Repression of basal transcription by vitamin D receptor: evidence for interaction of unliganded vitamin D receptor with two receptor interaction domains in RIP13Δ1

P P Dwivedi, G E O Muscat¹, P J Bailey¹, J L Omdahl² and B K May

Department of Biochemistry, University of Adelaide, Adelaide, South Australia 5005, Australia

¹University of Queensland, Centre for Molecular and Cellular Biology, Ritchie Research Laboratories B402, St Lucia, Queensland 4072, Australia

²Department of Biochemistry and Molecular Biology, University of New Mexico School of Medicine, Albuquerque, New Mexico 87131-5221, USA

(Requests for offprints should be addressed to J L Omdahl)

ABSTRACT

Repression of basal transcription of a 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) responsive 25-hydroxyvitamin D₃-24-hydroxylase (CYP24) promoter construct was observed in kidney cells in the absence of ligand and this repression was dependent on a functional vitamin D response element (VDRE). Basal repression was also seen with a construct where a consensus DR-3-type VDRE was fused to the thymidine kinase promoter. Expression of a dominant negative vitamin D receptor (VDR) isoform that strongly bound to the VDRE motif in the CYP24 promoter ablated basal repression. This VDR isoform lacked sequence in the hinge- and ligand-binding domains implicating one or both of these domains in basal repression. It is well known that thyroid hormone and retinoic acid receptors silence basal transcription of target genes in the absence of ligands and this repressor function can be mediated by the nuclear receptor corepressor N-CoR. Two variants of N-CoR have been described, RIP13a and RIP13Δ1. N-CoR and the variants contain two receptor interaction domains, ID-I and ID-II, which are identical

except region ID-II in RIP13Δ1 has an internal deletion. We have used the mammalian two hybrid system to investigate whether VDR, in the absence of ligand 1,25-(OH)₂D₃, can interact with these domains. The data showed that unliganded VDR does not interact with either ID-I or ID-II from RIP13a and RIP13Δ1, but does interact strongly with a composite domain of ID-I and ID-II from RIP13Δ1 (but not from RIP13a) and this strong interaction is abrogated in the presence of ligand. This finding implicates RIP13Δ1 in VDR-dependent basal repression of the promoter constructs under investigation. However, overexpression of RIP13Δ1 in kidney cell lines did not alter basal expression of the CYP24 promoter construct. It is concluded that either the level of endogenous RIP13Δ1 in these kidney cells permits maximal repression or that repression occurs by a mechanism that is independent of RIP13Δ1. Alternatively, repression may be dependent on RIP13Δ1 but requires an additional cofactor that is limiting in these cells.

Journal of Molecular Endocrinology (1998) **20**, 327–335

INTRODUCTION

In the absence of ligands, thyroid hormone receptor (TR) and retinoic acid receptor (RAR) can bind as heterodimers with retinoid X receptor (RXR) to repress basal transcription of target genes (Chen & Evans 1995, Hörlein *et al.* 1995, Horwitz *et al.* 1996). The silencing activity of TR and RAR is mediated by corepressor proteins. Two distinct but related corepressor proteins that have been identified,

N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator for retinoid and TRs; also referred to as TRAC-2 or TR associating cofactor), bind to unliganded TR or RAR (Chen & Evans 1995, Hörlein *et al.* 1995, Sande & Privalsky 1996). N-CoR contains two repressor domains at the amino terminus (Hörlein *et al.* 1995, Zamir *et al.* 1996) and a nuclear receptor interaction domain at the carboxyl terminus. The receptor interaction domain contains two sub-regions consisting of ID-I

(Hörlein *et al.* 1995) and ID-II (Seol *et al.* 1996). It has been demonstrated that ID-I in N-CoR is capable of interacting independently with either unliganded TR or RAR (Hörlein *et al.* 1995, Seol *et al.* 1996). However, these receptors interact more strongly with a region composed of ID-I and ID-II (Downes *et al.* 1996). Two N-CoR variants RIP13a and RIP13Δ1 have recently been characterized where the first N-terminal 1000 amino acids of N-CoR have been replaced with a unique short region (Seol *et al.* 1996). These variants retain ID-I and ID-II except that ID-II from RIP13Δ1 has an internal deletion of 120 amino acids (ID-IIΔ1) (Seol *et al.* 1996).

