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Selective Recognition of Vitamin D Receptor Conformations Mediates Promoter Selectivity of Vitamin D Analogs

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ABSTRACT

The transcription factor VDR is the nuclear receptor for 1α,25-dihydroxyvitamin D_3 (VD) and the mediator of all genomic actions of the nuclear hormone and its synthetic analogs. The sharp biological profile of the model VD analog 1(*S*),3(*R*)-dihydroxy-20(*R*)-(5′-ethyl-5′-hydroxy-hepta-1′(*E*),3′(*E*)-dien-1′-yl)-9,10-secopregna-5(*Z*),7(*E*),10(19)-triene (EB1089) (i.e., its high antiproliferative effect combined with low calcemic actions) has been correlated with the selectivity of EB1089 to activate heterodimeric complexes of VDR with its partner retinoid X receptor (RXR) on VD response elements (VDREs). These VDREs are formed by an inverted palindromic arrangement of two hexameric core binding motifs spaced by nine nucleotides (IP9) rather than VDREs that are formed by direct repeats with three intervening nucleotides (DR3). In this report, ligand-dependent gel-shift assays were used for a comparison of the ability of VD and EB1089 to stabilize VDR-RXR heterodimers on these two VDRE types. The gel-shift assays revealed EB1089 to be more sensitive for complexes on IP9-type VDREs than on DR3-type VDREs. In addition, a gel-shift clipping method was established to identify and compare complexes of ligand-stabilized VDR-RXR heterodimers on different VDREs. On each VDRE, two complexes could be discriminated that seemed to contain different functional conformations of the VDR and allowed a more differential view on DNA-complexed VDR-RXR heterodimers. The VDR-RXR conformation (which was more ligand-sensitive) gained through EB1089 a higher affinity (7-fold) for DNA binding and a more sensitive (9-fold) activation of an IP9-type VDRE than of a DR3-type VDRE, whereas with the natural hormone VD, no VDRE-type preference could be observed. This indicates that promoter selectivity of VDR ligands is based on their property to selectively increase affinity for VDREs and very sensitively stabilize VDR conformations in VDR-RXR-VDRE complexes.

As a physiologically active form of vitamin D_3, the nuclear hormone 1α,25-dihydroxyvitamin D_3 (VD) is a major regulator of calcium homeostasis (DeLuca et al., 1990) and is also involved in controlling cellular growth, differentiation, and apoptosis (Walters, 1992). The calcemic function of the hormone can cause side effects such as hypercalcemia, hypercalciuria, and soft tissue calcification (Vieth, 1990), but analogs of VD that display a more selective biological profile should have an interesting therapeutic potential against a variety of diseases, such as osteoporosis, cancer, and psoriasis (Pols et al., 1994). The VD analog 1(*S*),3(*R*)-dihydroxy-20(*R*)-(5′-ethyl-5′-hydroxy-hepta-1′(*E*),3′(*E*)-dien-1′-yl)-9,10-secopregna-5(*Z*),7(*E*),10(19)-triene (EB1089) (Mørk Hansen and Mäenpää, 1997) has a strong antiproliferative effect combined with a reduced calcemic action in vitro and in vivo (Colston et al., 1992; Mathiasen et al., 1993) and is presently investigated in clinical tests with different types of cancer (Gulliford et al., 1998). Moreover, in this and other studies (Carlberg et al., 1994; Nayeri et al., 1995; Quack et al., 1998a,b), EB1089 served as a model analog for the analysis of a selective activation of nuclear VD signaling.

The nuclear hormone VD and its analogs bind specifically to the VD receptor (VDR) (Pike, 1991; Carlberg, 1996a), which is a member of a superfamily of structurally related nuclear receptor transcription factors (Mangelsdorf et al., 1995). VDR binds as a dimer to specific sequences in the promoter of VD target genes, commonly referred to as VD response elements (VDREs) (Carlberg, 1995). The main partner receptor for the VDR is the retinoid X receptor (RXR) (Carlberg, 1996b), which is the nuclear receptor for 9-*cis*-retinoic acid (RA). Simple VDREs are formed by two hexam-

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ABBREVIATIONS: AF-2, (*trans*)activation function 2; ANF, atrial natriuretic factor; DR3, direct repeat spaced by 3 nucleotides; DR4, direct repeat spaced by 4 nucleotides; EB1089, 1(*S*),3(*R*)-dihydroxy-20(*R*)-(5′-ethyl-5′-hydroxy-hepta-1′(*E*),3′(*E*)-dien-1′-yl)-9,10-secopregna-5(*Z*),7(*E*),10(19)-triene; IP9, inverted palindrome spaced by 9 nucleotides; LBD, ligand-binding domain; RA, retinoic acid; RXR, retinoid X receptor; VD, 1α,25-dihydroxyvitamin D_3; VDR, 1α,25-dihydroxyvitamin D_3 receptor; VDRE, 1α,25-dihydroxyvitamin D_3 response element; VDR_{wt}, wild-type 1α,25-dihydroxyvitamin D_3 receptor; $VDR_{K413STOP}$, a 1α,25-dihydroxyvitamin D_3 receptor mutant that was truncated at its (*trans*)activation function 2 domain.

eric nuclear receptor binding sites and VDR-RXR heterodimers bind preferentially to directly repeated binding site arrangements with three spacing nucleotides (DR3-type VDREs) or to inverted palindromic structures with nine intervening nucleotides (IP9-type VDREs) (Carlberg, 1996b). Moreover, VDREs with direct repeats spaced by four nucleotides (DR4) and six nucleotides (DR6) type VDREs have been described (Carlberg, 1995). According to the model of multiple VD signaling pathways (Carlberg, 1996a), the pleiotropic function of VD is based on a variety of dimeric VDR complexes bound to different types of VDREs. The model assumes that each of these VDR-VDRE complexes may be representative for a group of primary VD responding genes that are involved in the regulation of a distinct proportion the pleiotropic action of the nuclear hormone. In support of this model, some VD analogs (e.g., EB1089) have shown the tendency to preferentially activate VDR-RXR heterodimers that are bound to IP9-type VDREs (Nayeri et al., 1995), whereas other analogs seem to prefer DR3-type VDRE-bound VDR complexes (Danielsson et al., 1997). This indication of promoter selectivity may be correlated with the observation that IP9-type VDREs have been found in some genes that are involved in the regulation of the cell cycle (Schräder et al., 1997).

Binding of VD or of a VD analog to the VDR results in stabilization of a functional conformation of its ligand-binding domain (LBD) (Nayeri et al., 1996a; Nayeri and Carlberg, 1997). According to crystal structures of nuclear receptor LBDs (e.g., of apo-RXRα; Bourguet et al., 1995) and all-*trans*-RA-bound RA receptor γ (Renaud et al., 1995), the binding of ligand mainly results in changing the position of the most carboxyl-terminal α-helix that contains the so-called activation function 2 (AF-2) domain. In general, the AF-2 domain provides an interface for interaction with coactivator proteins that mediate contacts to the basal transcriptional machinery (Horwitz et al., 1996; Jurutka et al., 1997; Masuyama et al., 1997). For the VDR, amino acid residues of the AF-2 domain also have been found to stabilize the high-affinity ligand-binding conformation of the LBD (Nayeri and Carlberg, 1997). Traditional ligand competition assays using radiolabeled ligand do not allow for visualization of receptor conformational changes (Mørk Hansen et al., 1996). In contrast, the limited protease digestion assay has been demonstrated to be a powerful method for characterizing functional VDR conformations (Nayeri et al., 1995, 1996a,b; Peleg et al., 1995, 1998; Nayeri and Carlberg, 1997). The interaction of monomeric VDR in solution with ligand protects the LBD against protease digestion. Under these conditions, with both VD and EB1089, two characteristic functional conformations of the LBD have been discriminated (Quack et al., 1998a,b). Moreover, some biologically potent VD analogs have demonstrated a higher functional affinity to VDR than the natural hormone with this method (Nayeri et al., 1996b). In addition, limited protease digestion has been used recently to monitor the kinetics of VDR stabilization by ligand (van den Bemd et al., 1996).

