expression during muscle differentiation (28–30,49), suggested that ROR could be functioning to regulate MyoD activity/function by directly interacting with MyoD or be part of a complex with MyoD and p300.

To determine the validity of this hypothesis we utilised the *in vitro* GST pull-down assay, in which glutathione-agarose-immobilised GST-MyoD was incubated with *in vitro*  $^{35}\text{S}$ -radiolabelled full-length p300 and ROR $\alpha$ 1. This assay confirmed the interaction of p300 with MyoD and clearly showed that ROR $\alpha$ 1 and MyoD were indeed capable of a direct interaction *in vitro* (Fig. 5A).

Since MyoD has independently been shown to interact with both p300 and ROR $\alpha$ 1, we investigated whether MyoD could simultaneously interact with p300 and ROR $\alpha$ 1 in the GST pull-down assay. Hence, we examined the ability of glutathione-agarose immobilised GST-MyoD to interact with a mixture of *in vitro*  $^{35}\text{S}$ -radiolabelled full-length native p300 and ROR $\alpha$ 1 (Fig. 5A, lane 7). We observed that GST-MyoD could simultaneously pull-down p300 and ROR $\alpha$ 1. We then investigated whether competitive binding between these interacting proteins was an issue with respect to the formation of a putative ternary complex. We examined the ability of increasing amounts of p300 (1, 3 and 10  $\mu\text{l}$ ; Fig. 5A, lanes 7–9) to affect the efficiency of interaction between GST-MyoD and radiolabelled ROR (Fig. 5A). We did not observe a reduction in the efficacy of binding among these interacting proteins when the quantity of p300 was substantially elevated, suggesting that these factors interact in a non-competitive manner. Furthermore, we observed similar results when the quantity of affinity resin was substantially reduced (data not shown).

### The N-terminal activation domain of MyoD mediates the interaction with the DNA binding domain of ROR $\alpha$ 1

To identify the domain within MyoD (Fig. 5B) that interacts with ROR $\alpha$ 1, we examined the ability of a number of GST-MyoD

fusion chimaera containing functional subdomains of MyoD and immobilised on glutathione-agarose beads [i.e. GST-MyoD N-terminus (amino acids 1–100), GST-bHLH (amino acids 102–161) and GST-MyoD C-terminus (amino acids 162–318)] to interact with  $^{35}\text{S}$ -radiolabelled ROR $\alpha$ 1 (Fig. 5B). The N-terminus of MyoD linked to GST interacted strongly with *in vitro* translated ROR $\alpha$ 1. In contrast, the GST-MyoD C-terminus and GST-bHLH regions did not support any significant interaction with ROR $\alpha$ 1. These experiments suggest that the N-terminal activation domain of MyoD, which is represented by the first 100 amino acids of the protein, is required for ROR $\alpha$ 1 binding. Interestingly, the N-terminal activation domain also mediates the interaction with the coactivator, p300.

We then investigated whether the C-terminus of ROR $\alpha$ 1 was necessary to support the interaction with MyoD. Hence, we examined the ability of GST-MyoD immobilised on glutathione-agarose beads to interact with  $^{35}\text{S}$ -radiolabelled ROR $\alpha$ 1 segments between amino acid residues 1 and 294 and 1 and 235 (Fig. 5C). It was observed that both C-terminally deleted fragments interact very efficiently with MyoD *in vitro*. This data suggests that the C-terminus of ROR $\alpha$ 1, which encodes the E region of ROR, is not required for binding (Fig. 5C).

To delimit whether the N-terminal AB region or the DBD mediated the interaction with MyoD *in vitro*, we examined the ability of the fragments between amino acid residues 1 and 140 and 1 and 75 to interact with MyoD, relative to the native full-length protein. In contrast to the ability of the region between 1 and 140 to support efficient binding, the fragment between amino acids 1 and 75 did not significantly bind MyoD (Fig. 5D).