The molecular action of 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) is mediated through the vitamin D receptor (VDR), a ligand-regulated transcription factor that binds as a heterodimer with RXR to specific vitamin D response elements (VDREs) in target-gene promoters. In this study, we examine the regulation by 1,25-(OH)₂D₃ of the gene for 25-hydroxyvitamin D₃ 24-hydroxylase (CYP24) (Hahn *et al.* 1994, Kerry *et al.* 1996), the key enzyme involved with 1,25-(OH)₂D₃ metabolism (Darwish & DeLuca 1996). In this communication, we have shown that VDR is involved in the repression of basal expression of a CYP24 promoter construct and also an unrelated promoter. These findings prompted us to investigate whether N-CoR or the N-CoR variants RIP13a and RIP13Δ1 interact with VDR to elicit this repression.

MATERIALS AND METHODS

Construction of plasmids

The GAL4 plasmids containing different interaction domains, the chloramphenicol acetyl transferase (CAT) reporter gene construct (pG5E1bCAT) and the VP16-TRα and VP16-RXRγ expression vectors employed in the mammalian two hybrid assay have been described earlier (Downes *et al.* 1996). The construct VP16-VDR was prepared by inserting an end-filled EcoRI fragment encoding the full length human VDR cDNA sequence into the end-filled XhoI site of pNLVP-16 containing the SV40 early promoter (Downes *et al.* 1996).

Two oligonucleotide PCR primers, with KpnI sites, were employed to amplify 186 bp of CYP24 promoter sequence (encompassing a VDRE at -150/-136 together with 74 bp of 5'-untranslated region) using as the template the plasmids pGL3WT (-298) and pGL3M1 (-298), the latter with a mutation in the proximal hexameric half site of the VDRE (5'-AGGTGA-3' to 5'-GCTTGA-3')

as described by Kerry *et al.* (1996). The PCR products for the wild type and mutant VDRE promoters were purified, digested with KpnI and ligated into the KpnI site of pGL3-Basic containing the firefly luciferase reporter gene (Promega, Madison, WI, USA), to generate pCYP24 WT-LUC and pCYP24M1-LUC. The construct pCYP24M2-LUC with -186 bp of promoter contained a mutation in the distal half site of the VDRE (5'-AGGGCG-3' to 5'-TTAGCG-3') and was synthesized by mutagenesis of the VDRE site in pCYP24WT-LUC using the Quik-Change mutagenesis protocol (Stratagene, La Jolla, CA, USA). Plasmid pSG5-VDRΔE contained a PCR-derived sequence that encodes amino acids 1-124 of the human VDR, which was constructed using the following oligonucleotide primers: 5'-GCGGAA TTCACCATGGAGGCAATGGCGGCCAGC-3' and 5'-CGCGGATCCAGCTTGGGCCGCAGACTG-3'. The 372 bp PCR product was cloned directionally into the EcoRI/BamHI sites of pSG5 (Stratagene) to give the pSG5-VDRΔE construct. The pRSV-hVDR clone has been previously described (Hahn *et al.* 1994), and contains the human VDR cDNA sequence cloned downstream of the Rous sarcoma virus (RSV).

Plasmid DNA was prepared by alkaline lysis and CsCl/ethidium bromide equilibrium density gradients. The plasmid DNA was quantified by spectrophotometry and analyzed by agarose gel electrophoresis to ensure experimental consistency and supercoiling.

Maintenance and transfection of cells

Monkey kidney fibroblast COS-1 and CV-1 cells and human embryonic kidney 293-T cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS). In preparation for electroporation, cells were grown in 175 cm² flasks to 60-70% confluency and removed by trypsinization. Transfections were performed by electroporation of cells (2 × 10⁶) in 500 µl ice cold electroporation buffer (Van den Hoff *et al.* 1992) containing 250 µg sheared salmon sperm DNA, and 2 pmol reporter plasmid. COS-1 and CV-1 cells were electroporated at 280 V and 960 µF while 293-T cells were electroporated at 200 V and 960 µF using a Bio-Rad Gene Pulser. A reference plasmid, pRSV-βgal, was included to correct for variations in transfection efficiency (Herbomel *et al.* 1984). Following electroporation, the samples were placed on ice for 10 min and plated in six-well plates containing DMEM and 10% FCS. Cells were allowed to recover for 24 h and media replaced with RPMI medium containing 10% charcoal-stripped

FCS. Cells were then further incubated or treated for 24 h with 10^{-7} M $1,25\text{-(OH)}_2\text{D}_3$ to activate CYP24 promoter expression, harvested in cell culture and lysis reagent (Promega) and luciferase activity determined as described previously (Kerry *et al.* 1996).

Gel mobility shift assays

A double-stranded oligonucleotide (CYP24-VDRE) was synthesized that encompassed the VDRE at $-150/-136$ in the rat CYP24 promoter (Kerry *et al.* 1996) and contained SalI and XhoI overhangs at the 5' and 3' ends respectively as shown.