In this study, a new method was developed, called a gel-shift clipping assay, that combines the advantages of the limited protease digestion assay and of the DNA-dependent gel-shift assay, which is closer to the in vivo condition. With the help of this technique, VD and EB1089 were compared for their ability to stabilize the VDR conformations in VDR-RXR heterodimers that were bound to either DR3- or IP9-type VDREs. On each VDRE, two VDR conformations were discriminated that show individual sensitivity to the different ligands. This indicates that the promoter selectivity of EB1089 is based on a stabilization of a highly ligand-sensitive VDR conformation of VDR-RXR heterodimers on an IP9-type VDRE.

Materials and Methods

Compounds. VD and EB1089 (for structures, see Fig. 1) were kindly provided by C. Mørk Hansen (LEO Pharmaceutical Products, Ballerup, Denmark). The ligands were dissolved in 2-propanol at 4 mM; dilutions were performed in ethanol.

DNA Constructs. The cDNA for human VDR and human RXRα were subcloned into the expression vector pSG5 (Stratagene, Heidelberg, Germany) (Carlberg et al., 1993). The VDR construct was used as template for a linear polymerase chain reaction using *Pfu* DNA polymerase (Stratagene) with a profile of 1 min at 94°C, 1 min at 55°C, and 11 min at 68°C for 16 cycles. The following primer pair was used for the K413STOP point mutations (K = lysine): K413STOP+ AGTGCAGCATGTAGCTAACGC and K413STOP− GCGTTAGCTACATGCTGCACT. After PCR, methylated template DNA was digested selectively with *Dpn*I and supercompetent *Epicurian coli* XL-1 (Stratagene) were transformed with nondigested, PCR-generated plasmid DNA. The point mutation was confirmed by sequencing.

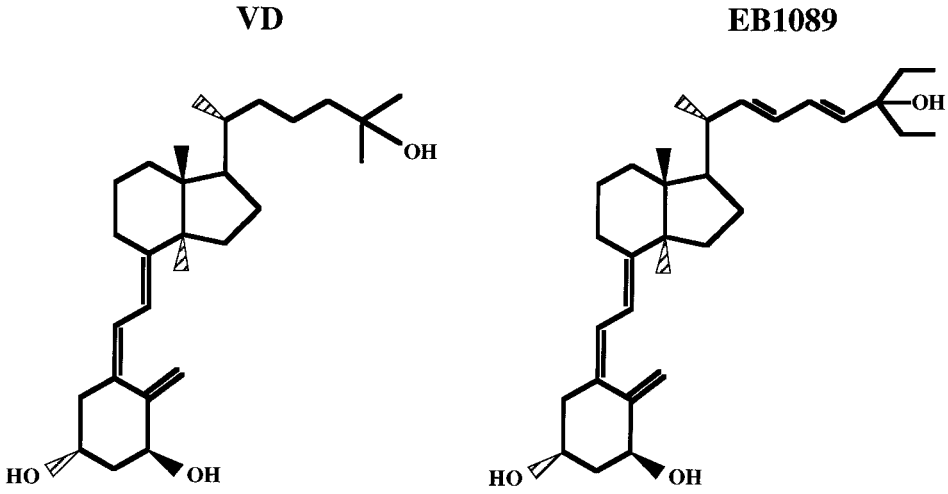

Fig. 1. Structure of EB1089 compared with the natural hormone VD. The side chain of EB1089 is extended by one carbon atom and contains each a double bond at positions C22 and C24 when comparing with VD.

