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Transcriptional repression by Rev-erbAα is dependent on the signature motif and helix 5 in the ligand binding domain: silencing does not involve an interaction with N-CoR

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ABSTRACT

Rev-erbAα is an orphan nuclear receptor that functions as a dominant transcriptional repressor. Tissue culture and *in situ* hybridisation studies indicated that Rev-erbAα plays an important role in mammalian differentiation and development. Previous studies have localised the silencing domain of Rev-erbAα to the D/E region of the orphan receptor. This study utilised the GAL4 hybrid system to demonstrate that efficient repression is mediated by 34 amino acids (aa) between aa 455 and 488 in the E region of the receptor. This domain contains the ligand binding domain (LBD)-signature motif [(F/W)AKxxxxFxxLxxxDQxxLL] and a region that, according to the recently published crystal structures of steroid receptors, would be predicted to form helix 5 of the canonical LBD structure. Fine deletions and site-specific mutagenesis indicated that both the LBD signature motif and helix 5 were necessary for efficient silencing. Utilising mammalian two hybrid technology, we have also demonstrated that Rev-erbAα does not associate with the interaction domain (aa 2218–2451) of the nuclear receptor co-repressor, N-CoR, that is known to interact with the thyroid hormone and retinoic acid receptors. This suggested that transcriptional repression by Rev-erbAα is not mediated through an interaction with N-CoR. In conclusion, we have identified and characterised the minimal domain of Rev-erbAα, that mediates transcriptional repression by this orphan receptor.

INTRODUCTION

The Rev-erb family of proteins are members of the type III/IV steroid/thyroid hormone nuclear receptor superfamily that act as ligand-inducible transcriptional regulators (1). This superfamily binds DNA through two highly conserved zinc-co-ordinated modules that contact specific bases within *cis*-acting regulatory elements of target genes, termed hormone response elements (HREs) (2). Binding specificity is determined by a short sequence within the DNA-binding domain (DBD), termed the P-box (3). Type II, III and IV steroid/thyroid receptors recognise the hexamer motif, AGGTCA. These nuclear receptors bind to a vast array of characterised hydrophobic ligands, such as thyroid hormone (T_3), all-*trans*-retinoic acid (all-*trans*-RA) and vitamin D, providing a direct link between extracellular hormonal signals and transcriptional responses. The Rev-erb family of receptors belong to a rapidly expanding subclass of this superfamily termed 'orphan receptors' which, although structurally and functionally related, have no identified ligand (4).

Two isoforms of the Rev-erb family have been isolated from mammalian genotypes: Rev-erbAα (5) [also known as Ear-1; (6)] and RVR (7) [also known as Rev-erbβ; (8,9) and BD73; (10)]. Major differences between the two isoforms occur within the hyper-variable A/B and D regions of the proteins (10). Both isoforms are expressed in a wide range of tissues and are present in all major organs. Rev-erbAα mRNA is upregulated during adipocyte differentiation but repressed during myogenesis (11,12). Addition of planar aromatic antioxidants (e.g. butylated hydroxytoluene) to HepG2 cells induces the expression of both isoforms of the Rev-erb family, with a profile similar to that of immediate early response genes (e.g. c-*fos* and c-*jun*) (10). The physiological and developmental role of the Rev-erb family during embryogenesis is largely unknown. Insight into their possible role and function, however, has been gained by the observation that constitutive expression of Rev-erbAα cDNA can block morphological and biochemical differentiation of mouse skeletal muscle cells (12).

Both receptors have been shown to bind as monomers to an asymmetric 11 base pair (bp), $(^{A}/_{T})_6$ AGGTCA motif (9,10,13). Rev-erbAα has also been demonstrated to bind to a novel HRE preferentially as a homodimer. The HRE consists of two tandemly arranged AGGTCA motifs separated by 2 bp with unique 5′ flanking and spacer nucleotides (RevDR-2) (14). Recently, a naturally occurring RevDR-2 has also been identified in the promoter of Rev-erbAα suggesting auto-regulation of its own transcript (15).

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Reports on the transcriptional properties of the Rev-erb family were initially conflicting. Rev-erbAα was first reported to act as a constitutive activator of transcription through its cognate monomeric asymmetric motif (13). More recently, we and other groups have demonstrated that members of the Rev-erb family are, in fact, constitutive repressors of transcription (7,9,10, 12,14). The Rev-erb family does not activate transcription and can repress *trans*-activation by RORs (retinoic acid-related orphan receptors) and RARs (retinoic acid receptors).

The ability of steroid receptors to repress basal transcription has long been established (16–18), although the mechanism by which steroid receptors achieve repression still remains unclear. The recent characterisation of two co-repressors, N-CoR and SMRT, that interact with unliganded thyroid hormone receptor (TR) and RAR has shed some light on the mechanism of nuclear receptor repression (19–21). We have demonstrated previously, using the GAL4 hybrid system, that Rev-erbAα contains an active transcriptional silencing domain located in the hinge/LBD (12). Therefore, we characterised further the repression domain of the Rev-erbAα receptor to a minimal region (34 amino acids) that is conserved within the Rev-erb family (97% homologous to RVR) and located in the E domain of the protein. The identified silencing domain was then examined in the context of the recently published crystal structures of steroid receptor LBDs (TR, RAR and the retinoid x receptor: RXR). Possible interaction between the recently characterised steroid receptor co-repressor, N-CoR, and Rev-erbAα was also studied utilising mammalian two hybrid technology.