5'-TCGAGCGGCGCCCTCACTCACCTCGCG-3'

'3-CGCCGCGGGAGTGAGTGGAGCGCAGCT-5'

CYP24-VDRE was labeled by end-filling with [$\alpha\text{-}^{32}\text{P}$]dCTP using Klenow fragment I enzyme and purified by 12% polyacrylamide gel electrophoresis. Nuclear extracts were prepared from COS-1 cells with or without co-transfection of pRSV-hVDR (or pSG5-VDR Δ E) expression vectors using the published protocol (Andrews & Faller 1991). COS-1 cells co-transfected with pRSV-hVDR were also treated with $1,25\text{-(OH)}_2\text{D}_3$ at 10^{-7} M. Binding reactions containing 5 μg nuclear protein, 1 μg poly (dI-dC) to a final volume of 12 μl in binding buffer (25 mM Tris-HCl, pH 7.6, 100 mM KCl, 0.5 mM dithiothreitol, 5 mM MgCl_2 , 0.5 mM EDTA and 10% glycerol) were incubated on ice for 10 min. Radiolabeled VDRE probe (200 000 c.p.m.) was added and reactions were further incubated on ice for 20 min. For gel shift inhibition assays, a VDR monoclonal antibody designated IgG2B (Affinity BioReagents Inc, Neshanic Station, NJ, USA) was employed. For supershift assays, RXR monoclonal antibody (4RX1D12) was kindly provided by Dr Pierre Chambon (Strasbourg, France). These antibodies were included in the binding reactions and incubated on ice for 10 min prior to addition of the probe. Retarded DNA nuclear protein complexes were resolved on a 4% non-denaturing polyacrylamide gel in low ionic strength running buffer ($0.5 \times \text{TBE}$) at 4°C . The gel was dried and exposed to Kodak X-Omat AR film with an intensifying screen at -70°C .

Mammalian two hybrid assay

Human choriocarcinoma cells, JEG-3, were cultured for 24 h in DMEM containing 5% charcoal-stripped FCS. Cells were co-transfected with 5 μg reporter plasmid (pG5E1bCAT) and 1 μg GAL-ID plasmids with either VP16 or VP16-VDR expression plasmid (1 μg) using the DOTAP procedure (Boehringer, Mannheim, Germany) in six-well

dishes as described previously (Downes *et al.* 1996). After 24 h, fresh medium was added and the cells were further cultured for another 24 h and then harvested for assaying of CAT activity.

Over-expression of RIP13A1

The expression clones for RIP13a (CDM-RIP13a) and RIP13A1 (CDM-RIP13A1) were kindly provided by Dr David Moore (Department of Molecular Biology, Massachusetts General Hospital, Boston, USA). These clones were over-expressed at 1, 2, 5 and 10 μg in the presence and absence of similar amounts of co-transfected pRSV-hVDR in COS-1, CV-1 and 293-T cells and expression of the constructs pCYP24WT-LUC and pDR3TK-LUC was investigated.

RESULTS

Promoter VDRE directed basal ligand-independent repression

We have investigated the expression of a construct (pCYP24WT-LUC) that contains rat CYP24 promoter sequence with a VDRE identified at $-150/-136$ on the non-coding strand (Fig. 1a; Kerry *et al.* 1996). Expression of this construct was induced about 7.5-fold by $1,25\text{-(OH)}_2\text{D}_3$ in COS-1 cells co-transfected with 1 μg VDR expression clone, pRSV-hVDR, to compensate for VDR deficiency (Fig. 1b). The construct pCYP24M1-LUC, in which the VDRE was mutated in the proximal half site, did not respond to hormone, thereby establishing that the VDRE was responsible for induction (Fig. 1b). Basal expression of pCYP24M1-LUC in the absence of added $1,25\text{-(OH)}_2\text{D}_3$ was found to be consistently increased 2.0–2.5-fold compared with the wild-type construct (pCYP24 WT-LUC) in cells co-transfected with pRSV-hVDR. A similar increase in basal expression was seen with the construct pCYP24M2-LUC in which the VDRE was mutated in the distal half site (Fig. 1b). This construct also did not respond to hormone (Fig. 1b).