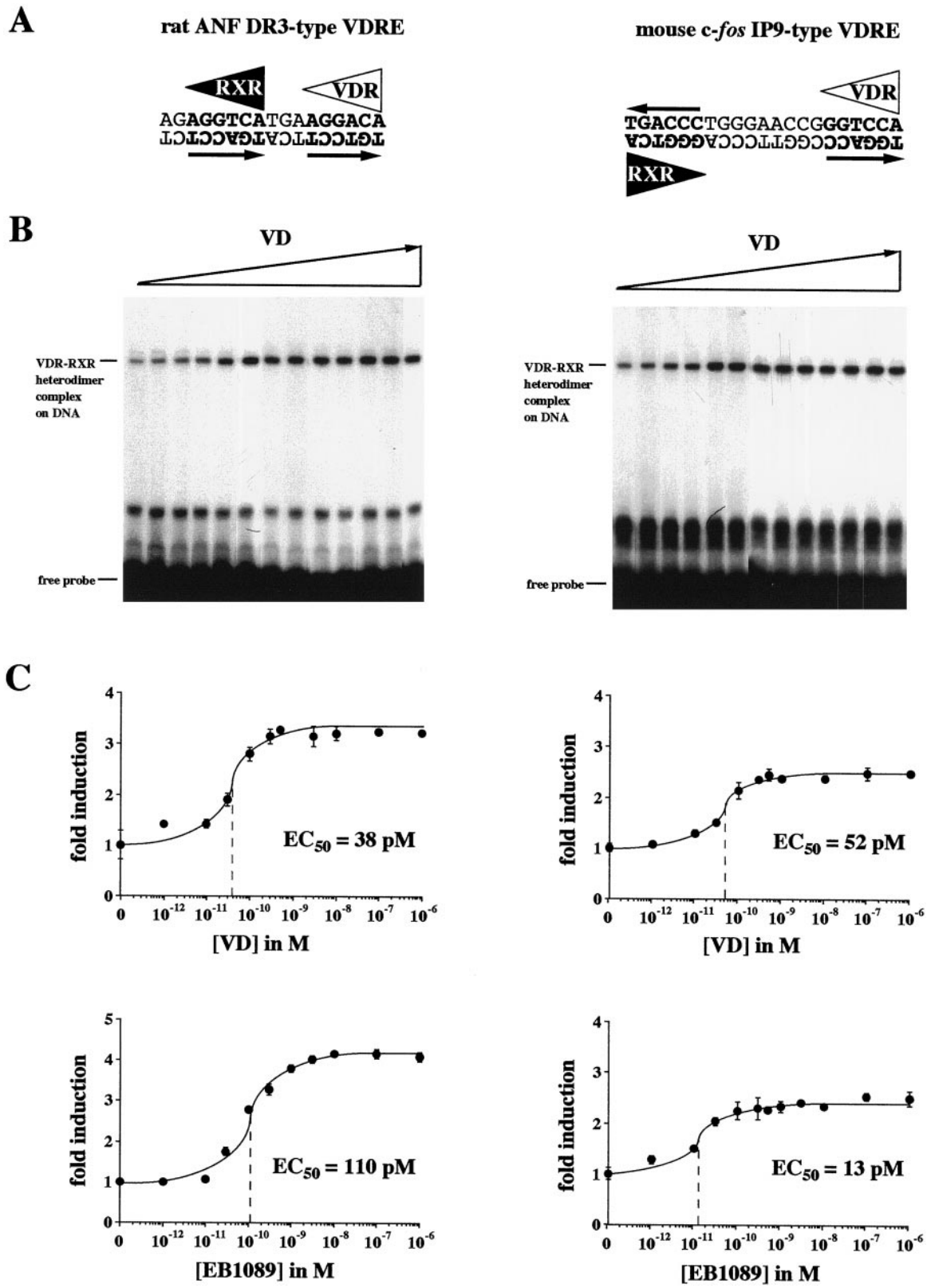

Fig. 2. Differential ligand stabilization of VDR-RXR heterodimer complexes on DR3- and IP9-type VDREs. A, the complex formation of the DNA binding domains for VDR and RXR on both VDRE types is schematically depicted (the core binding motifs are in bold), the DNA binding domains are represented by triangles to visualize their ordered orientation. Heterodimers of in vitro translated VDR and RXR protein were preincubated with graded concentrations of VD and EB1089. Gel-shift experiments were performed on either the ^{32}P-labeled DR3-type VDRE of the rat ANF gene promoter or on the IP9-type VDRE of the mouse c-*fos* gene promoter at an ionic strength of 100 mM KCl. B, VDR-RXR heterodimers were separated from free probe on an 8% nondenaturing polyacrylamide gel. Representative experiments are shown. C, the amount of protein-complexed VDREs was quantified on a BioImager in relation to nonliganded VDR-RXR heterodimers. Each data point represents the mean of triplicates and bars indicate S.D. EC_{50} values of VD and EB1089 were calculated from dose-response curves.

Ligand-Depend Gel-Shift and Gel-Shift Clipping Assays. Linearized DNAs of the pSG5-based constructs of wild-type VDR (VDR_{wt}), a VDR mutant that was truncated at its AF-2 domain ($VDR_{K413STOP}$), and RXRα were transcribed with T_7 RNA polymerase and translated using rabbit reticulocyte lysate as recommended by the supplier (Promega, Mannheim, Germany). Equal amounts of VDR (or VDR mutant) and RXR protein were mixed and incubated in the presence of indicated concentrations of VD or EB1089 (or ethanol as control) for 15 min at room temperature in a total volume of 20 μl of binding buffer [10 mM HEPES, pH 7.9, 1 mM dithiothreitol, 0.2 μg/μl poly(dI-C) and 5% glycerol]. The buffer had been adjusted to 100 mM monovalent cations by addition of KCl. The indicated DR3-type, DR4-type, and the IP9-type VDREs (for core sequences see Figs. 2A and 6A) were labeled by a fill-in reaction using [α-^{32}P]dCTP and the Klenow fragment of DNA polymerase I (Promega). Approximately 1 ng of labeled probe (50,000 cpm) was added to the receptor-ligand mixture and incubation was continued for 20 min. For DNA competition assays, 250- to 500-fold excess of nonlabeled VDRE was added for the times indicated in Figs. 3 and 4, respectively. Protein-DNA complexes were resolved on an 8 or 10% nondenaturing polyacrylamide gel (at room temperature) in 0.5 × Tris/boric acid/EDTA (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3).

For gel-shift clipping assays, the endoprotease trypsin (Promega) was added to a final concentration of 22 or 66 ng/μl and the incubation was continued for 10 min (or indicated times) at room temperature. For DNA competition assays, 250- to 500-fold excess of non-

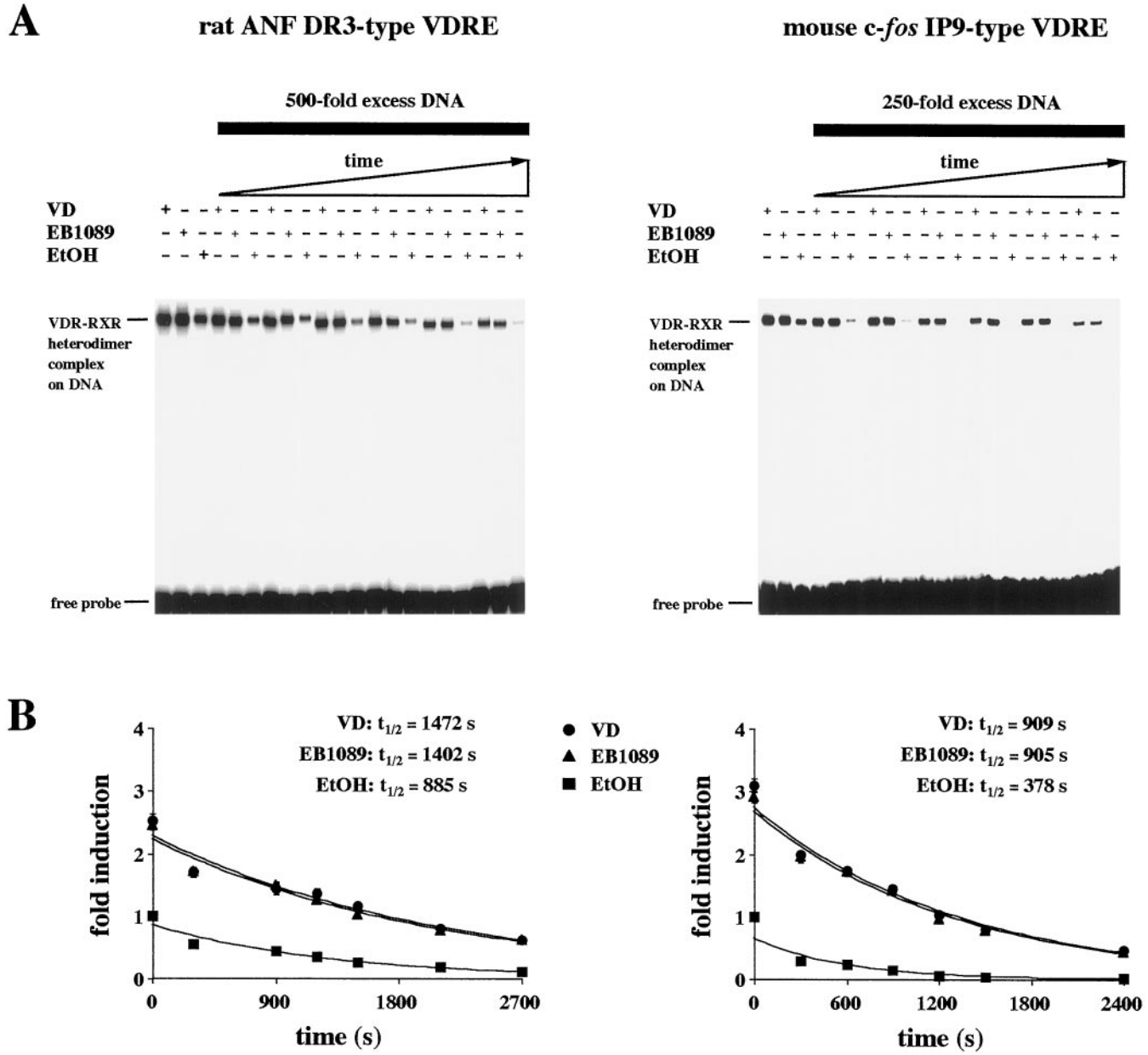

Fig. 3. DNA competition assay of ligand-induced VDR-RXR heterodimer-VDRE complexes. Heterodimers of in vitro translated VDR and RXR protein were preincubated with 1 μM VD or EB1089 (or ethanol as control). Gel-shift experiments were performed on either the ^{32}P-labeled DR3-type VDRE of the rat ANF gene promoter or on the IP9-type VDRE of the mouse c-*fos* gene promoter at an ionic strength of 100 mM KCl. After 20 min of incubation, a 500-fold excess of nonlabeled DR3-type VDRE or a 250-fold excess of nonlabeled IP9-type VDRE were added and incubation was continued for the indicated times. A, VDR-RXR heterodimers were separated from free probe on a 10% nondenaturing polyacrylamide gel. Representative experiments are shown. B, the amount of protein-complexed VDREs was quantified on a BioImager in relation to nonliganded VDR-RXR heterodimers. Each data point represents the mean of triplicates and bars indicate S.D. The half-lives ($T_{1/2}$) of VD-, EB1089- and solvent-stabilized VDR-RXR complexes were calculated form the extrapolation curves.

labeled VDRE was added for indicated times. The partially digested protein-DNA complexes were also then resolved on an 8 or 10% nondenaturing polyacrylamide gels.

