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FAST TRACK

1α,25-Dihydroxyvitamin D_3 Receptor as a Mediator of Transrepression of Retinoid Signaling

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Abstract The receptors for retinoic acid (RA) and for 1α,25-dihydroxyvitamin D_3 (VD), RAR, RXR, and VDR are ligand-inducible members of the nuclear receptor superfamily. These receptors mediate their regulatory effects by binding as dimeric complexes to response elements located in regulatory regions of hormone target genes. Sequence scanning of the tumor necrosis factor-α type I receptor (TNFαRI) gene identified a 3′ enhancer region composed of two directly repeated hexameric core motifs spaced by 2 nucleotides (DR2). On this novel DR2-type sequence, but not on a DR5-type RA response element, VD was shown to act through its receptor, the vitamin D receptor (VDR), as a repressor of retinoid signalling. The repression appears to be mediated by competitive protein–protein interactions between VDR, RAR, RXR, and possibly their cofactors. This VDR-mediated transrepression of retinoid signaling suggests a novel mechanism for the complex regulatory interaction between retinoids and VD. J. Cell. Biochem. 67:287–296, 1997.

Key words: vitamin D_3 receptor; regulation of transcription; retinoid signaling transrepression; tumor necrosis factor-α receptor type I

The biologically active forms of vitamins A and D, retinoic acid (RA), and 1α,25 dihydroxyvitamin D_3 (VD), are pleiotropic effectors that take an active functional role in many aspects of biology, including cellular development and differentiation, inhibition of proliferation, tumorigenesis, and embryogenesis [Chambon, 1994; Giguère, 1994; Walters, 1992]. Both the retinoids and VD act as transcriptional regulators via their specific nuclear receptors. There are two classes of retinoid receptors, RA receptors (RARs) and retinoid X receptors (RXRs), each with three subtypes and various splicing variants [Chambon, 1994; Giguère, 1994], whereas only one gene for the VD receptor (VDR) has been reported [Carlberg, 1996; Pike, 1991]. All-*trans* RA is a specific ligand of RARs, whereas 9-*cis* RA is a pan-agonist of all retinoid receptors. RAR, RXR, and VDR, as well as thyroid hormone (T_3) receptor (T_3R) form a subgroup within the nuclear receptor superfamily [Mangelsdorf et al., 1995]. Their common feature is a highly conserved DNA binding domain of 66–70 amino acids and a moderately homologous C-terminal ligand binding domain (LBD). This conservation is consistent with the binding of these nuclear receptors to similar hexameric core binding motifs. Simple hormone response elements are formed by an arrangement of two core binding motifs in a directly repeated, palindromic, or inverted palindromic orientation [Carlberg, 1995; Glass, 1994]. Specificity in response element recognition preference of different dimeric complexes is largely dictated by the specific core binding motif sequence, motif spacing, and orientation [Carlberg, 1995; Glass, 1994; Umesono et al., 1991]. Various studies have demonstrated that RAR acts mainly through a heterodimeric complex with RXR [Ladias and Karathanasis, 1991], which binds preferentially to directly repeated hexameric motifs (PuKKTCA) spaced by either 2 or 5 nucleotides (DR2 and DR5) or to inverted palindromes spaced by 8 nucleotides [Carlberg, 1995; Glass, 1994]. Moreover, RAR has also been shown to form homodimers [Carlberg et al., 1993; Schräder et al., 1993] and heterodimers with T_3R [Glass et al., 1989] and

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VDR [Schräder et al., 1993], which differ in their response element specificity [Schräder and Carlberg, 1994; Schräder et al., 1994].

In addition to gene regulation by direct protein-DNA interactions, protein-protein interactions that influence gene activity provide another dimension in gene regulation by nuclear hormones and their receptors, which is less well understood. Recent studies have shown that the VDR is capable of interacting with other nuclear receptors that are bound to DNA to influence gene transactivation through a protein–protein mechanism [Garcia-Villalba et al., 1996; Yen et al., 1996]. Transactivation of the growth hormone gene by T_3 and RA is inhibited by VDR, displacing T_3R and RAR from a perfect palindromic T_3 and RA responsive element [Garcia-Villalba et al., 1996]. Unliganded VDR can repress T_3R-mediated transcription on a DR4-type response element [Yen et al., 1996]. Moreover, VDR represses interleukin-2 activation by blocking the complex formation between the transcription factors $NFAT_p$ and AP-1 [Alroy et al., 1995]. These studies provide evidence for novel VDR activities incorporating protein–protein association, offering greater complexity and flexibility in transcriptional regulation.

This study reports the location of a retinoid responsive sequence from the 3′ region of the human TNFαRI gene (55-kDa form) [Garret et al., 1992]. This sequence has a DR2-type structure that binds RAR–RXR heterodimers but shows strong homology to the human osteocalcin VD response element. On this response element, VD and VDR are shown to repress retinoid signaling through a DNA-independent transrepression mechanism.

