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Vitamin D Receptor Displays DNA Binding and Transactivation as a Heterodimer With the Retinoid X Receptor, but Not With the Thyroid Hormone Receptor

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Abstract The vitamin D receptor (VDR) is a transcription factor believed to function as a heterodimer with the retinoid X receptor (RXR). However, it was reported [Schräder et al., 1994] that, on putative vitamin D response elements (VDREs) within the rat 9k and mouse 28k calcium binding protein genes (rCaBP 9k and mCaBP 28k), VDR and thyroid hormone receptor (TR) form heterodimers that transactivate in response to both 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and triiodothyronine (T_3). We, therefore, examined associations of these receptors on the putative rCaBP 9k and mCaBP 28k VDREs, as well as on established VDREs from the rat osteocalcin (rOC) and mouse osteopontin (mOP) genes, plus the thyroid hormone response element (TRE) from the rat myosin heavy chain (rMHC) gene. In gel mobility shift assays, we found no evidence for VDR-TR heterodimer interaction with any tested element. Further, employing these hormone response elements linked to reporter genes in transfected cells, VDR and TR mediated responses to their cognate ligands only from the rOC/mOP and rMHC elements, respectively, while the CaBP elements were unresponsive to any combination of ligand(s). Utilizing the rOC and mOP VDREs, two distinct repressive actions of TR on VDR-mediated signaling were demonstrated: a T_3-independent action, presumably via direct TR-RXR competition for DNA binding, and a T_3-dependent repression, likely by diversion of limiting RXR from VDR-RXR toward the formation of TR-RXR heterodimers. The relative importance of these two mechanisms differed in a response element-specific manner. These results may provide a partial explanation for the observed association between hyperthyroidism and bone demineralization/osteoporosis. J. Cell. Biochem. 75:462–480, 1999.

Key words: nuclear hormone receptor; 1,25-dihydroxyvitamin D_3; transcriptional activation; hormone response elements

The bioactivity of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the physiologically active metabolite of vitamin D, extends well beyond its traditional target tissues of bone, kidney, and intestine, wherein it plays a critical role governing the processes of bone remodeling, maintenance of inorganic phosphate and calcium homeostasis, and the prevention of rickets. The $1,25(OH)_2D_3$ hormone also apparently elicits a wide variety of responses in the immune, neurological, endocrine, and epithelial systems [Haussler et al., 1998]. The cellular responses to $1,25(OH)_2D_3$ are mediated through a specific nuclear protein, termed the vitamin D receptor (VDR), that belongs to the superfamily of nuclear transcription factors and includes the steroid, retinoid, and thyroid hormone receptors [Evans, 1988]. Transcriptional control of $1,25(OH)_2D_3$ responsive genes is achieved by binding of VDR to a specific vitamin D response element (VDRE), located within the 5′ promoter region of the regulated gene. Ironically, the first proteins discovered to be upregulated by $1,25(OH)_2D_3$, namely those coded for by the calbindin-D (CaBP) genes [Christakos et al., 1992], do not appear to be controlled by classic high affinity VDREs within their promoter re-

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gions. Only weak VDRE consensus sequences have been proposed as a result of studies of the 9k [Darwish and DeLuca, 1992] and 28k [Gill and Christakos, 1993] calbindin-D genes. However, a number of VDREs have been characterized in genes that display a more robust positive response to $1,25(OH)_2D_3$, such as rat and human osteocalcin [DeMay et al., 1990; Lian et al., 1999; MacDonald et al., 1991; Markose et al., 1990; Ozono et al., 1990], mouse osteopontin [Denhardt and Guo, 1993; Noda et al., 1990], avian integrin β_3 [Cao et al., 1993], and rat vitamin D 24-hydroxylase [Hahn et al., 1994; Zierold et al., 1994]. A comparison of the DNA sequences indicates that, in general, the natural VDRE for a positively regulated gene consists of a hexanucleotide direct repeat with a "spacer" of three nucleotides separating each half element (DR+3).

In many studies, using these natural DR+3 VDREs, VDR has been found to bind with greatest affinity as a heterodimer with the retinoid X receptor (RXR) [Haussler et al., 1997]. The usual orientation is such that RXR resides on the 5′ half-element and VDR occupies the 3′ half-element of the VDRE [Jin and Pike, 1996]. The thyroid hormone receptor (TR) and the retinoic acid receptor (RAR) have also been found to bind to their respective response elements primarily as heterodimers with RXR, on positively controlled DR+4 and DR+5 response elements, respectively [Zechel et al., 1994].

In addition to forming heterodimers with RXR, it has also been reported that, on the putative VDREs from the rat 9k calcium binding protein (rCaBP 9k) [Darwish and DeLuca, 1992] and the mouse 28k calcium binding protein (mCaBP 28k) [Gill and Christakos, 1993], VDR and TR can form heterodimers and activate transcription [Schräder et al., 1994]. On the putative rCaBP 9k VDRE, which has a spacer of three nucleotides, it was reported that TR could substitute for RXR as a 5′ active partner with VDR, and that this novel heterodimer mediated $1,25(OH)_2D_3$-responsive transcription was augmented by triiodothyronine (T_3) [Schräder et al., 1994]. Likewise, on the putative mCaBP 28k response element, which has a spacer of four nucleotides, VDR was claimed to serve as a 5′ active partner for TR in place of RXR, with transcription being stimulated by T_3 and enhanced further when $1,25(OH)_2D_3$ was added [Schräder et al., 1994].

These observations seemed to provide evidence, on a molecular level, for synergistic cross-talk between the thyroid and vitamin D hormonal systems, at least with respect to the expression of the 9k and 28k CaBPs. In an attempt to reproduce and possibly extend these findings, we utilized the availability of *Escherichia coli*-expressed and partially purified human VDR (hVDR), human RXRα (hRXRα), and rat thyroid hormone receptor ($rTR\alpha_1$) to determine by gel mobility shift analysis what types of dimeric protein complexes would form between these receptors on five selected hormone response elements. We also employed a cotransfection assay system to assess transcriptional responsiveness of these DNA binding protein complexes to the cognate ligands, $1,25(OH)_2D_3$ and T_3. This present work describes how these two experimental approaches were utilized to elucidate a molecular basis for cross-talk between the thyroid hormone and vitamin D pathways of gene control, but via mechanisms that do not involve direct TR-VDR heterodimerization.

MATERIALS AND METHODS

Proteins and Reagents

Rat $TR\alpha_1$ was overexpressed and purified as previously described [Kim et al., 1992] from BL21(DE3)pLysS *E. coli* cells containing pET-$TR\alpha_1$, a plasmid possessing a $rTR\alpha_1$ insert [Kim et al., 1992]. Human VDR and human RXRα were overexpressed and enriched to ≥ 95% and 75% purity, respectively, as previously described [Hsieh et al., 1995] from BL21(DE3)pLysS *E. coli* cells containing the respective protein-expressing plasmids, pT7–7hVDR and pT7–7hRXRα [Hsieh et al., 1995]. Klenow enzyme was purchased from Promega Corp. (Madison, WI). Poly(dI-dC) was obtained from Boehringer Mannheim (Indianapolis, IN). Synthetic oligonucleotides corresponding to the respective hormone response elements were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). [α-^{32}P]-dCTP (3,000 Ci/mmol) was purchased from Dupont-NEN Life Science Products (Boston, MA).

Labeling of Synthetic Oligonucleotides

Double-stranded versions of each of the tested response elements (direct repeat hexanucleotides are underlined), including attached *Hin*dIII or *Xba*1 overhangs (lowercase), were created by annealing the following single-stranded

synthetic oligonucleotides together with their complements (not shown). For the mouse osteopontin (mOP) VDRE [Noda et al., 1990], the oligonucleotide was 5′-agctACAAGGTTCACGAGGTTCACGTCT-3′. For the rat osteocalcin (rOC) VDRE [Terpening et al., 1991], the oligonucleotide was 5′-agctGCACTGGGTGAATGAGGACATTACA-3′. For the rat α-myosin heavy chain (rMHC) thyroid hormone response element (TRE) [Izumo and Mahdavi, 1988; Tsika et al., 1990], the oligonucleotide was 5′-agctTGGCTCTGGAGGTGACAGGAGGACAGCA-3′. For the putative rat calbindin-D 9k (rCaBP 9k) VDRE [Darwish and DeLuca, 1992], the sequence 5′-tctaGAGAGGGTGTCGGAAGCCCTG-3′ was used. For the putative mouse calbindin-D 28k (mCaBP 28k) VDRE [Gill and Christakos, 1993], the oligonucleotide was 5′-tctaGACTGGGGGATGTGAGGAGAAA-3′.

The annealed double-stranded oligomers were labeled by a fill-in reaction with the Klenow fragment of DNA polymerase I and [α-^{32}P]-dCTP to a specific activity of $\geq 10^8$ cpm/μg as described elsewhere [Nakajima et al., 1994]. Unincorporated label was removed by Sephadex G-25 (fine) spin column chromatography.

Gel Mobility Retardation Assays

Binding reactions [Thompson et al., 1998] were preincubated for 45 min at 22°C with the indicated *E. coli*-expressed proteins (1–4 pmol each) in 10 mM Tris-HCl, pH 7.6, 150 mM KCl, 1.0 mM DTT, 15% glycerol, 1 mg/ml acetylated bovine serum albumin, and 50 μg/ml poly(dI-dC) in a volume of 39 μl. One μl of DNA probe (0.5 ng DNA, or approximately 23 fmol, and 50,000–100,000 cpm) was added to give a final volume of 40 μl, and the reaction was allowed to continue for an additional 20 minutes at 22°C. The entire reaction was then subjected to nondenaturing electrophoresis on a 4% polyacrylamide gel at 13 mA for 75 min. The gel was dried and used to expose Kodak X-Omat film at -80°C.