In both cases, the gels were dried and exposed to a Fuji MP2040S imager screen (Fuji, Tokyo, Japan) overnight. The ratio of free probe to protein-probe complexes was quantified on a Fuji FLA2000 reader using Image Gauge software (Raytest, Sprockhövel, Germany). Each condition was analyzed, at least, in triplicate.

Results

Gel-shift experiments were performed using heterodimeric complexes that were formed by equal amounts of in vitro translated VDR and RXR proteins and the DR3-type VDREs of the rat atrial natriuretic factor (ANF) gene promoter (Kahlen and Carlberg, 1996) and the IP9-type VDRE of the mouse c-*fos* gene promoter (Schräder et al., 1997). Increasing concentrations of VD were found to enhance the amount of DNA-bound VDR-RXR heterodimers on both types of VDREs (Fig. 2B). Similar results were also obtained with graded concentrations of EB1089. When quantifying the ligand-stimulated enhancement of protein-DNA complex formation, typical dose response curves were obtained, from which half-maximal activation concentrations (EC_{50} values) could be determined (Fig. 2C). EC_{50} values of 38 pM for the DR3-type and 52 pM for the IP9-type VDRE with VD treatment were calculated, whereas in treatment with EB1089, the EC_{50} value for the DR3-type VDRE (110 pM) was found to be eight times higher than that for the IP9-type VDRE (13 pM). These results suggest that the previously described selectivity of EB1089 in activating IP9-type VDREs in reporter gene assays (Nayeri et al., 1995) is also apparent at the level of protein-DNA complex formation. DNA competition assays with nonlabeled VDREs allowed the determination of the half-lives of the different ligand-stabilized VDR-RXR heterodimer-VDRE complexes (Fig. 3). VD-stabilized VDR-RXR heterodimers showed a half-life of 1472 s for the DR3-type VDRE, which equals that of EB1089-stabilized complexes ($T_{1/2}$ = 1402 s). Also, on the IP9-type VDRE, VD- and EB1089-stabilized VDR-RXR heterodimeric complexes showed nearly identical half-lives of 909 and 905 s, respectively. Solvent-treated VDR-RXR heterodimers VDRE showed approximately 1.7-fold lower half-lives ($T_{1/2}$ = 885 s) for a DR3-type; for the IP9-type VDRE, the VDR-RXR heterodimers were even 2.4-fold less stable ($T_{1/2}$ = 378 s).

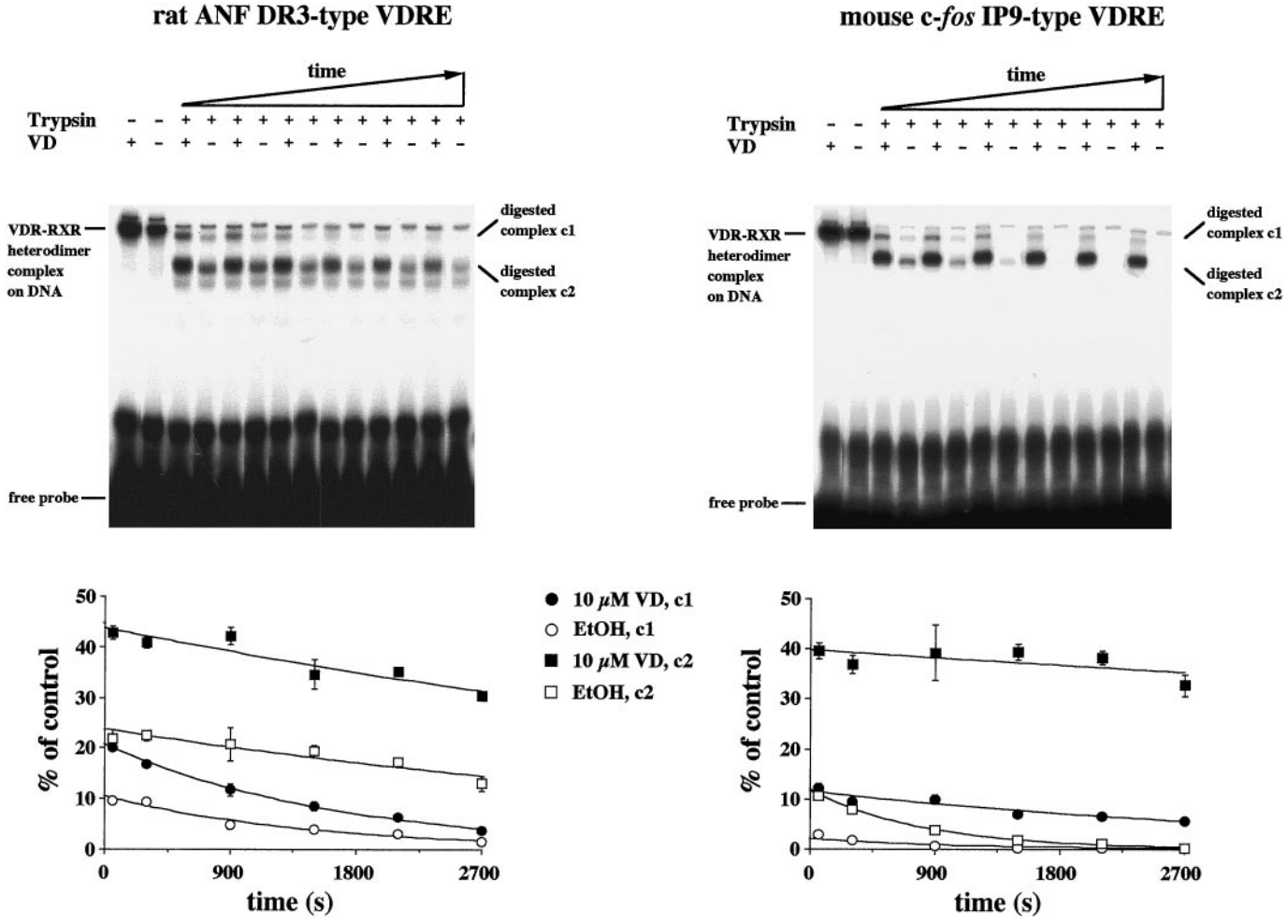

Fig. 4. Protease truncated VDR-RXR heterodimer-VDRE complexes indicate different conformations. Heterodimers of in vitro translated VDR and RXR were performed in the presence of 10 μM VD (or ethanol as control) on either the ^{32}P-labeled DR3-type VDRE or the IP9-type VDRE. Trypsin was then added to a final concentration of 66 ng/μl for the indicated incubation times. A, protein-DNA complexes were separated from free probe on an 8% nondenaturing gel. Representative experiments are shown. B, the amount of the ligand-bound, nondigested VDR-RXR heterodimer-VDRE complex was quantified on a BioImager and was taken as a reference for the quantification of two faster migrating, partially protease resistant protein-DNA complexes. ○, ●, complex c1; □, ■, complex c2. Each data point represents the mean of triplicates and bars indicate S.D.

