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Transcriptional repression by the orphan steroid receptor RVR/Rev-erbβ is dependent on the signature motif and helix 5 in the E region: functional evidence for a biological role of RVR in myogenesis

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ABSTRACT

RVR/Rev-erbβ/BD73 is an orphan steroid receptor that has no known ligand in the 'classical' sense. RVR binds as a monomer to an element which consists of an A/T-rich sequence upstream of the consensus hexameric half-site. However, RVR does not activate transcription and blocks transactivation of this element by ROR/RZR. The mechanism of RVR action remains obscure, hence we used the GAL4 hybrid system to identify and characterize an active transcriptional silencer in the ligand binding domain (LBD) of RVR. Rigorous deletion and mutational analysis demonstrated that this repressor domain is encoded by amino acids 416–449 of RVR. Furthermore, we demonstrated that efficient repression is dependent on the so-called LBD-specific signature motif, (F/W)AKxxxxFxxLxxxDQxxLL (which spans loop3–4 and helix 4) and helix 5 (H5; identified in the crystal structures of the steroid receptor LBDs). Although RVR is expressed in many adult tissues, including skeletal muscle, and during embryogenesis, its physiological function in differentiation and mammalian development remains unknown. Since other 'orphans', e.g. COUP-TF II and Rev-erbAα, have been demonstrated to regulate muscle and adipocyte differentiation, we investigated the expression and functional role of RVR during mouse myogenesis. In C2C12 myogenic cells, RVR mRNA was detected in proliferating myoblasts and was suppressed when the cells were induced to differentiate into post-mitotic, multinucleated myotubes by serum withdrawal. This decrease in RVR mRNA correlated with the appearance of muscle-specific markers (e.g. myogenin mRNA). RVR 'loss of function' studies by constitutive over-expression of a dominant negative RVRΔE resulted in increased levels of p21$^{Cip1/Waf1}$ and myogenin mRNAs after serum withdrawal. Time course studies indicated that expression of RVRΔE mRNA results in the precocious induction and accumulation of myogenin and p21 mRNAs after serum withdrawal. In addition, we demonstrated that over-expression of the COUP-TF II and Rev-erbAα receptors in C2C12 cells completely blocked induction of p21 mRNA after serum withdrawal. In conclusion, our studies identified a potent transcriptional repression domain in RVR, characterized critical amino acids within the silencing region and provide evidence for the physiological role of RVR during myogenesis.

INTRODUCTION

Members of the nuclear receptor (NR) 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 and appear to be the ancient progenitors of this receptor superfamily. The orphan receptor RVR/Rev-erbβ/BD73 is closely related to Rev-erbAα, ROR/RZRα (retinoic acid receptor-related orphan receptor) and the *Drosophila* orphan receptor E75A, particularly in the DNA binding domain (DBD) and the putative ligand binding domain (LBD). RVR, Rev-erbAα and ROR bind as monomers to an asymmetric $(^{A}/_{T})_6$RGGTCA motif. Furthermore, Rev-erbAα and RVR can repress constitutive transactivation from this motif by RORα (3,4). However, in contrast to ROR, RVR and Rev-erbAα do not activate transcription and mediate transcriptional repression of the Rev-erbAα promoter (3–9). RVR is expressed in the central nervous system, skeletal and dorsal muscles, spleen and mandibular and maxillar processes (3,4,6,7). During embryogenesis RVR is expressed in the notochord and neural tube, but its function/role during differentiation and mammalian development remains obscure.

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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 and express myogenic markers (reviewed in 10,11). 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*), which are muscle-specific transactivators that can direct cell fate, repress proliferation, activate differentiation and the contractile phenotype and function at the nexus of command circuits that control the mutually exclusive events of division and differentiation (reviewed in 10–12). Gene targetting studies have suggested that while MyoD and Myf-5 are required for determination (13), myogenin is specifically required for differentiation (14). MyoD forms heterodimers with the ubiquitously expressed E2A HLH gene products. MyoD–E2A heterodimers bind to an E box motif (CANNTG), present in muscle-specific enhancers (reviewed in 10,11). The MyoD heterodimeric complexes act in concert with a variety of other ubiquitous (e.g. Sp1, CTF and SRF) and tissue-specific (e.g. MEF-2) transcription factors to regulate myogenic promoters (reviewed in 10,11). Direct interaction of MyoD and myogenin with the nuclear retinoblastoma phosphoprotein (RB) has been observed (15,16) and the binding of RB to MyoD is necessary to stabilize the DNA-bound (MyoD–E2A protein) heteromeric complex. RB activity is controlled by cell division kinase (cdk) complexes with the D-cyclins (for a review see 17). The activity of cdks are regulated at the level of synthesis of the subunit partners (e.g. cyclins) of the complex, post-translational modification and by binding of inhibitors, including $p21^{Cip1/Waf1}$ (18,19). In C2 cells in culture, serum withdrawal induces differentiation, repression of cyclin D1 and induction of p21 mRNA/protein (15,20–23). The critical role of these cell cycle regulators in myogenesis has been demonstrated: (i) inhibition of myogenesis by forced expression of cyclin D1 results in phosphorylation and inhibition of MyoD function; (ii) ectopic expression of p21 in growing myoblasts results in cell cycle arrest (20,23,24).

Recently two orphan receptors have been shown to have a functional role in skeletal muscle differentiation. COUP-TF II and Rev-erbAα, a closely related isoform of RVR, antagonize myogenesis, repress MyoD mRNA expression and block induction of myogenin mRNA after serum withdrawal (25,26). Hence we investigated the transcriptional characteristics of RVR utilizing the GAL4 hybrid system and examined the expression/functional role of RVR in: (i) terminal skeletal muscle differentiation; (ii) regulation of MyoD/myogenin, cyclin D1 and p21 mRNAs with respect to the characterized link between these genes in differentiation and cell cycle control.

MATERIALS AND METHODS

Cell culture and transient transfections

COS-1 or JEG-3 (human choriocarcinoma) cells were cultured for 24 h in DMEM supplemented with 10% FCS in 6% CO_2 before transfection. Each 35 mm dish of COS-1 or JEG-3 cells (60–80% confluent) was transiently transfected with 2.5 μg reporter plasmid DNA (G5E1bCAT) expressing chloramphenicol acetyltransferase (CAT), mixed with 1 μg pGAL0-RVR or pGAL4-VP16-RVR chimeras by the DOTAP-mediated procedure as described previously (35). Mouse myogenic C2C12 cells were grown in DMEM supplemented with 20% FCS in 6% CO_2. Each 35 mm dish of myogenic C2C12 cells (80–90% confluent) was transiently transfected in DMEM supplemented with 2% FCS. Fresh medium was added to the cells after 24 h and cells were harvested for assay of CAT activity 48 h after transfection. Each transfection was performed at least three times to overcome variability inherent in transfections.