Construction of Expression and Reporter Plasmids

The cDNAs for hVDR, hRXRα, murine RXRβ (mRXRβ), and human placental TRβ$_1$ (hTRβ$_1$) were separately cloned into the *Eco*RI site of the expression vector pSG5 to yield pSG5hVDR [Hsieh et al., 1991], pSG5hRXRα, pSG5mRXRβ [MacDonald et al., 1993], and pSG5hTRβ$_1$, respectively. An additional hTRβ$_1$ expressing plasmid, pRSVhEACAT$^+$ [Markham et al., 1990], was a generous gift from Dr. Irwin Flink, of the University Heart Center, The University of Arizona. The vitamin D responsive (CT4)$_4$TK-GH growth hormone reporter plasmid, which contains four copies of the rat osteocalcin VDRE upstream of the viral thymidine kinase promoter in the vector pTK-GH, has been previously described [Terpening et al., 1991]. The reporter plasmids (mOP)$_4$TK-GH, (rCaBP 9k)$_4$TK-GH, (mCaBP 28k)$_4$TK-GH all contain four tandem copies of the respective VDREs, inserted into the *Hind*III site upstream of the thymidine kinase promoter of pTK-GH. The reporter plasmid (rMHC)$_2$TK-GH was constructed in a similar manner, but contains two tandem copies of the rat myosin heavy chain TRE.

Transient Transfection Studies

Transcriptional activity was measured in COS-7 cells transfected by the calcium phosphate DNA coprecipitation method (without glycerol shock) [Kingston, 1990]. Cells (700,000 per 60 mm dish) received 7 μg of one of the reporter plasmids (CT4)$_4$TK-GH, (mOP)$_4$TK-GH, (rCaBP 9k)$_4$TK-GH, (mCaBP 28k)$_4$TK-GH, or (rMHC)$_2$TK-GH in combination with the appropriate expression vector(s) pSG5hVDR (1 μg), pSG5hTRβ$_1$ (1 μg), or pRSVhEACAT$^+$ (2 μg). Experiments examining "rescue" by RXR involved the additional transfection of 0.3 μg of pSG5hRXRα or pSG5mRXRβ. Carrier DNA (pTZ18U) was added to bring the total DNA transfected to 30 μg per plate. Cells were incubated for 12–16 h with the precipitate, which was then removed by two washes with medium. Cells were replenished with serum-containing medium including the hormonal ligands, 1,25(OH)$_2$D$_3$ and/or T$_3$, at a concentration of 10^{-8} M. After 48 h of ligand or ethanol vehicle treatment, the amount of growth hormone secreted into the cell culture medium was determined with a radioimmunoassay kit (Nichols Institute, San Juan Capistrano, CA).

RESULTS

Response Elements Utilized

Figure 1 depicts the sequences of the response elements used in this study. The mOP and rOC elements represent the two main sub-

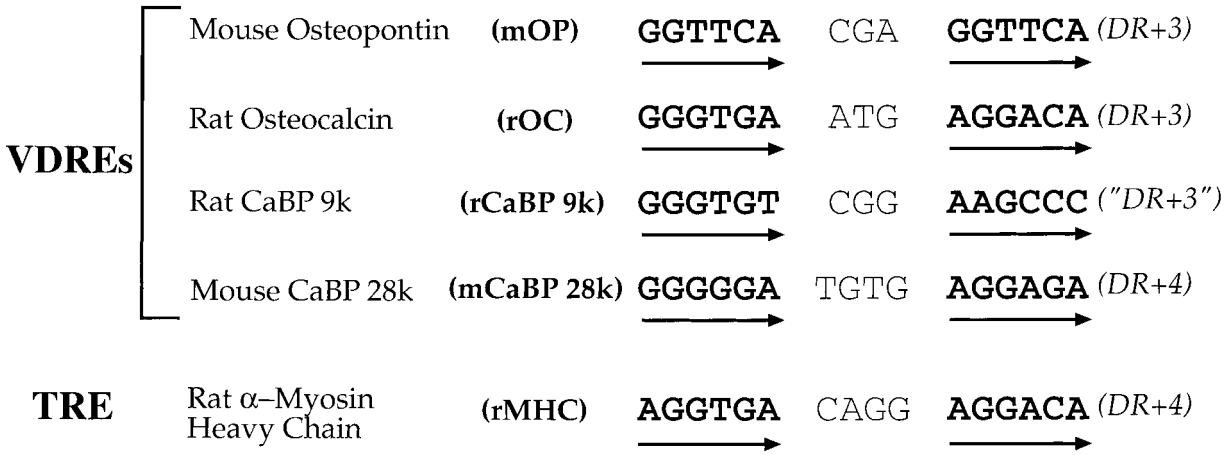

Fig. 1. Nucleotide sequence of vitamin D and thyroid hormone response elements employed in this study. The hexanucleotide half-sites constituting direct repeats (DR+3 or DR+4) are underlined with arrows.

types of positive DR+3 VDREs reported to date. The mOP VDRE is a perfect hexanucleotide direct repeat with a T present as the third base in each half element, whereas the rOC VDRE is an imperfect direct repeat with a G at this position. By comparison, the putative VDREs of the rCaBP 9k and mCaBP 28k genes are significantly different from the classical mOP and rOC VDREs. The 3′ half-site of the proposed rCaBP 9k VDRE not only is quite distinct from the corresponding mOP and rOC sequences, but also diverges considerably from the PuGTTCA consensus randomly determined for the VDRE 3′ half-element [Colnot et al., 1995; Nishikawa et al., 1994]. In contrast to the rCaBP 9k VDRE, both 5′ and 3′ half-sites in the putative mCaBP 28k VDRE display a high degree of sequence homology with the corresponding half-sites of rOC and the thyroid hormone responsive rMHC; however, the mCaBP 28k element has a spacer of four nucleotides (DR+4). Interestingly, the well-established rMHC TRE is a DR+4 with 5′ and 3′ half-site sequences almost identical to those in the rOC DR+3 VDRE.

VDR-TR Complexes Cannot Be Demonstrated in Gel Mobility Shift Analysis

Nuclear receptors hVDR (2 pmol), hRXRα (3 pmol), and rTRα_1 (1 pmol) were purified from an *E. coli* overexpression system (see Materials and Methods) and examined by gel mobility shift analysis for their ability to form DNA binding complexes on the hormone response elements listed in Figure 1. Figure 2A illustrates the DNA binding combinations formed when the indicated receptor(s) were incubated with 23 fmol of labeled mOP VDRE oligonucleotide. The major complex observed with the mOP VDRE is a heterodimer formed between VDR and RXR (Fig. 2A, lane 6), with TR and RXR forming a less intense, but still significant, DNA binding heterodimeric species (Fig. 2A, lane 5). No TR-VDR heterodimeric complex was generated on the mOP element (Fig. 2A, lane 4), and neither VDR (Fig. 2A, lane 1), RXR (Fig. 2A, lane 2), nor TR (Fig. 2A, lane 3) bound to this element in monomeric or homodimeric form. In contrast, results with the rOC VDRE (Fig. 2B) indicate that TR and RXR (Fig. 2B, lane 5) form the predominant heterodimer, with the expected VDR-RXR heterodimer also displaying significant DNA binding to this element (Fig. 2B, lane 6). Although VDR alone (Fig. 2B, lane 1) and RXR (Fig. 2B, lane 2) do not retard the mobility of the rOC VDRE, TR alone (Fig. 2B, lane 3) yields what appear to be monomeric complexes, with the less retarded bands presumably representing partially degraded TR species binding to DNA. In Figure 2B the migration pattern of TR+VDR (Fig. 2B, lane 4) is identical to that of TR alone (Fig. 2B, lane 3); therefore, we conclude that, as with the mOP VDRE, TR and VDR do not form a DNA binding heterodimer on the rOC VDRE. Figure 2C displays two similar experiments examining DNA binding combinations formed on the putative rCaBP 9k and mCaBP 28k response elements. A TR-RXR heterodimer that associates with the mCaBP 28k element (Fig. 2C, lane 11) represents the only major binding species interact-

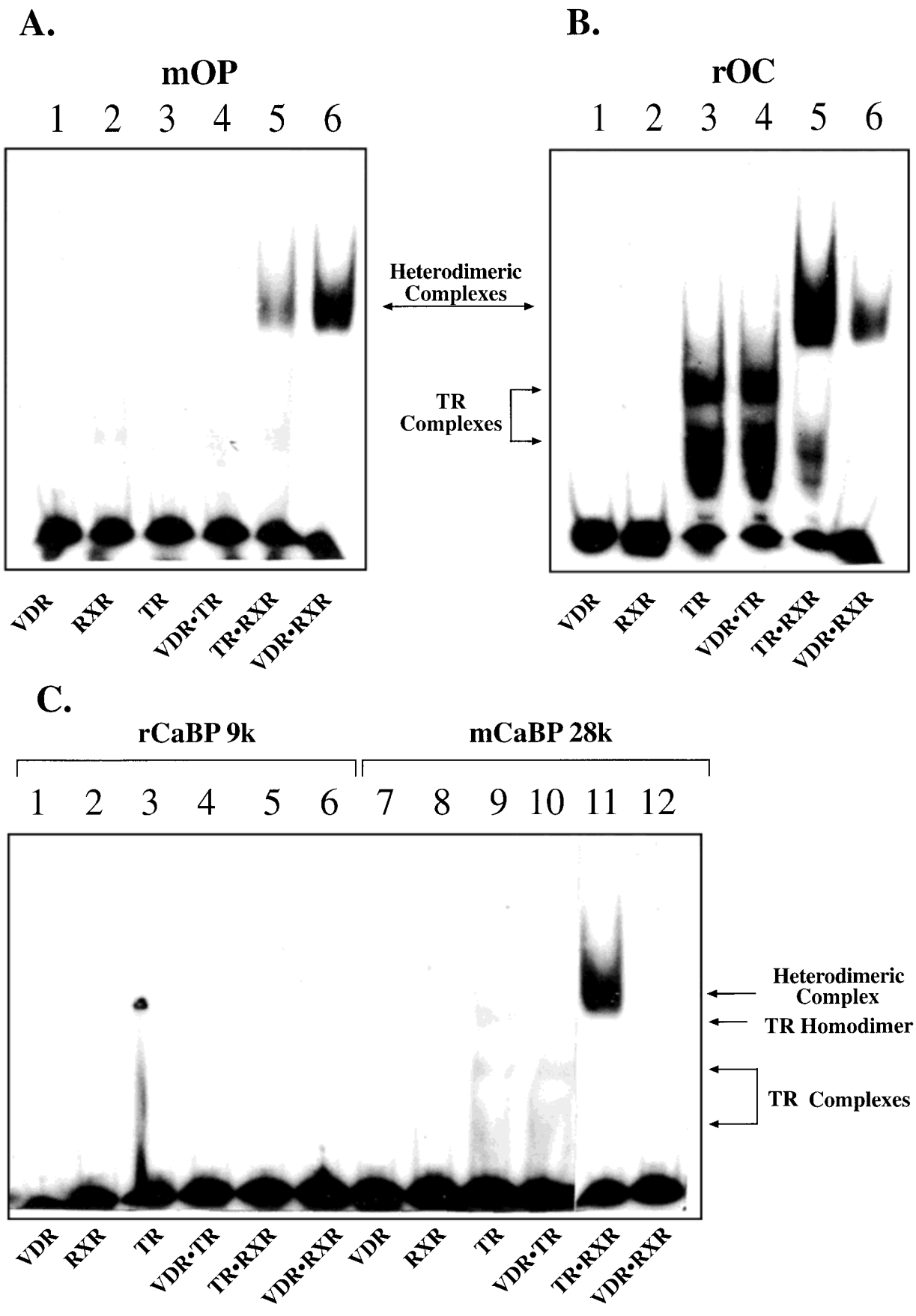
A.
mOP
1 2 3 4 5 6
VDR
RXR
TR
VDR•TR
TR•RXR
VDR•RXR
Heterodimeric Complexes
TR Complexes
B.
rOC
1 2 3 4 5 6
VDR
RXR
TR
VDR•TR
TR•RXR
VDR•RXR
C.
rCaBP 9k
mCaBP 28k
1 2 3 4 5 6 7 8 9 10 11 12
VDR
RXR
TR
VDR•TR
TR•RXR
VDR•RXR
VDR
RXR
TR
VDR•TR
TR•RXR
VDR•RXR
Heterodimeric Complex
TR Homodimer
TR Complexes