MATERIALS AND METHODS

Primer sequences

GMUQ142 5′-TCCAGCGGATCCTCCCCCAGCCGGACC-3′
GMUQ132 5′-CGGGATCCGGGCGGGTCACTGGGCGTCC-3′
GMUQ171 5′-GCGCGTCGACATATGGAAGACAGCAGCCGA-3′
GMUQ172 5′-GGCGTCGACTATTGCCGGGCGGGTCACTGGGCGTCC-3′
GMUQ173 5′-GCGGAATTCACCATGTATGGAAATTATTCCCAC-3′
GMUQ176 5′-GCGGAATTCCTCAGGTGATCTGCAGTGGGGTCT-3′
GMUQ296 5′-GCGAATTCACCATGGTNAA$^{A}/_{G}$$^{A}/_{T}$CNAA$^{G}/_{A}$- AA$^{G}/_{A}$CA-3′
GMUQ297 5′-GCGAATTCACCNC$^{A}/_{T}$$^{A}/_{G}TCN^{G}/_{C}$$^{A}/_{T}NA^{A}/_{G}$N- GT$^{T}/_{C}TC^{G}/_{A}TA^{T}/_{C}$TG-3′
GMUQ301 5′-GCGCGTCGACATATG$^{T}/_{A}$CTG$^{G}/_{T}$$^{A}/_{G}CA^{T}/_{G}$G- A$^{A}/_{G}$ATCTGGGAAG-3′
GMUQ302 5′-GCGTCTAGATGA$^{A}/_{C}$GCAAA$^{T}/_{G}$CG$^{T}/_{C}$ACCAT- $^{T}/_{C}$A$^{A}/_{G}$$^{A}/_{C}$A-3′
GMUQ303 5′-GCGCGTCGACATATGTTTGC$^{A}/_{C}$AA$^{G}/_{A}$$^{A}/_{C}$$^{G}/_{A}$- GAT$^{T}/_{C}CC^{T}/_{C}$GGC-3′
GMUQ304 5′-GCGTCTAGAAGC$^{T}/_{C}$TT$^{T}/_{A}$A$^{A}/_{G}$CAG$^{A}/_{G}$$^{T}/_{G}$T- $^{G}/_{C}$ACCTG-3′
GMUQ307 5′-GCGCGTCGACATATGGCTGGTGCT$^{A}/_{C}$$^{G}/$AG- AT$^{T}/_{C}CC^{T}/_{C}$GGCTTC-3′
GMUQ308 5′-GCGCGTCGACATATGGCTGATGCT$^{A}/_{C}$$^{G}/_{A}$G- AT$^{T}/_{C}CC^{T}/_{C}$GGCTTC-3′
GMUQ327 5′-GCGCGTCGACATATGTTTGCCGCACACATC
GMUQ328 5′-GCGCGTCGACATATGTTTGCCAAACACATCCCCGGCGCCCGTGACC
GMUQ329 5′-GCGCGTCGACATATGTTTGCCAAACACATCGCCGGCGCCCGTGACC

All GAL; VP16 and GALVP16-Rev-ebAα and GAL-N-CoR constructs were sequenced to confirm the reading frame using a Pharmacia sequencing kit. Amplification using these primers was with Ultima DNA polymerase (Perkin Elmer) or Pfu DNA polymerase (Stratagene) according to the manufacturer's protocols.

Plasmids

The expression plasmids pGALO (22), pNLVP16 (23), GAL4/VP16 (12), pGEX-cTRα (24) and pG5E1b-CAT (25) have been described elsewhere. pGAL0 contains the GAL4 DBD and pGAL4/VP16 contains the GAL4 DBD linked to the acidic activation domain of VP16.

The construct VP16-mRXRγ was amplified using primers GMUQ173 and GMUQ176 to amplify the coding region from pSG5-RXRγ. The resulting fragment was cleaved with *Eco*RI, end filled with Klenow then cloned into *Nde*I-digested, Klenow end-filled pNLVP16.

The construct VP16-cTRα was created by digestion of pGEX-cTRα with *Eco*RI. The resulting fragment was end filled with Klenow and ligated into *Nde*I-cleaved, Klenow end-filled pNLVP16.

GAL-NCoR (ID) was created by amplifying the NCoR interaction domain from a C2C12 muscle cell cDNA library in λZAP II using the primers GMUQ296 and GMUQ297. The resulting product was cleaved with *Eco*RI and ligated with *Eco*RI-cleaved pGAL0.

pGAL4/VP16-Rev-erbAα (GV-Rev) and VP16-Rev-erbAα (VP16-Rev) chimeras were created by inserting fragments of Rev-erbAα into the pGAL4/VP16 and pNLVP16 vectors respectively. To construct VP16-Rev aa 107–614, the primers GMUQ171 and GMUQ172 were used to amplify a region from pBS-rRev-erbAα. The resulting fragment was cleaved with *Nde*I and ligated with *Nde*I-digested pNLVP16. GV-Rev aa 437–614 and GV-Rev aa 557–614 were constructed by cleaving Rev-erb cDNA with *Bgl*II–*Bam*HI and *Eco*RI–*Bam*HI respectively. The resulting fragments were end filled with Klenow and ligated into *Nde*I-cleaved, Klenow end-filled GAL4/VP16.

VP16-Rev aa 437–614 was constructed by cleaving Rev-erb cDNA with *Bgl*II–*Bam*HI end filling the resulting fragment with Klenow and ligating to *Xho*I-cleaved, Klenow end-filled pNLVP16. To construct VP16 Rev aa 509–614, GAL-Rev aa 21–614 (12) was digested with *Sac*I, end filled with T4 DNA polymerase and ligated into *Xho*I-cleaved, Klenow end-filled pNLVP16. To construct GV-Rev aa 509–614, VP16 Rev aa 509–614 was digested with *Sal*I/*Xba*I, the resulting fragment was then ligated into *Sal*I/*Xba*I-cleaved pGAL4/VP16. To construct VP16 Rev aa 21–614, the primers GMUQ142 and GMUQ132 were used to amplify a region from 63 to 1847 bp. The resulting fragment was cleaved with *Bam*HI, end filled with Klenow and ligated into *Nde*I-cleaved, Klenow end-filled pNLVP16. To construct VP16 Rev aa 290–614, Rev-erbAα cDNA was digested with *Eco*RV/*Bam*HI, end filled with Klenow and ligated to *Xho*I-cleaved, Klenow end-filled pNLVP16.

To construct VP-Rev aa 437–509 and GV-Rev aa 437–509, VP16-Rev aa 437–614 was cleaved with *Sac*I, and ligated into *Sac*I-cleaved pGAL4/VP16. The resulting vector was cleaved with *Sal*I/*Xba*I and the Rev-erbAα fragment was cloned into *Sal*I/*Xba*I-digested pNLVP16 respectively.