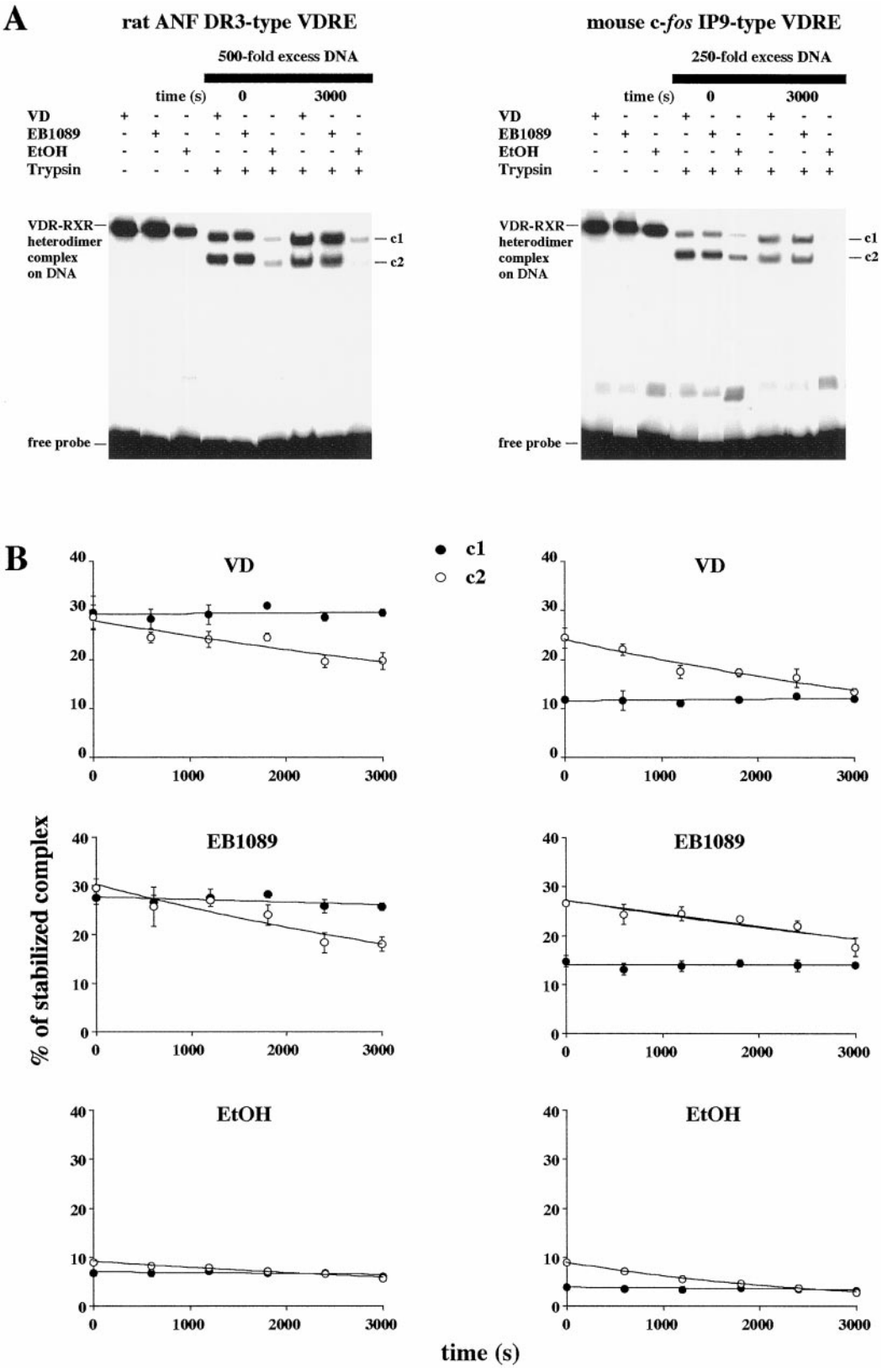

Fig. 5. DNA competition assay of ligand-induced, protease-truncated VDR-RXR heterodimer-VDRE complexes. Heterodimers of in vitro translated VDR and RXR were formed and incubated with 1 μM VD or EB1089 (or ethanol as control). Twenty minutes after the addition of either the ^{32}P-labeled DR3-type VDRE or the IP9-type VDRE, the VDR-RXR heterodimer-VDRE complexes were incubated with trypsin (22 ng/μl) for 10 min. Then a 500-fold excess of nonlabeled DR3-type VDRE or a 250-fold excess of nonlabeled IP9-type VDRE was added and incubation was continued within the indicated time-frame. A, protein-DNA complexes were separated from free probe on a 10% nondenaturing gel. Representative experiments are shown. B, the amount of the ligand-bound, nondigested VDR-RXR heterodimer-VDRE complex was taken as a reference for the quantification of complexes c1 and c2. ●, complex c1; ○, complex c2. Each data point represents the mean of triplicates and bars indicate S.D.

A gel-shift clipping method was developed to investigate the mechanisms of this ligand-activated, VDRE-selective VDR-RXR heterodimer complex formation. This new assay system combines the well established gel-shift assay as a detection method for protein-DNA interactions and the limited protease digestion assay as a method for the analysis of functional nuclear receptor conformations. In this assay, VDR-RXR heterodimer complexes were first formed on VDREs under gel-shift assay reaction conditions followed by application of a limited concentration of an endoprotease, such as trypsin. Separation of the reaction products on a nondenaturing polyacrylamide gel provided two protein-DNA complexes (c1 and c2) that migrated faster (i.e., that seemed to be of lower molecular mass) than nondigested VDR-RXR heterodimers (Fig. 4A). This ligand-induced stabilization of these two complexes was further investigated in a time course experiment on both VDRE types (Fig. 4B). The results indicated that complete digestion of the original VDR-RXR heterodimers into the smaller complexes 1 and 2 was achieved upon incubation with trypsin after only 1 min. However, a high proportion of the original complex seemed completely digested, because the sum of the amounts of complexes 1 and 2 was lower than that of the input. Interestingly, in the presence of VD, 2- to 10-fold higher amounts of complexes 1 and 2 were stabilized than with the ethanol solvent control. This ratio was observed to be higher on the DR3-type VDRE than on the IP9-type VDRE and was additionally enhanced through extended incubation with the protease. An incubation time of 10 min was defined as standard for all following experiments. DNA competition assays with nonlabeled VDREs allowed the determination of the half-lives of the different ligand-stabilized VDR-RXR heterodimer-VDRE complexes (Fig. 5). Interestingly, the stability of these truncated VDR-RXR complexes, in particular that of complex c1, was found to be much higher than that of the comparable nondigested complexes. Compared with the solvent control, the half-lives of complex c1 (and complex c2) were stabilized through the ligands VD and EB1089 on the DR3-type VDRE by factors of 5.0 (1.2) and 1.3 (0.8), respectively, and on the IP9-type VDRE by factors of 2.9 (1.8) and 19.2 (3.0), respectively.