Figure 2.

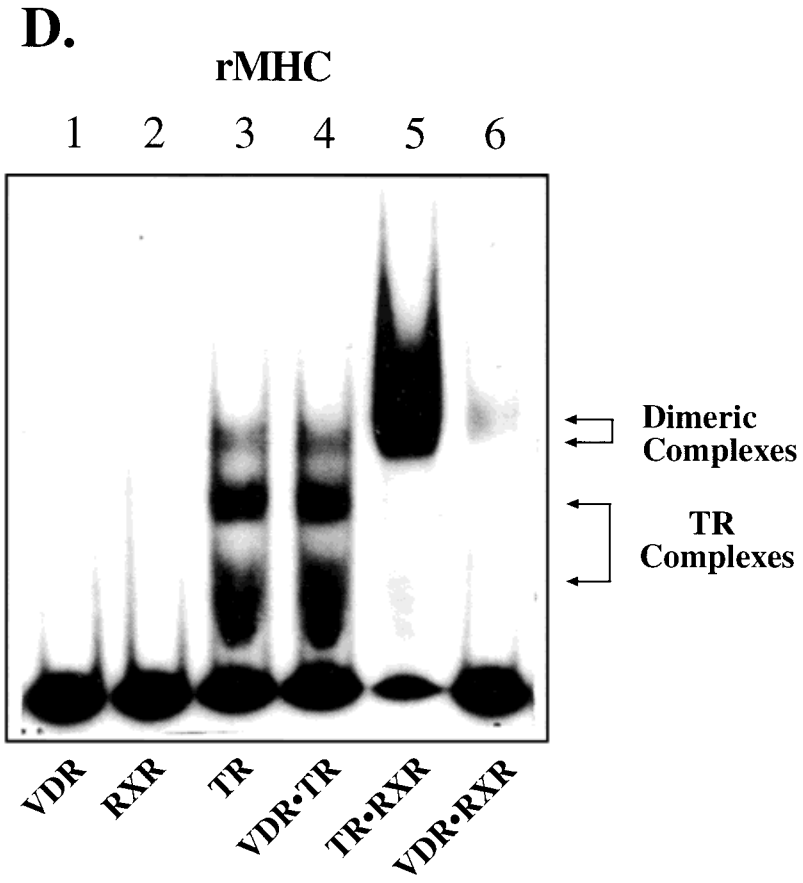

Fig. 2. (Continued) Monomeric and dimeric complexes formed by hVDR, hRXRα, and rTRα$_1$ on the rOC, mOP, rCaBP 9k, mCaBP 28k, and rMHC response elements. *E. coli*-expressed hVDR (2 pmol), hRXRα (3 pmol) and rTRα$_1$ (1 pmol) were analyzed for their ability to form DNA binding complexes in 150 mM KCl on mOP (**A**) and rOC (**B**) VDREs at a response element concentration of 0.58 nM as described in Materials and Methods. No hormonal ligands were included in these gel mobility shift experiments [Thompson et al., 1998]. **Lanes 1, 2,** and **3** depict the complexes formed on the element by hVDR, hRXRα and rTRα$_1$ alone (no added partner), respectively, while the DNA binding species that form with VDR+TR, TR+RXR and VDR+RXR are shown in **lanes 4, 5,** and **6**, respectively. The same gel shift conditions and sequence of lanes were employed as described in A and B to determine the ability of VDR, RXR, TR, and combinations thereof, to form DNA binding complexes on the putative rCaBP 9k (**lanes 1–6**) and mCaBP 28k (**lanes 7–12**) VDREs (**C**), as well as on the rMHC TRE (**D**). Each of the four panels illustrates data representative of three independent experiments.

ing with either of these sequences. In addition, faint complexes could be observed in TR-containing lanes (Fig. 2C, lanes 9 and 10) in the case of mCaBP 28k that presumably contain either TR monomers or possibly a homodimer. Contrary to a previous report [Schräder et al., 1994], no TR-VDR heterodimer was observed on either the rCaBP 9k (Fig. 2C, lane 4) or mCaBP 28k (Fig. 2C, lane 10) response elements. Figure 2D illustrates the receptor binding preferences exhibited by the rMHC TRE. As expected, this element avidly associates with TR-RXR heterodimers (Fig. 2D, lane 5), and binds VDR-RXR only very weakly (Fig. 2D, lane 6). Similar to results in Figure 2C with the mCaBP 28k element, lane 3 of Figure 2D representing TR alone appears to contain multiple complexes, possibly comprising homodimeric, monomeric intact, and partially degraded receptors. As with the mOP, rOC, rCaBP 9k, and mCaBP 28k response elements, there was no evidence for association of the rMHC element with a TR-VDR heterodimer since the VDR + TR lane (Fig. 2D, lane 4) displayed the same band pattern as that seen in lane 3 with TR alone (Fig. 2D).

Ability of Tested Response Elements to Serve as VDREs

The capacity of all five elements to mediate a transcriptional response to 1,25(OH)$_2$D$_3$ was evaluated in the presence of hVDR and

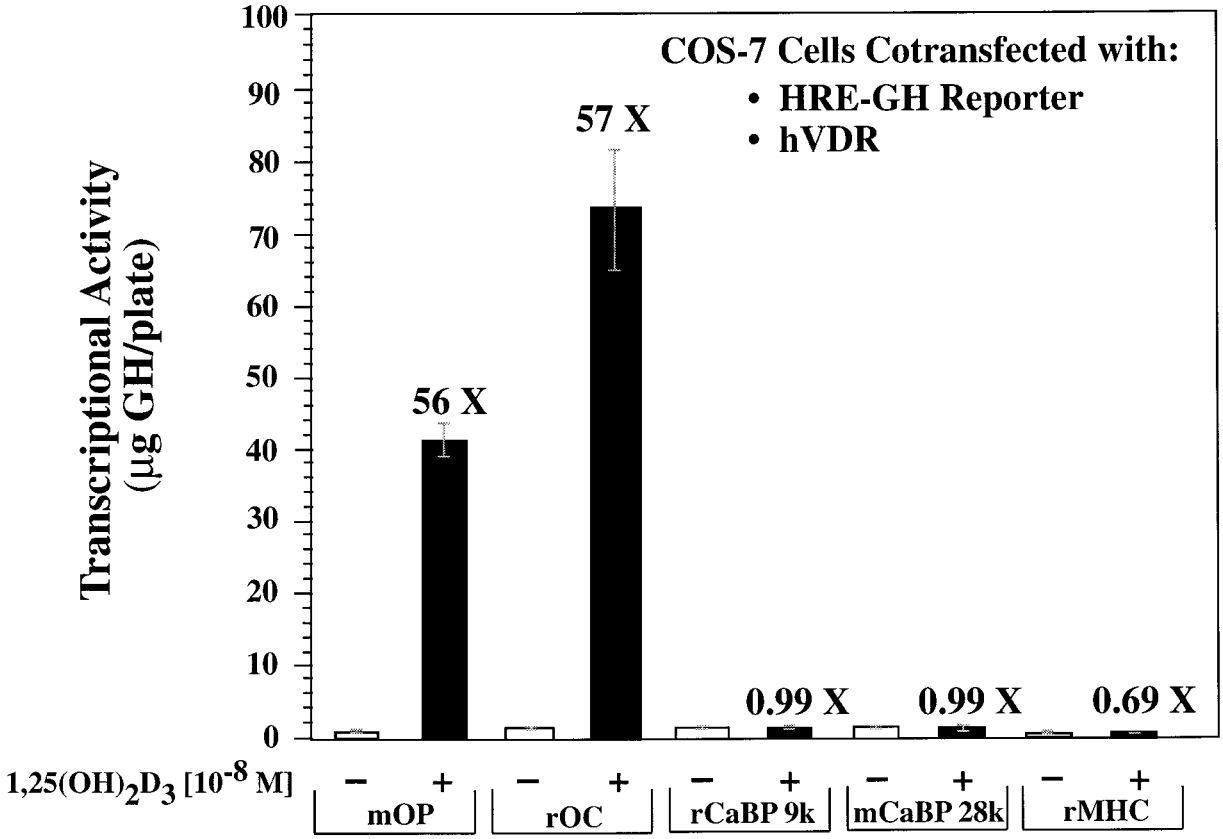

Fig. 3. Transcriptional responses to $1,25(OH)_2D_3$ mediated by mOP, rOC, rCaBP 9k, mCaBP 28k, or rMHC hormone response elements (HREs). COS-7 cells were cotransfected with 7 μg of reporter plasmid containing the respective response element in combination with 1 μg of pSG5hVDR, as described in Materials and Methods. After transfection, cells were treated for 48 hours with $1,25(OH)_2D_3$ at a concentration of 10^{-8} M. Cell media were then assayed for human growth hormone (GH) by RIA (Nichols Institute) and results were expressed as μg GH produced/plate. The calculated fold induction by $1,25(OH)_2D_3$ is given at the top of the solid bar in each treatment group. Error bars represent mean ± standard deviation, with n = 3 in each case. The data presented are representative of at least three replicate experiments.