Construction of stable cell lines

C2C12 cells were stably transfected at ~40% confluence using the DOTAP (Boehringer Mannheim)-mediated procedure as described previously (36). Briefly, a 1 ml DNA/DOTAP mixture (containing 20 μg pSG5-RVRΔE, 1.5 μg pCMV-NEO, 150 μl DOTAP in 20 mM HEPES, 150 mM NaCl, pH 7.4) was added to the cells in 25 ml fresh culture medium. The cells were then grown for a further 24 h to allow cell recovery and for high level pCMV-NEO expression before selection. Stable transfectants were isolated after 7–14 days selection in DMEM supplemented with 20% FCS and 400 μg/ml G418. The Rev-erbAα and COUP-TF II cell lines have been previously described (25,26).

Primer sequences and plasmids

GMUQ251, 5′-CGCGGATCCCACCATGGAGCTGAACGCAGGAGG-3′
GMUQ252, 5′-CGCGGATCCTTAAGGATGAACTTTAAAGGC-3′
GMUQ265, 5′-CGCGGATCCGTTCACGAGATGCTGTTCGAT-3′
GMUQ301, 5′-GCGCGTCGACATATGT/ACTGG/TA/GCAT/GGAA/GATCTGGGAAG-3′
GMUQ302, 5′-GCGTCTAGATGAA/CGCAAAT/GCGT/CACCATT/CAA/GA/CA-3′
GMUQ303, 5′-GCGCGTCGACATATGTTTGCA/CAAG/AA/CG/AGATT/CCCT/CGGC-3′
GMUQ304, 5′-GCGTCTAGAAGCT/CTTT/AAA/GCAGA/GT/GTG/CACCTG-3′
GMUQ307, 5′-GCGCGTCGACATATGGCTGGTGCTA/CG/AGATT/CCCT/CGGCTTC-3′
GMUQ308, 5′-GCGCGTCGACATATGGCTGATGCTA/CG/AGATT/CCCT/CGGCTTC-3′

Two primers, GMUQ251 and GMUQ252, were used to PCR amplify the 1731 bp open reading frame of RVR from the parent plasmid pCMXRVR (4) with *UlTma* DNA polymerase (Perkin Elmer). This gave a fragment containing the 1731 bp open reading frame of RVR with primer-derived *Bam*HI ends. This PCR fragment was cloned into *Sma*I-digested pBS and was called pBS-RVR. pGAL-RVR and pGAL4-VP16-RVR (GV-RVR) chimeras were created by inserting fragments of RVR into the pGAL0 (37) and pGAL4-VP16 (25) vectors. pGAL0 contains the GAL4 DBD and pGAL4-VP16 contains the GAL4 DBD linked to the acidic activation domain of VP16. The 1745 bp fragment of *Bam*HI-digested pBS-RVR was end-filled with Klenow and ligated with *Sal*I-digested, Klenow end-filled pGAL0 and pGAL4-VP16. To construct pGAL-RVR(1–88) and GV-RVR(1–88), the 1745 bp fragment of *Bam*HI-digested pBS-RVR was digested with *Hin*fI and the 273 bp fragment was end-filled with Klenow and cloned into *Sal*I-digested, Klenow end-filled pGAL0 and pGAL4-VP16. pGAL-RVR(1–276) and GV-RVR(1–276) were created by inserting the Klenow end-filled, 837 bp fragment of *Sph*I/*Bgl*II digestion of the 1745 *Bam*HI fragment from pBS-RVR into *Sal*I-digested, Klenow end-filled pGAL0 and pGAL4-VP16. To construct pGAL-RVR(170–576), a PCR fragment was prepared for insertion into pGAL0. Two primers, GMUQ265 and GMUQ252, were used to PCR amplify this region from the parent plasmid pCMX-RVR, as above, with *UlTma* DNA polymerase. This fragment was digested with *Bam*HI and cloned into *Bam*HI-digested $pBSK^+$ and was called pBSK-RVR(170–576). The 1236 bp insert generated by *Bam*HI

digestion of pBSK-RVR(170–576) was cloned into *Bam*HI-digested pGAL0. pVP16-RVR(170–576) was prepared by ligating the end-filled, 1236 bp *Bam*HI fragment of pGAL-RVR(170–576) into *Xho*I-digested, Klenow end-filled pNLVP16 (38). GV-RVR(170–576) was prepared by ligating the 1274 bp *Sal*I–*Xba*I fragment of VP16-RVR(170–576) into *Sal*I/*Xba*I-cleaved pGAL4-VP16. To construct pVP16-RVR(178–353) and pVP16-RVR(355–576), the 1236 bp insert generated by *Bam*HI digestion of pGAL-RVR(170–576) was digested with *Eco*RI and the 564 and 675 bp fragments were end-filled with Klenow and cloned into *Xho*I-digested, Klenow end-filled pNLVP16. GV-RVR(178–353) and GV-RVR(355–576) were created by ligating the *Sal*I–*Xba*I fragment of pVP16-RVR(178–353) and the *Sal*I–*Xba*I fragment of pVP16-RVR(355–576) into *Sal*I/*Xba*I-cleaved pGAL4-VP16.

For construction of the following GV-RVR chimeras, the following primers were used to PCR amplify with *UlTma* DNA polymerase regions of RVR from GV-RVR(355–576): GV-RVR(394–449), GMUQ301 and GMUQ302; GV-RVR(416–449), GMUQ303 and GMUQ302; GV-RVR(394–437), GMUQ301 and GMUQ304; GV-RVR(416–437), GMUQ303 and GMUQ304. The following primers were used to create mutations in the FAK regions of GV-RVR(416–449): GV-RVR AGAR, GMUQ307 and GMUQ302; GV-RVR ADAN, GMUQ308 and GMUQ302. PCR amplification (Pfu DNA polymerase, Stratagene) products from GV-RVR(355–576) containing primer-derived *Sal*I 5′-ends and *Xba*I 3′-ends were digested with *Sal*I/*Xba*I and ligated to *Sal*I/*Xba*I-digested pGAL4-VP16.

pSG5-RVR was created by ligating the *Bam*HI-cleaved 1745 bp fragment of pBS-RVR into *Bam*HI-digested pSG5. Sense and antisense clones were screened by *Eco*RI digestion. To create pSG5-RVRΔE, *Sph*I/*Bgl*II-digested pGEX-1 RVR was end-filled with Klenow and the 837 bp *Bam*HI fragment was ligated into *Bgl*II-digested, end-filled, *Bam*HI-digested pSG5. Double-stranded sequencing of ligation junctions confirmed authenticity and that the foreign protein was being expressed in-frame.