A broader range of VDREs was then analyzed for ligand-dependent complex formation using both gel-shift assays and gel-shift clipping assays. In addition to the DR3-type VDRE of the rat *ANF* gene, the well known DR3-type VDRE of the mouse osteopontin gene (Noda et al., 1990) was also tested. Moreover, an additional IP9-type VDRE from the human calbindin D_{9k} gene (Schräder et al., 1995) and the DR4-type VDRE of the rat *Pit-1* gene (Rhodes et al., 1993) were also included in the selection (Fig. 6A). Complex formation of

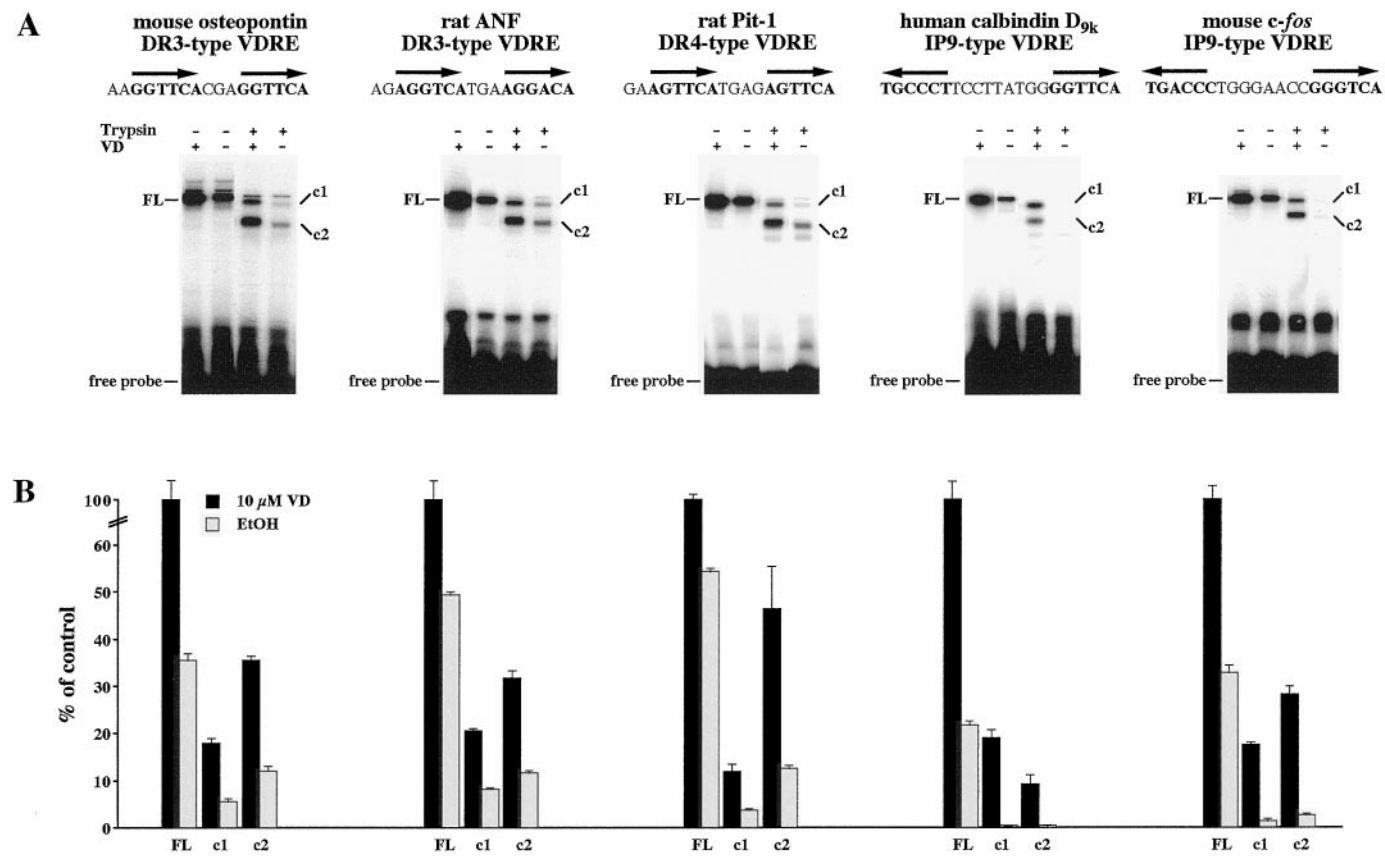

Fig. 6. Gel-shift clipping on different types of VDREs. Heterodimers of in vitro translated VDR and RXR were performed in the presence of 10 μM VD (or ethanol as control) on the ^{32}P-labeled DR3-type VDRE from the rat ANF gene promoter, the DR3-type VDRE from the mouse osteopontin gene promoter, the DR4-type VDRE from the rat *Pit-1* gene promoter, the IP9-type VDRE from the human calbindin D_{9k} gene promoter and the IP9-type VDRE from the mouse c-fos gene promoter. Trypsin was then added to a final concentration of 66 ng/μl and the incubation was continued for 10 min. A, protein-DNA complexes were separated from free probe on an 8% nondenaturing gel, representative experiments are shown. B, the amounts of digested VDR-RXR heterodimer-VDRE complexes (c1 and c2) were quantified on a BioImager in relation to the respective ligand-induced, nondigested VDR-RXR heterodimer (FL). Each column represents the average of triplicates and bars indicate S.D.

