Journal of Cellular Biochemistry 61:325–337 (1996)

ARTICLES

High Activity, Soluble, Bacterially Expressed Human Vitamin D Receptor and Its Ligand Binding Domain

David G. Mottershead, Patsie Polly, Ruth J. Lyons, Robert L. Sutherland, and Colin K.W. Watts

CRC for Biopharmaceutical Research (D.G.M., R.J.L., R.L.S., C.K.W.W.), Cancer Biology Division (D.G.M., R.J.L., R.L.S., C.K.W.W.), and Bone and Mineral Research Division (P.P.), Garvan Institute of Medical Research, St. Vincent's Hospital, Darlinghurst, Sydney, N.S.W. 2010 Australia

Abstract The effects of 1α,25(OH)$_2$vitamin D$_3$ on cell growth and differentiation are primarily mediated by the nuclear vitamin D receptor (VDR). In order to study aspects of receptor function and ultimately the structural basis of the VDR–ligand interaction, it is necessary to produce large quantities of purified VDR. To achieve this, we have expressed the human VDR and its ligand binding domain in *E. coli* as fusion proteins with the maltose binding protein using the expression vector pMal-c2. In this system high level expression of both fusion proteins in a soluble form was achieved, whereas previous attempts to express the VDR in *E. coli* have resulted in an insoluble product. After affinity purification on amylose resin, the fusion proteins were isolated with yields of 10–20 mg/l of culture. Both forms of the recombinant receptor bound 1α,25(OH)$_2$vitamin D$_3$ with high affinity; estimated K_d values from Scatchard analysis for the purified full-length receptor and the ligand binding domain were 0.16 ± 0.07 nM and 0.04 ± 0.02 nM, respectively. The nonhypercalcemic analogs of vitamin D, MC903 and Δ22-1,25*S*,26(OH)$_3$vitamin D$_3$, bound the recombinant fusion proteins with a similar affinity to the native ligand, 1α,25(OH)$_2$vitamin D$_3$. In addition, the full-length VDR fusion protein was shown by gel shift analysis to bind weakly to the human osteocalcin gene vitamin D response element, an interaction greatly facilitated by addition of RXRα. These results show that the bacterial expression system detailed here is readily able to produce soluble and functional VDR and its ligand binding domain in high yield. These proteins are easily purified and should be suitable for further structural and functional analysis.

Key words: vitamin D receptor, 1α,25(OH)$_2$vitamin D$_3$, pMal, ligand binding, gel shift analysis, VDRE, osteocalcin gene promoter, fibronectin gene promoter

The hormonal metabolite of vitamin D, 1α,25(OH)$_2$vitamin D$_3$ (1,25D$_3$), is active in numerous cell types and exhibits a wide range of biological effects, including those involved in calcium homeostasis and the regulation of cell growth and differentiation [Walters, 1992; Studzinski et al., 1993; Eisman, 1994; Bouillon et al., 1995]. These effects are mediated primarily by interaction of 1,25D$_3$ with the nuclear vitamin D receptor (VDR), a member of the steroid–thyroid hormone receptor superfamily of ligand-inducible transcription factors [MacDonald et al., 1994]. The binding of the VDR to its specific DNA response elements (VDREs) to initiate specific gene transcription is a process involving protein–ligand, protein–protein, and protein–DNA interactions [Breen et al., 1994; Kuiper and Brinkmann, 1994]. Protein–protein interactions include formation of heterodimers with retinoid receptors (RXR and RAR) and thyroid hormone receptors, or homodimer formation between VDR monomers [Glass, 1994; Schrader et al., 1994a,b]. The receptor–ligand interaction appears to influence the equilibrium between the formation of homodimers and heterodimers [Cheskis and Freedman, 1994], which differ in their response element selectivity [Freedman et al., 1994; Nishikawa et al., 1994].

In common with other members of the hormone receptor superfamily, VDR function is mediated by discrete regions within the receptor's subunit or domain structure. Sequence comparison to other family members and mutagenesis studies have provided some definition of the VDR regions involved in ligand binding, DNA binding, receptor dimerization, and gene transactivation [MacDonald et al., 1994]. Full

Received September 15, 1995; accepted October 26, 1995.

Address reprint requests to Colin K.W. Watts, Garvan Institute of Medical Research, St. Vincent's Hospital, Darlinghurst, Sydney, N.S.W. 2010 Australia.

structural definition of these regions is likely to be achieved by X-ray crystallographic analysis. Although DNA binding domain structures of the glucocorticoid receptor, estrogen receptor, retinoic acid receptor (RAR) β, and retinoid X receptor (RXR) α [Hard et al., 1990; Luisi et al., 1991; Katahira et al., 1992; Lee et al., 1993, 1994; Schwabe et al., 1993] and the ligand binding domain of the RXRα [Bourguet et al., 1995] have been studied in this way or by NMR, no such information is yet available for the VDR.

Ligand binding domain structural information would be particularly useful for rational drug design. VDR ligands, including $1,25D_3$, have potential applications in a number of areas, notably cancer therapy, inhibiting cell proliferation and causing the expression of a more differentiated phenotype in malignant cells of various types [Eisman, 1994]. However, a major problem with the use of $1,25D_3$ is the associated development of hypercalcemia. This has led to the development of nonhypercalcemic analogs of $1,25D_3$ [Bikle, 1992; Jones and Calverley, 1993; Pols et al., 1994; Bouillon et al., 1995]. To develop further such analogs for these or other specialized functions, an understanding of the structural basis for the interaction between $1,25D_3$ or various analogs and the VDR is required, ultimately by X-ray crystallographic analysis of the receptor–ligand complex. This will require a plentiful supply of receptor in an active hormone binding state. Previously, the VDR has been produced in various expression systems, including baculovirus [MacDonald et al., 1991; Ross et al., 1991], yeast [Sone et al., 1990], and adenovirus [Smith et al., 1991]. For ease of production bacterial expression is preferable, but to date the VDR has been insoluble when expressed in *E. coli,* requiring the use of denaturing conditions for solubilization and subsequent purification [Kumar et al., 1992; Nakajima et al., 1993; Towers et al., 1993; Cheskis et al., 1994; Elaroussi et al., 1994]. We report here the production of the VDR receptor and its ligand binding domain in *E. coli* as soluble fusion proteins with the maltose binding protein (MBP). The fusion proteins are expressed at high level, can be readily purified from the soluble cell extract, bind ligands with high affinity and specificity, and, in the presence of RXRα, recognize a synthetic DNA sequence corresponding to the osteocalcin gene VDRE DNA sequence.

MATERIALS AND METHODS

Materials

$1,25D_3$ and the nonhypercalcemic analog, Δ22-$1,25S,26(OH)_3$vitamin D_3, were generously supplied by Dr. M. Uskokovic (Hoffmann La Roche, Nutley, NJ). Calcipotriol (MC903) was kindly supplied by Dr. L. Binderup (Leo Pharmaceuticals, Ballerup, Denmark). $1\alpha,25(OH)_2$[26,27-^{3}H]vitamin D_3 ($1,25[^3H]D_3$, 10 μCi/ml, 180 Ci/mmol) was from Amersham International (Buckinghamshire, UK). The anti–vitamin D receptor monoclonal antibody (9A7) was from Affinity BioReagents (Neshanic Station, NJ) and the anti-maltose binding protein antibody was from New England Biolabs (Beverly, MA).