MATERIALS AND METHODS

DNA Constructs

OSCAT1 consists of a part of the human osteocalcin promoter (−344/+34) driving the chloramphenicol acetyltransferase (CAT) gene [Morrison et al., 1989] and serves a basal promoter for heterologous reporter construction as it confers high transcription in NIH 3T3 cells and is nonresponsive to VD treatment. OSLAC8 is a reporter construct that contains the 5′ *Hin*dIII/*Sal*I human osteocalcin promoter region including the VD response element (−512/−486) fused to the 5′ *Hin*dIII/*Eco*RI fragment from pCH110 (Pharmacia) containing the β-galactosidase (LacZ) gene. Complementary oligonucleotides representing the 27-base pair (bp) sequence structure located in the 3′ region of the human TNFαRI gene were synthesized with *Hin*dIII ends and annealed to form a double-stranded DNA fragment, which was subcloned in three copies into the *Hin*dIII site of OSCAT1 (TNF33.3R.OSCAT1). For comparison, complementary oligonucleotides representing the DR5-type RA response element of the human RARβ gene [de Thé et al., 1990], were synthesized with *Hin*dIII ends, annealed and subcloned as a single copy into the *Hin*dIII site of OSCAT1.

Cell Culture, Transfections, and Reporter Gene Assays

Mouse fibroblast NIH 3T3 cells were cultured and transfected by calcium phosphate precipitation as described previously [Polly et al., 1996]. Transfected NIH 3T3 cells were treated alone or in combination with the indicated concentrations of 9-*cis* RA, all-*trans* RA, and VD. NIH 3T3 cells were harvested 16 h after treatment and assayed for CAT activity with a nonchromatographic method and β-galactosidase activities were determined as described [Polly et al., 1996]. The CAT activities were normalized to β-galactosidase activity and induction factors were calculated as the ratio of CAT activity of ligand-stimulated cells to that of mock-induced controls.

Gel-Shift Analysis

Wild-type and mutated (Δ) DNA probes were prepared by annealing complementary oligonucleotides and radiolabeling with [^{32}P]-dCTP to 10^8 cpm/μg by fill-in reactions. All probes were gel purified through a 15% polyacrylamide gel. Linearized cDNAs for human VDR, human RXRα, and human RARα were used for in vitro translation by the TNT® T7 coupled reticulocyte lysate system (Promega, Madison, WI). DNA probes (30,000 cpm/reaction) were incubated with the indicated amounts of in vitro translated or bacterially expressed human RXRα, human RARα, and human VDR [Mottershead et al., 1996] in 20 μl binding buffer (10 mM Hepes, pH 7.9, 50 mM KCl, 0.5 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT, and 0.3 μg/μl poly dI-dC) for 20 min at room temperature. Gel-shift experiments were performed either with bacterially expressed or with in vitro translated human VDR, human RARα, and hu-

man RXRα. Two different methods of protein production were used to validate receptor–DNA binding in vitro. RAR–RXR heterodimer formation was assessed on the complete TNFαRI sequence (27mer), on the DR2-type TNFαRI sequence (14mer), on the human RARβ RA response element, and on the human osteocalcin VD response element. In competition experiments, increasing amounts (0.05–1.5 μg) of full-length VDR or of VDR-LBD were added after RAR-RXR complex formation. Gel pre-electrophoresis and autoradiography was performed as outlined previously [Polly et al., 1996].

RESULTS

The sequence of the human TNFαRI gene (accession no. M85145) was aligned with the nuclear receptor core binding motif consensus sequence RRKNSA (R = A or G, K = G or T, S = C or G) [Carlberg, 1995]. A sequence in the 3′ region of the gene, which contained four potential core binding motifs in close proximity to each other, showed strong homology to the complete human osteocalcin VD response element [Morrison et al., 1989] (Fig. 1A). The four core binding sites of the TNFαRI sequence (27mer) can be viewed as a palindrome with no spacing (P0), an inverted palindrome spaced by one nucleotide (IP1) and direct repeats spaced by either 2 or 7 nucleotides (DR2 and DR7). In gel shift experiments using both bacterially expressed and in vitro translated human RARα and human RXRα receptors the binding of RAR–RXR heterodimers to the complete (27mer, Fig. 1B) and DR2-type (14mer, Fig. 1C) TNFαRI 3′ located sequence was observed. Gel-shift analysis using wild-type and mutated versions of the DR2-type response element and bacterially expressed human VDR, human RXRα and human RARα showed binding of RAR–RXR heterodimers to the wild-type, but not to the mutated DR2-type, sequence (Fig. 1D). Consistent binding of other hetero- or homodimeric nuclear receptor complexes was not observed.