$1,25(OH)_2D_3$. COS-7 cells were co-transfected with the respective reporter plasmid in combination with the expression vector for hVDR, and then treated with $1,25(OH)_2D_3$ at a final concentration of 10^{-8} M. Figure 3 indicates that, in the presence of hVDR, only the rOC and mOP response elements function as VDREs by transducing a transcriptional response to $1,25(OH)_2D_3$, while cells transfected with the rCaBP 9k and mCaBP 28k elements exhibited only basal levels of transactivation following treatment with ligand. These data, combined with those of Figure 2C, indicate that neither the rCaBP 9k nor the mCaBP 28k sequences are capable of functioning as VDREs, at least when multiple copies of minimal oligonucleotides are evaluated in the context of the tested reporter constructs. As expected, the rMHC TRE was not transcriptionally responsive to $1,25(OH)_2D_3$ in the presence of hVDR (Fig. 3), despite the fact that a miniscule association of VDR-RXR with this TRE apparently occurs, in vitro (Fig. 2D, lane 6).

Ability of Tested Response Elements to Function as TREs

The transcriptional response of each of the element-containing reporter plasmids to TR/T_3 treatment was also evaluated by cotransfection of COS-7 cells. As depicted in Figure 4, only the rMHC functions as a TRE in mediating a transcriptional response to T_3 (7.8-fold). The marginal response displayed by mOP in this experiment (1.45-fold above basal) was not reproducible in any subsequent experiments (Fig. 5A, for example) and therefore is not considered significant. The lack of response of the rOC and mCaBP 28k response elements to T_3 is noteworthy in view of their strong binding to the TR-RXR heterodimer (Fig. 2B and C, respectively). This observation argues that the TR-RXR heterodimer represents a transcrip-

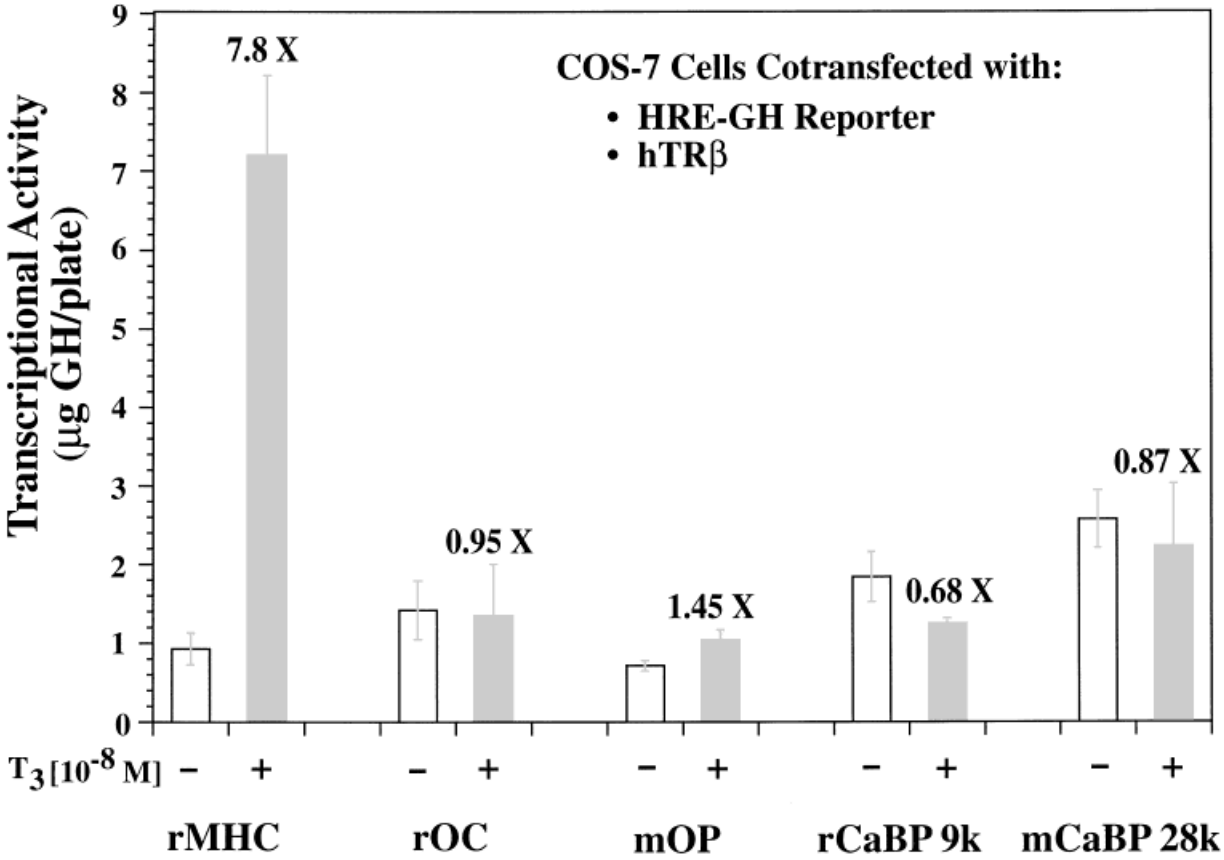

Fig. 4. Transcriptional responses to T_3 in the presence of TR. COS-7 cells were cotransfected with the indicated response element-containing reporter plasmids, in combination with 2 μg of the hTRβ_1 expression vector pRSVhEACAT$^+$, and then treated with T_3 (10^{-8} M). GH assays and data expression are as in Figure 3. The data presented are representative of at least three replicate experiments.

tionally inactive, possibly conformationally nonfunctional, species when associated with these DNA elements.

Transactivation From VDREs and TREs Is Repressed by the Non-Cognate Receptor and Its Ligand

To determine if VDR and TR could display any type of molecular cross-talk at the transcriptional level, COS-7 cells were co-transfected with vectors expressing VDR and TR, and then treated with various combinations of the cognate ligands at a concentration of 10^{-8} M. Figure 5A illustrates that the presence of TR and T_3 results in a repression of the transcriptional responses to $1,25(OH)_2D_3$ displayed by both the rOC and mOP VDREs, decreasing to levels approximately 36% of that observed with $1,25(OH)_2D_3$ alone. No transactivation of either VDRE reporter was detected with T_3 treatment alone. Figure 5B indicates that the presence of $1,25(OH)_2D_3$ and VDR results in a much more modest repression of the transcriptional response of the rMHC TRE to T_3, with an attenuation to approximately 80% of the response observed with T_3 alone. Figure 5B again illustrates that under the present experimental conditions of using isolated response elements out of their native promoter context, neither the rCaBP 9k nor the mCaBP 28k reporter constructs respond to any tested combination of VDR or TR and their ligands. These findings strongly suggest that the putative calbindin-D hormone response elements [Darwish and DeLuca, 1992; Gill and Christakos, 1993; Schräder et al., 1994] do not represent direct targets for nuclear receptor mediated transcriptional control by either $1,25(OH)_2D_3$ or T_3.

Dose Effect of Unliganded TR on Transactivation From the rOC and mOP VDREs

As seen in Figure 5A, the presence of TR+T_3 represses the transcriptional response of the rOC and mOP VDREs to $1,25(OH)_2D_3$. In order