CAT assays

Cells were harvested and aliquots of the cell extracts were incubated at 37°C with 0.1–0.4 µCi [^{14}C]chloramphenicol (ICN, Cleveland, OH) in the presence of 5 mM acetyl-CoA and 0.25 M Tris–HCl, pH 7.8. After a 0.2–4 h incubation period, the samples were analysed on silica gel thin layer chromatography plates as described previously (35). Quantitation of CAT assays was performed with an AMBIS β-scanner.

Western blots

Rabbit anti-mouse cyclin D1 antibody (Santa Cruz no. sc-717) was used at a concentration of 1 µg/ml for 1 h at room temperature. Rabbit anti-GAL4 antiserum (Santa Cruz no. sc-428) was used at a concentration of 1 µg/ml overnight at 4°C. Extract preparation, electrophoresis, transfer, non-specific blocking, washing and further steps were carried out with Boehringer Mannheim ECL Western blotting detection reagents according to the manufacturer's protocols, as described previously (25).

RNA extraction and Northern hybridization

Total RNA was extracted by the acid guanidinium thiocyanate/phenol/chloroform method (39). Poly(A)$^+$ RNA was extracted using an mRNA isolation kit (Boehringer Mannheim) from total RNA using biotin-linked oligo(dT) and streptavidin-linked magnetic beads. Northern blots, random priming and hybridizations were performed as described previously (40). The actin probes used were as described by Bains *et al.* (41). The mouse myogenin (42) and MyoD (43) cDNAs were excised from the pEMSVscribe (Moloney sarcoma virus)-based expression vectors. Mouse cyclin D1 was excised from pGEX-3X-CYL1 (44) and mouse p21 was excised from pCMW35, an unpublished clone encoding mouse p21 from the Vogelstein laboratory. The RVR cDNA probe was the sequence spanning bp 508–1731, encompassing the D and E regions, and was excised from GAL4-RVR(170–576) with *Bam*HI.

RESULTS

RVR functions as a dominant transcriptional repressor: identification of an active transcriptional silencer located between amino acids 394 and 449 in the E region

The literature to date on RVR indicated that this orphan receptor repressed transcription of the Rev-erbAα promoter and suppressed the ability of RORα to transactivate gene expression. These experiments were all based on transfection assays. To elucidate the molecular basis of these transcriptional characteristics, we investigated the potential of RVR to modulate transcription by utilizing the GAL4 hybrid system, whereby a putative transactivator is fused to the DBD of the well-characterized yeast GAL4 protein. If active, the putative transactivator induces transcription of the CAT reporter placed downstream of the GAL4 binding sites. The system utilized an SV40 promoter expression vector with a multiple cloning site downstream of the GAL4 DBD (amino acids 1–147) from which the activation domain had been deleted. We fused various domains of the RVR protein to the GAL4 DBD to examine their effects on the basal level of expression from an E1b promoter downstream of five copies of a 17mer GAL4 binding site linked to the CAT reporter.

As shown in Figure 1A, the GAL4-RVR chimeras did not activate transcription of the G5E1bCAT plasmid. This implies that RVR does not contain any modular activation domains. However, it should be noted that RVR activity may be ligand dependent and to date no ligand has been identified or characterized. There is a growing body of evidence that the transactivating activity of steroid receptors can be modulated in a ligand-independent manner by phosphorylation events (27,28). It has been demonstrated that the N-terminus of Rev-erbAα, which contains 50 serine/threonine residues out of 131, possesses a phosphorylation-dependent N-terminal activation domain (25). The N-terminal AB region of RVR is serine rich (27/102 amino acids) and thus may be a potential target for kinases. The above transfections were repeated in the presence of 8-Br-cAMP, which activates cAMP-dependent protein kinases. This treatment did not improve the ability of the GAL4-RVR chimeras to activate transcription and suggested that phosphorylation by cAMP-dependent protein kinases does not activate RVR (data not shown).

To further understand the transcriptional properties of RVR, we utilized the GAL4-VP16 chimera, a potent transcriptional activator, to study the activity of this protein. To examine whether RVR possessed the ability to repress a potent functional transactivator, we sub-cloned segments of RVR cDNA into the GAL4-VP16 expression vector and examined the effect on activation of the CAT reporter gene linked to GAL4 binding sites. A similar investigative approach utilizing the GAL4-VP16

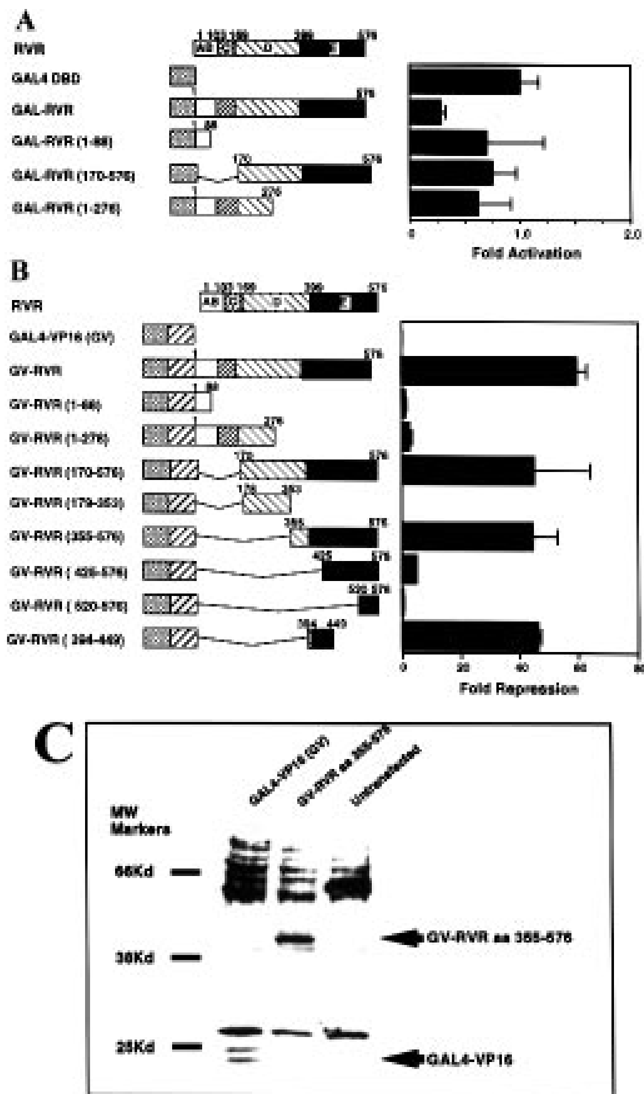

Figure 1. Analysis of potential transcriptional activation and repression domains of RVR. Various regions of RVR were sub-cloned into a multiple cloning site in-frame with and 3′ of the GAL4 DBD (**A**) or GAL4-VP16 (GV) coding region (**B**) respectively. COS-1 cells were co-transfected with pG5E1bCAT reporter (2.5 µg) and GAL-RVR (A) or GV-RVR (B) chimeras (1 µg) and assayed for CAT activity. Results shown are mean ± SD and were derived from three independent experiments. (A) Results represent fold activation compared with that of GAL4 DBD alone. (B) Transcriptional repression is expressed relative to GAL4-VP16 alone. (**C**) Whole cell extracts from untransfected COS-1 cells and COS-1 cells transfected with GAL4-VP16 or GV-RVR(355–576) were analysed on Western blots using a polyclonal antibody to the GAL4 DBD. The positions of the transfected proteins are indicated.

chimera has been utilized to analyse the thyroid hormone receptor (29) and Rev-erbAα (25). The GAL4-VP16 protein, which contains the yeast GAL4 DBD and the transactivating domain of herpes simplex virus VP16, is a potent transcriptional activator of GAL4 binding sites linked to CAT.