A moderate increase in basal expression of pCYP24 WT-LUC (1.6-fold) was consistently observed in COS-1 cells not co-transfected with pRSV-hVDR (and therefore containing low endogenous levels of VDR) compared with cells co-transfected with pRSV-hVDR (Fig. 1b). By contrast, the higher basal expression of pCYP24M1-LUC and pCYP24M2-LUC was not altered by VDR over-expression (Fig. 1b). It is evident, therefore, that over-expression of VDR in COS-1 cells represses basal expression of pCYP24 WT-LUC in a manner that requires a functional

VDRE. Similar results were obtained in CV-1 cells although somewhat greater levels of repression were observed. In these experiments, CV-1 cells were co-transfected with increasing amounts of pRSV-hVDR. It can be seen that in the absence of co-transfected pRSV-hVDR, basal expression of pCYP24M1-LUC was 2.5-fold higher than

pCYP24WT-LUC presumably reflecting the inhibitory action of endogenous VDR on the wild-type construct. However, expression of pCYP24WT-LUC was substantially reduced with increasing amounts of pRSV-hVDR so that at 10 µg vector, expression was decreased about 60% (Fig. 1c). In these experiments, expression of pCYP24M1-LUC was not altered by exogenously expressed pRSV-hVDR (Fig. 1c).

We next determined whether basal repression is observed on another 1,25-(OH)₂D₃ responsive promoter. A thymidine kinase promoter construct, either with or without an upstream consensus VDRE of the DR3-type (two AGGTCA motifs separated by 3 bp), was investigated in COS-1 cells transfected with pRSV-hVDR (Fig. 2a). Basal repression was decreased about 40% in the presence of the VDRE (Fig. 2b), although the level of repression was the same in the presence or absence of pRSV-hVDR. This latter finding may reflect a much greater binding affinity of the consensus DR3-type VDRE for VDR/RXR compared with the weaker VDRE from the CYP24 promoter (Jin & Pike 1996) so that maximal repression on the DR3-type VDRE can be achieved with endogenous levels of VDR/RXR. Basal repression is therefore exhibited by two VDREs of the DR3-type with different sequence, strand orientation and location, either in a natural or artificial promoter environment.

FIGURE 1. Effect of unliganded VDR on basal expression of wild-type and mutated VDRE-CYP24 promoter constructs. The constructs shown diagrammatically in (a) are the wild-type pCYP24WT-LUC, pCYP24M1-LUC with a mutation in the proximal half site of the VDRE and pCYP24M2-LUC with a mutation in the distal half site of the VDRE. In (b) COS-1 cells were transfected with 2 pmol of either pCYP24WT-LUC, pCYP24M1-LUC or pCYP24M2-LUC in the presence (+) and absence (-) of 1 µg expression clone pRSV-hVDR and 1,25-(OH)₂D₃ at 10⁻⁷ M. The luciferase activity of pCYP24WT-LUC in the presence of exogenous VDR is taken as 1.0 and activities are expressed relative to this as fold expression. In (c) CV-1 cells were transfected with 2 pmol pCYP24WT-LUC (and 1, 5 or 10 µg pRSV-hVDR) or pCYP24M1-LUC (and 1 or 5 µg pRSV-hVDR). The luciferase activity of pCYPWT-LUC in the absence of exogenous VDR is taken as 100% and activities are expressed relative to this as % expression. As an internal control in (b) and (c), cells were co-transfected with 5 µg β-galactosidase expression vector, pRSV-βgal. Luciferase activities were determined in cell lysates and normalized for transfection efficiency using β-galactosidase activity. Data are the mean ± s.d. of three independent experiments.

FIGURE 2. Effect of unliganded VDR on basal expression of TK promoter constructs with and without a consensus VDRE. The constructs are shown diagrammatically (a). In (b) COS-1 cells were transfected with 2 pmol pTK-LUC or pDR3TK-LUC in the presence (+) or absence (-) of 1 μ g pRSV-hVDR. As an internal control, cells were co-transfected with 5 μ g β -galactosidase expression vector, pRSV- β gal. Luciferase activities were determined in cell lysates and normalized for transfection efficiency using β -galactosidase activity. The luciferase activity of pTK-LUC in the presence of exogenous VDR is taken as 100% and activities are expressed relative to this as % expression. Data are the mean $\pm$ S.D. of three independent experiments.

Effect of a dominant negative VDR on basal expression

Basal expression of pCYP24 WT-LUC in COS-1 cells, co-transfected with pRSV-hVDR, was also investigated using a dominant negative isoform of human VDR, designated VDR Δ E where the ligand binding domain and most of the hinge region were deleted. Using gel shift analysis, a major protein complex was observed using the probe CYP24-VDRE and nuclear extracts from COS-1