Construction of Plasmids

The pMal-c2 expression vector (New England Biolabs, Beverly, MA) was used to express the VDR and its ligand binding domain (VDRlbd) as fusion proteins with the MBP [Maina et al., 1988]. The full-length human VDR cDNA was excised from pSP65 VDR [Baker et al., 1988] by digestion with *Eco*RI and ligated into the *Eco*RI site of pMal-c2, generating the plasmid pMalVDRfl. The VDRlbd was amplified by polymerase chain reaction using the template VDR cDNA, incorporating 5′ *Bam*HI and *Bgl*II sites and a 3′ *Bam*HI site (5′ primer, CGCGGATCCAGATCTAAGGAGTTCATTCTGA, 3′ primer, CCGGATCCTCATAGTCAGGAGATCT) in the fragment encoding VDR amino acids 91–427. This fragment was ligated into *Bam*HI-digested pMal-c2, generating the plasmid pMalVDRlbd. The correct orientation of the VDR and VDRlbd fragments was confirmed by restriction endonuclease digestion.

Production of Fusion Proteins

The expression vectors pMalVDRfl and pMalVDRlbd were transformed into *E. coli* strain BL21(DE3)plysS (26). For production of the fusion proteins, an overnight culture was inoculated into LB medium supplemented with ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml), and grown to an absorbance at 600 nm of 0.4–0.5. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.05 mM. Cells were harvested after 3 hr by centrifugation and stored at −70°C. The cell pellet was thawed on ice, resuspended in 0.1 volumes of lysis buffer (50 mM Tris · HCl, pH

7.4, 1.5 mM EDTA, 5 mM DTT, 50 mM NaCl, 10% glycerol, 0.5 mM PMSF), and disrupted by sonication. The lysate was centrifuged at 35,000 *g* for 30 min and stored at −70°C. The production of the fusion proteins was confirmed by Coomassie brilliant blue staining after SDS-PAGE of the cell lysate.

Affinity Purification of MBP-VDRfl and MBP-VDRlbd

The MBP-VDR fusion proteins were purified by affinity chromatography on amylose resin (New England Biolabs, Beverly, MA). In brief, soluble extract was diluted fivefold with column buffer (50 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, 5 mM DTT, 500 mM NaCl, 10% glycerol, 0.5 mM PMSF, 0.25% Tween 20) and allowed to mix with amylose resin overnight at 4°C. After washing with column buffer to remove unbound proteins, the amylose resin was resuspended in elution buffer (lysis buffer plus 10 mM maltose) and mixed for 2–3 hr at 4°C. The purified fusion proteins were quantitated by Bradford assay or absorbance at 280 nm, aliquoted, and stored at −70°C.

Ligand Binding Assays

1,25[^{3}H]D_3 was dried under vacuum and dissolved in binding buffer (50 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, 5 mM DTT, 50 mM NaCl, 10% glycerol, 0.5 mM PMSF, 0.1% BSA). Bacterial extracts or purified receptor preparations were incubated with 1,25[^{3}H]D_3 for 18–24 hr at 4°C. Bound 1,25[^{3}H]D_3 was separated from free ligand by charcoal absorption. Nonspecific binding was defined as binding of ligand in the presence of a 200-fold molar excess of unlabeled ligand. For Scatchard analysis, correction for nonspecific binding was applied using the method of Chamness and McGuire [Chamness and McGuire, 1976], and the dissociation constant (K_d) determined by the graphics program CA-Cricket Graph III. Competitive binding assays were carried out using a concentration of 1,25[^{3}H]D_3 of 0.3 nM, with increasing concentrations of unlabeled competing ligands, with bound and free ligand being separated as for Scatchard analysis. Results for K_d and IC_{50} are presented as mean ± SEM, n = 3.

Gel Shift Analysis

Oligonucleotides were synthesized to represent the human osteocalcin gene VDRE and surrounding DNA sequences 5′GCTGCCTTTG-GTGACTCACCGGGTGAAC-GGGGGCATT-GCGAGGC3′ (nt −457/−521) [Morrison and Eisman, 1993], and a 65 bp fragment of the murine fibronectin gene promoter 5′-ATCAG-CATCTCTTTTGTTCGCGGCGAACCCACC-GTACCCCGTGACGTCACCCGGACTC-TGGG-3′ (nt −206/−145) [Polly and Nicholson, 1993]. Complementary oligonucleotides were annealed and labeled with ^{32}P-dCTP to 10^8 cpm/μg by Klenow fill-in, and gel purified through a 15% acrylamide gel. Probes (30,000 cpm/reaction) were incubated with recombinant VDR fusion proteins ± purified RXRα-GST fusion protein [Manglesdorf et al., 1991] or MBP-RARα (Shen et al., manuscript in preparation) in 10 mM Tris · HCl/HEPES, pH 7.9, 50 mM KCl, 0.5 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT, and 6 μg poly dI-dC for 20 min at room temperature [Sagami et al., 1986]. Antibody competition was performed with a monoclonal rat anti-chicken VDR antibody that recognizes the human VDR (9A7, Affinity Bioreagents, Neshanic Station, NJ). VDR and RXRα proteins were preincubated with antibody (1–4 μg) for 15 min at room temperature, prior to addition of probe. Gel pre-electrophoresis was performed for 15 min at 4°C. The reaction mixtures were electrophoresed at 35 mA through a 5% nondenaturing polyacrylamide gel containing 1.6% glycerol prepared in 0.5× TBE buffer (89 mM Tris base, 89 mM boric acid, 4 mM EDTA, pH 7.4) [Garner and Revzin, 1981; Baldwin, 1990]. Gels were dried under vacuum at 80°C and autoradiographed overnight with intensifying screens at −70°C.