Six chimeric GAL4-VP16 (GV)-RVR expression plasmids were constructed; GV-RVR (amino acids 1–576), GV-RVR ABCD (amino acids 1–276), GV-RVR AB (amino acids 1–88), GV-RVR DE (amino acids 170–576), GV-RVR D (amino acids 179–353) and GV-RVR E (amino acids 355–576). These were co-transfected with the reporter ($pG_5E1bCAT$) into COS-1 cells and the CAT activity assayed. Full-length RVR, the DE region and the E region of RVR when linked to the GAL4-VP16 chimera very efficiently repressed (40- to 60-fold) transactivation by GAL4-VP16 (Fig 1B). This suggested that RVR cDNA contains an active and potent transcriptional repressor in the E region. The AB (amino acids 1–88), ABCD (amino acids 1–276) and D (amino acids 179–353) regions of RVR had no effect on the ability of the GAL4-VP16 protein to transactivate gene expression.

To further characterize this repression domain, three more chimeric GAL4-VP16 (GV)-RVR expression plasmids that contained sub-domains of the E region were constructed: GV-RVR(425–576), GV-RVR(520–576) and GV-RVR(394–449). These were co-transfected with the reporter (pG5E1bCAT) and CAT activity assayed. RVR(394–449) when linked to GAL4-VP16 very efficiently repressed (≥40-fold) transactivation by GAL4-VP16 to a similar level as the RVR E region (amino acids 355–576) (Fig. 1B). This suggests that all the silencing ability of the C-terminal region of RVR may be due to the region between amino acids 394–449 in RVR. In contrast, RVR(520–576) had no effect on the ability of the GAL4-VP16 protein to transactivate gene expression (Fig. 1B). RVR(425–576), which contains only part of RVR(394–449), weakly repressed transcription of GAL4-VP16.

To verify that repression of GAL4-VP16 by RVR was not due to the different stabilities or expression levels of the GV-RVR chimeric proteins, we analysed whole cell extracts from transfected COS-1 cells on Western blots using a polyclonal antibody to the GAL4 DBD. The GV-RVR(355–576) protein, which demonstrated strong repression of GAL4-VP16, was expressed efficiently with respect to the GAL4-VP16 protein (Fig. 1C). This indicates that the ability of the region between amino acids 355–576 of the RVR protein to repress transactivation by GAL4-VP16 is not due to differences in protein levels relative to GAL4-VP16 protein.

To determine if this transcriptional repression could be relaxed by phosphorylation, the transfections were repeated in the presence of 8-Br-cAMP. This stimulator of cAMP-dependent protein kinases did not affect the ability of the C-terminal domain to repress transcription (data not shown). In conclusion, these experiments demonstrate that the C-terminal region of RVR (between amino acids 394 and 449), within the E region, encodes an active transcriptional silencer.

Repression is dependent on the LBD-specific signature motif and helix 5 in the E region located between amino acids 416 and 449

Recent publication of the crystal structures for the LBDs of three members of the steroid/thyroid receptor superfamily, thyroid hormone (TR), retinoic acid (RAR) and retinoid X (RXR) receptors, have revealed a conserved structure consisting of 12 α-helices (30–32). The smallest characterized repression domain of RVR (amino acids 394–449) identified would encompass H3, L3–4, H4 and H5 (see Fig. 2A). The most conserved amino acids, or the so-called LBD-specific signature [(F/W)AKxxxxFxxL-xxxDQxxLL], for the superfamily spans H3, L3–4 and H4 of the LBD domain (33). It has been proposed that this motif contributes to stabilization of the LBD canonical structure. Therefore, we decided to investigate the contribution of H3, H4 and H5 as well as the LBD-specific signature to the ability of RVR to repress transcription.