cells transfected with pRSV-hVDR (Fig. 3a, lane 2; Kerry *et al.* 1996) while with nuclear extracts from COS-1 cells over-expressing pSG5-VDR Δ E a strong band of faster mobility was detected (Fig. 3a, lane 3). Neither complex was observed with nuclear extracts from COS-1 cells that had not been transfected (Fig. 3a, lane 1). To characterize retarded complexes, we employed monoclonal antibodies to VDR and RXR. With VDR nuclear extracts, VDR antibody prevented complex formation (Fig. 3b, lanes 1 and 2) and the complex was supershifted with RXR antibody (lane 3) as described (Kerry *et al.* 1996) thus demonstrating the presence of VDR and RXR in the complex. By contrast, the neutralizing VDR antibody prevented formation of the complex seen with VDR Δ E extracts (Fig. 3b, lanes 4 and 5), but the supershifting RXR antibody did not react with the complex (lane 6). Therefore, it is evident that the complex formed with the VDR Δ E extracts contains VDR (but not RXR) and is most likely a VDR homo-dimer, a conclusion that agrees with the work of Nishikawa *et al.* (1995).

Transfection of COS-1 cells with the expression clone for VDR Δ E (together with pRSV-hVDR) increased basal expression of pCYP24WT-LUC to a level that was the same as pCYP24M1-LUC (Fig. 3c). In the absence of pRSV-hVDR, basal expression of the wild-type construct was elevated somewhat (1.6-fold) while over-expression of VDR Δ E increased basal expression to the level of pCYP24M1-LUC (Fig. 3c). However, VDR Δ E did not influence basal expression of the mutated VDRE construct pCYP24M1-LUC either in the presence or absence of pRSV-hVDR (Fig. 3c). The action of the dominant negative VDR Δ E to completely reverse VDR-mediated basal repression of the wild-type construct, confirms the dependency of basal inhibition upon a functional VDRE.

Interaction of VDR with a region composed of ID-I and ID-II from RIP13 Δ 1

The corepressor N-CoR has been shown to repress basal expression of the TR and RAR inducible genes. To evaluate whether basal repression by VDR of vitamin D-inducible genes also involves N-CoR, we employed the mammalian two hybrid system to test for an interaction between VDR and N-CoR and its variants RIP13a and RIP13 Δ 1 (Downes *et al.* 1996, Seol *et al.* 1996). In these experiments, the yeast GAL4 DNA binding domain was fused to the interaction domains (ID-I and ID-II) from the corepressors N-CoR, RIP13a and RIP13 Δ 1, and expressed in transfected cells together with the full length human VDR protein linked to the transactivation domain of

FIGURE 3. Binding to the CYP24-VDRE using nuclear proteins from COS-1 cells transfected with either wild-type VDR or the dominant negative VDR (VDRΔE) (a, b) and the effect of VDRΔE on basal expression of pCYP24WT-LUC and pCYP24M1-LUC (c). The radiolabeled oligonucleotide (CYP24-VDRE) encompassing the VDRE was incubated with nuclear extracts of COS-1 cells either mock transfected (COS-1) or transfected with 5 μg pRSV-hVDR (COS-1+VDR) or 5 μg pSG5-VDRΔE (COS-1+VDRΔE). The major retarded complexes unique to pRSV-hVDR and pSG5-VDRΔE are arrowed (a). For neutralization and supershift assays (b) nuclear extracts were incubated with (+) or without (–) VDR-neutralizing monoclonal antibody (VDRmab) or RXR-supersifting monoclonal antibody (RXRmab). The upper arrow (starred) indicates the supershifted complex. In (c) COS-1 cells were transfected with 2 pmol pCYP24WT-LUC or pCYP24M1-LUC in the presence (+) and absence (–) of 1 μg pRSV-hVDR or pSG5-VDRΔE. As an internal control, cells were co-transfected with 5 μg β-galactosidase expression vector, pRSV-βgal. Luciferase activities were determined in cell lysates and normalized for transfection efficiency using β-galactosidase activity. The luciferase activity of pCYP24WT-LUC in the presence of exogenous VDR is taken as 1·0 and activities are expressed relative to this as fold expression. Data are the mean ± s.d. of three independent experiments.

herpes simplex virus VP16 (i.e. VP16-VDR). Trans-activation of a CAT reporter gene downstream of four GAL4 binding sites fused to the E1b promoter (pG5E1bCAT) is only achieved when the corepressor interaction domains and receptor interact physically. We used chimeric GAL4 plasmids that contained either ID-I or ID-II or ID-I+II, and these were designated

GAL4-ID-I, GAL4-ID-II and GAL4-ID-I+II. We also used GAL4 constructs with GAL4-ID-IIΔ1 and GAL4-ID-I+IIΔ1. ID-I corresponded to the region between amino acids 2218/2451 of N-CoR (Hörlein *et al.* 1995) and 1164/1397 in RIP13a (Downes *et al.* 1996, Seol *et al.* 1996), while ID-II corresponded to the region between amino acids 1848/2163 of N-CoR and 794/1109 in RIP13a (Downes *et al.* 1996, Seol *et al.* 1996). ID-II from RIP13Δ1 (ID-IIΔ1) has an internal deletion of 120 amino acids and lacks amino acids 805–925 from RIP13a (Seol *et al.* 1996). Seol *et al.* (1996) mapped the minimal ID-II domain between amino acids 1010/1089 and 2063/2142 in RIP13a and N-CoR respectively, hence our ID-II region encompasses this minimal domain. These interaction domains are shown in Fig. 4a.