For construction of the following GV-Rev chimeras, the following primers were used to amplify regions of Rev-erbAα from GV-Rev aa 437–614; GV-Rev aa 437–476 (GMUQ301 and GMUQ304); GV-Rev aa 455–476 (GMUQ303 and GMUQ304) and GV-Rev aa 455–488 (GMUQ303 and GMUQ302). The following primers were used to create mutations in the FAKH and L3-4 regions of GV-Rev aa 455–488; GV-Rev AGAR (GMUQ307 and GMUQ302); GV-Rev ADAQ (GMUQ308 and GMUQ302); (GV-Rev FAA (GMUQ 327 and GMUQ 302); GV-Rev L3–4 A (GMUQ 328 and GMUQ 302) and GV-Rev L3–4 AA (GMUQ 329 and GMUQ 302). These fragments containing primer-derived 5′ *Sal*I and 3′ *Xba*I sites were digested with *Sal*I/*Xba*I and ligated to *Sal*I/*Xba*I-digested pGAL4/VP16. VP16 Rev aa 455–488 was constructed by amplifying this region of Rev-erbAα from GV-Rev aa 437–614 using the primers GMUQ303 and GMUQ302, digesting the product with *Sal*I/*Xba*I and ligating to *Sal*I/*Xba*I-digested pNLVP16.

Double-stranded sequencing of ligation junctions confirmed authenticity and that the foreign proteins were being expressed in frame.

Cell culture and transfection

COS-1 or JEG-3 cells were cultured for 24 h in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% (v/v) fetal calf serum (FCS) before transfection. Each 35 mm dish (Falcon) of COS-1 cells (60–70% confluence) was transiently transfected with 2.5 μg of reporter plasmid DNA (G5E1b-CAT) expressing chloramphenicol acetyl transferase (CAT), mixed with the appropriate amount of expression vector (1 μg GAL4/VP16 chimeras) by the DOTAP (Boehringer Mannheim) mediated procedure as described previously (24,26). The DNA/DOTAP mixture was added to the cells in 3 ml of fresh medium. After a period of 24 h, fresh medium was added to the cells. Cells were harvested for the assay of CAT enzyme activity 24–72 h after the transfection period. Each transfection was performed at least three times to overcome the variability inherent in transfections.

Mouse myogenic C2C12 cells (27,28) were grown in DMEM supplemented with 20% (v/v) FCS in 6% CO_2. Prior to and during transfection, this cell line was induced to biochemically and morphologically differentiate into multinucleated myotubes by serum withdrawal medium [DMEM supplemented with 2% (v/v) FCS]. Each 35 mm dish of myogenic C2C12 cells (90–100% confluence) was transiently transfected as described above. After 24 h, fresh medium was added to the cells. Cells were harvested for the assay of CAT enzyme activity 24 h after the addition of fresh medium. Each transfection experiment was performed at least three times to overcome the variability inherent in transfections.

Mammalian two hybrid assay

Plasmids ([G5E1bCAT reporter, 3 μg) and GAL NCoR (ID: interaction domain: aa 2218–2446) were co-transfected/expressed into JEG-3 cells cultured for 24 h in 3 ml DMEM containing 5% charcoal stripped FCS, in 6% CO_2 at 37°C prior to transfection (using DOTAP as described previously) with one of the following vectors; VP16, VP16-cTRα, VP16-mRXRγ, VP16-Rev aa 21–614, VP16-Rev aa 107–614, VP16-Rev aa 290–614, VP16-Rev aa 437–614, VP16-Rev aa 509–614 or VP16-Rev aa 455–488. The DNA/DOTAP mixture was added to the cells in 3 ml of fresh medium. After a period of 24 h, fresh medium was added. Cells were harvested after a further 24 h and assayed for the ability to *trans*-activate the reporter (GE1bCAT). Each transfection was performed at least three times in order to overcome the variability inherent in transfections.

CAT assays

Cells were harvested, normalised to protein concentration (29) and CAT activity measured as previously described (30). Aliquots of the cell extracts were incubated at 37°C, with 0.1–0.4 mCi [^{14}C]chloramphenicol (ICN) in the presence of 5 mM Acetyl CoA in 0.25 M Tris–HCl pH 7.8. After a 1–4 h incubation period, the reaction was stopped by the addition of 1 ml ethyl acetate which was used to extract the chloramphenicol and its acetylated forms. Extracted materials were analysed on Silica gel thin layer chromatography plates as described previously (30). Quantitation of all CAT assays (except Fig. 2D) was performed by an AMBIS β-scanner. Please note that Figure 2D was quantitated on a BioRad Molecular Image System, GS-363, which has increased sensitivity. This helped to discriminate between the subtle effects of different point mutations.

Western blots

Cells were washed in PBS, scraped into 1% SDS and protein content was determined using the BCA Protein Assay (Pierce). Total cell extract (30 μg) was boiled in Laemmli sample buffer and electrophoresed on 10% polyacrylamide resolving gels before being electrophoretically transferred onto nitrocellulose (Hybond-C Super, Amersham). Non-specific binding sites were blocked by immersing membranes in 5% skim milk in PBS for 1–2 h at room temperature followed by incubation with Rabbit anti-GAL 4 antisera (Santa Cruz # sc-428) in (1 μg/ml) 1% skim milk in PBS overnight at 4°C. Membranes were then washed three times in PBS containing 0.05% Tween 20. Further steps involving HRP secondary antibodies and the catalysed oxidation of luminol were carried out with Amersham ECL Western blotting detection reagents according to the manufacturer's protocols.

RESULTS

Rev-erbAα contains an independent transferable repression domain within the E region of the protein

Previously, we have mapped an active transcriptional silencing function of Rev-erbAα to the C-terminal putative ligand binding domain (LBD) or D/E region between aa 290 and 614 (12). To characterise further the transcriptional silencing properties of Rev-erbAα and to delimit the repression domain, we investigated the ability of this orphan receptor to repress *trans*-activation by the GAL4/VP16 chimera, a potent activator of gene expression. We sub-cloned segments of the Rev-erbAα cDNA into the GAL4/VP16 expression vector and examined the activation of the CAT reporter gene linked to GAL4 binding sites. A similar investigative approach utilising the GAL4/VP16 chimera has been utilised to analyse the thyroid hormone receptor (31) and Rev-erbAα (12). The GAL4/VP16 protein, which contains the yeast GAL4 DBD and *trans*-activating domain of the herpes simplex virus, VP16, is a potent transcriptional activator of the GAL4 DNA binding sites linked to CAT.