Treatment of TNF33.3R.OSCAT1 (three copies of the TNFαRI 3′ region sequence in the context of the human osteocalcin basal promoter) transfected NIH 3T3 cells with 9-*cis* RA or all-*trans* RA produced a 4.1- or 3.9-fold induction of CAT activity, respectively, whereas the combined treatment of 9-*cis* RA with VD or of all-*trans* RA with VD resulted in approximate 50% repression of reporter gene activity to 2.3-fold (Fig. 2A). To confirm the transrepressive activity of VD on the TNFαRI sequence, retinoid signalling was examined in parallel with VD/retinoid co-stimulatory activity using the OSLAC8 LacZ reporter construct (containing the complete human osteocalcin promoter including the VD response element) and the RAREOSCAT1 reporter construct (containing the RARβ DR5-type RARE) cotransfected into NIH 3T3 cells. Addition of VD alone was sufficient to increase β-galactosidase activity by 3.6-fold and the combination of VD with 9-*cis* RA or all-*trans* RA resulted in enhancement of reporter gene activity 4.6- and 5.2-fold, respectively (Fig. 2B). Treatment of pRAREOSCAT1 transfected NIH 3T3 cells resulted in reduced retinoid stimulated transcription after treatment with VD. Downregulation of all-*trans* RA stimulated gene transcription from 11.6- to 8.6-fold and of 9-*cis* RA stimulated gene transcription from 12.1- to 8.9-fold was observed (Fig. 2C). However, when VD-mediated transrepression was compared between the TNFαRI sequence and the RARβ RA response element, the magnitude of the repressive effect on the RARβ RA response element was not as great as that seen with the TNFαRI RA sequence (Fig. 2A,C).

Gel-shift experiments with bacterially expressed receptors showed competition of the RAR–RXR heterodimer binding by addition of VDR (0.2–1.5 μg) without apparent DNA binding of VDR–RAR or VDR–RXR heterodimers to the complete TNFαRI 3′ region sequence (27mer, Fig. 3A). Using in vitro translated receptors, the addition of VDR resulted in the dissociation of RAR–RXR heterodimers from the TNFαRI DR2-type sequence (14mer, Fig. 3B). This shows that bacterially expressed and in vitro translated VDR resulted in equivalent reduction of RAR–RXR heterodimer binding to the complete and the DR2-type sequence. Interestingly, also the addition of bacterially expressed VDR–LBD dissociated RAR–RXR heterodimers from the complete TNFαRI 3′ region sequence (Fig. 3C). Competition of RAR–RXR heterodimers by post-addition of VDR is also seen on the human osteocalcin VD response element; however, either VDR–RAR or VDR–RXR heterodimeric binding subsequently appears as a lower-molecular-weight complex (Fig. 3D). By contrast, RAR-RXR heterodimer binding on the human RARβ RA response element