RESULTS

Expression of the Full-Length Vitamin D Receptor and Its Ligand Binding Domain

The full-length human vitamin D receptor and the ligand binding domain, including the receptor hinge region [MacDonald et al., 1994], were produced as fusion proteins with MBP by insertion of the respective cDNAs into the pMal-c2 bacterial expression vector [Maina et al., 1988]. This vector utilizes the IPTG-inducible P_{tac} promoter and results in cytoplasmic expression of the recombinant fusion proteins. Bacterial cultures transformed with the plasmids pMalVDRfl and pMalVDRlbd were induced with IPTG at 37°C and incubated at this temperature for 3 hr to enable accumulation of the induced fusion protein. Samples from the in-

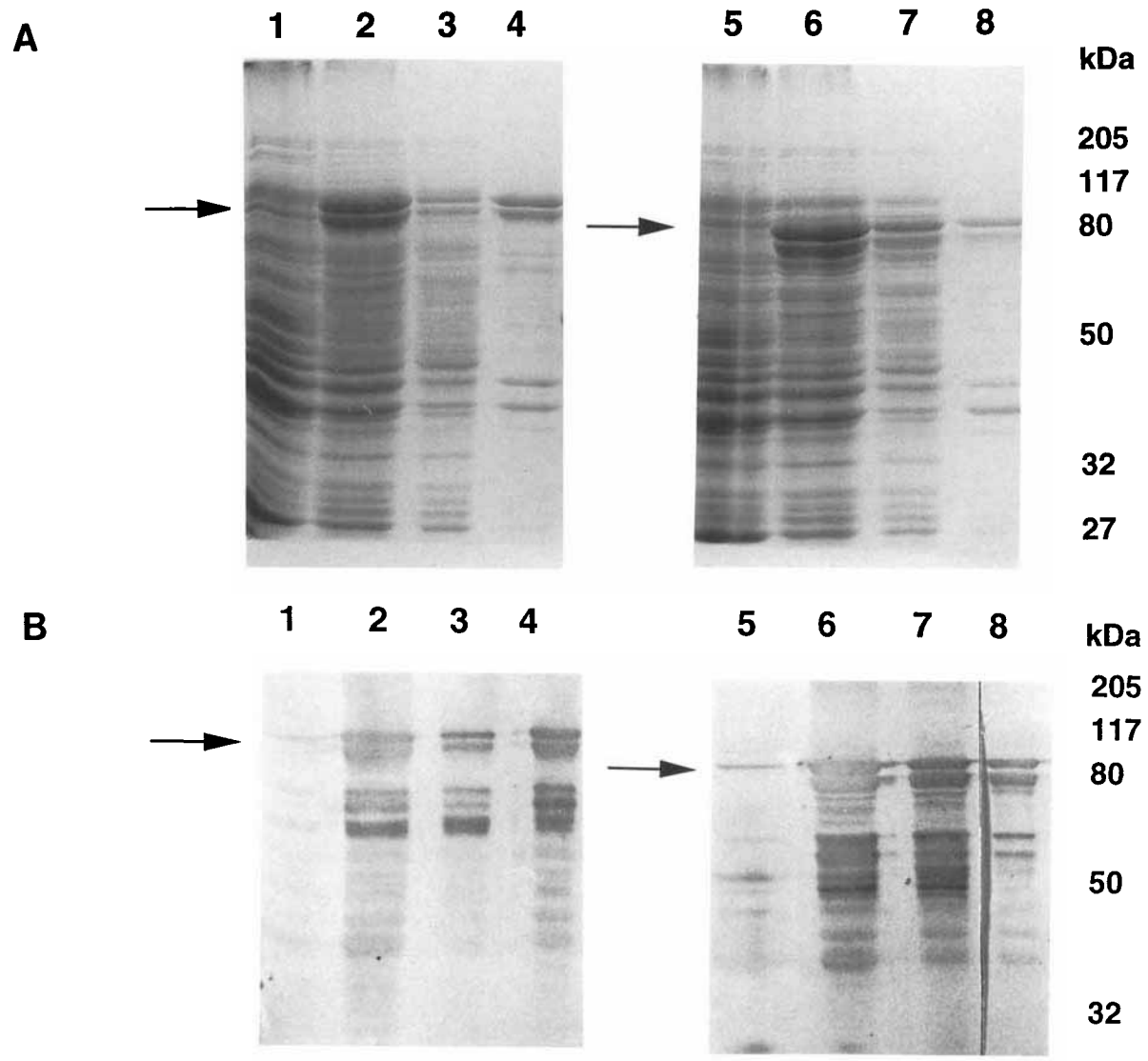

Fig. 1. Expression of MBP-VDRfl and MBP-VDRlbd fusion proteins. **A:** Coomassie blue–stained SDS-PAGE gel. **B:** Western blot of samples prepared from bacteria induced at 37°C. *Lanes 1–4:* Samples from pMalVDRfl induction. *Lanes 5–8:* Samples from pMalVDRlbd induction. Lanes 1 and 5: uninduced total cell extract. Lanes 2 and 6: Induced total cell extract. Lanes 3 and 7: soluble cell extract. Lanes 4 and 8: Insoluble cell extract. *Arrows* identify the MBP-VDRfl and MBP-VDRlbd fusion proteins. The Western blot was probed with an anti-VDR monoclonal antibody (9A7).

duced cells analyzed by 10% SDS-PAGE (Fig. 1A) indicated high level production of fusion proteins, evidenced by new protein products at 80–90 kDa (Fig. 1A, lane 2) and 70–80 kDa (Fig. 1A, lane 6) representing the MBP-VDRfl and MBP-VDRlbd products, respectively. The larger molecular weight product in the case of both fusion proteins corresponds to the correct molecular weight for the complete VDR (48 kDa) or the VDRlbd (40 kDa) fused to the MBP (42 kDa). All induced protein products visible by Coomassie blue staining were immunoreactive with a monoclonal antibody specific for the VDR (Fig. 1B). Lower molecular weight immunoreactive species produced upon IPTG induction of pMalVDRfl and pMalVDRlbd possibly result from in vivo proteolysis or premature termination of transcription or translation. Comparison of soluble and insoluble induced cell extracts by SDS-PAGE (Fig. 1A) and Western analysis (Fig. 1B) indicate that the MBP-VDRlbd protein is more soluble than the MBP-VDRfl protein when produced at 37°C. To maximize the level of soluble protein, the effect of temperature and

TABLE I. Optimization of pMalVDRfl Induction Conditions*

Induction conditions	Specific binding (pmoles 1,25[^{3}H]D $_3$)
37°C/0.4 mM IPTG	14
30°C/0.4 mM IPTG	60
30°C/0.1 mM IPTG	74
30°C/0.05 mM IPTG	86

*Bacteria transformed with pMalVDRfl were induced with varying concentrations of IPTG at either 30°C or 37°C. Expression levels of soluble VDRfl were obtained by determining the specific binding of 1,25[^{3}H]D_3 in 0.5 μl of bacterial extract.

IPTG concentration on the level of production of MBP-VDRfl was investigated. Binding of 1,25[^{3}H]D_3 to induced bacterial cell extracts was used to monitor the level of soluble protein. The results (Table I) indicate that a temperature of 30°C and an IPTG concentration of 0.05 mM are optimal, with temperature being a more important factor than IPTG concentration in determining the level of soluble protein. A further decrease in either of these induction parameters gave no further increase in specific 1,25D_3 binding (data not shown). These optimal conditions were used for the production of recombinant VDR in all subsequent experiments.