A total of four chimeric GAL4/VP16-Rev-erbAα (GV-Rev) receptor expression plasmids were constructed; GV-Rev aa 437–614, GV-Rev aa 509–614, GV-Rev aa 557–614, GV-Rev aa 437–509. The previously described GV-Rev aa 290–614 (12) was used as a comparison in these experiments. These constructs were co-transfected with the reporter, G5E1b-CAT, into COS-1 cells and the CAT activity assayed.

Efficient transcriptional repression (~60–80-fold) was observed when the regions of Rev-erbAα aa 437–614 or 437–509 were fused to the GAL4/VP16 chimera (Fig. 1A). In contrast, the aa 509–614 and 557–614 of Rev-erbAα had no effect on the ability of the GAL4/VP16 protein to *trans*-activate gene expression (Fig. 1A). The aa 290–614 portion of Rev-erbAα linked to GAL4/VP16 as published previously (12), also repressed (~40–60-fold) the transcription of GAL4/VP16. In summary, this deletion analysis indicated that repression was mediated by a short region between aa 437 and 509 in Rev-erbAα.

To verify that the weak activity of the GV-Rev chimeric proteins was not due to different stability or expression levels relative to the GAL4/VP16 chimera, we analysed via Western blot analysis whole cell extracts from untransfected COS-1 cells, and COS-1 cells transfected with GAL4/VP16 alone or GV-Rev aa 437–509 (which demonstrated strong repression). The GV-Rev aa 437–509 (~28.4 kDa) protein was expressed at higher levels

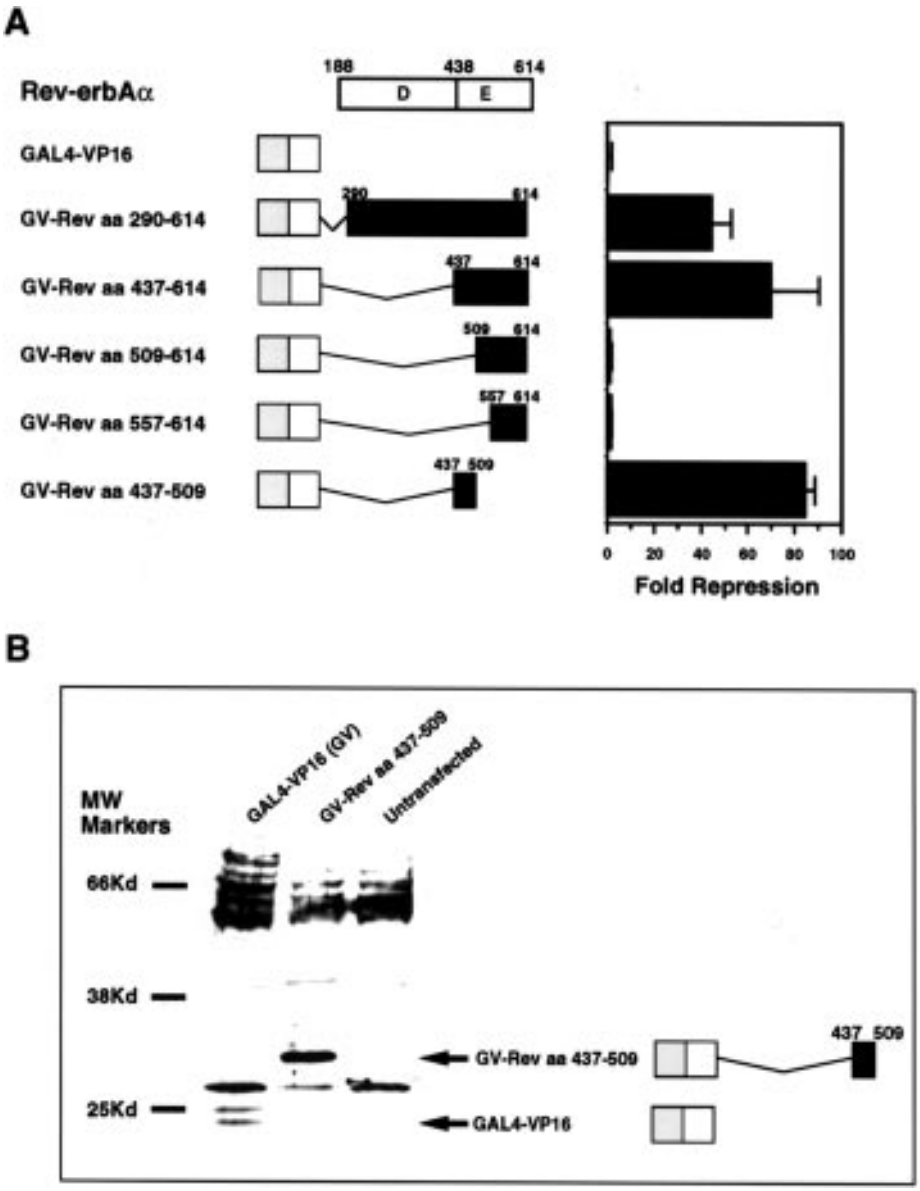

Figure 1. Localisation of a repression domain of Rev-erbAα. Various regions of RVR were sub-cloned into the multiple cloning site of GALVP16 in frame and 3′ of the GAL4/VP16 coding region. (**A**) COS-1 cells were co-transfected with pG5E1bCAT reporter (2.5 µg) and GV-Rev chimeras (1 µg) and assayed for CAT activity. Results shown are mean ± standard deviation (SD) and were derived from a triplicate experiment. Transcriptional repression is expressed relative to GAL4/VP16 alone. (**B**) Whole cell extracts from untransfected COS-1 cells and COS-1 cells transfected with GAL4/VP16 or GV-Rev aa 437–509 were analysed on Western blots using a polyclonal antibody to the GAL4 DBD. The positions of the transfected proteins are indicated.