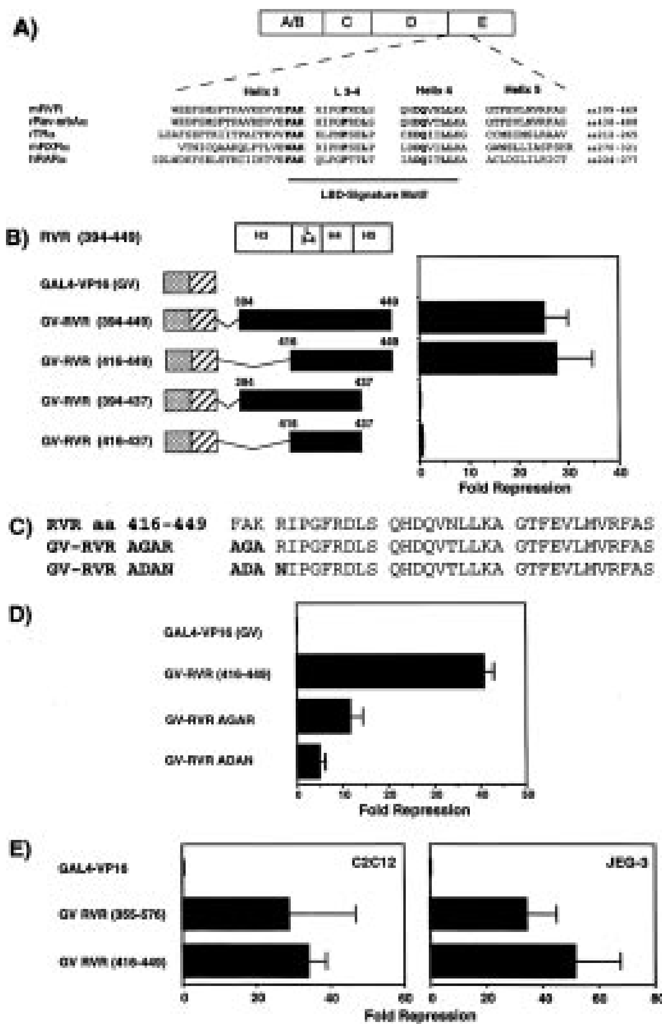

Figure 2. Characterization of the transciptional repression domain in the E region of RVR. (**A**) Alignment of mRVR, rRev-erbAα, rTRα, mRXRα and hRARα showing the region from H3 to H5 in the E region. Helical assignments for rTRα (32), rRev-erbAα, mRXRα and mRVR are based upon the helices denoted for the hRXRα and hRARα crystal structures (31). Conserved amino acids in the LBD-specific signature motif (underlined) are in bold. (**B**) COS-1 cells were co-transfected with pG5E1bCAT reporter (2.5 µg) and GV-RVR chimeras (1 µg) and assayed for CAT activity. Results shown are mean ± SD and were derived from three independent experiments. Transcriptional repression is expressed relative to GAL4-VP16 alone. (**C**) Amino acid residues of the RVR repression domain (416–449) and mutations induced in the FAKR region. Mutated amino acid residues are shown in bold. (**D**) Two chimeric GV-RVR expression plasmids containing mutations in the repression domain (see B) were co-transfected with the reporter (G5E1bCAT) into COS-1 cells and the CAT activity assayed. Results shown are mean ± SD and were derived from three independent experiments. Transcriptional repression is expressed relative to GAL4-VP16 alone. (**E**) C2C12 (mouse myogenic cells) and JEG-3 cells (human choriocarcinoma) were co-transfected with pG5E1bCAT reporter (2.5 µg) and GAL4-VP16-RVR chimeras (1 µg) and assayed for CAT activity. Results shown are mean ± SD and were derived from three independent experiments. Transcriptional repression is expressed relative to GAL4-VP16 alone.

We constructed three chimeric GAL4-VP16 (GV)-RVR expression plasmids: GV-RVR(416–449) (FAK–H5), GV-RVR(394–437) (H3–H4) and GV-RVR(416–437) (FAK–H4). These were co-transfected with the reporter ($pG_5E1bCAT$) into COS-1 cells and CAT activity assayed.

The FAK–H5 region of RVR(416–449) when linked to GAL4-VP16 repressed transactivation by GAL4-VP16 as efficiently as the RVR E region (amino acids 394–449) (Fig. 2B). This suggested that most of H3 was not required for repression of transcription by RVR. In contrast, RVR(394–437) (H3–H4) and RVR(416–437) (FAK–H4) did not efficiently repress the ability of the GAL4-VP16 protein to transactivate gene expression. This data demonstrates that H5 is absolutely necessary for active transcriptional silencing.

Crystal structures for the LBDs of steroid receptors have determined that the LBD signature is essential in preserving the canonical structure of the LBD. The conserved aromatic amino acid phenylalanine (F) in the FAK motif has been shown to set the spatial limits of this hydrophobic cluster and the conserved lysine (K) in the FAK motif (at the beginning of the LBD-specific signature) has been shown to interact with the glutamine (Q) in H4 (amino acids K418–Q432 in RVR) (33). Therefore, we decided to mutate the FAK in the LBD-specific signature to determine the role of the FAK motif in transcriptional repression by RVR(419–449). We constructed two chimeric GV-RVR expression plasmids containing mutations in the repression domain, changing the FAKR sequence to AGAR (GV-RVR AGAR) or ADAN (GV-RVR ADAN) (see Fig. 2C). These were co-transfected with the reporter ($pG_5E1bCAT$) into COS-1 cells and CAT activity assayed.

Neither the GV-RVR AGAR (~12-fold) nor the GV-RVR ADAN (~5-fold) construct efficiently repressed transcriptional activation by GAL4-VP16 in comparison with GV-RVR(416–449) (~40-fold) (Fig. 2D). These results demonstrate that the amino acids FAKR of RVR are important in transcriptional repression and that the LBD signature motif is necessary for efficient transcriptional silencing. Interestingly, the impact of the ADA versus AGA mutation on repression is more significant, probably due to mutation of a neutral alanine (A) to a charged aspartic acid (D).

Combined with the previous observations, we can conclude that RVR contains an active transcriptional silencing domain in the E region of the protein that is active when transferred to a heterologous DBA. The minimal region necessary to confer active transcriptional repression lies between amino acidss 416 and 449, which includes the LBD-specific signature (spanning FAK, L3–4 and H4) and H5. Furthermore, the data indicate that the LBD-specific signature motif, in the absence of H5, cannot mediate active transcriptional silencing.

Active transcriptional silencing by RVR is not cell specific and occurs in mouse myogenic cells

Next we wished to examine whether repression by RVR was cell specific. We examined the ability of GV-RVR(355–576) and GV-RVR(416–449), both of which exhibit strong repression ability in COS-1 cells, to repress transcription in C2C12 (mouse myogenic) and JEG-3 (human choriocarcinoma) cells. In both cell lines we observed that both RVR(355–576) and RVR(416–449) strongly repressed transactivation by the GAL4-VP16 protein (~40- to 60-fold) (Fig. 2E). This suggests that the cofactors involved in active transcriptional silencing by RVR are not cell specific and are present in different cell types.