Affinity Purification of MBP-VDRfl and MBP-VDRlbd Fusion Proteins

The recombinant fusion proteins MBP-VDRfl and MBP-VDRlbd were purified from bacterial cell extracts by amylose affinity chromatography. Soluble cell extracts were incubated with amylose affinity resin overnight and, after washing to remove nonspecifically bound proteins, the MBP fusion proteins were eluted by competition with 10 mM maltose. The eluted proteins were analyzed by SDS-PAGE and Western blot (Fig. 2). The purified MBP-VDRfl product migrated as a single band of 90 kDa and a doublet of 80–85 kDa. Similarly, the purified MBP-VDRlbd product migrated as a single band of 90 kDa and a doublet of 70–75 kDa. All these products were immunoreactive, both to a polyclonal antibody specific for the MBP and to a monoclonal antibody specific for the VDR (Fig. 2B). Between 1 and 2 mg of protein was recovered from 1 ml of amylose resin after incubating the resin with the equivalent of 10 ml of induced bacterial cell extract. This corresponds to a level of production of soluble fusion protein of 10–20 mg/l of induced bacterial culture.

Ligand Binding Analysis

Saturation binding analysis was conducted with 1,25[^{3}H]D_3 on soluble extracts from induced bacteria and compared with 1,25[^{3}H]D_3 binding to the purified MBP-VDRfl and MBP-VDRlbd proteins (Fig. 3). Both the full-length receptor and the ligand binding domain fusion proteins exhibited specific, high affinity, saturable binding of 1,25[^{3}H]D_3. The purified receptors exhibited a similar binding affinity to the unpurified receptors in the soluble cell extract, with the K_ds for the cell extract and purified MBP-VDRfl protein being 0.08 ± 0.01 nM and 0.16 ± 0.07 nM, respectively, and the K_ds for the cell extract and purified MBP-VDRlbd being 0.02 ± 0.01 nM and 0.04 ± 0.02 nM, respectively. The addition of hRXRα, either as the purified protein or as the GST fusion protein in a bacterial cell extract, had no effect on the ligand binding affinity or capacity of the MBP-VDR fusion proteins (data not shown).

Competition by Nonhypercalcemic Vitamin D Analogs

The ligand binding properties of the purified MBP-VDRfl and MBP-VDRlbd proteins were further analyzed by competitive displacement experiments with nonhypercalcemic analogs of vitamin D (Fig. 4). The nonhypercalcemic analog MC903 competed as efficiently for VDR binding as the natural ligand 1,25D_3, both in the case of the full-length fusion protein MBP-VDRfl and the ligand binding domain MBP-VDRlbd. The IC_{50} values for 1,25D_3 and MC903 binding to MBP-VDRfl were 1.4 ± 0.4 nM and 0.8 ± 0.2 nM, respectively. The IC_{50} values for binding to MBP-VDRlbd for 1,25D_3 and MC903 were 0.7 ± 0.2 nM and 0.5 ± 0.03 nM, respectively. The trihydroxylated derivative, Δ22-1,25*S*,26(OH)$_3$ vitamin D_3, demonstrated a similar ability to compete for binding to the full-length VDR as 1,25D_3, with an IC_{50} value of 2.1 nM (n = 2). A slight decrease in efficiency of binding of the Δ22-1,25*S*,26(OH)$_3$ vitamin D_3 analog was detected when binding to the MBP-VDRlbd protein, an IC_{50} value of 2.7 nM (n = 2) being measured.

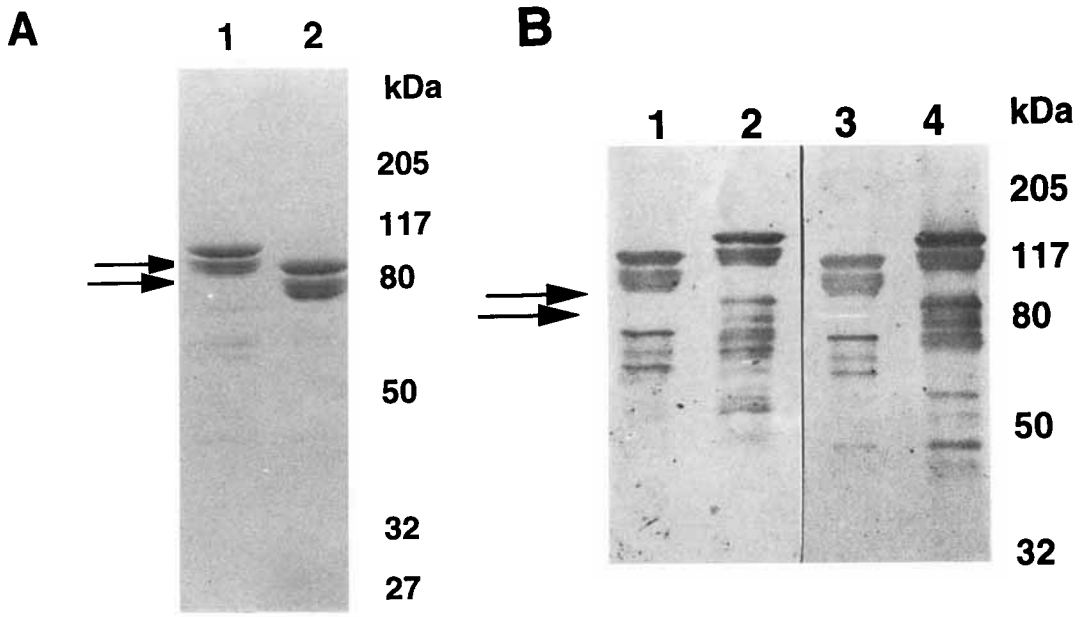

Fig. 2. Affinity-purified MBP-VDRfl and MBP-VDRlbd. **A:** Coomassie blue–stained SDS-PAGE gel of MBP-VDRfl (*lane 1*) and MBP-VDRlbd (*lane 2*) purified by affinity chromatography on amylose resin. **B:** Western blot of purified MBP-VDRfl (*lanes 2* and *4*) and MBP-VDRlbd (*lanes 1* and *3*). Lanes 1 and 2 were probed with a polyclonal anti-MBP antibody and lanes 3 and 4 were probed with the anti-VDR monoclonal antibody 9A7. Arrows identify the MBP-VDRfl and MBP-VDRlbd full-length products.

Binding of Recombinant VDR to a Synthetic VDRE

Gel shift analysis of the binding of MBP-VDRfl to a ^{32}P-dCTP-labeled double-stranded oligonucleotide, corresponding to VDRE sequences of the human osteocalcin gene promoter, showed the formation of a protein–DNA complex (complex *a*) using either bacterial lysate containing MBP-VDRfl (Fig. 5, lane 4) or amylose affinity–purified MBP-VDRfl (Fig. 5, lane 8) in the presence of purified RXRα-GST fusion protein. Complexes in approximately the same position were also present with VDRfl alone or in the presence of MBP-RARα, but could be seen only after prolonged exposure and thus are not apparent in Figure 5 (lanes 2, 3, 6, 7). Two other major complexes were formed (complexes *b*, *c*), but these are likely to be nonspecific protein–DNA complexes as they were present in all lanes where protein was present including lanes containing the MBP-VDRlbd, which does not have receptor sequences responsible for specific DNA binding (lanes 5, 9). The identity of the complex in lane 8, presumed to represent the VDRfl-RXRα heterodimer, was confirmed with an anti-VDR antibody, which at 4 μg/reaction was able to inhibit complex formation (Fig. 6, lane 6, complex *a*). Other, nonspecific, complexes were unaffected. Only these nonspecific complexes were observed in gel shifts with RXRα-GST alone (not shown).