The ability of co-expressed VP16-VDR to transactivate gene expression in the two hybrid assay was investigated in JEG3 cells. The plasmids VP16-TRα and VP16-RXRγ were used as positive and negative controls, since these receptors interact strongly and weakly respectively, with N-CoR/RIP13a (Hörlein *et al.* 1995, Downes *et al.* 1996, Seol *et al.* 1996). It was observed that VP16-VDR did not significantly interact with either ID-I (Fig. 4b), as originally reported (Hörlein *et al.* 1995) or with ID-II, or ID-I+II (Fig. 4c and d). However, a substantial increase of about 40-fold in CAT activity was observed when VP16-VDR was transfected with GAL4-ID-I+IIΔ1 (Fig. 4f) while GAL4-ID-II Δ1 alone was ineffective (Fig. 4e). These results demonstrate that VDR strongly interacts with a composite domain of ID-I and ID-IIΔ1 from RIP13Δ1. Treatment of cells with 1,25-(OH)₂D₃ at 10⁻⁷ M ablated completely the

FIGURE 4. VDR interaction with N-CoR, RIP13a and RIP13Δ1 in a mammalian two hybrid assay. (a) Alignment of the interaction domains (ID) for N-CoR, RIP13a and RIP13Δ1: ID-I (dark grey area), ID-II (light grey area), the region from 805 to 925 in RIP13a is deleted in RIP13Δ1. The region 228–275 in RIP13a is deleted. The striped areas in the N-termini of RIP13a and RIP13Δ1 represent unique regions. Regions of proteins containing interaction domains of RIP13a and RIP13Δ1 used in the mammalian two hybrid assay are shown in black. The amino acids in the constructs refer to the corresponding amino acids in RIP13a. (b–f) JEG3 cells were cotransfected with 1 μg of each GAL4 chimera and 1 μg of either VP16 or VP16 chimeras as indicated, together with 3 μg reporter plasmid, pG5E1bCAT. In (f) cells were also treated with 1,25-(OH)₂D₃ at 10⁻⁷ M or 9-cis retinoic acid (RA) at 10⁻⁷ M. Fold activation is expressed relative to CAT activity measured after transfection of respective GAL4 IDs and VP16-vector, arbitrarily set to 1.0. Results shown are the mean ± s.d. from at least three independent experiments.

interaction between VDR and the composite interaction domain (Fig. 4f).

CAT activity increased about 10-, 40- and 90-fold when the VP16-TR α construct was co-transfected with either the GAL4-ID-I, GAL4-ID-II or GAL4-ID-I+II constructs respectively (Fig. 4b–d). This observation was consistent with previous studies, which demonstrated that TR interacts significantly with either domain but most strongly with a composite domain containing both ID-I and ID-II interaction domains (Downes *et al.* 1996, Seol *et al.* 1996). The interaction of RXR γ with ID-I, ID-II, ID-I+II or ID-II Δ 1 was weak and less than 5-fold (Fig. 4b–e), as found previously (Downes *et al.* 1996). However, co-transfection of VP16-RXR γ with GAL4-ID-I+II Δ 1 resulted in a substantial increase ($\sim$ 20-fold) in CAT expression (Fig. 4f), as reported by Downes *et al.* (1996). This interaction, however, was not affected by the RXR ligand 9-cis retinoic acid at 10^{-7} M (Fig. 4f), and the significance of the interaction remains unclear.

Over-expression of RIP13 Δ 1

It is evident from the two-hybrid-assay data (Figs 1b and c and 2b) that the VDR-directed basal repression of promoter activities may be due to the corepressor action of RIP13 Δ 1. To directly examine this possibility, RIP13 Δ 1 was over-expressed in COS-1 cells and the effect on basal expression of the constructs pCYP24WT-LUC and pDR3TK-LUC measured. A RIP13 Δ 1 expression clone, at concentrations of 1, 2, 5 and 10 μ g did not alter basal expression in the presence or absence of pRSV-hVDR (data not shown). A similar observation was made in CV-1 cells and human kidney 293-T cells.