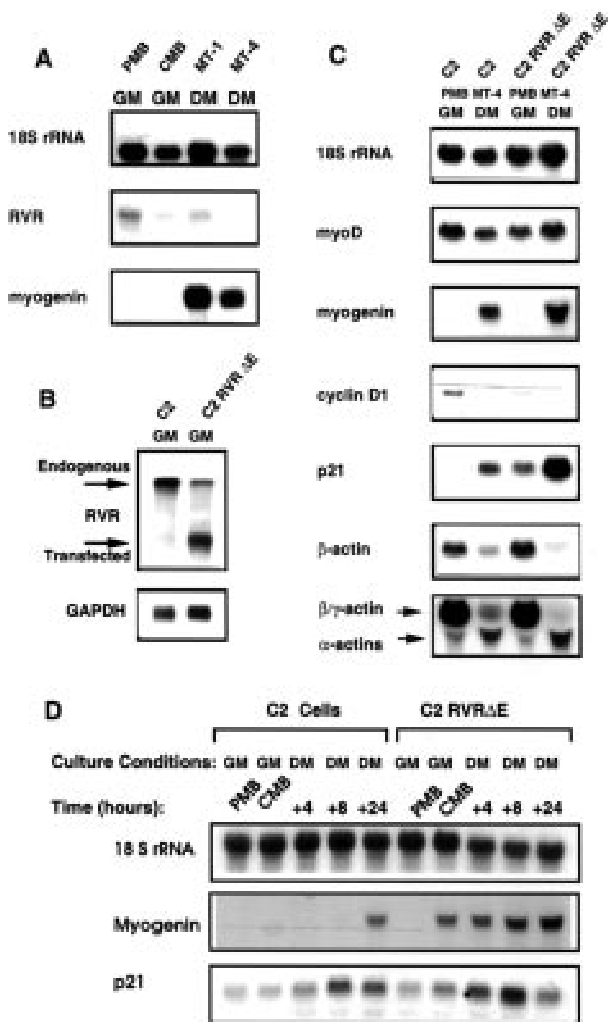

Figure 3. Expression of RVR during myogenesis and Northern analysis of stable transfectants expressing a dominant negative RVRΔE transcript. (**A**) Poly(A)$^+$ RNA was extracted from proliferating myoblasts (PMB) and confluent myoblasts (CMB) in growth medium (GM) and myotubes 1 and 4 days (MT-1 and MT-4) after serum withdrawal in differentiation medium (DM). RNA (2.5 µg) was blotted and probed for RVR and myogenin, using random primed cDNA probes, and 18S rRNA, using an oligonucleotide probe. (**B**) Poly(A)$^+$ RNA was extracted from proliferating myoblasts of C2C12 and stable transfectant (C2:RVRΔE) cells. RNA (2.5 µg) was blotted and probed for RVR and GAPDH using random primed cDNA probes. (**C**) Total RNA was isolated from parent C2C12 and C2:RVRΔE cells as proliferating myoblasts (PMB) in growth medium (GM) and myotubes 96 h after serum withdrawal (MT-4) in differentiation medium (DM). RNA (20 µg) was blotted and probed for myoD, myogenin, cyclin D1, p21, β-actin and α-actins (cytoskeletal and sarcomeric), using cDNA probes, and 18S rRNA, using an oligonucleotide probe. (**D**) Total RNA was isolated from parent C2C12 cells and C2:RVRΔE cells as proliferating myoblasts (PMB), confluent myoblasts (CMB) and 4, 8 and 24 h after addition of differentiation medium (DM). RNA (20 µg) was blotted and probed for myogenin and p21, using cDNA probes, and 18S rRNA, using an oligonucleotide probe.

RVR mRNA is repressed during myogenic differentiation

A biological function for RVR in development and differentiation has not been found, although the mRNA is expressed during embryogenesis and ubiquitously expressed in many adult tissues. However, RVR is expressed in muscle and functions as a transcriptional repressor in myogenic cells, hence we investigated its physiological role during C2C12 myogenesis, a well-characterized paradigm of mammalian differentiation. To study what role RVR might play in muscle differentiation we investigated the expression of RVR mRNA during myogenic differentiation in the mouse C2C12 myoblast cell line. Proliferating C2C12 myoblasts can be induced to biochemically and morphologically differentiate into post-mitotic, multinucleated myotubes by serum withdrawal in culture over a 48–96 h period. This transition from a non-muscle phenotype to a contractile phenotype is associated with repression of non-muscle proteins and activation/expression of a structurally diverse group of genes. This gene activation encodes a functional sarcomere responsible for the major activity of this specialized cell type, i.e. contraction. These events are characterized by a sequence of transitions typified by the *actin* multigene family. During myogenesis the cytoskeletal/non-muscle β- and γ-actins are down-regulated; in contrast, the sarcomeric cardiac and skeletal α-actins are induced. These isoform transitions correlate with repression of cyclin D1 and activation of myogenin and p21 mRNAs.

Total RNA and poly(A)$^+$ RNA were isolated from proliferating myoblasts, confluent myoblasts and post-mitotic myotubes after 1 and 4 days of serum withdrawal and examined by Northern blot analysis. RVR mRNA was abundantly expressed in myoblasts, however, this transcript is suppressed (2.5- to 4-fold, relative to 18S rRNA and GAPDH mRNA respectively; data not shown) as myoblasts exit the cell cycle and fuse to form differentiated multinucleated myotubes that have acquired a muscle-specific phenotype (Fig. 3A). Down-regulation of RVR mRNA correlated with the recent observations that the mRNA for two other orphan steroid receptors, COUP-TF II and Rev-erbAα, is repressed during myogenesis. These orphans have been demonstrated to antagonistically regulate myogenesis, repress MyoD mRNA expression and abrogate the induction of myogenin mRNA after serum withdrawal (25,26).

Concomitant with this decrease in RVR mRNA was the induction of myogenin mRNA (5- to 6-fold, relative to 18S rRNA and GAPDH mRNA respectively), which confirmed that these cells had terminally differentiated (Fig. 3A). Repression of RVR (Fig. 3A) and cytoskeletal non-muscle β- and γ-actin mRNA and cyclin D1 (relative to the equivalent levels of 18S rRNA) and the induction of p21, sarcomeric α-actins and myogenin mRNA (Fig. 3C) confirmed that these cells had terminally differentiated. Expression of MyoD mRNA in myoblasts and myotubes confirmed the myogenic nature of these cells. The differential expression of RVR mRNA suggested that this orphan receptor may regulate the process of differentiation and/or maintenance of myoblast proliferation in a similar manner to COUP-TFII and Rev-erbAα.

Constitutive expression of a 'dominant negative' RVR (RVRΔE) in myogenic cells stimulates muscle-specific markers of differentiation

The Northern analyses demonstrated that RVR mRNA repression correlates with the biochemical and morphological differentiation of myogenic cells that results in transition from a non-muscle phenotype to a contractile phenotype. To examine the role of RVR and to identify the probable target(s) of this orphan receptor in muscle cells we proceeded to examine the effect of knocking out RVR function by constitutive over-expression of a

dominant negative RVRΔE expression vector that lacked the E region (which encodes the repression domain) in the C2C12 cell line. The construct pSG5-RVRΔE (which contained RVRΔE cloned in the sense orientation into pSG5) was co-transfected with pCMV-NEO. Stable transfectants were isolated as a polyclonal pool of G418-resistant colonies (comprised of >20 individual resistant colonies). This cell line was called C2:RVRΔE. The C2:RVRΔE cell line produced abundant amounts of the exogenous/transfected 0.9 kb RVRΔE mRNA transcript, relative to the endogenous full-length 4.5 kb transcript (Fig. 3B). Interestingly, the level of full-length RVR transcript is reduced 2.8-fold in the C2:RVRΔE cell line relative to the GAPDH control. Furthermore, we observed that bacterially expressed RVRΔE protein bound the optimal monomeric $(A/T)_6$AGGTCA motif more efficiently than the full-length native protein (data not shown).

To examine the effect of constitutive dominant negative RVR expression on factors involved in determination (e.g. MyoD) and differentiation (e.g. myogenin) we isolated total RNA from C2:RVRΔE and normal C2C12 proliferating myoblasts (PMB) and myotubes (MT-4) before and after 96 h of serum withdrawal respectively. These RNAs were Northern blotted and probed with 18S rRNA, cytoskeletal/non-muscle β- and γ-actins and sarcomeric/striated α-skeletal and α-cardiac actin, MyoD, myogenin, cyclin D1 and p21 labelled cDNAs (Fig. 3C). These probes enabled us to determine the impact of constitutive dominant negative RVR expression on important markers of myogenesis.