In contrast, MBP-VDRfl showed significant levels of binding to an oligonucleotide corresponding to VDRE sequences of the murine fibronectin gene promoter, even in the absence of RXRα (Fig. 7, lane 2, complex *a*). Addition of RXRα-GST (lane 5) had little effect on the intensity of this complex in comparison to its effect on the binding of VDR to the osteocalcin sequence. MBP-RARα (lane 6) was also unable to promote VDR binding. The presence of VDR in complex *a* was confirmed by competition with 9A7 antibody (not shown). These results are consistent with preferential binding of the VDR homodimer by the fibronectin promoter VDRE sequence.

DISCUSSION

In this report we describe the high level expression of the full-length human vitamin D receptor and its ligand binding domain as soluble fusion proteins with maltose binding protein in *E. coli*. The presence of the MBP in the recombinant products enabled a simple and efficient purification of the fusion proteins by binding to

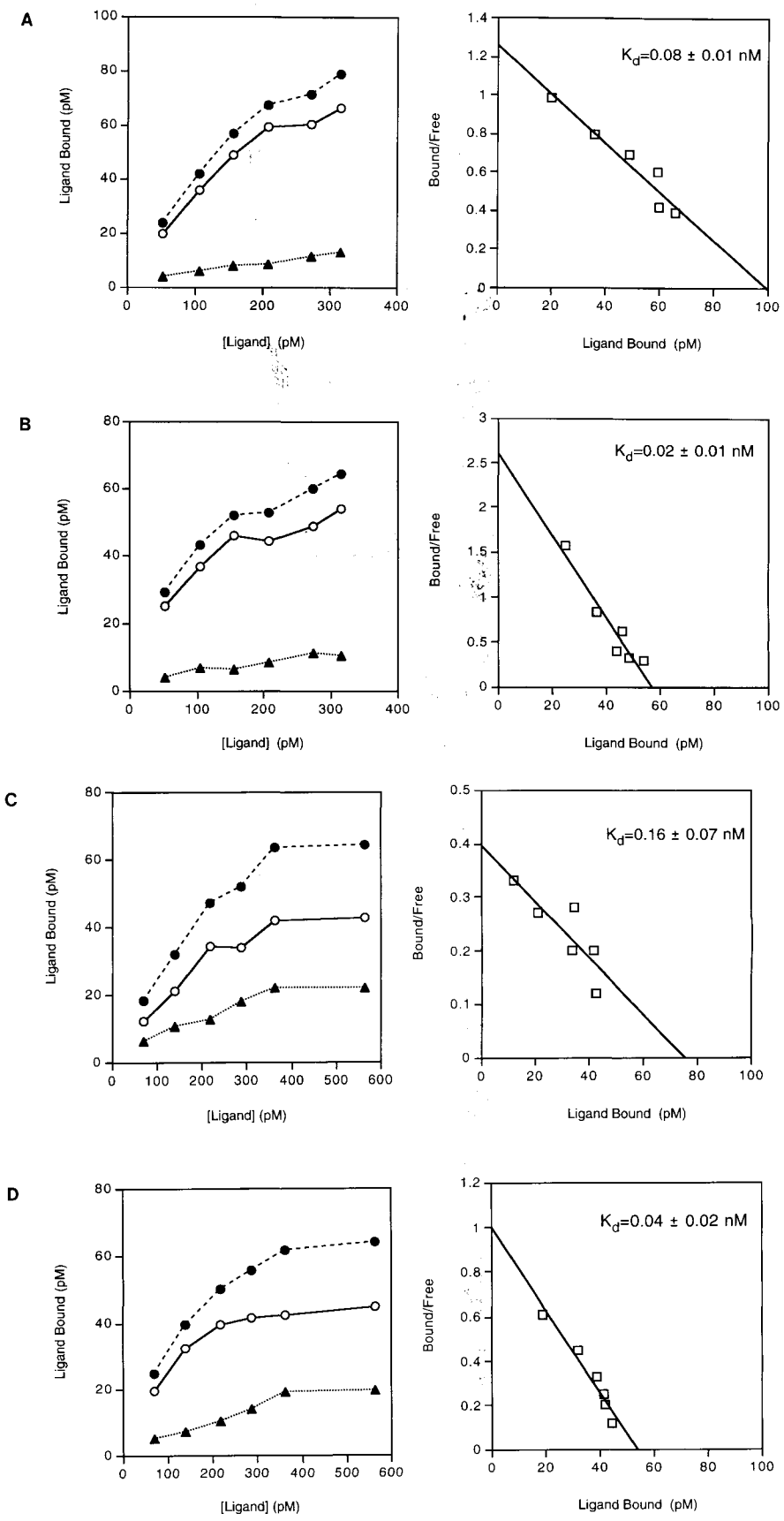

Fig. 3. Saturation binding curves (*left*) and Scatchard plots (*right*) of 1,25[^{3}H]D_3 binding. **A** and **B:** Soluble extracts of MBP-VDRfl and MBP-VDRlbd, respectively. The figure shows a representative experiment; the K_d ± SEM was calculated from three independent experiments. **C** and **D:** Affinity-purified MBP-VDRfl and MBP-VDRlbd, respectively. The figure shows a representative experiment; the K_d ± SEM was calculated from four independent experiments. Total 1,25[^{3}H]D_3 binding activity (●), nonspecific binding activity (▲), and calculated 1,25[^{3}H]D_3 specific binding activity (○) are represented.

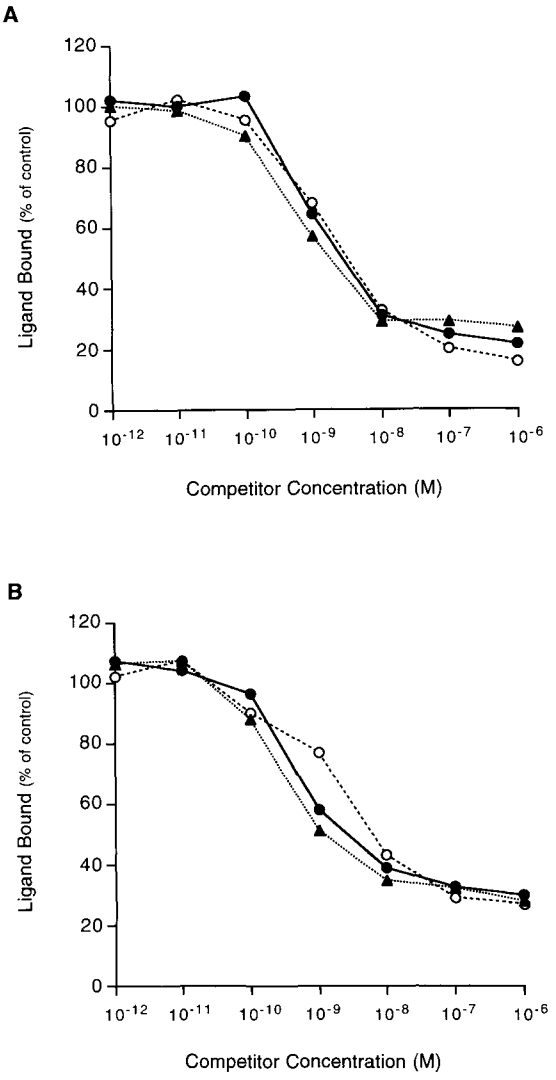

Fig. 4. Competition displacement curves of 1,25[^{3}H]D_3 by nonhypercalcemic vitamin D analogs. **A:** Affinity-purified MBP-VDRfl. **B:** Affinity-purified MBP-VDRlbd. Representative displacement curves for 1,25D_3 (●), and the analogs MC 903 (▲) and Δ22-1,25*S*,26$(OH)_3$ vitamin D_3 (○) are shown.