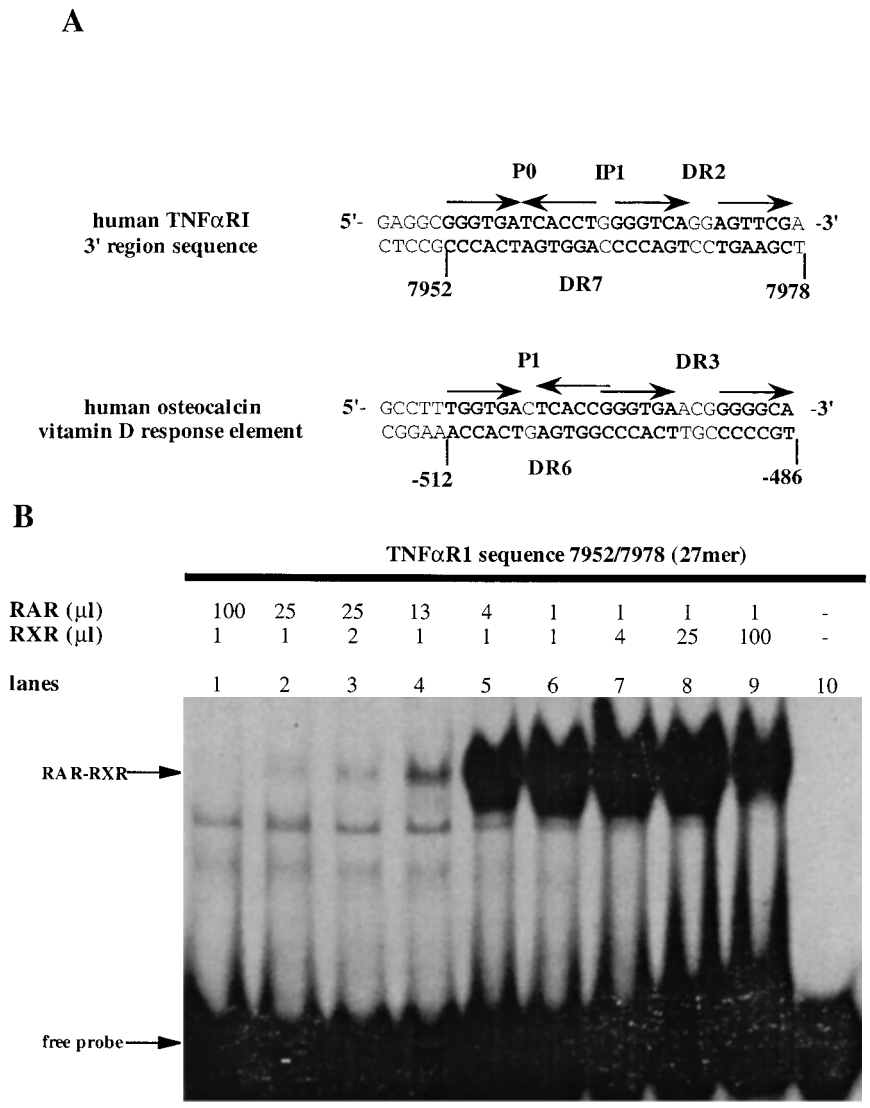

Fig. 1. A DR2-type sequence structure located in the human TNFαRI gene binds RAR-RXR. Sequence alignment of the human TNFαRI gene sequence with a nuclear receptor consensus binding site located a sequence in the 3′ region of the gene that has high homology with the complete VD response element of the human osteocalcin gene aligned below (**A**). Boldface, hexameric core binding motifs; arrows, orientation. Gel-shift experiments were performed using different volumetric ratios of bacterially expressed (**B**) and in vitro translated (**C**) human RXRα, human RARα, human VDR, and [^{32}P]-labeled complete TNFαRI 3′ region sequence (**B,** 27mer) and DR2-wild-type TNFαRI sequence or DR2-mutant type TNFαRI sequence (**D,** 14mer). RAR-RXR heterodimer complexes (arrow) were separated from nonspecific (NS) complexes (●). Data shown are representative of three independent experiments.

was essentially unchanged by increasing amounts of competitor VDR (Fig. 3E).

DISCUSSION

In this report a novel functional DR2-type sequence was identified in a 3′ enhancer position of the human TNFαRI gene. The response element is bound by RAR–RXR heterodimers and shows functionality in a heterologous promoter context in NIH 3T3. The current model of retinoid signaling suggests that DR2- and DR5-type sequences are occupied in 5′-RXR–RAR-3′ polarity by RXR–RAR heterodimers [Kurokawa et al., 1993, 1994; Perlmann et al., 1993; Predki et al., 1994; Zechel et al., 1994a,b]. The observed VDR-mediated repression of retinoid signalling described in this report could result from VDR sequestering either RXR or RAR from the RXR–RAR complex that binds the DR2-type sequence. A similar squelching phe-