(~2-fold) than that of GAL4/VP16 (~21 kDa) verifying that the perceived ability of the region aa 437–509 of Rev-erbAα to repress the activation of the CAT construct was not simply due to a reduction in the amount of GAL4/VP16 chimeric construct being expressed.

Interestingly, we note that the region between aa 437 and 509 in the Rev-erbAα protein that mediates repression, exhibits ~90% homology to a similar region in the RVR protein (aa 399–470) indicating that the repression domain is strongly conserved between these two closely related genes (see Fig. 2A). These experiments have identified an independent, transferable and highly conserved repression domain within the E region of the Rev-erbAα (aa 437–509) protein.

Transcriptional repression by Rev-erbAα is dependent on the nuclear receptor-specific signature motif and helix 5 in the ligand binding domain: helix 5 is necessary for transcriptional silencing

Recent publication of the crystal structures for the LBD of three members of the steroid/thyroid receptor superfamily, TR, RAR and RXR revealed a conserved canonical structure in the LBD, consisting of 12 α-helices (32–34). The availability of this structural data prompted us to align Rev-erbAα with TR, RAR and RXR. The smallest identified repression domain of the Rev-erbAα (aa 438–509) encompasses a region that forms α-helices 3 (H3), Loop3–4 (L3–4), 4 (H4), 5 (H5) and the β-sheet

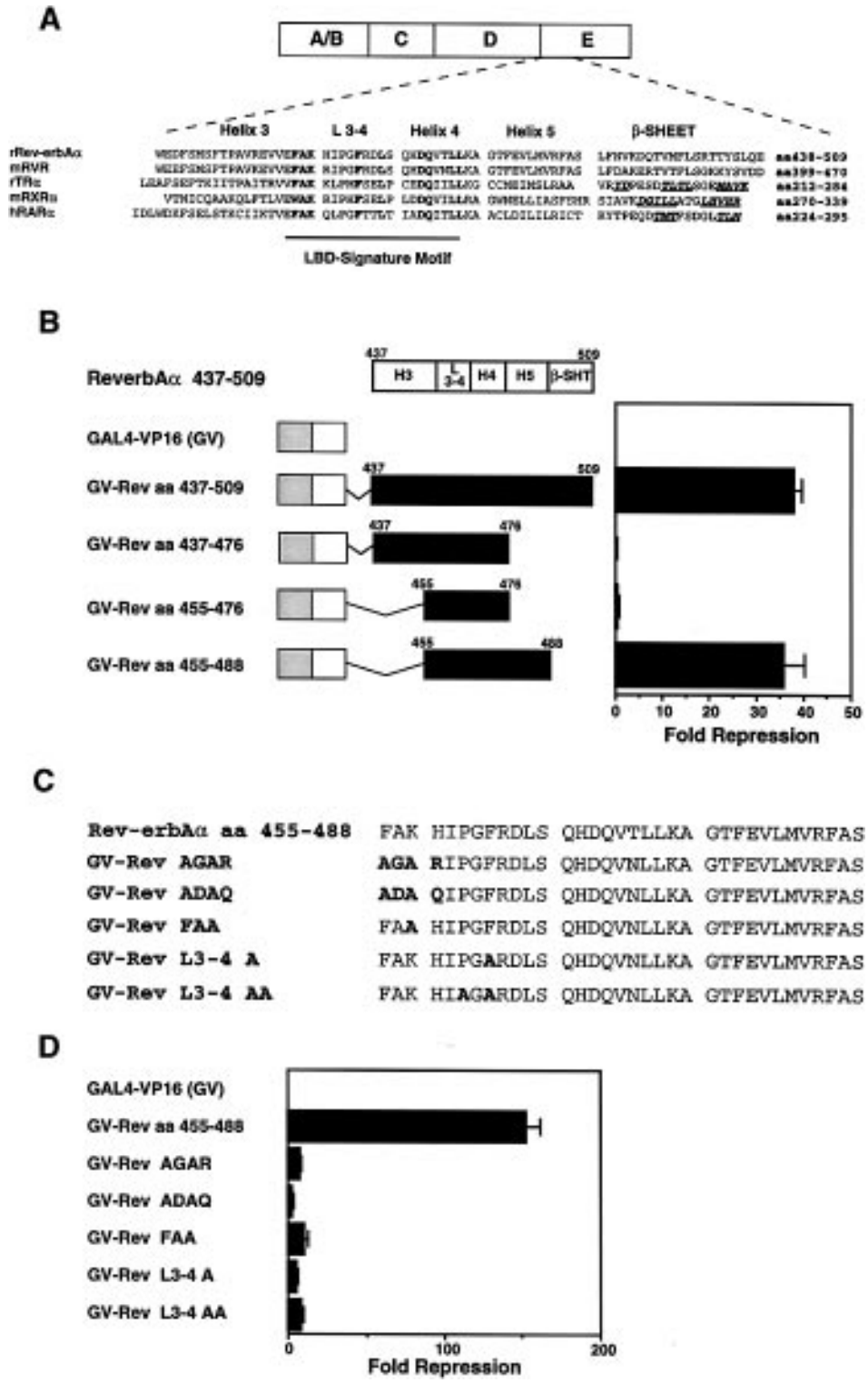

Figure 2. Analysis of the Rev-erbAα repression domain in the E region. (**A**) Alignment of rRev-erbAα, mRVR, rTRα, mRXRα and hRARα showing the region from H3 to H5 and β-sheet in the E region. Helical assignments for rTRα (32), rRev-erbAα, mRXRα and mRVR are based upon the helices denoted for the hRXRα and hRARα crystal structures (33). Conserved amino acids in the LBD-specific signature motif (underlined) are in bold type. (**B**) COS-1 cells were co-transfected with pG5E1bCAT reporter (2.5 μg) and or GV-Rev-erbAα chimeras (1 μg) and assayed for CAT activity. Results shown are mean ± SD and were derived from a triplicate experiment. Transcriptional repression is expressed relative to GAL4/VP16 alone. (**C**) Sequence of Rev-erbAα aa 455–488 and mutations induced in the FAK and L3–4 regions. Mutated amino acid residues are shown in bold type. (**D**) The chimeric GV-Rev expression plasmids containing mutations in the repression domain depicted in (C) were co-transfected with the reporter (G5E1b-CAT) into COS-1 cells and the CAT activity assayed. Results shown are mean ± SD and were derived from a triplicate experiment. Transcriptional repression is expressed relative to GAL4/VP16 alone.

formation in TR, RAR and RXR (see Fig. 2A). This region includes the 'LBD-specific signature motif', [(F/W)AKxxxxFxx-LxxxDQxxLL] that is highly conserved among the nuclear hormone receptors, and lies within the E-region of most steroid receptors spanning H3, L3–4 and H4 of the LBD (35). It has been proposed that this motif contributes to stabilisation the canonical structure of the LBD (35). Interestingly, this region is found in the Rev-erbAα repression domain. Based on this information, we decided to investigate the role of H3, L3–4, H4 (that span the LBD-specific signature motif), H5 and the β-sheet in repression of transcription by Rev-erbAα.