amylose resin. Produced in this manner, the VDR and its ligand binding domain can be purified under native conditions with yields of 10–20 mg/l of bacterial culture. Previous protocols for the production of the VDR from various species in *E. coli* have all necessitated the use of denaturants or detergents to solubilize the receptor prior to purification [Kumar et al., 1992; Nakajima et al., 1993; Towers et al., 1993; Cheskis et al., 1994; Elaroussi et al., 1994]. Other successful approaches to overexpression of soluble VDR have included adenoviral [Smith et al., 1991], yeast [Sone et al., 1990], and baculoviral [MacDonald et al., 1991] expression systems, although yields were comparatively low. High level expression of soluble rat VDR has been achieved in a baculovirus system [Ross et al., 1991]. However, the bacterial expression system described here is considerably simpler in terms of production and purification of the receptor protein.

A fusion protein, approximately 10 kDa smaller, is also produced upon induction of either pMalVDRfl or pMalVDRlbd. These proteins are possibly carboxy-terminal truncated fusion proteins (perhaps resulting from proteolysis or premature termination of translation) as they react with both the MBP-specific antibody and the anti-VDR monoclonal antibody, the epitope of which is located at the amino-terminus of the ligand binding domain (amino acid residues 89–105) [McDonnell et al., 1989]. Furthermore, these fusion proteins copurify with the full-length products on the amylose resin. A similar result has been reported during the expression and purification of the RARβ receptor as a maltose binding protein fusion [Lombardo et al., 1994]. In the case of the VDR fusion proteins reported here, the presence of truncated products is unlikely to affect the analysis of ligand binding; e.g., by altering affinity, as removal of as little as 5 kDa of sequence from the carboxy-terminus of the VDR destroys ligand binding [Allegretto et al., 1987; McDonnell et al., 1989; Nakajima et al., 1994].

The MBP-VDRfl and MBP-VDRlbd fusion proteins exhibited specific, high affinity binding of 1,25D_3, with a K_d for the purified proteins of 0.16 ± 0.07 and 0.04 ± 0.02, respectively. The measured binding affinity for 1,25D_3 of both recombinant forms of the VDR did not significantly alter upon purification. Further, the ligand binding of the purified receptor preparation was stable over a period of at least 3 months at −70°C. The measured affinities for the purified MBP-VDRfl and MBP-VDRlbd proteins are comparable or higher than those for the VDR produced in other expression systems, which have exhibited a K_d ranging from 0.7 nM for baculoviral [MacDonald et al., 1991] and adenoviral VDR [Smith et al., 1991] to 0.2 nM for the VDR produced in yeast [Sone et al., 1990]. Native VDR extracted from tissue sources has yielded a receptor with a measured K_d of 0.01–0.2 nM [MacDonald et al., 1994], these higher affinities perhaps reflecting the influence of accessory proteins. In our study the K_d measured

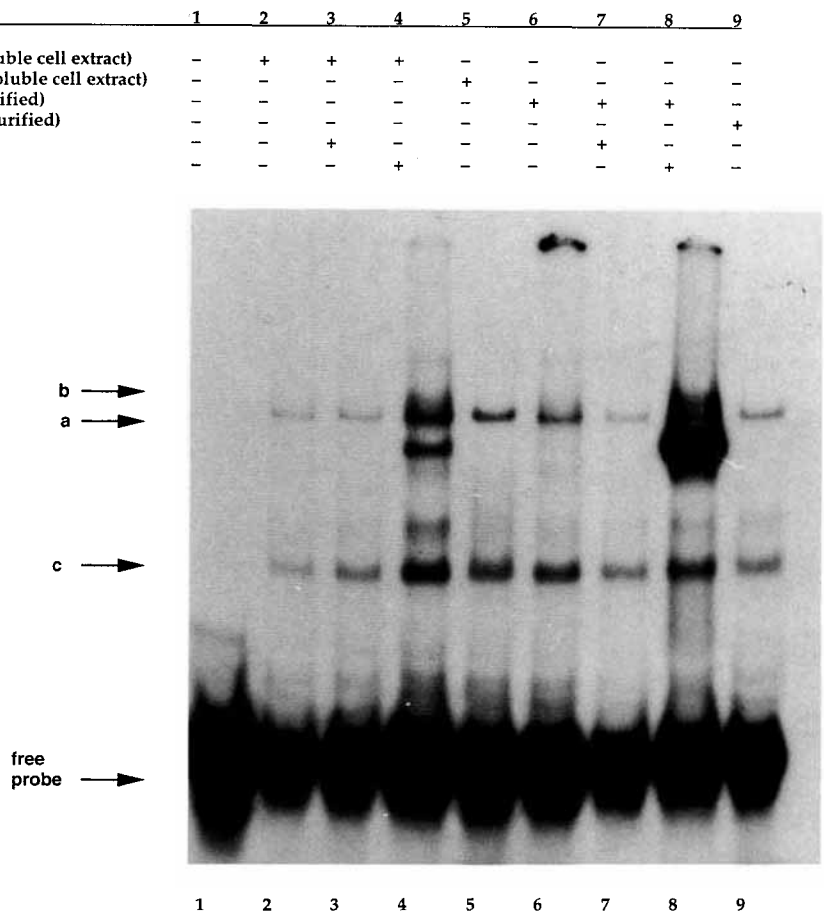

Fig. 5. Gel shift analysis of binding of MBP-VDRfl to a vitamin D receptor DNA response element. MBP-VDR fusion proteins were incubated with a radiolabeled oligonucleotide sequence corresponding to the VDRE of the human osteocalcin gene promoter ± recombinant MBP-RARα or RXR-GST fusion proteins, and the reactions subjected to nondenaturing 5% polyacrylamide gel electrophoresis. Complex *a* is the VDR-RXR heterodimer; complexes *b* and *c* are nonspecific.

for the ligand binding domain of the VDR has consistently indicated a 4–5-fold higher binding affinity for $1,25D_3$ compared to the full-length receptor. This implies that the conformation of the ligand binding domain is altered when the rest of the molecule is present. If this is the situation for the VDR, it does not seem to be so for the retinoic acid receptors (RARs), where we have found that the ligand binding domains exhibit a similar binding affinity for ligand as the full-length receptors (Shen et al., manuscript in preparation).