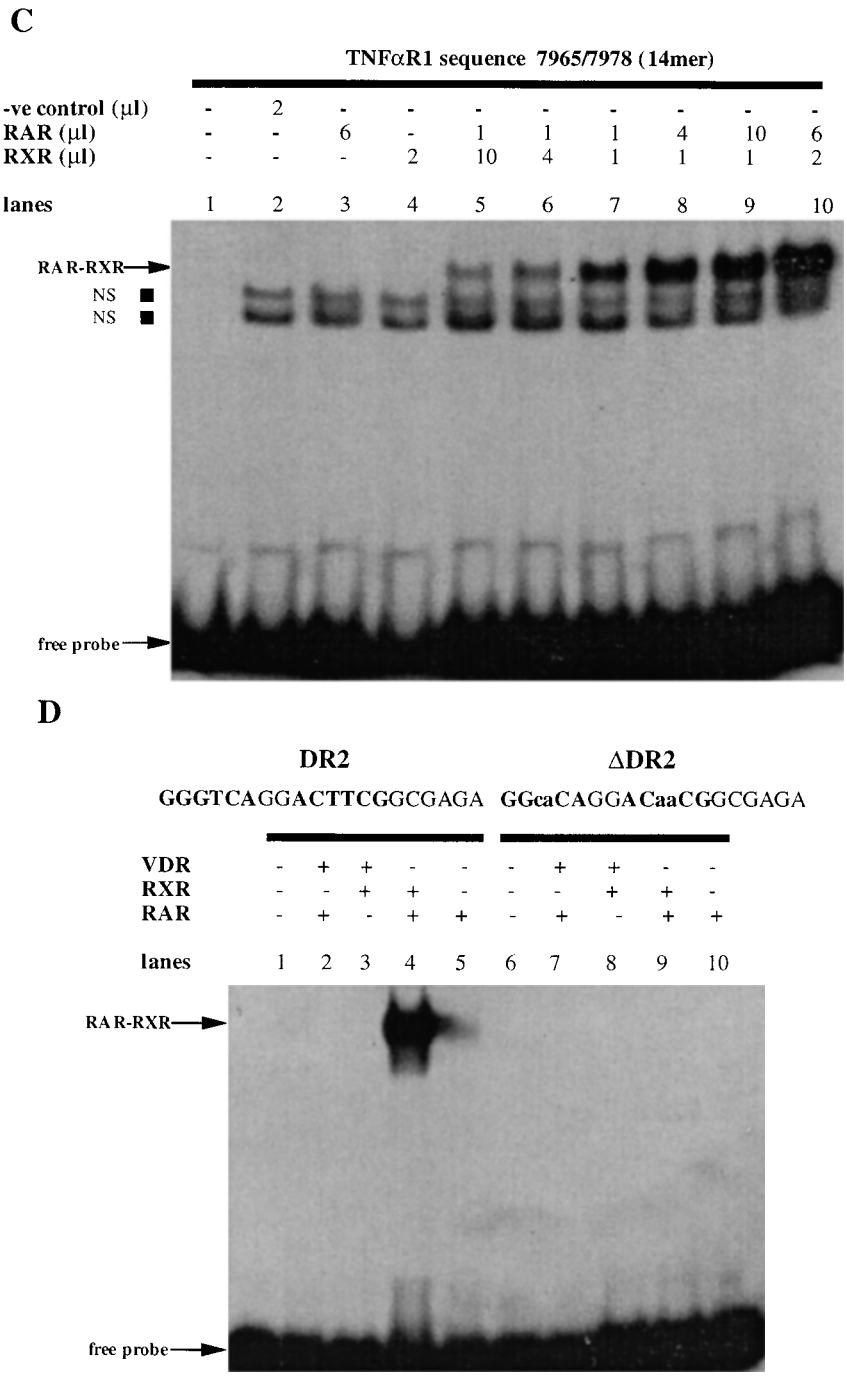

Fig. 1. (Continued.)

nomena has already been described for RXR in T_3 and VD signaling [Lehmann et al., 1993; Schräder et al., 1995]. RXR or RAR may reassociate with VDR either in solution or on an appropriate response element (as shown here on the human osteocalcin VD response element). The gel-shift competition experiments presented in this report indicate that VDR competes for either RXR or RAR, but the respective VDR–RAR and VDR–RXR heterodimers differ in their DNA binding specificity from RAR–RXR heterodimers and do not bind to the DR2-type sequence. The binding affinity of RAR–RXR heterodimers to DR5-type sequences (as shown here for the human RARβ gene) appears to be higher than the DR2-type sequence tested,