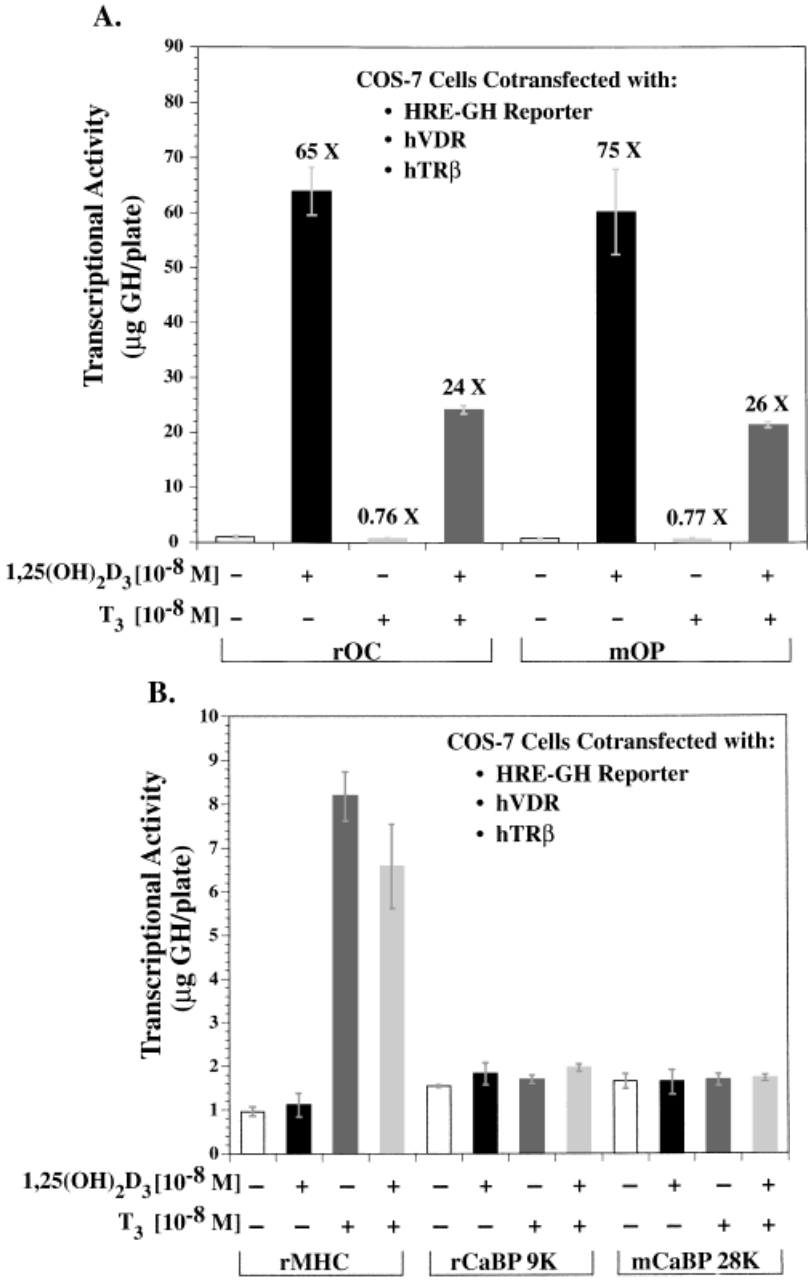

Fig. 5. Transcriptional responses of tested elements to $1,25(OH)_2D_3$ alone, T_3 alone, and $1,25(OH)_2D_3+T_3$ in the presence of both VDR and TR. COS-7 cells were transfected with the indicated response element reporter plasmids in combination with the expression vectors pSG5hVDR and pRSVhEACAT$^+$. The cells were then treated with vehicle, $1,25(OH)_2D_3$, T_3 or $1,25(OH)_2D_3+T_3$. **A:** T_3 effects on transcription from the rOC and mOPs VDREs. **B:** Effects of $1,25(OH)_2D_3$ on T_3-TR signaling from the rMHC TRE, as well as the influence of these ligands on signaling from the putative rCaBP 9k and mCaBP 28k response elements. Error bars represent mean ± standard deviation, with n = 3 in each case. The data presented are representative of at least two replicate experiments; however, it should be noted that while the modest repression by $1,25(OH)_2D_3$ of T_3-TR action on the rMHC TRE was reproducible, the apparent miniscule increases in transactivation from the rCaBP 9k element seen with $1,25(OH)_2D_3$ and/or T_3 were not repeatable (see Figs. 3 and 4).

to determine if there is a T_3-independent component to this repression, the effect of unliganded TR on the transcriptional responsiveness of rOC and mOP to $1,25(OH)_2D_3$ was evaluated. Figure 6 illustrates that unliganded $TR\beta_1$ elicits a strong, dose-dependent reduction in rOC-mediated, $1,25(OH)_2D_3$-stimulated transcription, a phenomenon beginning even with the smallest amount (0.4 μg) of $pSG5hTR\beta_1$ expression vector, and intensifying at the highest

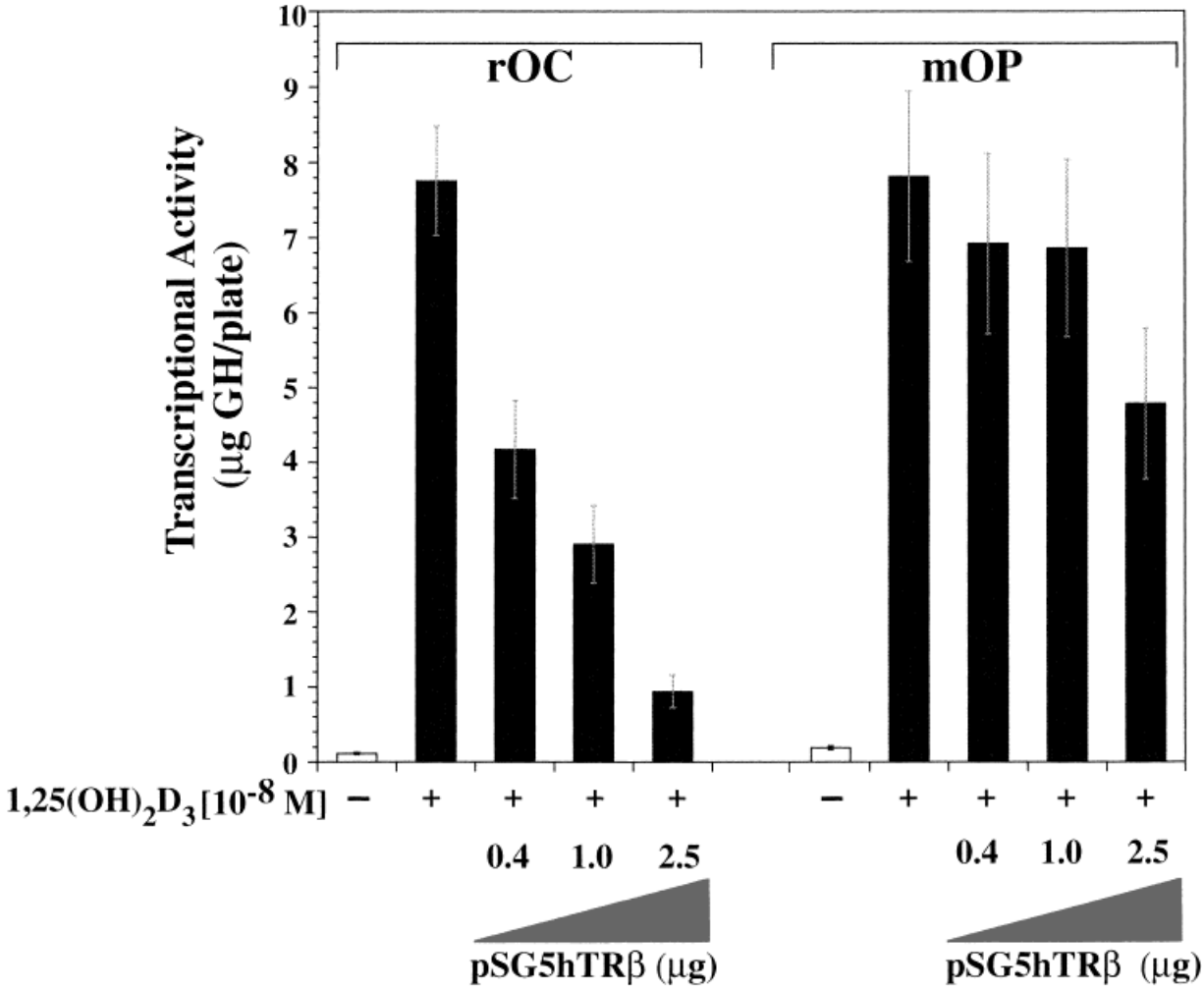

Fig. 6. Transcriptional responses mediated by VDR and $1,25(OH)_2D_3$ from the rOC and mOP VDREs are repressed in the presence of unliganded TR. COS-7 cells were cotransfected with 1 μg of the pSG5hVDR expression vector, 7 μg of the appropriate VDRE-containing reporter vector and the indicated amounts of the expression vector pSG5hTRβ_1. The cells were then treated with $1,25(OH)_2D_3$ at a concentration of 10^{-8} M. GH assays and data expression are as in Figure 3.

amount (2.5 μg) to only 12% of the transcription level seen with VDR-$1,25(OH)_2D_3$ alone. In contrast, the same amounts of unliganded TR had little effect on the transcriptional response to $1,25(OH)_2D_3$ from the mOP VDRE, with a significant decrease in transactivation observed only with the highest concentration of TRβ_1 expression vector employed (likely expressing amounts of hTRβ_1 that are far above the physiologic range). The results in Figure 6, in combination with the data contained in Figures 2A, B, suggest that unliganded TRβ_1 forms a strong RXR complex on the rOC VDRE and a weaker one on the mOP VDRE. However, this heterodimeric complex is transcriptionally unresponsive to T_3 (see also Fig. 4). The ability of this unliganded TR-RXR complex to inhibit the transcriptional response to $1,25(OH)_2D_3$ is largely limited to the higher affinity rOC VDRE (Fig. 6) and this effect appears to constitute competition by TR-RXR with VDR-RXR for association with the DNA element.

Effect of Liganded vs. Unliganded TR on Transactivation From the rOC and mOP VDREs

COS-7 cells were co-transfected with the expression vectors for VDR alone or VDR+TRβ_1 (1 μg each) in combination with reporter plasmids containing either the rOC or mOP VDREs. Cells were treated with $1,25(OH)_2D_3$ or $1,25(OH)_2D_3 + T_3$ as indicated in Figure 7, with all ligands at a concentration of 10^{-8} M. As was observed in Figure 6, cells receiving 1 μg of TRβ_1 expression plasmid displayed a substantial (60%) repression in $1,25(OH)_2D_3$-mediated transactivation from the rOC VDRE (Fig. 7). If