DISCUSSION

We have investigated the basal expression of two promoter constructs containing functional VDREs. One construct with promoter sequence from the rat CYP24 gene, encompassed an active DR3-type VDRE (5'-AGGTGAgtgAGGGCG-3') at -150/-136 on the non-coding strand while the other construct contained a consensus VDRE of the DR-3 type (5'-AGGTCAgtgAGGTCA-3') fused upstream of the thymidine kinase promoter. We observed that basal expression of these constructs was consistently increased when the VDRE was inactivated in the CYP24 promoter or deleted in the TK promoter. The findings implied that these promoters were subject to a repression mechanism that was dependent on an intact VDRE. There was the possibility that an activator protein adventitiously bound to the altered VDRE in the mutated

pCYP24 promoter and was responsible for the increased basal expression observed. However the evidence argues against this proposal, since basal expression of the CYP24 promoter construct was increased to about the same extent when the VDRE was altered in either of the two half sites by insertion of different mutated sequences.

Basal repression of the wild-type CYP24 promoter construct in CV-1 cells was shown to be increased with increased levels of cellular VDR and this repression was dependent on a functional VDRE. In keeping with the necessity of a functional VDRE for the observed repression, over-expression of a dominant negative VDR isoform (that binds strongly to a VDRE motif *in vitro*), completely alleviated basal repression of the wild-type CYP24 promoter. Since this VDR isoform lacked the ligand binding domain E and most of domain D (hinge region), it can be proposed that one or both of these domains are involved in the repression mechanism.

It has been demonstrated that the corepressor protein N-CoR mediates transcription silencing of unliganded TR or RARs bound as heterodimers with RXR to their respective sites on target genes (Hörlein *et al.* 1995, Horwitz *et al.* 1996, Sande & Privalsky 1996). The N-CoR variants, RIP13a and RIP13 Δ 1, have recently been characterized (Seol *et al.* 1996) and two distinct steroid receptor interaction domains, ID-I and ID-II, identified in both N-CoR and the variant proteins. In view of the basal repression observed here and the role of VDR, we re-examined the question of whether VDR interacts with N-CoR/RIP13a, since the apparent lack of interaction reported previously was investigated using only ID-I (Hörlein *et al.* 1995). Based upon the mammalian two hybrid system, we have clearly demonstrated a strong interaction between VDR and a composite domain of ID-I and ID-II from RIP13 Δ 1 (but not from RIP13a or N-CoR). This interaction by VDR is completely inhibited in the presence of its ligand 1,25-(OH) $_2$ D $_3$. Interestingly, none of ID-I, ID-II, or ID-II Δ 1 could interact independently with VDR. It can be concluded, therefore, that the region between amino acids 805/925 in RIP13a, which is absent in the RIP13 Δ 1 isoform, can selectively discriminate between nuclear receptors such as TR and VDR.

On the basis of the data from the two hybrid assay, it can be proposed that the basal repression observed in the present work is directed by RIP13 Δ 1. To investigate this directly, experiments were performed in which an expression clone for full length RIP13 Δ 1 was introduced into kidney cells. However, the levels of basal expression of the wild-type construct containing the CYP24 promoter and the construct pDR3TK-LUC were

not affected. It is possible that basal repression was unaltered because of already high endogenous levels of RIP13Δ1 in the cells. Alternatively, a cofactor for repressor activity may be limiting. It has recently been suggested that a complex of N-CoR with other proteins (mSin3 and RPD1) is responsible for transcription repression by modification of chromatin configuration through local deacetylation (Heinzel *et al.* 1997). In this regard, it is interesting that RIP13Δ1 lacks an mSin3 interaction domain suggesting that if this protein is functional *in vivo* it does not repress via recruitment of this deacetylase complex. Furthermore, since chromatin formation on plasmids is poor in transient expression assays, the basal repression observed in this study most likely reflects a mechanism of repression that is independent of deacetylase activity.

It should also be noted that while VDR-dependent basal repression has been reported previously (Yen *et al.* 1996), repression of an osteocalcin VDRE-driven reporter gene construct was not observed by Masuyama *et al.* (1997). Hence, the repression effect of unliganded VDR may be promoter context or responsive element specific. This phenomenon and the possible role of RIP13Δ1 in repression remains to be fully elucidated. A naturally occurring dominant negative isoform of VDR has been reported (Ebihara *et al.* 1996) and thus basal and hormone induced levels of certain 1,25-(OH)₂D₃-responsive genes in different cells may be dependent upon the presence and amount of both negative and positive coregulators.