Previously, it has been reported that overexpressed VDR from bacterial and baculoviral sources does not exhibit saturable, high affinity binding of ligand, unless rat liver nuclear extract is included in the ligand binding assays [Nakajima et al., 1993]. It was suggested that a component of the rat liver nuclear extract, possibly RXRs, was necessary to enable the recombinant VDR to bind ligand with high affinity. Our results demonstrate saturable, high affinity $1,25D_3$ binding to an *E. coli*-expressed VDR without the addition of any additional factors, such as RXRs. Further, the addition of hRXRα, either as the purified protein or as the GST fusion protein in a bacterial cell extract, had no effect on ligand binding by the MBP-VDR fusion proteins. Possibly the recombinantly expressed VDR in these other systems is inherently unstable with regard to ligand binding activity because of incorrect folding, and proteins in nuclear extracts that interact with the VDR such as the RXRs are able to stabilize the protein. In the case of the MBP-VDR fusion proteins, the presence of the MBP may serve a similar function to

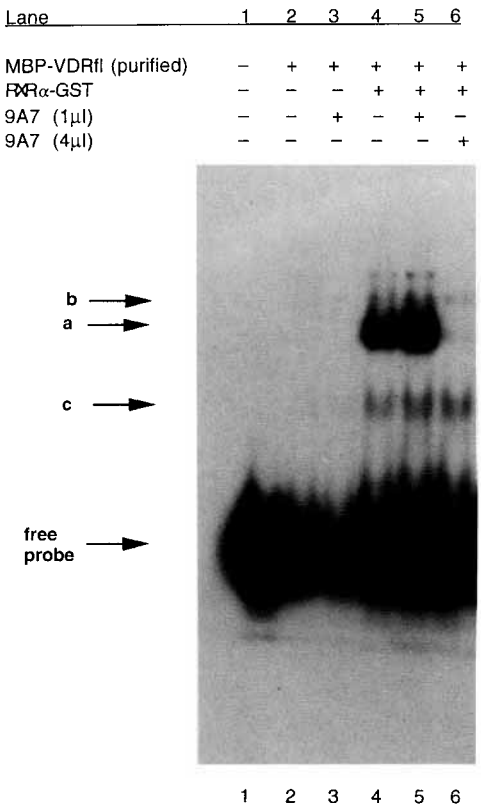

Fig. 6. Inhibition of binding of MBP-VDRfl to a vitamin D receptor DNA response element by an anti-VDR antibody. Purified MBP-VDRfl fusion protein was incubated with a radiolabeled oligonucleotide sequence corresponding to the VDRE of the human osteocalcin gene promoter. To promote formation of a specific DNA binding complex, purified RXRα-GST fusion protein was included in some lanes; the presence of VDR in this complex is shown by inclusion of the VDR monoclonal antibody 9A7 in the reaction mix, inhibiting complex formation. Reactions were subjected to nondenaturing 5% polyacrylamide gel electrophoresis. Complex *a* is the VDR-RXR heterodimer; complexes *b* and *c* are nonspecific.

stabilize the ligand binding properties of the VDR.

Competition displacement studies with nonhypercalcemic analogs of vitamin D and the MBP-VDR fusion proteins further suggest that the ligand binding pocket of the MBP-VDRfl and MBP-VDRlbd proteins is correctly formed. The analog MC903 competed for the binding of 1,25[^{3}H]D_3 to the MBP fusion proteins with a similar efficiency to the native ligand, 1,25D_3. The analog Δ22-1,25*S*,26(OH)$_3$ vitamin D_3 demonstrated a slightly reduced ability to compete for the binding of 1,25[^{3}H]D_3 to MBP-VDRlbd, compared to 1,25D_3. Previous studies [Binderup and Bramm, 1988; Pols et al., 1991; Colston et al., 1992] have indicated that the analog MC903 has a similar ability to bind to the VDR as does 1,25D_3. Our study confirms this for a recombinantly produced VDR and indicates that, like 1,25D_3, the ability of the analog MC903 to bind to the VDR is not dependent on the presence of an auxiliary factor such as an RXR.

Functional analysis of MBP-VDRfl using gel shift analysis showed that the protein binds its cognate DNA response element, an interaction greatly facilitated in the presence of RXRα, presumably through heterodimer formation. The presence of VDR in this complex was demonstrated by inhibiting its formation with the chicken VDR monoclonal 9A7 antibody. This antibody recognizes the hinge region between the DNA binding and ligand binding domains [Pike et al., 1982] and would thus be expected to interfere with DNA binding. Similarly, 9A7 abolished the binding of a baculovirus-expressed human VDR to the rat osteocalcin VDRE sequence [MacDonald et al., 1991], although in that system a small quantity of a supershifted species appeared, which we did not observe. Our results are also consistent with other studies showing that in vitro binding of recombinant VDR, produced by a variety of methods, to the human osteocalcin VDRE and related sequences is much enhanced in the presence of auxiliary nuclear factors including RXRs [Sone et al., 1990; Yu et al., 1991; Freedman et al., 1994; Nakajima et al., 1994]. However, Carlberg's group found that in vitro–translated VDR binds human osteocalcin sequences preferentially in the homodimeric form and that binding is not enhanced by RXRα [Carlberg et al., 1993; Schrader et al., 1994a]. These contrasting results may perhaps be explained by variations in gel shift methodologies or the VDRE sequences used, although the possibility that differences between recombinant VDR proteins are responsible cannot be discounted. The ability of MBP-VDRfl to discriminate between various VDREs has been preserved, however, as we have shown that the murine fibronectin gene DR6-type VDRE preferentially binds MBP-VDRfl as a homodimer and not as a VDR-RXRα heterodimer, in agreement with results obtained using in vitro–translated VDR and RXRα [Polly et al., in press].