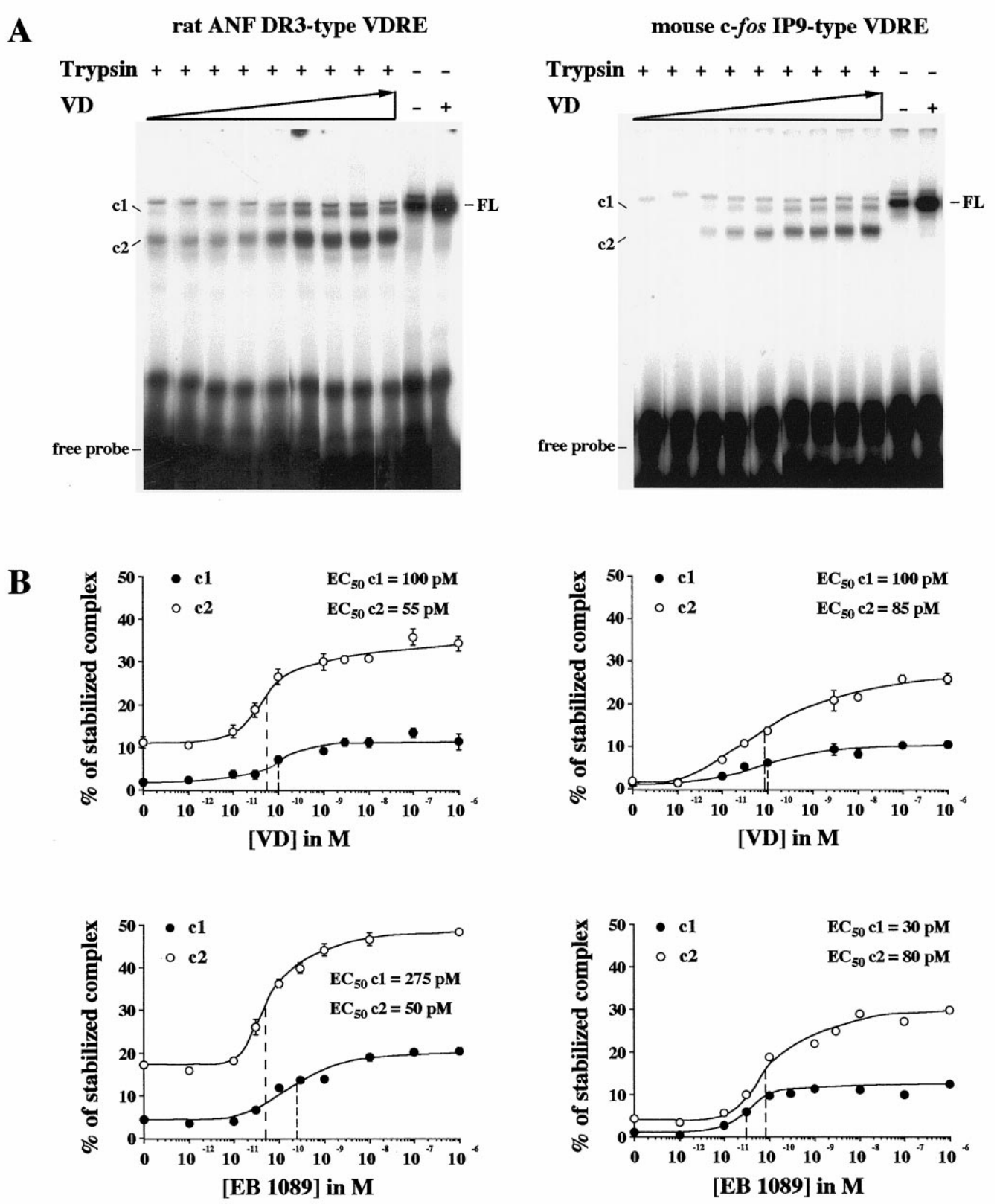

Fig. 7. Selective stabilization of VDR-RXR heterodimer-VDRE complexes. Gel-shift clipping experiments were performed by forming heterodimers between in vitro translated VDR and RXR in the presence of graded concentrations of VD or EB1089 on the ^{32}P-labeled DR3-type VDRE of the rat *ANF* gene promoter or the IP9-type VDRE of the mouse c-*fos* gene promoter. Trypsin was then added to a final concentration of 66 ng/μl and incubation was continued for 10 min. A, protein-DNA complexes were separated from free probe on an 8% nondenaturing gel. Representative experiments are shown. B, the amounts of digested VDR-RXR heterodimer-VDRE complexes (c1 and c2) were quantified on a BioImager in relation to the respective ligand-induced, nondigested VDR-RXR heterodimer (FL). Each data point represents the mean of triplicates and bars indicate S.D. EC_{50} values of VD and EB1089 were calculated from the dose-response curves.

VDR-RXR heterodimers was enhanced by ligand on all five VDREs (Fig. 6B), demonstrating that the IP9-type VDREs displayed a higher ligand inducibility (3- and 4.6-fold) than the two DR3-type VDREs (2.0- and 2.8-fold) and the DR4-type VDRE (1.8-fold). This tendency was confirmed by gel-shift clipping assays, where the overall ligand-inducibility of the digested complexes 1 and 2 (2.5- to 12.7-fold) was found to be higher than that of nondigested complexes in the gel-shift assay. On a given VDRE, complexes 1 and 2 showed comparable inducibility that was again found to be higher on IP9-type VDREs than on DR4-type and DR3-type VDREs.

Investigation of the dose-dependent stabilization of complexes 1 and 2 on a DR3-type and an IP9-type VDRE with graded concentrations of VD and EB1089 allowed the EC_{50} values for each condition to be determined (Fig. 7). Upon VD treatment, the EC_{50} values for complex 1 were found to be 100 pM on both VDRE types; for complex 2, the EC_{50} values were 55 pM for the DR3-type VDRE and 85 pM for the IP9-type VDRE. Treatment with EB1089 demonstrated a similar property for complex 2; the EC_{50} values were determined as 50 pM on the DR3-type VDRE and 80 pM on the IP9-type VDRE. However, complex 1 displayed an interesting selectivity; the EC_{50} value on the DR3-type VDRE was found to be 275 pM, whereas the IP9-type VDRE readily stabilized a concentration that was 10-fold lower (EC_{50} value of 30 pM). Interestingly, the selective stabilization of complex 1 with EB1089 on an IP9-type VDRE parallels the preferential induction of VDR-RXR heterodimer formation on the same VDRE (see Fig. 2).

The role of the AF-2 domain in stabilizing the high-affinity ligand binding conformation of the VDR in DNA-bound VDR-RXR heterodimers was investigated by comparing VDR_{wt} in a combined gel-shift/gel-shift clipping assay on both a DR3-type and an IP9-type VDREs with $VDR_{K413STOP}$ (Fig. 8). The basal binding levels (i.e., the DNA-binding ability of nonliganded, digested, or nondigested VDR-RXR heterodimers) were found to be comparable for both VDR_{wt} and $VDR_{K413STOP}$. High VD concentrations (10 μM) provided both VDR forms with a similar enhancement of VDR-RXR complex formation on DNA. Lower VD concentrations (1 nM), which are known to provide the same effect on the stimulation of complex formation of VDR_{wt} (Fig. 2C), were far less