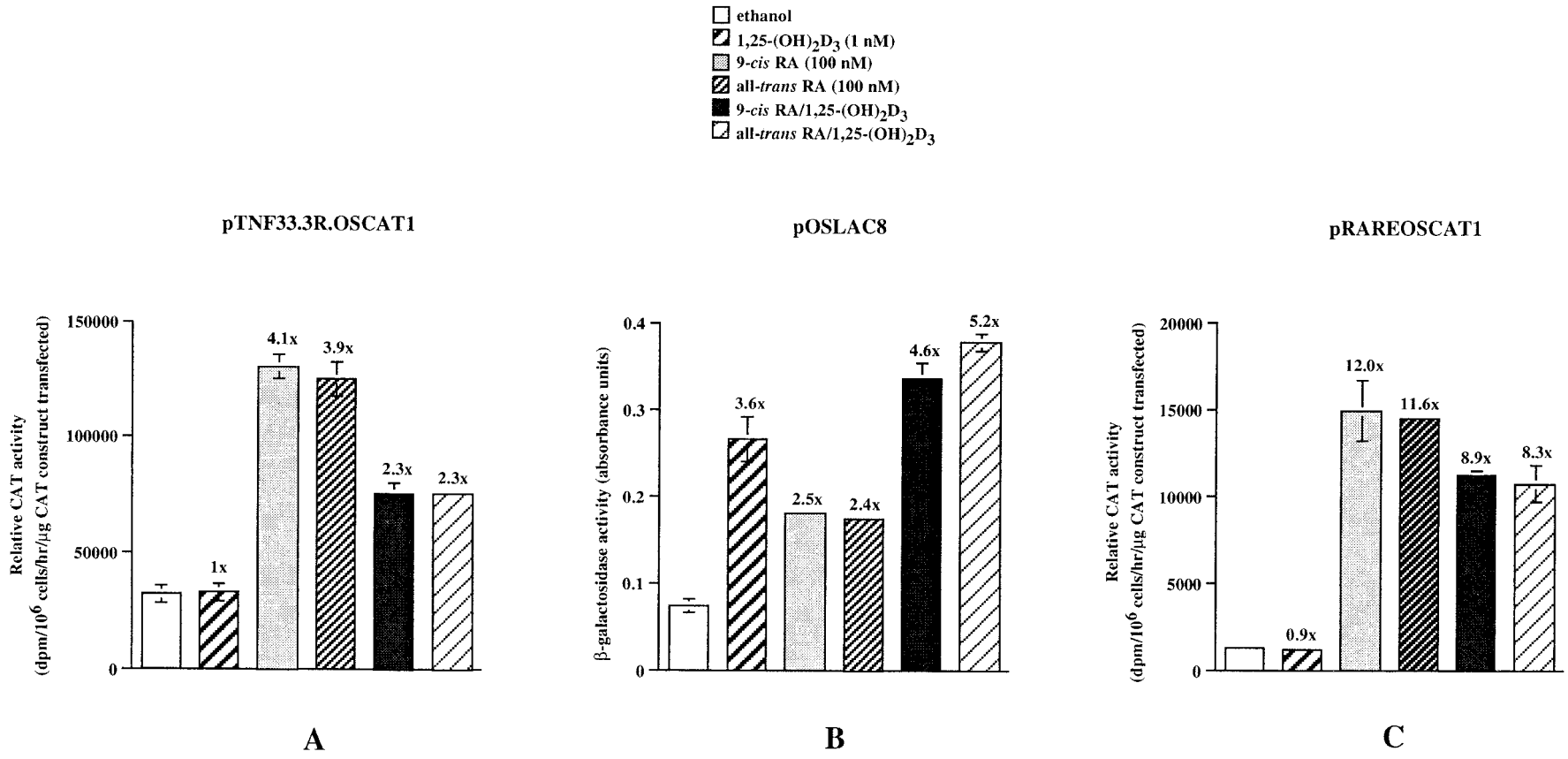

Fig. 2. Repression of retinoid signalling on the human TNFαRI sequence. NIH 3T3 cells were transfected with the CAT and LacZ reporter constructs TNF33.3R.OSCAT1, and OSLAC8 and treated with 1 nM VD, 100 nM 9-*cis* RA, 100 nM all-*trans* RA alone or in combination, as indicated. CAT (**A**) and β-galactosidase (**B**) activity was determined after 16 h of incubation. Combined 9-*cis* RA and VD or all-*trans* RA and VD treatment resulted in apparent repression of retinoid signaling from pTNF33.3R.OSCAT1 and pRAREOSCAT1 (**A,C**), whereas retinoid signaling from pOSLAC8 was further stimulated (**B**). Fold induction was calculated in comparison to solvent-treated controls. Data shown are representative of three independent experiments. Columns are means of triplicates. Bars = SD.