We constructed three chimeric GV-Rev receptor expression plasmids; GV-Rev aa 455–488 (FAK, L3–4, H4 and H5), GV-Rev aa 437–476 (H3, L3–4 and H4) and GV-Rev aa 455–476 (FAK, L3–4 and H4), the previously described GV-Rev aa 437–509 (H3 to β-sheet) was used as a control in this experiment. These were co-transfected with the reporter G5E1b-CAT, and the CAT activity assayed (Fig. 2B).

The region of Rev-erbAα between aa 455 and 488 (FAK, L3–4, H4 and H5) very efficiently repressed (~40-fold) GAL4/VP16 mediated *trans*-activation (Fig. 2B). This suggested that most of helix 3 and the β-sheet were not required for repression of transcription by Rev-erbAα. In contrast, the aa 437–476 (H3, L3–4 and H4) and 455–476 (FAK, L3–4 and H4) regions of Rev-erbAα had no effect on the ability of the GAL4/VP16 protein to *trans*-activate gene expression (Fig. 2B). This strongly indicated that helix 5 was absolutely necessary for efficient repression. The aa 437–509 (H3 to β-sheet) portion of Rev-erbAα linked to GAL4/VP16 as earlier described, also repressed (~40-fold) the transcription of GAL4/VP16.

These fine deletion studies suggested that the LBD-specific signature motif (that spans FAK, L3–4 and H4) and H5 mediated active transcriptional silencing. The aa of H3, N-terminal of the FAK residues and the β-sheet region are not necessary for repression. Deletion of H5 abolished repression, suggesting it was absolutely required for repression: furthermore, the data indicated that the LBD-specific signature motif (FAK, L3–4 and H4), in the absence of H5, cannot mediate active transcriptional silencing.

The highly conserved amino acid residues of the LBD specific signature motif are required for efficient transcriptional repression by the Rev-erbAα orphan receptor

The LBD-specific signature motif [(F/W)AKxxxxFxxLxxx-DQxxLL] has been assigned many functions including dimerisation and *trans*-activation. The conserved aromatic amino acid (F) has been shown to set the spatial limits of this hydrophobic cluster (on F455 in Rev-erbAα), and the conserved lysine (K) in the FAK motif has been demonstrated to interact with the glutamine residue in H4 (aa K457-Q470 in Rev-erbAα) respectively (Fig. 2A) (35). Furthermore, the predominantly hydrophobic amino acids in this region cluster around an invariant phenylalanine in the L3–4 sequence (HIPGFRDLS in Rev-erbAα). Therefore, we decided to mutate the FAK sequence and the invariant phenylalanine in the LBD-specific signature motif to determine the contribution of these aa to the function of the silencing domain.

We constructed five new chimeric GV-Rev expression plasmids containing mutations in the repression domain, changing the FAKH sequence (in H3) to AGAR (GV-Rev AGAR), ADAQ (GV-Rev ADAQ) and FAAH(GV-Rev FAA); and changing the L3–4 sequence (HIPGFRDLS) to HIPGARDLS (GV-Rev L3–4 A) and HIAGARDLS (GV-Rev L3–4 AA) (Fig. 2C). The GV-Rev aa 455–488 (FAK to H5) construct was used as a comparison of repression ability. These were co-transfected with the reporter, G5E1b-CAT, and the CAT activity assayed.

Complete mutation of the FAKH motif to either AGAR or ADAQ inhibited the ability of Rev-erbAα to repress transcriptional activation by GAL4/VP16 (see GV-Rev AGAR and GV Rev-ADAQ; Fig. 2C and D). Interestingly, the impact of the ADAQ versus AGAR mutation on repression is more significant, probably due to the mutation of a neutral alanine (A) to a charged aspartic acid (D). Furthermore, the specific mutation of the lysine (K457) to an alanine (A) in the FAK motif significantly reduced the ability of Rev-erbAα to repress *trans*-activation by GALVP16 (see GV-Rev FAA; Fig. 2C and D). Specific mutation of the invariant phenylalanine (F462) in loop 3–4 or the simultaneous mutation of the invariant F and the highly conserved proline (P460) in loop 3–4 significantly reduced the ability of Rev-erbAα to repress *trans*-activation by GALVP16 (see GV-Rev L3–4 A and GV-Rev L3–4 AA; Fig. 2C and D).

These results demonstrated that the FAK residues and the invariant phenylalanine (F) in L3–4 of Rev-erbAα, are essential for efficient repression of *trans*-activation by the GAL4/VP16 protein. Combined with our previous observations, we can conclude that the conserved residues in the LBD-specific signature motif (spanning FAK, L3–4 and H4) and helix 5 between aa 455 and 488 are required for repression of transcription by Rev-erbAα.

The independent/transferable repressor domain functions in different cell types: the co-factors involved in transcriptional silencing are not cell-specific

We then investigated whether co-factors involved in repression by Rev-erbAα were cell-specific. We examined whether GV-Rev aa 437–614 and GV-Rev aa 455–488, both of which exhibit strong repression of transcription in COS-1 cells, were able to repress transcription efficiently in C2 (mouse myogenic) and JEG-3 (choriocarcinoma) cells. In both cell lines, we observed strong repression by both the GV-Rev aa 437–614 or the GV-Rev aa 455–488 constructs (~40–60-fold) (Fig. 3A), suggesting that different cell types possess co-factors that interact with Rev-erbAα to mediate repression.