ACKNOWLEDGEMENTS

The authors wish to thank Drs Wongi Seol and David D Moore for the plasmids containing full length RIP13a and RIP13Δ1 that were used to construct various GAL4 chimeras. The studies on VDR in G Muscat's laboratory were supported by a block grant from the National Heart Foundation for 1996–1997.

REFERENCES

- Andrews NC & Faller DV 1991 A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Research* **19** 2499.
- Chen JD & Evans RM 1995 A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature* **377** 454–457.
- Darwish HM & DeLuca HF 1996 Recent advances in the molecular biology of vitamin D action. *Progress in Nucleic Acid Research and Molecular Biology* **53** 321–344.
- Downes M, Burke LJ, Bailey PJ & Muscat GEO 1996 Two receptor interaction domains in the corepressor N-CoR/RIP13 are required for an efficient interaction with Rev-erbA-α and RVR-physical association is dependent on the E region of the orphan receptors. *Nucleic Acids Research* **24** 4379–4386.
- Ebihara K, Masuhiro Y, Kitamoto T, Suzawa M, Uematsu Y, Yoshizawa T, Ono T, Harada H, Matsuda K, Hasegawa T, Masuhige S & Kato S 1996 Intron retention generates a novel isoform of the murine vitamin D receptor that acts in a dominant negative way on the vitamin D signaling pathway. *Molecular and Cellular Biology* **16** 3393–3400.
- Hahn CN, Kerry DM, Omdahl JL & May BK 1994 Identification of a vitamin D responsive element in the promoter of the rat cytochrome P450₂₄ gene. *Nucleic Acids Research* **22** 2410–2416.
- Heinzel T, Lavinsky RM, Mullen T-M, Soderstrom M, Laherty CD, Torchia J, Yang W-M, Brard G, Ngo SD, Davie JR, Seto E, Eisenman RN, Rose DW, Glass CK & Rosenfeld MG 1997 A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. *Nature* **387** 43–48.
- Herbomel P, Bourachot B & Yaniv M 1984 Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. *Cell* **39** 653–662.
- Hörlein AJ, Näär AM, Heinzel T, Torchia J, Gloss B, Kurokawa R, Ryan A, Kamei Y, Söderström M, Glass CK & Rosenfeld MG 1995 Ligand independent-repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature* **377** 397–403.
- Horwitz KB, Jackson TA, Bain DL, Richer JK, Takimoto GS & Tung L 1996 Nuclear receptor coactivators and corepressors. *Molecular Endocrinology* **10** 1167–1177.
- Jin CH & Pike W 1996 Human vitamin D receptor-dependent transactivation in *Saccharomyces cerevisiae* requires retinoid X receptor. *Molecular Endocrinology* **10** 196–205.
- Kerry DM, Dwivedi PP, Hahn CN, Morris, HA, Omdahl JL & May BK 1996 Transcriptional synergism between vitamin D-responsive elements in the rat 25-hydroxyvitamin D 24-hydroxylase (CYP24) promoter. *Journal of Biological Chemistry* **271** 29715–29721.
- Masuyama H, Jefcoat SC & MacDonald PN 1997 The N-terminal domain of transcription factor IIB is required for direct interaction with the vitamin D receptor and participates in vitamin D-mediated transcription. *Molecular Endocrinology* **11** 218–228.
- Nishikawa J, Kitaura M, Imagawa M & Nishihara T 1995 Vitamin D receptor contains multiple dimerization interfaces that are functionally different. *Nucleic Acids Research* **23** 606–611.
- Sande S & Privalsky ML 1996 Identification of TRACs a family of co-factors that associate with and modulate the activity of nuclear hormone receptors. *Molecular Endocrinology* **10** 813–825.
- Seol W, Mahon MJ, Lee YK & Moore DD 1996 Two receptor interacting domains in the nuclear hormone receptor corepressor RIP13/N-CoR. *Molecular Endocrinology* **10** 1646–1655.
- Van den Hoff MJB, Moorman AFM & Lamers WH 1992 Electroporation in 'intracellular' buffer increases cell survival. *Nucleic Acids Research* **20** 2902.
- Yen PM, Liu Y, Sugawara A & Chin WW 1996 Vitamin D receptors repress basal transcription and exert dominant negative activity on triiodothyronine-mediated transcriptional activity. *Journal of Biological Chemistry* **271** 10910–10916.
- Zamir I, Harding HP, Atkins B, Hörlein A, Glass CK, Rosenfeld MG & Lazar MA 1996 A nuclear hormone receptor corepressor mediates transcriptional silencing by receptors with different repression domains. *Molecular and Cellular Biology* **16** 5458–5465.

RECEIVED 22 September 1997