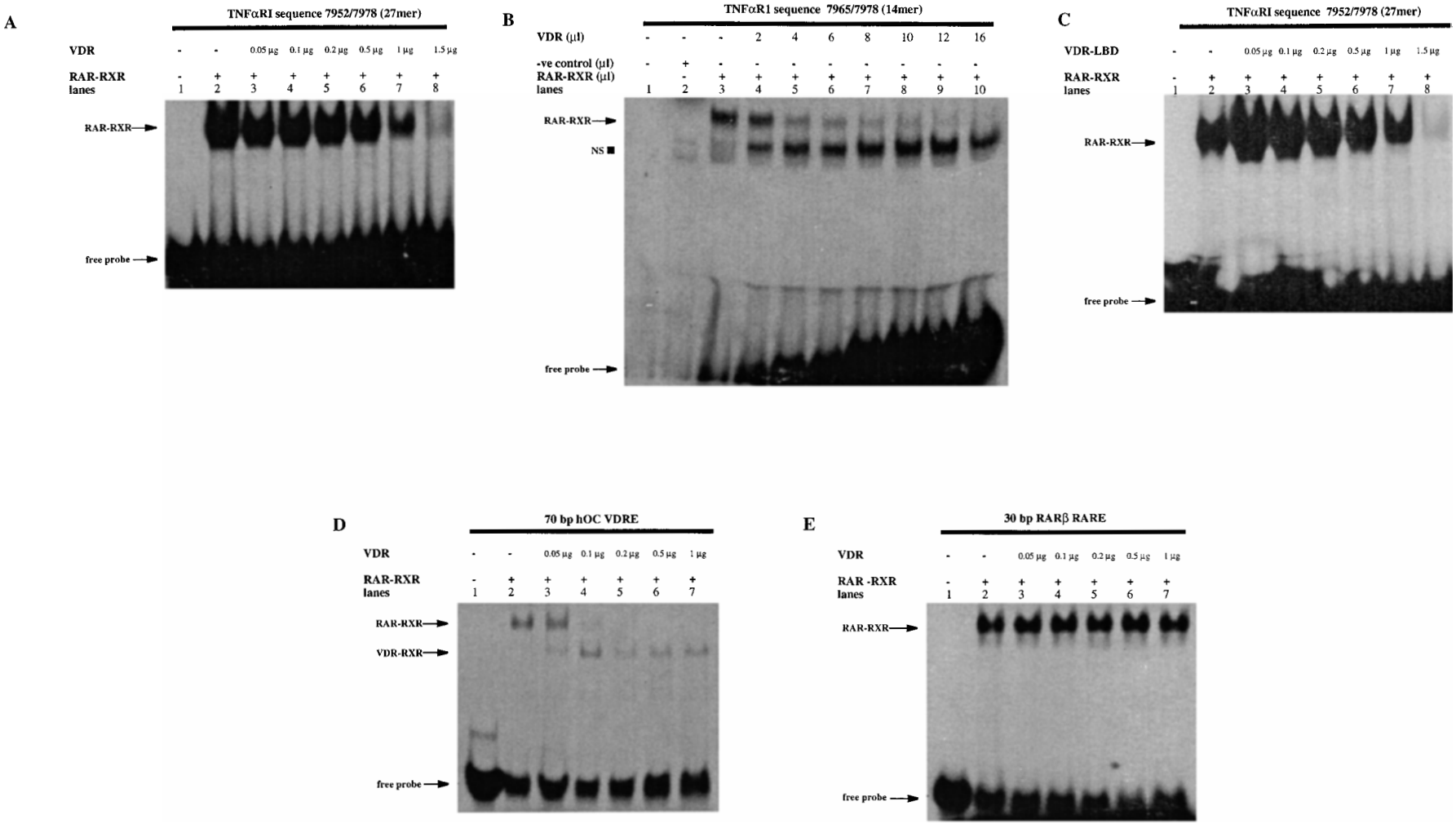

Fig. 3. Competition binding analysis. Gel-shift experiments were performed by incubating bacterially expressed and in vitro translated human RXRα and human RARα with [^{32}P]-labeled response elements from the human genes TNFαRI (**A–C**), osteocalcin (**D**), and RARβ (**E**). Increasing amounts of bacterially expressed competitor VDR (**A,D,E**), in vitro translated competitor VDR (**B**), or bacterially expressed competitor VDR-LBD (**C**) were added after RAR–RXR heterodimer formation. RAR–RXR heterodimer complexes (arrow) were separated from putative VDR–RXR heterodimer or nonspecific (NS) complexes (●). A representative from four independent experiments for each gel shift panel is shown.