Repression by Rev-erbAα is not mediated by N-CoR *in vivo*

The molecular mechanisms responsible for nuclear receptor transcriptional silencing are not well understood. However, the nuclear receptor co-repressor, N-CoR, a 270 kDa protein, has been characterised as a co-repressor for the steroid receptors, TR and RAR (19,20). N-CoR mediates ligand-independent inhibition of gene transcription by direct binding in the unliganded state and regulation of AF-2 function. In an effort to understand the mechanism of repression by Rev-erbAα, we used the mammalian two hybrid system to investigate the possible interaction of Rev-erbAα and N-CoR. The two hybrid system was originally developed in yeast to investigate protein–protein interactions and has since been adapted for use in mammalian cells. A chimeric receptor consisting of the yeast GAL4 DNA binding domain fused to the characterised interaction domain (ID) (aa 2218–2451) of N-CoR (20) was expressed in cells with a second chimeric receptor containing full length or various deletions of the Rev-erbAα receptor linked to the *trans*-activation domain of

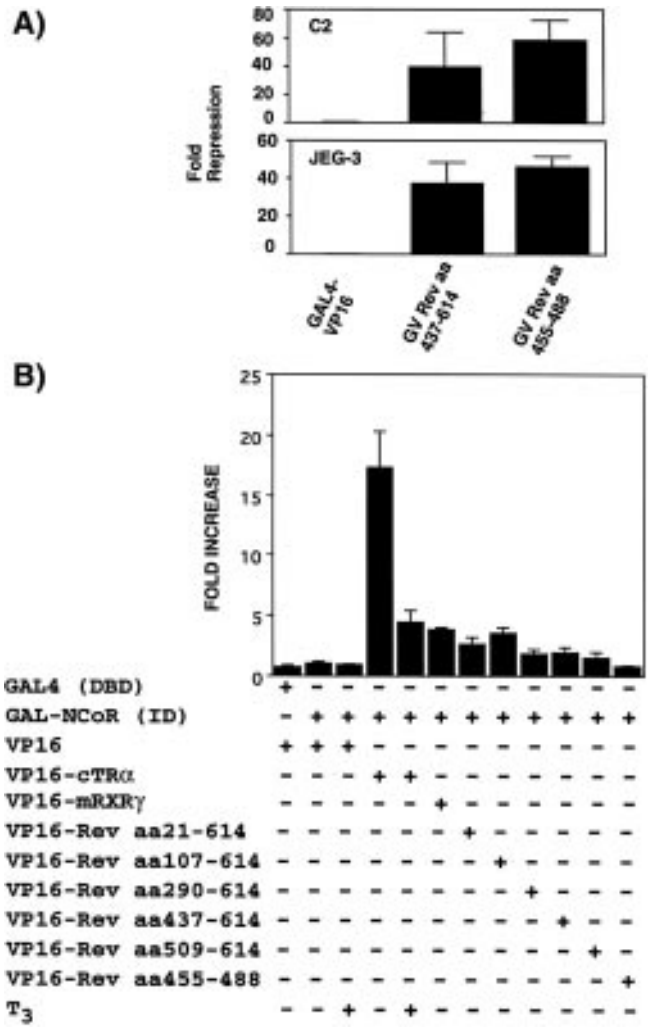

Figure 3. (**A**) Analysis of the repression domain of Rev-erbAα in different cell types. C2C12 (mouse myogenic cells) and JEG-3 cells were co-transfected with pG5E1bCAT reporter (2.5 μg) and GV-Rev chimeras (1 μg) and assayed for CAT activity. Results shown are mean ± SD and were derived from a triplicate experiment. Transcriptional repression is expressed relative to GAL4/VP16 alone. (**B**) Rev-erbAα does not interact efficiently with the interaction domain of the co-repressor, N-CoR, *in vivo*. JEG3 cells were co-transfected with pGAL0 or GAL4-mN-CoR (ID) and VP-Rev chimeras as indicated in the presence of the reporter pG5E1bCAT. Positive and negative controls VP16-cTRα (±T_3) and VP16-mRXRγ are included. The mean CAT activity values and SDs (bars) were derived from a triplicate experiment. CAT activity is represented as fold increase over activity after transfection of GAL-NCoR (ID) and VP16.

VP16. In these assays, *trans*-activation of a CAT reporter gene downstream of GAL4 binding sites fused to the E1b promoter is only achieved when the co-expressed receptors physically interact. In this study, we used the chimeric VP16 constructs containing TRα and RXRγ that have been shown to interact strongly and very weakly respectively, with N-CoR as positive and negative controls respectively.

Consistent with previous interaction studies, we saw a 15–20-fold increase in CAT activity when the VP16-cTRα construct was transfected with the GAL-N-CoR (ID) construct (19,20). In the presence of T_3, the ligand for TR, this activity, as expected, was reduced to a level equivalent to that seen with VP16-mRXRγ (3–4-fold) indicating very weak interaction of TR with N-CoR in the presence of it cognate ligand (Fig. 3B). Little or no CAT activity was observed when any of the VP16-Rev-erbAα (full length or delimited) constructs were co-transfected with GAL-N-CoR (ID) suggesting inefficient or no interaction of the Rev-erbAα receptor with the characterized interaction domain (aa 2218–2451) of N-CoR in intact cells.

Transcriptional activity of Rev-erbAα is modified by agents that stimulate phosphorylation (12). Therefore, we also tested whether the phosphorylation of Rev-erbAα could induce the interaction of Rev-erbAα with N-CoR. In the presence of 8-Br-cAMP no significant interaction between Rev-erbAα and N-CoR was observed in intact cells (data not shown).

These results suggest that Rev-erbAα does not physically contact/associate with the interaction domain of N-CoR (aa 2218–2451) and that transcriptional repression by Rev-erbAα involves a mechanism that functions independently of N-CoR.