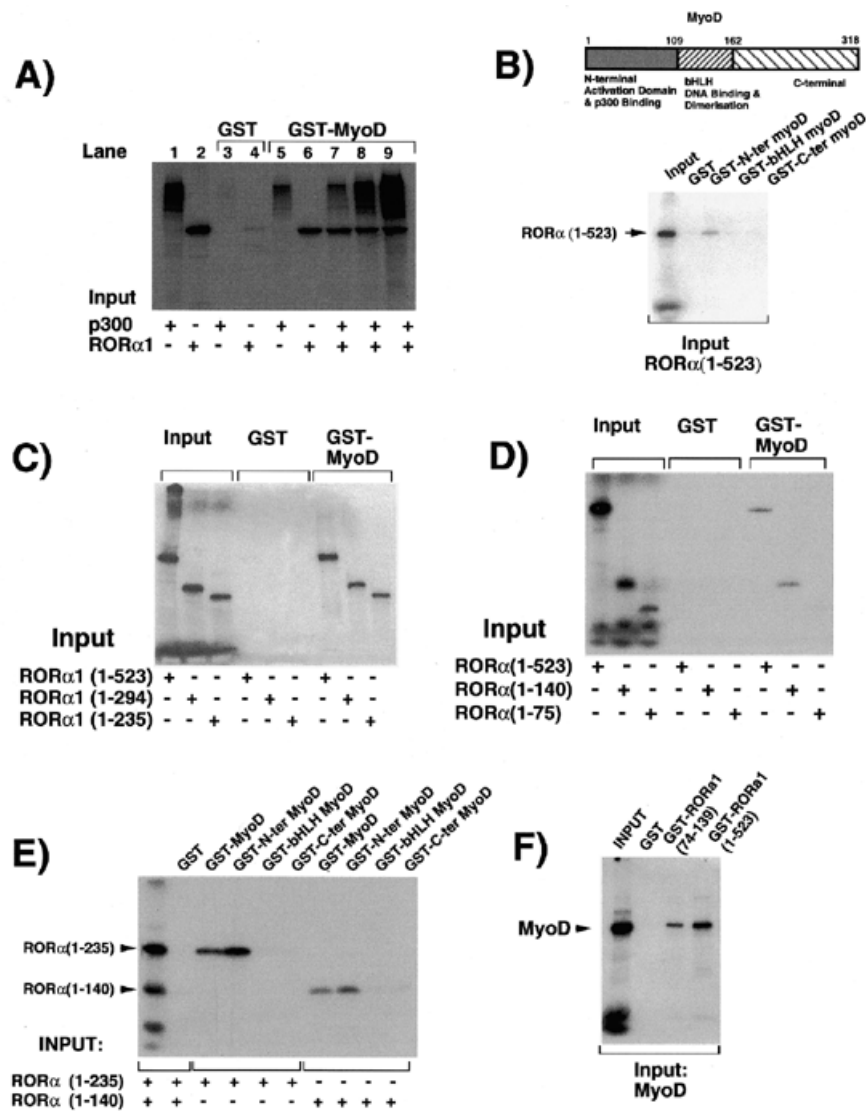
We similarly examined the ability of the region between amino acid residues 1 and 140, which encode the N-terminal AB region and the C region/DBD, relative to the amino acid residues between 1 and 294, which encode the ABCD region of ROR, to interact with various sub-domains of MyoD (Fig. 5E). We observed that the ABC region (amino acids 1–140) was sufficient to support the interaction with MyoD. Furthermore, it was sufficient to mediate the specificity of the interaction with the N-terminal region of MyoD linked to GST (Fig. 5E). This suggested that the C region of ROR was involved in mediating binding to MyoD.

Consistent with the observations above we also demonstrated a direct interaction between the DBD of ROR $\alpha$ 1 and MyoD in the GST pull-down assay. We showed that the C region/DBD alone between amino acids 74 and 139 of ROR efficiently interacted with the  $^{35}\text{S}$ -radiolabelled full-length MyoD, relative to native ROR protein linked to GST and immobilised on glutathione-agarose beads (Fig. 5F). The *in vitro* translated *myoD* product interacted with similar efficiency with GST-ROR $\alpha$  and GST-ROR $\alpha$ (74–139).

This suggests that the C region of ROR that encodes the DBD is necessary for the interaction with MyoD. Moreover, it suggests that this region also functions as a dimerization interface.