We noted that the rate of differentiation was enhanced upon serum withdrawal and more multinucleated myotubes were formed in cells overexpressing RVRΔE mRNA compared with the parent C2C12 cell line. The absolute levels of myogenin and p21 mRNA were significantly enhanced in C2:RVRΔE cells after 96 h of serum withdrawal (MT-4 myotubes 4 days) (Fig. 3C), in accordance with the enhanced morphological differentiation of these cells. The level of cyclin D1 mRNA in proliferating myoblasts was lower, relative to the level in native cells. Furthermore, expression of p21 was elevated in both myoblasts and in myotubes (on a background of normal MyoD mRNA levels), reflecting the increased ability to differentiate and demonstrating that p21 mRNA levels are influenced by RVR during myogenesis.

Expression of RVRΔE results in precocious induction and accumulation of myogenin and p21 mRNAs after serum withdrawal

To examine whether the elevated levels of myogenin and p21 mRNAs in the C2:RVRΔE cell line were due to an acceleration of terminal differentiation, we conducted a time course study. We isolated total RNA from C2 and C2:RVRΔE cells as proliferating myoblasts (PMB), confluent myoblasts (CMB, harvested 24 h after the harvesting of PMB cells) and +4, +8 and +24 h after serum withdrawal in differentiation medium (DM) [(i.e. 4, 8 and 24 h after harvesting of the CMB sample in growth medium (GM)]. Northern analysis clearly demonstrated that terminal differentiation is accelerated in the C2:RVRΔE cell line. Myogenin is strongly expressed after 4 h in the C2:RVRΔE cell line, whereas in native C2 cells expression is not observed until 24 h after serum withdrawal (Fig. 3D). In fact, myogenin mRNA is spontaneously induced by cell contact in the C2:RVRΔE cell line in growth medium (GM). p21 mRNA is induced 4 h after serum withdrawal in the C2:RVRΔE line, in contrast to the native C2 cell line, where p21 mRNA is not induced until 8 h after serum withdrawal. These studies demonstrate that terminal differentiation is accelerated in the C2:RVRΔE cell line and the markers of myogenic differentiation are precociously induced.

Constitutive expression of the orphan steroid receptors COUP-TF II and Rev-erbAα in C2 cells increases cyclin D1 levels in myoblasts and blocks induction of p21 mRNA after serum withdrawal during myogenesis

As previously mentioned, two other 'orphans', COUP-TF II and Rev-erbAα, that are expressed in proliferating myoblasts have recently been demonstrated to antagonistically regulate muscle differentiation in culture, repress MyoD mRNA expression and abrogate the induction of myogenin mRNA after serum withdrawal (25,26). We decided to investigate whether over-expression of sense (S) and antisense (AS) COUP-TF II and Rev-erbAα cDNAs in the cell lines C2:COUP-TF II S, C2:COUP-TFII AS, C2:Rev-erbAα S and C2:Rev-erbAα AS (described previously; 25,26) affected p21 mRNA and cyclin D1 protein expression during myogenesis.

In the cell line C2:COUP-TFII S, stably transfected with pSG5 COUP-TFII S, the induction of p21 mRNA by serum withdrawal was completely blocked (Fig. 4A). Interestingly, the levels of cyclin D1 protein in the myoblasts of this cell line are elevated relative to the levels in normal C2 cells (Fig. 4B). These observations correlate with the absence of MyoD and myogenin mRNA in the C2:COUP-TFII S cell line (26). Curiously, constitutive expression of antisense COUP-TFII in the cell line C2:COUP-TF II AS did not lead to greater induction of p21 mRNA after serum withdrawal, but resulted in a slight reduction in cyclin D1 protein levels in these myoblasts compared with cyclin D1 levels in native C2 cells.

In the cell line C2:Rev-erbAα S, overexpressing Rev-erbAα, induction of p21 mRNA by serum withdrawal was completely blocked (Fig. 4C). Interestingly, unlike the effect of COUP-TF II in these cells, the level of cyclin D1 protein in this cell line was unaffected (Fig. 4D). However, constitutive expression of antisense Rev-erbAα cDNA in the cell line C2:Rev-erbAα AS did not significantly alter the levels of cyclin D1 protein in myoblasts and p21 mRNA after serum withdrawal.

These studies demonstrated that constitutive over-expression of two other orphan steroid receptors, COUP-TF II and Rev-erbAα, which are normally expressed in proliferating myoblasts affected expression of p21 mRNA during myogenesis.

DISCUSSION

Previous studies have demonstrated that Rev-erbAα (closely related to RVR, 97% in the DBD and 68% in the LBD) possesses both an N-terminal phosphorylation-dependent activation domain (25) and a strong repressor domain located in the C-terminus (25,34). Our work with the GAL4 hybrid system indicates that RVR does not possess an activation domain, but does contain a potent transcriptional silencing domain within the C-terminal putative LBD/E region (between amino acids 416 and 449). Repression by this region was not relieved by an activator of cAMP-dependent kinases, 8-Br-cAMP. Although RVR contains serine-rich regions in the N-terminus, the lack of sequence homology in the N-terminal regions between RVR and Rev-erbAα