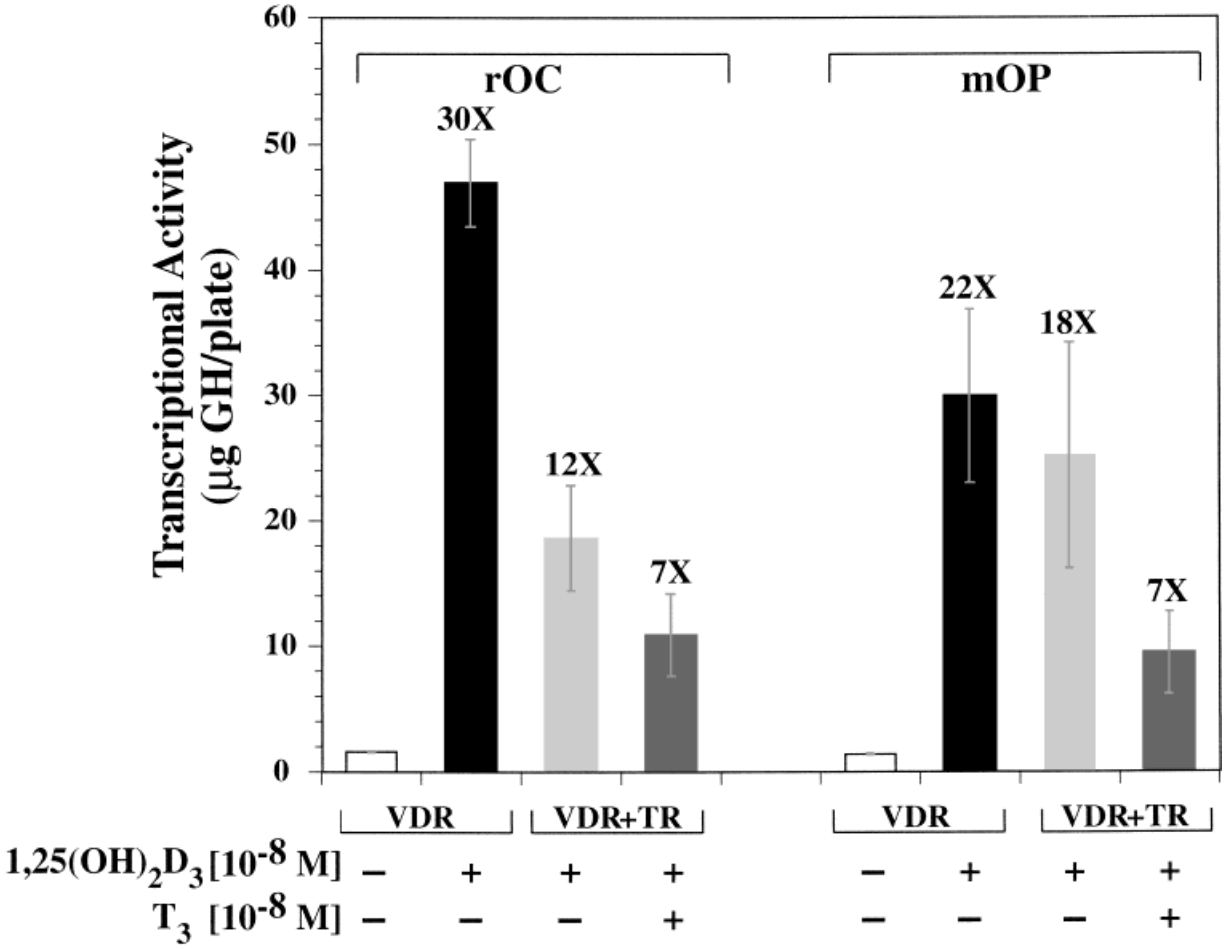

Fig. 7. Effect of liganded vs. unliganded TR on rOC and mOP VDRE mediated transactivation. COS-7 cells were cotransfected with 7 μg of either the rOC or mOP VDRE-containing reporter vector together with the expression vector pSG5hVDR (1 μg) or pSG5hVDR in combination with pSG5hTRβ_1 (1 μg). The cells were then treated as indicated with 1,25$(OH)_2D_3$ or 1,25$(OH)_2D_3$+T_3, with all ligands at a concentration of 10^{-8} M. Transactivation was assessed by GH assay as described in Figure 3.

this TRβ_1 is then liganded, rOC-mediated transactivation is further depressed to levels that are approximately 23% of those observed with 1,25$(OH)_2D_3$ treatment in the presence of VDR alone. In contrast, the mOP VDRE transcriptional response to 1,25$(OH)_2D_3$ is relatively unaffected by the presence of unliganded TRβ_1 (as was seen earlier in Fig. 6), but the addition of T_3 in combination with TRβ_1 results in a dramatic reduction to approximately 32% of the activity for mOP-mediated transactivation by 1,25$(OH)_2D_3$.

T_3/TR Inhibition of VDR Transactivation Is Relieved by Supplying Exogenous RXR

In order to assess whether competition for association with the common RXR coreceptor might be responsible for the repressive effects of TR seen in Figures 5A, 6, and 7, an experiment was performed to test the ability of excess exogenous RXR to overcome this repression. COS-7 cells were cotransfected with either the rOC (Fig. 8A) or the mOP (Fig. 8B) VDRE-reporter plasmids in combination with both the VDR and TRβ_1 expression vectors (1 μg each). Certain cells also received 0.3 μg of the expression vectors for either hRXRα or mRXRβ. The cells were then treated with 1,25$(OH)_2D_3$ or

Fig. 8. Ability of either RXRα or RXRβ to overcome T_3/TR inhibition of VDRE mediated transactivation. COS-7 cells were cotransfected with either the rOC (**A**) or mOP (**B**) reporter plasmids (7 μg each) in combination with both the pSG5hVDR (1 μg) and pSG5hTRβ_1 (1 μg) expression vectors and, where indicated, the expression vectors for either hRXRα or mRXRβ (0.3 μg each). The cells were then treated with 1,25$(OH)_2D_3$ or 1,25$(OH)_2D_3$+T_3, with all ligands at a concentration of 10^{-8} M. Figures 8A and 8B each represent an average of five independent experiments ± the standard deviation, with n = 3 in each separate experiment.

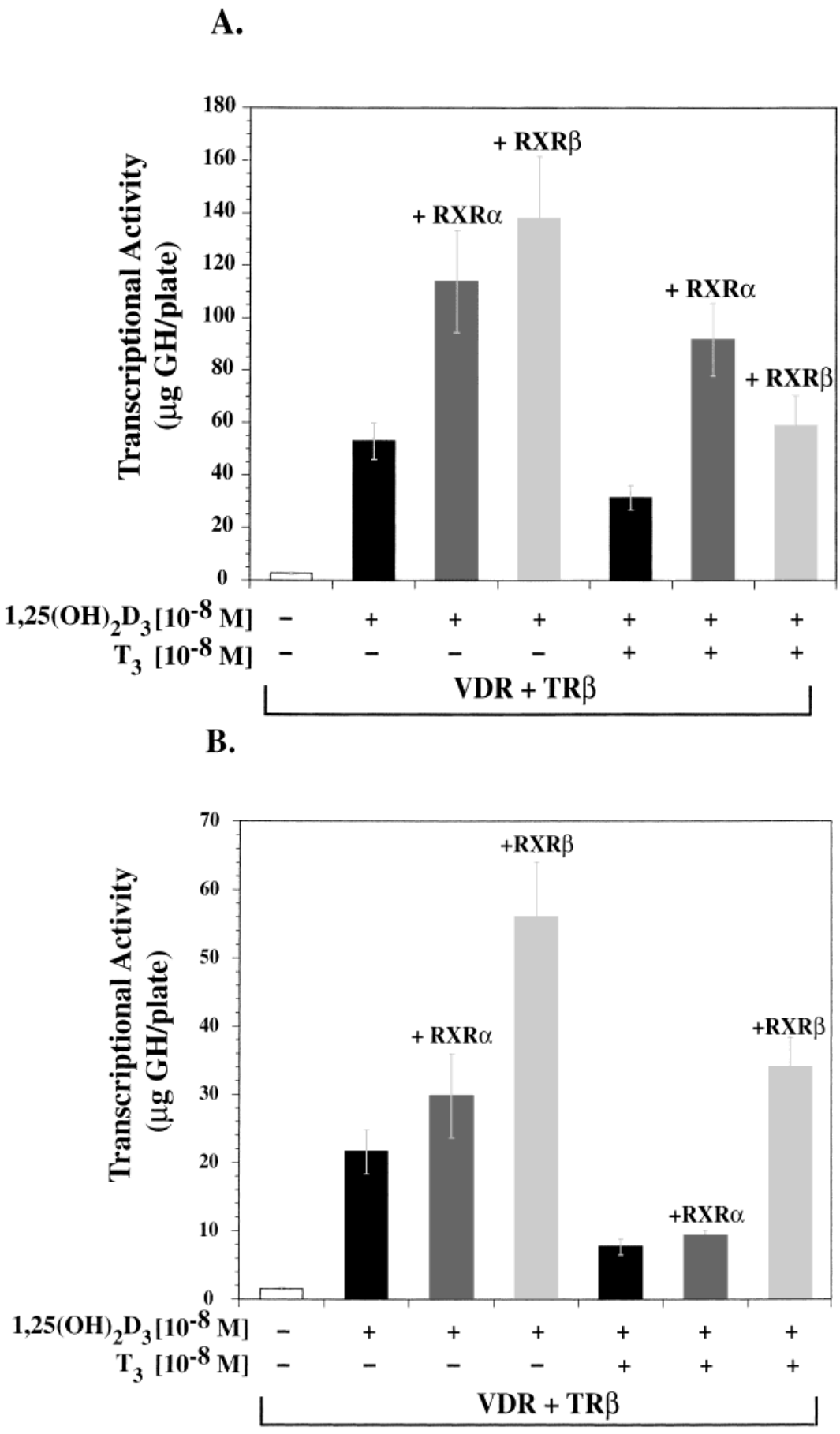
A.
Transcriptional Activity
(μg GH/plate)
180
160
140
120
100
80
60
40
20
0
+ RXRβ
+ RXRα
+ RXRα
+ RXRβ
1,25(OH)2D3 [10-8 M]
T3 [10-8 M]
VDR + TRβ
B.
Transcriptional Activity
(μg GH/plate)
70
60
50
40
30
20
10
0
+RXRβ
+ RXRα
+RXRβ
+RXRα
1,25(OH)2D3 [10-8 M]
T3 [10-8 M]
VDR + TRβ

Figure 8.

$1,25(OH)_2D_3+T_3$. As seen in Figure 8, supplying either RXRα or RXRβ generally augments the VDR-mediated transcriptional response to $1,25(OH)_2D_3$. Interestingly, when tested on the rOC VDRE (Fig. 8A), RXRα is more potent than RXRβ in overcoming T_3/TR repression. In contrast, T_3/TR inhibition of mOP mediated transactivation by $1,25(OH)_2D_3$ (Fig. 8B) is reversed more efficiently by RXRβ, with the RXRα isoform having relatively little effect on the transcriptional responses to $1,25(OH)_2D_3$ or $1,25(OH)_2D_3+T_3$. These results are indeed consistent with a mechanism for T_3-dependent repression that involves a diversion of the RXR partner away from the VDR-RXR heterodimer toward the TR-RXR complex. That the rOC and mOP VDREs display a distinct isoform preference for the particular RXR partner for VDR was an unexpected, but potentially significant finding.