## DISCUSSION

Exogenous expression of the ROR $\Delta\text{E}$  dominant negative expression vector in C2 myogenic cells significantly reduced the expression of MyoD, myogenin and p21 (and endogenous ROR $\alpha$ ) mRNA levels after the induction of differentiation by serum withdrawal. Furthermore, the extent of morphological differentiation as assayed by immunocytochemistry was similarly effected. This suggested that native ROR $\alpha$ 1 functions to positively regulate myogenesis. These observations were entirely consistent with



**Figure 5.** ROR and p300 directly interact with MyoD *in vitro*. (A) ROR and p300 were radiolabelled with [<sup>35</sup>S]methionine by *in vitro* transcription/translation and assayed for their ability to interact with glutathione immobilised GST and GST-MyoD. Glutathione immobilised GST-MyoD protein was incubated with <sup>35</sup>S-radiolabelled ROR and either 1, 3 or 10 μl of radiolabelled p300. In each pull-down, the input lanes represent ~10% of the total protein. (B) The N-terminal acid-rich activation domain of MyoD mediates the interaction with ROR II *in vitro*. GST pull-down showing an interaction between <sup>35</sup>S-radiolabelled ROR and glutathione-agarose immobilised GST and GST-MyoD N-terminus, GST-MyoD C-terminus and GST-MyoD bHLH. The input lanes represent ~10% of the total protein. (C) The E region of ROR is not required for MyoD binding. GST pull-down showing an interaction between <sup>35</sup>S-radiolabelled ROR(1-523), (1-294) and (1-235) and glutathione-agarose immobilised GST and GST-MyoD. The input lanes represent ~10% of the total protein. (D) The C region/DBD of ROR functions as a dimerization interface. GST pull-down showing an interaction between <sup>35</sup>S-radiolabelled ROR(1-523), (1-140) and (1-75) and glutathione-agarose immobilised GST and GST-MyoD. The input lanes represent ~10% of the total protein. (E) The ABC region of RORα1 supports the interaction with the N-terminal acid-rich activation domain of MyoD *in vitro*. GST pull-down showing an interaction between <sup>35</sup>S-radiolabelled ROR(1-235) and (1-140) and glutathione-agarose immobilised GST, GST-MyoD, GST-MyoD N-terminus, GST-MyoD C-terminus and GST-MyoD bHLH. The input lanes represent ~10% of the total protein. (F) The DBD of RORα binds MyoD. GST pull-down showing an interaction between <sup>35</sup>S-radiolabelled MyoD with glutathione-agarose immobilised GST, GST-RORα1 and GST-RORα1(74-139). The input lanes represent ~10% of the total protein.

several observations, including: (i) the constitutive expression of RORα1 during myogenesis; (ii) the antagonistic effects of exogenous Rev-erb α (15) and RVR (16) expression on muscle differentiation (i.e. the closely related but oppositely acting orphan nuclear receptors); (iii) the exogenous expression of dominant negative RVRΔE (16) leading to increased levels of p21<sup>Waf-1/Cip-1</sup> and myogenin mRNAs after serum withdrawal and precocious biochemical and morphological differentiation.

This study also indicated that closely related orphan nuclear receptors, RORα, Rev-erbA and RVR, all target expression of the

bHLH proteins, MyoD and myogenin, and the cdk inhibitor p21 during myogenesis (15-17). Rev-erbA and RVR have antagonistic effects on muscle differentiation, whereas RORα has a positive influence on myogenesis. The opposing functional roles on similar gene targets that have critical effects in differentiation correlates with the contrasting properties of these nuclear receptors in transcriptional regulation.

Interestingly, exogenous expression of RORΔE in myogenic cells leads to repression of endogenous RORα mRNA; this is identical to the effect of exogenous RVRΔE expression in



myogenic cells, which resulted in repression of endogenous RVR mRNA expression. As discussed, this is not unexpected and quite a common observation in the myogenic system due to the regulation of mRNA pool sizes by a sensor system in myogenic cells that is receptive to mRNA produced from the endogenous and exogenous loci (37,40–44). This process in muscle serves to make exogenous dominant negative expression vectors quite a useful tool in the myogenic system because it results in very high levels of the transfected product relative to the endogenous target.