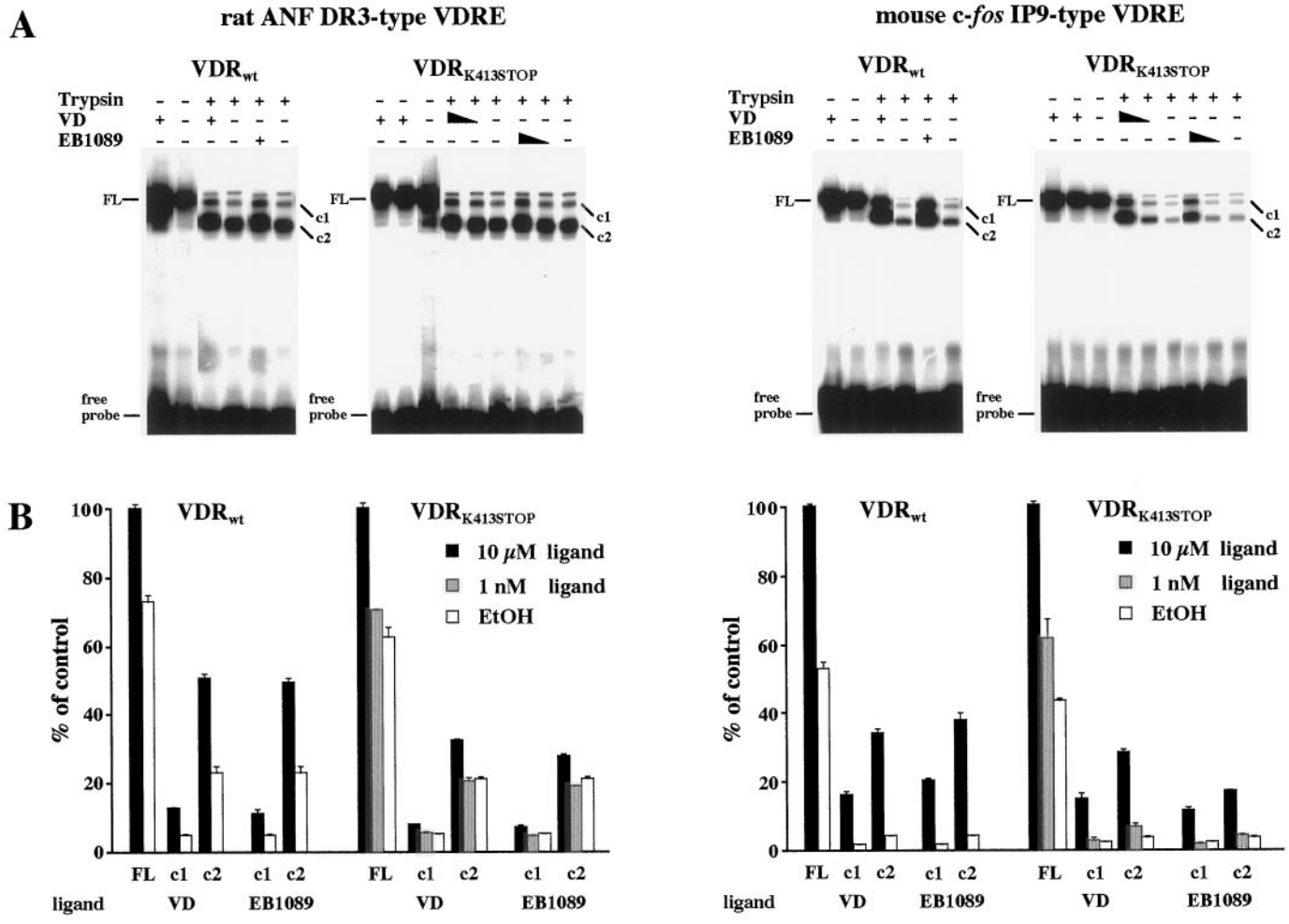

Fig. 8. The role of the VDR AF-2 domain in VDR-RXR heterodimer-VDRE complex stabilization. Gel-shift clipping experiments were performed by forming heterodimers between in vitro translated VDR_{wt} or $VDR_{K413STOP}$ and in vitro translated RXR in the presence of the indicated concentrations of ligands (or ethanol as control) either on the ^{32}P-labeled DR3-type VDRE or the IP9-type VDRE. Trypsin was then added to a final concentration of 66 ng/μl and incubation was continued for 10 min. A, protein-DNA complexes were separated from free probe on an 8% nondenaturing gel. Representative experiments are shown. B, the amounts of digested VDR-RXR heterodimer-VDRE complexes (c1 and c2) were quantified on a BioImager in relation to the respective ligand-induced, nondigested VDR-RXR heterodimer (FL). Each column represents the average of triplicates and bars indicate S.D.

effective with $VDR_{K413STOP}$. In contrast with $VDR_{K413STOP}$, the ligand stabilization of the digested VDR-RXR complexes c1 and c2 was found to be reduced even at high concentrations of VD and EB1089 (10 μM) and almost abolished at a lower concentration (1 nM). Taken together, the gel-shift clipping assay once again displayed higher sensitivity in the detection of ligand-activated effects on protein-DNA complexes than the gel-shift assay. Moreover, the critical role of the AF-2 domain on ligand effects at low concentrations was confirmed by both methods.

Discussion

Nuclear receptors are the central components of the complex signaling processes mediated by nuclear hormones. The specific interaction of a nuclear receptor, such as the VDR, with response elements of promoter regions determines which genes will be regulated by the respective nuclear hormone (i.e., in the case of VDR, which genes are primary VD-responding genes). The complex formation between VDR-RXR heterodimers and their specific VDREs is therefore a central molecular step in the specific activation of VD-responding genes. Investigation of the selective modulation of this complex formation on different types of VDREs by VDR ligands was therefore the subject of this study.

The selective biological profile of EB1089 [i.e., its potent antiproliferative potential combined with a reduced calcemic effect (Nayeri et al., 1995; Danielsson et al., 1997)] was associated with the higher selectivity (approximately 15-fold) of the analogs to activate IP9-type VDREs than DR3-type VDREs (Nayeri et al., 1995). It was hypothesized that primary VD-responding genes that are involved in mediating growth arrest should preferentially contain IP9-VDREs in their promoter region (Carlberg, 1996a,b). Because of the relatively low number of known primary VD-responding genes with characterized VDREs, this idea has not yet been statistically proven; interestingly, however, the genes of mouse c-*fos* and human and mouse $p21^{WAF1/CIP1}$ each contain a VDRE in their regulatory regions (Carlberg, 1997). This suggests that the latter genes should be activated selectively by EB1089, which is the analog with the highest preference for the activation of IP9-type VDREs within a group of approximately 30 analogs that have presently been analyzed for promoter selectivity (Carlberg and Polly, 1998; Quack et al., 1998a,b). Moreover, promoter selectivity seems to be closely linked to the exact structure of EB1089, because metabolites and close structural relatives of the analog were found to have lost this property almost entirely (Quack et al., 1998a,b).

In this study, a VDRE-selective in vitro stabilization of VDR-RXR heterodimers was demonstrated for the first time. Ligand-dependent gel-shift assays showed that EB1089 mediated the stabilization of VDR-RXR heterodimers on IP9-VDREs at approximately 8-fold lower concentrations than on DR3-type VDREs. In contrast, the natural hormone VD showed no significant selectivity. Interestingly, the EB1089-induced more highly sensitive complex formation of VDR-RXR heterodimers (observed in gel-shift assays) on an IP9-type VDRE does not seem to be based on an increased DNA-binding affinity. Gel-shift clipping assays demonstrated similar results and allowed for the differentiation between two ligand-stabilized complexes of DNA-bound VDR-RXR heterodimers. On an IP9-type VDRE, VDR-RXR complex 1 was found to be stabilized by EB1089 at approximately 9-fold lower concentrations than on a DR3-type VDRE. This selectivity could not be observed with the second truncated VDR-RXR heterodimer complex c1 or with VD as ligand. In contrast to gel-shift assays, DNA competition in the context of gel-shift clipping assays showed that EB1089-stabilized VDR-RXR heterodimers have an affinity for IP9-type VDREs that is approximately 7 times higher than VD-stabilized VDR-RXR heterodimers. The truncated VDR-RXR complexes that were obtained in the gel-shift clipping assay represent a subset of all DNA-bound VDR-RXR heterodimers observed in the gel-shift assay. VDR-RXR heterodimer complex 1 seems to be the most critical subset; it demonstrates that effects of EB1089 are not only mediated by an increased sensitivity, but also through an increased affinity for IP9-type VDREs. Moreover, the analysis of the functional role of the AF-2 domain of the VDR by gel-shift clipping assays suggests that complex c1 represents a subset of VDR-RXR heterodimers that shows a profile similar to that of the high-affinity ligand-binding conformation 1 of VDR monomers. This finding has already been characterized by limited protease digestion (Nayeri et al., 1996a; Nayeri and Carlberg, 1997).

Taken together, the selective activation of IP9-type VDREs by EB1089 (i.e., the observation of promoter selectivity of a VD analog) was found to be based on the enhanced DNA-binding affinity of a subset of all VDR-RXR heterodimers that are selectively stabilized by EB1089 at concentrations as low as 30 pM. This suggests that promoter selectivity is based on the stabilization of VDR-RXR heterodimers in a high-affinity ligand-binding conformation that involves the AF-2 domain of the VDR. These findings link the interaction of VD and its analogs with functional conformations of monomeric VDR in solution, that were obtained by limited protease digestion assays, with the conformations of DNA-bound VDR-RXR heterodimers. This provides further insight into the mechanisms of nuclear VD signaling, thus allowing a parallel, detailed analysis of the molecular action of biologically potent VD analogs.

In summary, the gel-shift clipping assay was developed in this study as a novel, very potent method for the analysis of ligand-stabilized, functional conformations of the VDR. The ligand-dependent gel-shift assay was also demonstrated to be a powerful method. In the latter assay, the complete protein-DNA complex is quantified, not only digestion products, which makes its interpretation easier. However, both assay systems allow not only the analysis of functional effects of VD and its synthetic analogs but can also be applied for the characterization of VDREs. Moreover, both methods have the potential to be used with other nuclear receptors and maybe also with other regulators of conditional gene expression.

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