DISCUSSION

Cross-talk between the thyroid and vitamin D signaling systems could have important ramifications for physiology and development in mammalian systems, as well as in clinical states of hyperthyroidism and hypervitaminosis D. In an attempt to demonstrate a molecular basis for such cross-talk, Schräder and coworkers [Schräder et al., 1994], using in vitro translated receptors, reported the formation of TR-VDR heterodimers that bound to putative response elements in the rCaBP 9k and mCaBP 28k genes. Further, transient transfection studies employing VDRE-tkCAT reporter constructs appeared to show that the transcriptional responsiveness of these TR-VDR heterodimers was optimal only in the presence of both $1,25(OH)_2D_3$ and T_3. Although RXR is a master heteropartner for type II nuclear receptors such as VDR, TR, and the retinoic acid receptor [Lala et al., 1996], heterodimerization amongst other nuclear receptors is a rare phenomenon and, to our knowledge, only the type I mineralocorticoid and glucocorticoid receptors have been shown to form such complexes [Liu et al., 1995; Trapp et al., 1994].

Herein, experiments utilizing overexpressed and partially purified receptors in gel retardation assays are described in which we were unable to detect TR-VDR heterodimers on any of five tested response elements, including the putative rCaBP 9k and mCaBP 28k VDREs. The absence of TR-VDR binding to both CaBP sequences has also been reported (in data not shown) by others [Yen et al., 1996]. In a more recent study [Raval-Pandya et al., 1998] TR-VDR was found not to bind to the rat CaBP 9k element by gel shift analysis. In addition, TR-VDR association could not be detected in solution using the yeast two hybrid assay system [Raval-Pandya et al., 1998]. Based upon the current data, plus the results of these two other investigations, both of which were published since the appearance of the paper by Schräder and associates [Schräder et al., 1994], it can be concluded that the reported TR-VDR heterodimer is not a biochemically or physiologically relevant complex. Moreover, the absence of any detectable VDR-RXR binding or transcriptional response to either T_3 and/or $1,25(OH)_2D_3$ in any of our experiments using oligonucleotides encompassing the rCaBP 9k and mCaBP 28k elements, even in multiple copies, leads to the further conclusion that these sequences do not represent classical VDREs or TREs, or even novel elements that require the presence of both hormonal ligands. The contrasting data of Schräder and coworkers [Schräder et al., 1994] may have resulted from markedly different experimental conditions, including receptor sources/amounts and the cell types employed, or these previous findings may simply be in error. However, additional probing of the rCaBP 9k and mCaBP 28k genes, especially of the intact, natural promoter, is still warranted in order to elucidate their potentially cell-specific, hormonal regulation as mediated by members of the nuclear receptor superfamily. In fact, the rCaBP 9k gene is known to be negatively regulated by glucocorticoids in intestine [Christakos et al., 1992], and estrogen positively modulates the transcription of this gene in the uterus [L'Horset et al., 1994].

Despite the fact that VDR-TR heterodimers apparently do not occur, we did observe that TR-RXR heterodimers, in addition to binding to a TRE from the rMHC gene, crossed over to bind strongly to both the rOC VDRE and the mCaBP 28k sequences, while forming a weaker, but still detectable complex on the mOP VDRE (Fig. 2). However, transient transfection data (Figs. 4–6) demonstrated that the TR-RXR heterodimer, while mediating the expected transcriptional response to T_3 on the rMHC TRE, is transcriptionally inactive when bound to the rOC, mCaBP 28k, and mOP sequences. Further, consistent with a lack of evidence for func-

tional VDR-TR heterodimers in the present study, we did not observe enhancement of $1,25(OH)_2D_3$ activity by T_3, using any combination of receptor(s) on any of the elements tested (Fig. 5A and B). Rather, instead of synergism, an inhibition in the level of the VDR-mediated $1,25(OH)_2D_3$ response was observed with the rOC VDRE when unliganded TR was present. This repression by unliganded TR was not seen on the mOP VDRE, at least when 0.4 to 1.0 μg of $TR\beta_1$ expressing plasmid was transfected (Fig. 6); however, the inclusion of T_3 in addition to TR did elicit a marked reduction in the transcriptional activity of this VDRE, while further repressing the transactivation of the rOC VDRE (Fig. 7). These results, in concert with the data obtained from gel mobility retardation experiments (Fig. 2), suggest that the $1,25(OH)_2D_3$-dependent activity of bone expressed genes such as osteocalcin and osteopontin is negatively modulated by the thyroid hormone receptor via both T_3-dependent and T_3-independent mechanisms.

As presented in the summary model (Fig. 9), considerable research has shown that VDR-RXR heterodimers form transcriptionally active complexes on the mOP and rOC VDREs in response to $1,25(OH)_2D_3$ [Haussler et al., 1998], while TR-RXR heterodimers do the same on TREs (e.g., from the rMHC gene) in the presence of T_3 [Leng et al., 1994; Wong and Shi, 1995]. TR has also been shown to bind a number of different hormone response elements in the absence of ligand [Chin and Yen, 1996]. Indeed, in the current study, unliganded TR-RXR heterodimers (and even TR alone in the absence of complementary RXR) exhibits binding to the rOC and mCaBP 28k sequences, but only as transcriptionally inactive complexes (see Figs. 2 and 4). While the overall effects of this binding on CaBP 28k gene expression in vivo are unclear, unliganded TR, presumably as a heterodimer with RXR, does appear capable of repressing the $1,25(OH)_2D_3$ responsiveness of the rOC VDRE by competition with VDR-RXR for binding to the DNA. In related studies, unliganded TR has been observed to repress the activity of other members of the nuclear receptor superfamily by competitive DNA binding. For example, unliganded TR has been shown to form a repressive complex on both the estrogen response element [Glass et al., 1988] and on a palindromic TRE [Graupner et al., 1991]. In addition, unliganded TR has been observed to repress glucocorticoid receptor mediated transcriptional activation [Spanjaard et al., 1995] in an isoform specific manner, possibly again by competitive DNA binding (although the dissimilarity of the TRE and GRE half-element sequences suggests that this effect may occur through squelching of a common transcription factor). A DNA-based mechanism of negative regulation seems to be employed by COUP-TF, which has been shown to antagonize retinoic acid receptor, RXR, and TR activity by competitive DNA binding [Tran et al., 1992]. Therefore, like COUP-TF, TR appears to be capable of functioning as a cross-repressor of the action of multiple nuclear receptors.

Does the reverse of the above phenomenon occur? In other words, can VDR attenuate TR signaling? That VDR may be capable of inhibiting T_3 signaling is implied by the report [Yen et al., 1996] that VDR can repress T_3-mediated transcription by, at least in part, forming transcriptionally inactive VDR-RXR heterodimers competing with TR-RXR for TRE binding. The present results do indicate a relatively weak binding of VDR-RXR to the MHC TRE, and modest repression by liganded VDR of T_3-mediated transactivation. This VDR suppression of TR action possibly results from titration of the common RXR partner in favor of formation of VDR-RXR heterodimers (see below), but could be caused by inactive VDR-RXR occluding the rMHC TRE. Therefore, the results summarized above reveal the potential (under certain cellular conditions) for either VDR or TR to inhibit transactivation by the other receptor via competing for DNA binding, especially if there is high half-element homology in the relevant response elements, as there is here between the rOC VDRE and the rMHC TRE. The fact that osteocalcin and α-myosin heavy chain represent two major expressed genes in bone and muscle, respectively, may be significant in terms of musculoskeletal physiology and its control by $1,25(OH)_2D_3$ and T_3.

Another notion illustrated by Figure 9 is that the presence of both TR and T_3 results in a diversion of RXR away from $1,25(OH)_2D_3$-regulated pathways toward formation of a transcriptionally active TR-RXR heterodimers. Sequestration of limiting RXR could, therefore, inhibit $1,25(OH)_2D_3$ responses from the rOC, mOP, or indeed any VDRE. Thus, transactivation mediated by the rOC VDRE is likely repressed by both T_3-independent (competitive DNA bind-