Rev-erbA and RVR function as dominant repressors of gene expression, silencing transcription via an interaction with the nuclear receptor co-repressor, N-CoR (17,50–56). Rev-erbA and RVR are expressed in proliferating mononucleated myoblasts, then suppressed during the differentiation of these cells into post-mitotic multinucleated cells that have acquired the contractile phenotype. Our study revealed that the cofactor, p300, a functional integrator/coactivator of diverse signal transactivation pathways (57), mediated ROR $\alpha$ 1-dependent transactivation. These observations are consistent with the functions of the Rev-erb family and ROR orphan nuclear receptors, as dominant repressors and activators of transcription, respectively. Furthermore, we have demonstrated that the N-terminal 149 amino acids of p300 directly interact with the E region of ROR $\alpha$ . This correlates with the requirements for transactivation by this receptor and strongly suggests that the function of this orphan receptor is modulated by positive cofactor interactions *in vivo*. The effect of the dominant negative expression vector in myogenic cells also correlates with the inability of this ROR product to interact with p300. Sartorelli and colleagues have demonstrated that p300 functions as an essential coactivator for the functions of MyoD and MEF-2 during myogenic conversion and transactivation (29). Puri and colleagues also demonstrated a requirement for p300 in MyoD-mediated/dependent cell cycle arrest (30). Subsequently, Puri, Sartorelli and colleagues demonstrated that p300 has a critical role in the recruitment of the histone acetyltransferase PCAF to the MyoD regulatory complex (32). These reports support the effects of the dominant negative ROR construct on MyoD, myogenin and p21 mRNA expression and the cell cycle.

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* and *MRF-4*). 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; 18–23), control the cell cycle (e.g. RB; 58) and regulate chromatin accessibility and architecture (p300 and PCAF; 29–32). These protein–protein interactions regulate cellular proliferation and activate myogenic-specific transcription that results in the contractile phenotype.

Our study suggests that one of the mechanisms involved in the functional role of ROR in myogenesis is a direct interaction between MyoD and ROR $\alpha$ 1. The interaction is mediated by the N-terminal activation domain of MyoD (which also mediates p300 binding) and the C region of ROR that encodes the DBD. The fact that the DBD (C region) of ROR $\alpha$ 1 mediates the interaction with MyoD is novel, and perhaps surprising. However, during the course of this investigation two other reports have demonstrated direct interactions between nuclear receptors (RXR and COUP-TF II) and MyoD (48,47). With respect to the requirement for the ROR $\alpha$ 1 DBD as a dimerization interface for MyoD, it should be mentioned that this domain contains the DR

and T box motifs, which have been implicated as dimerization interfaces in RXR (and other nuclear receptors). The DR box has been strongly implicated in the heterodimerization of TR and RAR with RXR (furthermore, ROR and RAR belong to the same nuclear receptor sub-family; 59,60). Furthermore, the T box sequence that forms a third helix in RXR has been implicated in homo- and heterodimerization (61–65). Recently, COUP-TF II and SpI have been demonstrated to synergistically regulate transcription of the HIV type I LTR (66). Consistent with our observations Rohr *et al.* demonstrated that the *in vitro* and *in vivo* physical interaction with SpI is mediated by the DBD of COUP-TF. Moreover, the Octamer transcription factors are recruited by the C region (DBD) of the glucocorticoid receptor (67) and the RXR DBD functions as a dimerization interface for PCAF (68).

Although we have demonstrated that MyoD can directly interact with ROR and p300 and that the bHLH proteins can simultaneously pull down the coactivator and the orphan nuclear receptor, the presence of a ternary complex has not been identified directly. Furthermore, our investigation did not provide any indication of competitive binding between these components of the activation complex. Hence, we propose a structure/model involving multiple contacts among these components that produces an active transcription complex that mediates transactivation of ROR-responsive genes during myogenesis. Finally, it demonstrates direct crosstalk between the orphan nuclear receptor and bHLH pathways that 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.

In conclusion, ROR1-mediated regulation of myogenic transcription involves the cofactor p300 and the bHLH factor MyoD. Whether 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. Current investigations examining the direct and indirect effects of ROR $\alpha$ 1 on the expression of and activity of the promoters of *myoD*, *myogenin* and *p21* will hopefully illuminate the entire role of ROR $\alpha$ 1 in myogenesis.

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