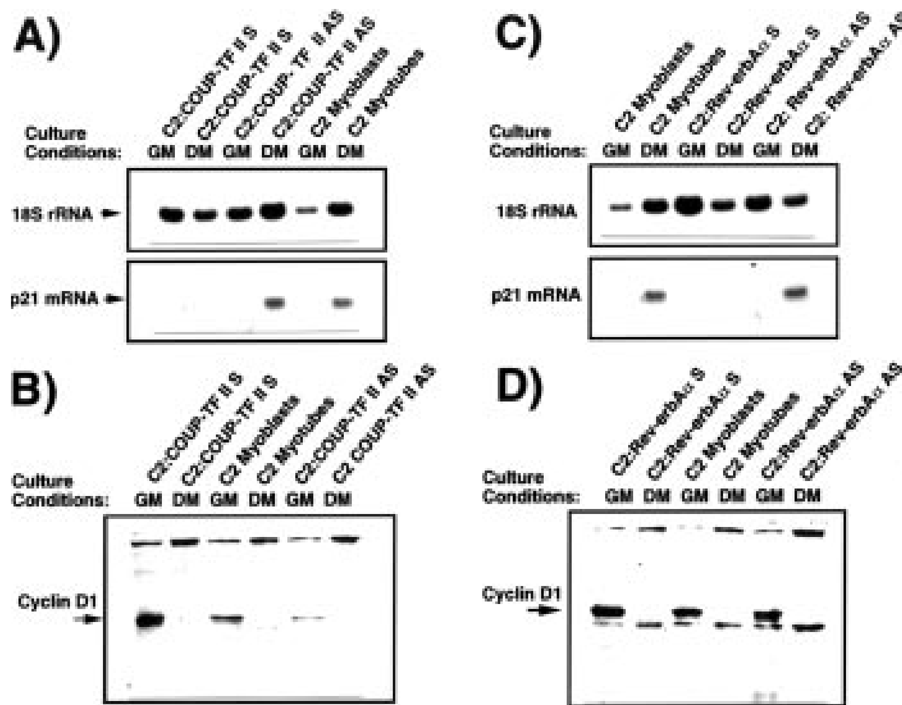

Figure 4. Effects of overexpression of COUP-TF II and Rev-erbAα in C2C12 cells on cyclin D1 and p21 expression. Northern (**A** and **C**) and Western (**B** and **D**) analysis of C2C12 cells stably tranfected with pSG5-COUP-TF II sense (S) and antisense (AS) constructs (A and B) or with pSG5-Rev-erbAα sense (S) and antisense (AS) constructs (C and D). For Northern analysis, total RNA was isolated from parent C2C12 cells and the stably transfected cells as proliferating myoblasts in growth medium (GM) and myotubes/cells 72 h after serum withdrawal in differentiation medium (DM). RNA (20µg) was blotted and probed for p21, using a cDNA probe, and 18S rRNA, using an oligonucleotide probe. For Western analysis, 20µg of whole cell extracts from untransfected cells and C2 cells transfected with the indicated chimeras were analysed with a polyclonal cyclin D1 antibody.

and the low percentage of glutamines and prolines may explain the differences in *trans*-acting potential.

Our studies indicate that RVR possesses a potent transcriptional repression domain in the E region of the orphan receptor that functions in different cell types. Recently reported crystal structural studies on the TR, RXR and RAR LBDs and detailed nuclear receptor (NR) amino acid residue alignments have identified a NR-specific signature in this region (33). This motif, (F/W)AKxxxxFxxLxxxDQxxLL, contains most of the conserved amino acid residues that stabilize the core of the canonical fold of NR LBD domains. The amino acids that encode the repression function of RVR (amino acids 416–449) are found in a domain that forms α-helices 3, 4 and 5 (and L3–4) of the putative LBD region and encompasses the LBD signature motif. It has been proposed that this region forms a hydrophobic pocket in the LBD core. Our data confirms the importance of this motif, as mutation of the FAK residues impairs the silencing effects of this region. Furthermore, our studies demonstrated that H5 was necessary for RVR function. The importance of H5 to NR function is highlighted from natural mutations in this region of other NRs that lead to generalized resistance to thyroid hormone, partial and complete androgen insensitivity syndrome and testicular feminization (33 and references therein). Structural analysis of TR/RAR/RXR indicates that this region is buried inside the receptor. Whether this is the case with RVR, which does not contain H12 (the AF-2 domain), remains to be determined. Curiously, this domain is very highly conserved (Fig. 2A) within the Rev-erb family, with only one amino acid difference found in the Rev-erbAα receptor (amino acids 455–488). This is in agreement with the reported transcriptional repression properties of both orphan receptors. Whether the domain we have defined between amino acids 416–449 of RVR directly represses transcription or functions as a repression interface for another molecule is not currently clear.

Our studies also indicate a biological role for RVR in mammalian differentiation. We demonstrated that proliferating C2C12 myoblasts expressed RVR mRNA, which was repressed when cells were induced to differentiate by serum withdrawal into multinucleated myotubes that express a contractile phenotype. RVR 'loss of function' studies in a cell line that constitutively expressed an RVRΔE mRNA (which lacked the identified functional repression domain) indicated that the process of differentiation was accelerated. This observation correlated with increased levels and the precocious induction of $p21^{Cip1/Waf1}$ and myogenin mRNA in these cells, which encode a cdk inhibitor and HLH protein respectively. These RVR 'loss of function' studies indicated that RVR plays a significant role in the cascade of events that antagonistically regulate myogenesis. The importance of functional orphan receptor expression in the regulation of myogenesis was highlighted by the inhibition of p21 mRNA induction after serum withdrawal in cell lines that constitutively over-express either COUP-TF II or Rev-erbAα. Over-expression of these orphan receptors has been previously demonstrated to abrogate induction of myogenin mRNA after serum withdrawal (25,26). Furthermore, in a cell line that constitutively expressed antisense COUP-TF II cDNA (26), we observed significantly

increased levels of myogenin mRNA after serum withdrawal, similar to the effect of over-expression of RVRΔE in C2C12 cells. These studies suggest that this group of orphan steroid receptors (COUP-TF II, Rev-erbAα and RVR), which have been demonstrated to function as transcriptional repressors, may play a co-ordinated role in the antagonistic regulation of myogenesis and maintenance of the proliferative state. This group of closely related 'orphan' repressors directly or indirectly regulate and target induction/expression of p21 and myogenin mRNA. The expression of these genes, as demonstrated by many other studies, is critical to cell cycle exit and transactivation of the myogenic programme respectively (14,20,23).

It has been demonstrated that MyoD may induce terminal cell cycle arrest and maintenance of the post-mitotic state during muscle differentiation by increasing the expression of p21 (20,21). The possibility exists that the orphan steroid receptors (RVR, COUP-TF II and Rev-erbAα) indirectly block induction of p21 during myogenesis, via suppression of MyoD mRNA. Over-expression of COUP-TF II and Rev-erbAα suppresses the levels of MyoD mRNA and blocks cell cycle exit and biochemical differentiation (25,26). However, we note that in the cell line C2:RVRΔE expression of p21 mRNA was precociously induced after serum withdrawal in a background of normal MyoD mRNA levels. The cdk inhibitor p21 is induced during myogenesis (20–22), as well as in other cells undergoing terminal differentiation, including cartilage, skin and nasal epithelium (22). Over-expression of p21 in C2C12 cells inhibits myoblast proliferation and reverses the inhibition of muscle-specific gene expression in mitogen-rich medium (20,23). Thus the regulation of p21 expression by the 'orphans' may subsequently affect the function of the cyclin D–cdk4 complex. Certainly, in cells over-expressing a dominant negative RVR (that had an accelerated rate of differentiation) the levels of cyclin D1 mRNA were lower. Analogously, cells that over-expressed COUP-TF II expressed higher levels of cyclin D1 protein in proliferating myoblasts. Whether, the effects on p21 are mediated purely by inhibition of MyoD expression/function is not clear at present.

In conclusion, RVR, COUP-TF II and Rev-erbAα function as transcriptional repressors that antagonistically regulate HLH and p21 gene expression during muscle differentiation by influencing the decision to 'divide or differentiate'.

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