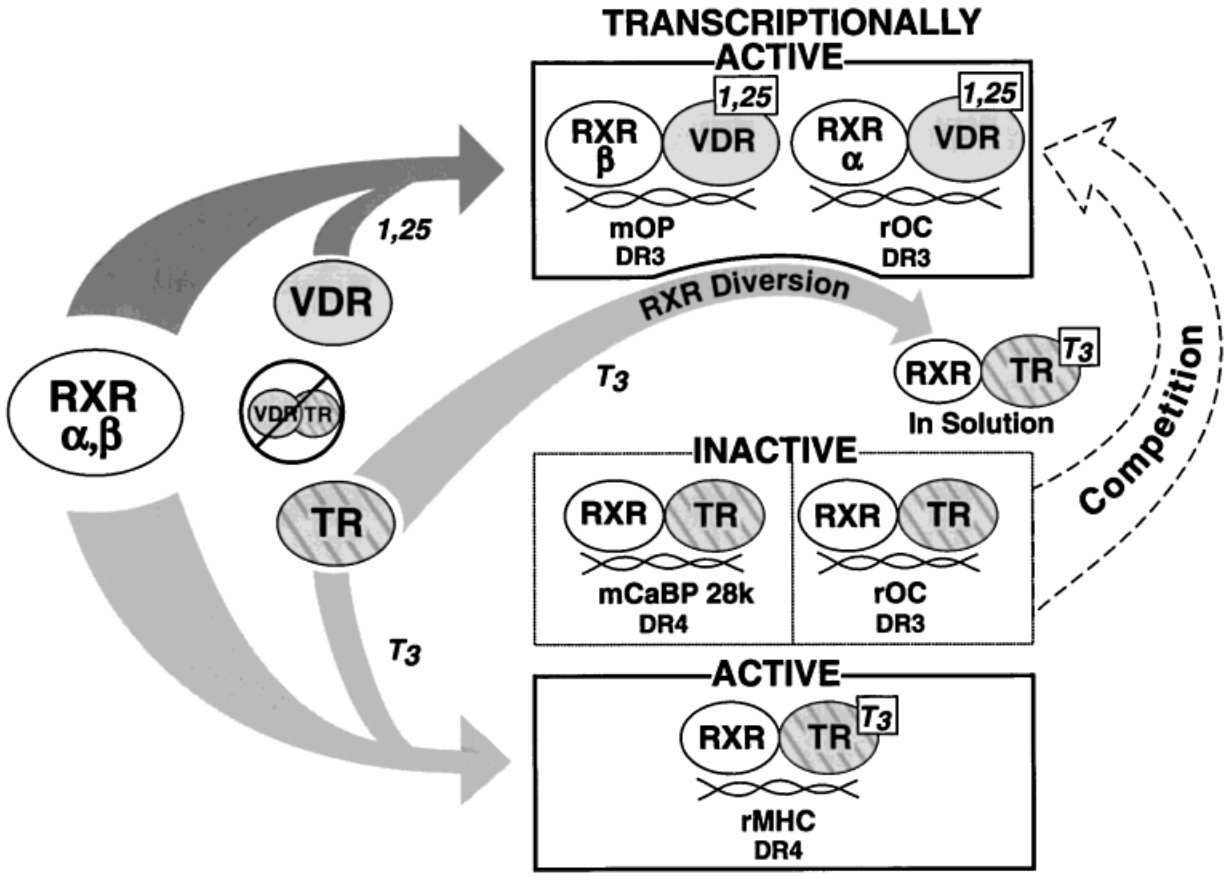

Fig. 9. Model for molecular crosstalk between the $1,25(OH)_2D_3$ and T_3 hormonal pathways. This hypothesis explains how TR can repress the VDR-mediated transcriptional response to $1,25(OH)_2D_3$ of the bone remodeling proteins osteocalcin and osteopontin, via both T_3-dependent and T_3-independent mechanisms. In a process enhanced by the presence of their respective cognate ligands, VDR and TR form heterodimeric complexes with RXR, but not with each other (**left**). VDR-RXR complexes then activate transcription from the mOP or rOC VDREs (top box). Likewise, TR-RXR heterodimers transactivate from the rMHC TRE (bottom box). Repression apparently can occur by two means: (1) Direct competition for DNA binding. The formation of an inactive TR-RXR complex on the rOC VDRE likely results in repression of the $1,25(OH)_2D_3$-mediated transcriptional response because of direct exclusion of the active VDR-RXR complex from the DNA. This could occur even in the absence of T_3, assuming sufficient TR is present in a given cell (see Fig. 6). Although the mechanism by which the mCaBP 28k gene is transcriptionally upregulated in response to $1,25(OH)_2D_3$ has yet to be fully characterized, the binding of the inactive TR-RXR complex to the DR+4 in this gene may also suppress transactivation. (2) Diversion of limiting RXR. Both VDR and TR share a common heterodimeric partner. The presumably limiting supplies of RXR within a cell, therefore, may be effectively diverted from one hormonal pathway to another. Thus, TR could repress the $1,25(OH)_2D_3$ transcriptional responses of both the mOP and rOC genes, in a T_3-dependent manner, by diverting RXR away from VDR to instead form TR-containing heterodimers (RXR diversion arrow). The converse repression by VDR of TR signaling from the rMHC element (not shown in figure) was only modest in our system (Fig. 5B), but has been reported by others (see Discussion).

ing) and T_3-dependent (RXR diversion) mechanisms, while mOP-dependent transactivation appears to be inhibited predominantly by the RXR diversion pathway (Fig. 9). Significant support for the RXR diversion portion of this hypothesis is provided by the data in Figure 8A and B, which demonstrate that excess RXR can relieve TR-dependent repression of $1,25(OH)_2D_3$ mediated signaling.

Other investigators have reported analogous examples of signaling repression via diversion of limiting RXR. Inhibition of the T_3 response has been attributed to 9-*cis* retinoic acid ligand-induced squelching of the RXR heterodimeric partner of TR toward the formation of RXR homodimers [Lehmann et al., 1993]. TR-mediated transactivation was also shown to be inhibited by competition with peroxisome proliferator activated receptor (PPAR) for RXR binding [Juge-Aubry et al., 1995], while TR is known to suppress RAR activity by titration of the common RXR partner [Barettino et al., 1993]. Simi-

lar to our findings, it has been recently reported [Raval-Pandya et al., 1998] that the presence of TR represses the $1,25(OH)_2D_3$ responses of reporter vectors containing the rat vitamin D 24-hydroxylase promoter and the rOC VDRE. Their study also included RXR "rescue" experiments similar to those depicted in Figure 8A and B, providing independent evidence for the recruitment of limiting RXR partner toward formation of TR-RXR heterodimers during TR repression of VDR action. Although not observed by this other group [Raval-Pandya et al., 1998] in probing the rOC VDRE, the present results (Figs. 5A and 7) demonstrate a clear additional effect of T_3 on TR-mediated repression of $1,25(OH)_2D_3$ signaling. This raises the possibility that relatively high levels of expression of TR may have existed in the experiments of Raval-Pandya and coworkers, with a resultant squelching of RXR that possibly masked a requirement for T_3 in the overall inhibition of $1,25(OH)_2D_3$-mediated activity. Another difference between the conclusions from this other investigation [Raval-Pandya et al., 1998] and those of the present work is that, based partly on experiments with a TR mutant lacking a functional DNA binding domain, those authors concluded that suppression by TR did not involve competitive DNA binding [Raval-Pandya et al., 1998]. However, again, their TR DNA-binding mutant may have been so highly overexpressed that it could sequester RXR and obscure the DNA binding component of the effect. Our gel retardation assay data do, in fact indicate that unliganded TR-RXR can bind strongly to the rOC (while displaying less affinity for the mOP VDRE). Thus, the present data remain consistent with a model (Fig. 9) of $1,25(OH)_2D_3$ transcriptional repression by TR, transpiring through both a T_3-independent mechanism involving competitive DNA binding to the rOC, as well as T_3-dependent diversion of RXR from both the rOC and mOP VDREs to form TR-RXR heterodimers.

The rescue experiments depicted in Figure 8A and B also indicate that, when complexed to a specific VDRE, VDR exhibits distinct preferences for particular RXR isoforms. Thus, VDR on the rOC VDRE displays a slight preference for RXRα "rescue," while transcriptional activity mediated by VDR from the mOP VDRE is clearly more efficiently restored by RXRβ. The molecular basis for this RXR isoform selectivity may be related to the finding that VDR assumes distinct conformations when bound to the rOC and mOP VDREs, a phenomenon that is reportedly determined by dinucleotide differences between the two sequences [Staal et al., 1996]. In contrast to our findings, however, it was reported that, in extracts of ROS 17/2.8 osteoblastic cells, the endogenous heterodimeric partner for VDR is RXRα on both the rOC and mOP VDREs [Staal et al., 1996]. The reason for this difference is unclear, but it should be emphasized that the present rescue experiments involve the addition of exogenous RXR. Thus, the isoform selectivity observed herein may not reflect the actual participation of a particular endogenous RXR partner in the in vivo setting. Nevertheless, the present data do demonstrate that the rOC and mOP VDREs confer upon VDR a distinct selectivity for the α or β isoform of its RXR partner, provided both isoforms are present in comparable amounts. A third isoform of RXR, namely RXRγ (not used in this study), has been shown in transformed yeast cells to confer a higher VDR transcriptional activity from a vitamin D 24-hydroxylase VDRE reporter construct than does either RXRα or RXRβ [Jin and Pike, 1996; Kephart et al., 1996]. RXRβ. In fact, using the yeast system, RXRβ seemed largely unable to enhance the $1,25(OH)_2D_3$-dependent transactivation [Kephart et al., 1996]. Heterodimers containing different retinoid receptor isoforms also have been reported by others to show distinct activation patterns with various response elements, and the responsiveness of a given heterodimer to varying concentrations of ligand has been demonstrated to be dependent upon the response element to which it is bound [La Vista-Picard et al., 1996]. The sum of these reports and the current data argue that both the RXR isoform and the particular VDRE sequence can significantly influence the nature of the positive VDR response to $1,25(OH)_2D_3$. To date, however, these factors, as well as potential T_3 effects, have not been studied in genes that are negatively regulated by $1,25(OH)_2D_3$, such as collagen 1A1 [Bedalov et al., 1998; Pavlin et al., 1994].

There have been other reports of cross-talk between the VDR and TR signaling pathways that appear to involve mechanisms beyond competitive DNA binding and titration of the common RXR partner. As with other nuclear receptors, unliganded TR is known to interact with transcriptional corepressors [Chen and Evans, 1995; Horlein et al., 1995], and with compo-

nents of the basal transcriptional machinery [Fondell et al., 1993]. Unliganded TR has been shown to bind directly to TFIIB, and thereby has the potential to prevent formation of a preinitiation complex with VDR [Blanco et al., 1995; Guo et al., 1997; Masuyama et al., 1997]. In a related finding, Yen and coworkers [Yen et al., 1996] have reported that, in addition to competitive DNA binding by VDR-RXR to the TRE, VDR could also repress T_3-mediated transcription in CV1 cells by titration of a common associated protein which did not appear to be RXR. The titration of such a common associated protein may also be the basis for the reduction by $1,25(OH)_2D_3$ of T_3 stimulated growth hormone secretion in pituitary GH_4C_1 cells [Garcia-Villalba et al., 1996].

All of the above described results taken together strongly support the concept of cross-talk between the T_3 and $1,25(OH)_2D_3$ hormonal pathways, not as a result of TR-VDR heterodimerization, but instead as a delicate interplay between the relative levels of ligands, receptors, RXR isoform expression, common associated proteins, and also the nature of the respective response elements. Physiologic evidence for such cross-talk includes the observation that syndromes of resistance to thyroid hormone are also associated with skeletal defects and growth retardation [Refetoff et al., 1993]. Conversely, excess thyroid hormone in animal models [Ongphiphadhanakul et al., 1992], and in humans [Duncan et al., 1994; Faber and Galloe, 1994; Mosekilde et al., 1990], elicits accelerated bone mineral loss and thus confers the potential risk of premature osteoporosis. Therefore, the present data illustrating that TR can repress the activity of $1,25(OH)_2D_3$ responsive genes such as osteopontin and osteocalcin through both T_3-independent and T_3-dependent mechanisms may have clinical implications, such as providing a possible mechanism for the bone demineralization associated with excessive thyroid replacement therapy.

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