DISCUSSION

This work reports on the characterisation of the repression domain in the Rev-erbAα receptor. We have shown that the Rev-erbAα receptor contains a potent transcriptional silencing domain in the C-terminal region of the protein that abrogates the ability of the potent *trans*-activator, GAL4/VP16, to activate gene expression. Deletion analyses narrowed the repression domain to a region encompassing aa 437–509 of the Rev-erbAα protein. These results are contrary, in some aspects, to those obtained by Harding and Lazar which indicated that Rev-erbAα contains two repression domains located between aa 200 and 289 and aa 376 and 614 that are dependent on each other for maximal repression (14). Furthermore, that report indicated that the region between aa 432 and 614 of Rev-erbAα exhibited no transcriptional repression; this is not in agreement with the results obtained from our study (aa 437–614 showed strong repression of GAL4/VP16 transcriptional activity ~60–80-fold). Differences observed in the two studies may be accounted for by the choice of assay system used, GAL4 rather than GAL4/VP16, and the reporter system employed, pGL2-luciferase (SV40 minimal promoter) versus G5E1b-CAT (E1b TATA box). It is unlikely however, that these differences could account for the marked discrepancies between the two studies.

We have defined this silencing domain further in the context of the recently published LBD consensus structure for steroid receptors (35). The publication of crystal structures for the LBD of TR, RAR and RXR have revealed that the LBD forms a conserved canonical structure consisting of 12 α-helices. The repression domain identified in Rev-erbAα encompassed a region that would putatively form helices 3–5 (H3–H5) and includes a region which contains β-sheet formation. This region also contains the highly conserved LBD-signature motif (F/W) AKxxxxFxxLxxxDQxxLL, that is present in most (>80) steroid receptors (35). It has been proposed that these conserved residues hold together helices H3, H4, H5, H8 and H9, and the loops to form a hydrophobic pocket, the LBD core. The structural analysis of TR/RAR/RXR indicates that this region is buried inside the receptor. Whether this is the case with Rev-erbAα which does not contain helix 12 (AF-2 domain) remains to be determined. Furthermore, the predominantly hydrophobic amino acids in this region cluster around an invariant phenylalanine in the L3–4 sequence. The conserved aromatic amino acid, phenylalanine,

and the conserved lysine in the FAK motif have been shown to set the spatial limits of this hydrophobic cluster and to interact with the glutamine residue in H4 respectively (aa K457 and Q470 in Rev-erbAα) (Fig. 2A) (35).

Fine deletion analysis demonstrated that the LBD-signature motif (i.e. FAK, L3–4 and H4) and H5 are sufficient and necessary to mediate transcriptional repression by Rev-erbAα, delimiting the minimal region required for active transcriptional silencing of gene transcription to a 34 amino acid region between aa 455 and 488, located in the E-region of the Rev-erbAα receptor. This domain is conserved within the Rev-erb family with only one aa difference found in the RVR/Rev-erbβ receptor (aa 416–449). To date, the reported transcriptional repression properties of both the Rev-erbAα and RVR have been identical (9,10,15).

Site-specific mutagenesis demonstrated and verified the importance of the FAK motif, the conserved lysine (K457) in the FAK motif and the invariant phenylalanine (F462) in the L3–4 sequence (HIPGFRDLS) of Rev-erbAα. Interestingly, mutation of the FAK motif, to ADA versus AGA had a more significant impact on the ability of this region to repress transcription, suggesting that a charged amino acid (e.g. D) disrupts the structure of this domain.

We also demonstrated that the repression domain of Rev-erbAα is functional in myogenic cells (mouse C2C12 muscle cells) and choriocarcinoma cells adding to list of cell types (JEG-3, HepG2, NIH3T3 and 293T cells) in which transcriptional repression by Rev-erbAα has been demonstrated (14). This data supports the hypothesis that co-factors involved in repression by Rev-erbAα are not cell-specific. Furthermore, we have shown that Rev-erbAα acts as a transcriptional repressor in a cell type where it has been demonstrated to function in an antagonistic manner (12).

Interestingly, both Rev-erbAα (14) and RVR (15) have been demonstrated to repress basal transcription when bound, as a homodimer, to their cognate DR-2 element, but not however, when bound as a monomer to the characterised asymmetric 11 bp motif. It is possible, therefore, that two copies of the repression domain are required for function *in vivo*. Consistent with this hypothesis is the knowledge that GAL4 DBD binds to DNA as a dimer (36) and therefore our chimeric constructs would presumably also bind DNA as dimers. The repression domain we have identified as discussed earlier, contains the LBD-signature motif which appears to be necessary for the function of the repression domain. This motif has also been characterised as important in protein dimerisation of steroid receptors and similar mutations to the ones performed in this study (mutation of the aa FAK) are known to disrupt dimerisation of other steroid receptors (37–39).

In this report a potential mechanism of repression by Rev-erbAα was also investigated utilising the mammalian two hybrid system to study whether the recently characterised nuclear receptor co-repressor, N-CoR, interacted with the Rev-erbAα receptor. We found that, in contrast with TR which interacted strongly with aa 2218–2451 of N-CoR in a ligand-dependent manner (19), Rev-erbAα did not strongly associate with the characterised interaction domain of N-CoR (aa 2218–2451). Interaction between Rev-erbAα and N-CoR was not observed after the addition of a PKA stimulating agent (8-Br-cAMP). These results suggest that Rev-erbAα does not mediate repression through an interaction with N-CoR. However, we cannot rigorously rule out that other regions/domains of N-CoR may interact with this orphan receptor. This does not discount the possibility that novel co-repressors may mediate active transcriptional silencing by the Rev-erbAα receptor.

Whether the domain we have defined between aa 455 and 488 of Rev-erbAα directly represses transcription or functions as a repression interface for another molecule is not clear. Another possible mechanism by which Rev-erbAα could be mediating active transcriptional silencing of gene transcription is by interaction with the basal transcription factors such as TATA-binding protein or TFIIB, both of which have been demonstrated to interact with other nuclear steroid receptors in a ligand-dependent manner (40–42). It has been suggested that the interaction of these basal transcription factors with steroid receptors may be, at least partially, responsible for repression of basal transcription by steroid receptors (40–42). We are currently investigating whether the Rev-erbAα receptor can interact with any of these basal transcription factors.

In conclusion, we have characterised an independent, dominant, transferable transcriptional silencer in the E region of the orphan steroid receptor, Rev-erbAα, that mediates transcriptional repression through an, as yet, unknown mechanism.

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