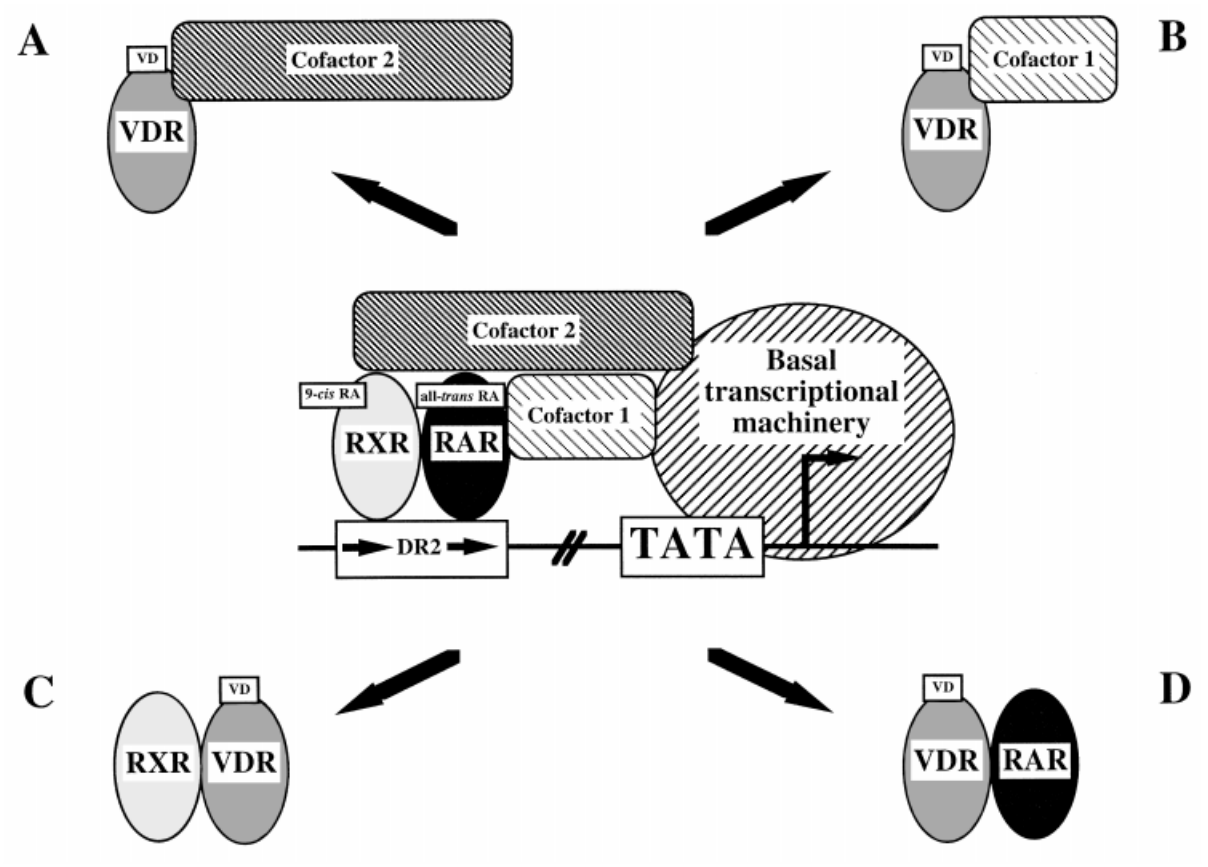

Fig. 4. Model of retinoid signaling transrepression by the VDR. Ligand-activated RAR–RXR heterodimers, bound to a DR2-type sequence, communicate their transactivation signal to the basal transcriptional machinery by the RAR-specific cofactor 1 and/or the RXR-specific cofactor 2. Ligand-activated VDR may interfere with this retinoid signaling process via competition for cofactor 2 (**A**), cofactor 1 (**B**), RXR (**C**), or RAR (**D**). In the case in which one of the four proteins has a limiting concentration and VDR shows protein–protein affinity, this interference will result in transrepression.

as VDR shows a significant transrepression on retinoid signalling only on the latter. This observation may explain the results seen in transcription assays, that is, why the transrepressive potential of VDR on DR5-type response elements appears to be weaker than that on DR2-type response elements (Fig. 2A,C). Taken together, these data suggest a protein–protein interaction model of VDR-mediated transrepression. Moreover, this mechanism appears to play a more prominent regulatory role on weak and medium-strength RA response elements than on strong elements.

Binding of ligand to nuclear receptors results in the presentation of a short amphipathic α-helix at the C-terminal LBD of the nuclear receptor, referred to as activation function-2 (AF-2) domain [Danielian et al., 1992; Durand et al., 1994]. The AF-2 domain is an interface for the interaction with cofactors that mediate the activation signal to the basal transcriptional machinery [Bourguet et al., 1995; Renaud et al., 1995] (Fig. 4). In recent studies, nuclear receptors have been shown to display a rather broad spectrum of promiscuous heterodimeric interactions with other nuclear receptors [Green, 1993]. It has been shown that a given nuclear receptor is able to contact different cofactors such as CREB-binding protein (CBP) and conversely, that a given cofactor can interact with different nuclear receptors, e.g., by using distinct domains CBP directly interacts with the LBD of many nuclear receptors [Kamei et al., 1996]. CBP is a cointegrator that can cooperate with coactivators of nuclear receptors to contact the basal transcriptional machinery and integrate distinct signal transduction pathways. The model proposed in Figure 4 illustrates the possible interaction of liganded VDR with RXR, RAR, and cofactors 1 and 2. Both cofactors are assumed to show specificity for the interaction with, e.g., liganded RXR and

RAR. Retinoid receptor partners or cofactors exchange with VDR via interactive association with the AF-2 interface of RXR or RAR, this would result in transrepression of retinoid signaling by way of communication with the basal transcriptional machinery.

When VDR has comparable or higher affinity than that of RAR or RXR for heterodimeric partner proteins or for one or the other cofactor, it will compete for binding (Fig. 4). The amount of free VDR, i.e., VDR that is not complexed by other proteins, appears to be comparable to that of the heterodimeric retinoid receptor complexes, as excessive molar amounts of VDR appear to be necessary to dissociate response element-bound RAR–RXR heterodimers. This competition will then result in a disturbance of retinoid signaling. This indicates that, as with other transrepressive processes, the relative level of endogenous, cellular expression of the involved receptors is of central importance. The fact that the VDR–LBD, which contains the AF-2 domain, showed a competitive effect that was comparable to that of full-length receptor indicated that VDR transrepression may be mediated mainly by the strong protein–protein interaction interfaces located in the ligand binding domain and is consistent with the hypothesis that transrepression is mediated by protein–protein interactions.

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