In conclusion, the MBP bacterial fusion protein expression system described here provides an efficient method for the recombinant production of the human VDR. This material can be

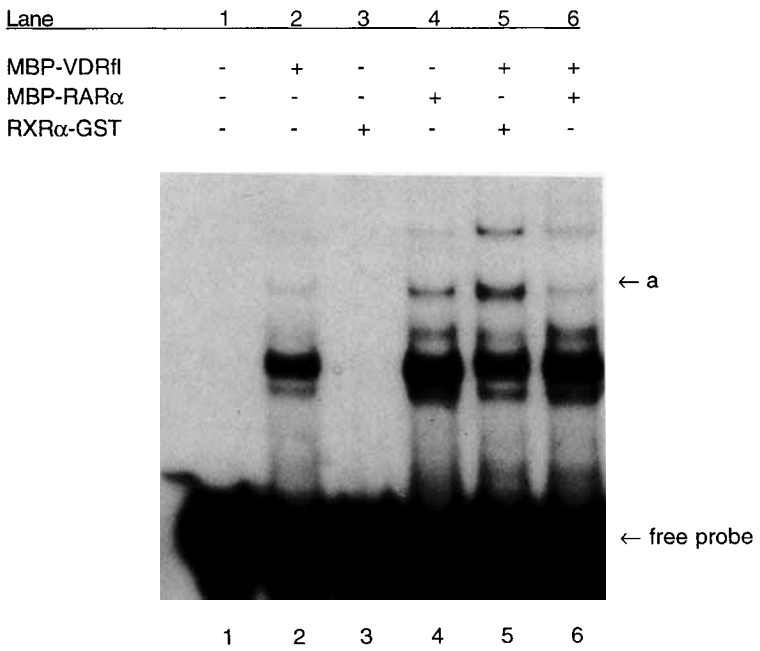

Fig. 7. MBP-VDR fusion proteins were incubated with a radiolabeled oligonucleotide sequence corresponding to the VDRE of the murine fibronectin gene promoter ± recombinant MBP-RARα or RXR-GST fusion proteins, and the reactions subjected to nondenaturing 5% polyacrylamide gel electrophoresis. Complex a is the VDR-VDR homodimer.

rapidly purified by amylose affinity chromatography with yields of 10–20 mg of soluble protein reproducibly obtained from 1 l of bacterial culture. The product retains ligand binding and DNA binding activities comparable to the native receptor. This material has already been used to demonstrate that accessory proteins are not required for high affinity ligand binding and to confirm that the human osteocalcin and murine osteocalcin VDREs preferentially bind VDR-RXRα heterodimers and VDR homodimers, respectively. It is likely that this system will be suitable for the production of the quantities of pure VDR necessary for structural studies.

ACKNOWLEDGMENTS

This work was supported by the Australian Government's Co-operative Research Centre Program, and the National Health and Medical Research Council of Australia.

REFERENCES

Allegretto EA, Pike JW, Haussler MR (1987): C-terminal proteolysis of the avian 1,25-dihydroxyvitamin D3 receptor. Biochem Biophys Res Commun 147:479–485.

Baker AR, McDonnell DP, Hughes M, Crisp TM, Mangelsdorf DJ, Haussler MR, Pike JW, Shine J, O'Malley BW (1988): Cloning and expression of full-length cDNA encoding human vitamin D receptor. Proc Natl Acad Sci USA 85:3294–3298.

Baldwin AS (1990): Analysis of sequence-specific DNA binding proteins by the gel mobility shift assay. In Siebenmann F, Oxender DL (eds): "DNA and Protein Engineering Techniques." New York: Wiley-Liss, Inc, pp 73–76.

Bikle DD (1992): Clinical counterpoint: Vitamin D: New actions, new analogs, new therapeutic potential. Endocr Rev 13:765–784.

Binderup L, Bramm E (1988): Effects of a novel vitamin D analogue MC903 on cell proliferation and differentiation in vitro and on calcium metabolism in vivo. Biochem Pharmacol 37:889–895.

Bouillon R, Okamura WH, Norman AW (1995): Structure–function relationships in the vitamin D endocrine system. Endocr Rev 16:200–257.

Bourguet W, Ruff M, Chambon P, Gronemeyer H, Moras D (1995): Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-α. Nature 375:377–382.

Breen EC, van Wijnen AJ, Lian JB, Stein GS, Stein JL (1994): In vivo occupancy of the vitamin D responsive element in the osteocalcin gene supports vitamin D–dependent transcriptional upregulation in intact cells. Proc Natl Acad Sci USA 91:12902–12906.

Carlberg C, Bendik I, Wyss A, Meier E, Sturzenbecker LJ, Grippo JF, Hunziker W (1993): Two nuclear signalling pathways for vitamin D. Nature 361:657–660.

Chamness GC, McGuire WL (1976): Scatchard plots: Common errors in correction and interpretation. Steroids 26: 538–542.

Cheskis B, Freedman LP (1994): Ligand modulates the conversion of DNA-bound vitamin D3 receptor (VDR) homodimers into VDR–retinoid X receptor heterodimers. Mol Cell Biol 14:3329–3338.

Colston KW, Chander SK, Mackay AG, Coombes RC (1992): Effects of synthetic vitamin D analogues on breast cancer cell proliferation in vivo and in vitro. Biochem Pharmacol 44:693–702.

Eisman JA (1994): Vitamin D and cancer: New insight into vitamin D physiology and potential for cancer therapy. In Heersche JNM, Kanis JA (eds): "Bone and Mineral Research." Amsterdam, New York, Oxford, Elsevier Science BV, pp 45–76.

Elaroussi MA, Prahl JM, DeLuca HF (1994): The avian vitamin D receptors: Primary structures and their origins. Proc Natl Acad Sci USA 91:11596–11600.

Freedman LP, Arce V, Perez FR (1994): DNA sequences that act as high affinity targets for the vitamin D3 receptor in the absence of the retinoid X receptor. Mol Endocrinol 8:265–273.

Garner M, Revzin A (1981): A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: Application to components of the *Escherichia coli* lactose operon regulatory system. Nucleic Acids Res 9:3047–3060.

Glass CK (1994): Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers. Endocr Rev 15:391–407.

Hard T, Kellenbach E, Boelens R, Maler BA, Dahlman K, Freedman LP, Carlstedt-Duke J, Yamamoto KR, Gustafsson JA, Kaptein R (1990): Solution structure of the glucocorticoid receptor DNA-binding domain. Science 249:157–160.

Jones G, Calverley M (1993): Newer vitamin-D drugs for use in bone disease, psoriasis and cancer. Trends Exp Med 4:297–303.

Katahira M, Knegtel RM, Boelens R, Eib D, Schilthuis JG, van der Saag PT, Kaptein R (1992): Homo- and heteronuclear NMR studies of the human retinoic acid receptor beta DNA-binding domain: Sequential assignments and identification of secondary structure elements. Biochemistry 31:6474–6480.

Kuiper GG, Brinkmann AO (1994): Steroid hormone receptor phosphorylation: Is there a physiological role? Mol Cell Endocrinol 100:103–107.

Kumar R, Schaefer J, Wieben E (1992): The expression of milligram amounts of functional human 1,25-dihydroxyvitamin D3 receptor in a bacterial expression system. Biochem Biophys Res Commun 189:1417–1423.

Lee MS, Kliewer SA, Provencal J, Wright PE, Evans RM (1993): Structure of the retinoid X receptor alpha DNA binding domain: A helix required for homodimeric DNA binding. Science 260:1117–1121.

Lee MS, Sem DS, Kliewer SA, Provencal J, Evans RM, Wright PE (1994): NMR assignments and secondary structure of the retinoid X receptor alpha DNA-binding domain. Evidence for the novel C-terminal helix. Eur J Biochem 224:639–650.

Lombardo A, Costa E, Chao WR, Toll L, Hobbs PD, Jong L, Lee MO, Pfahl M, Ely KR, Dawson MI (1994): Recombinant human retinoic acid receptor beta. Binding of synthetic retinoids and transcriptional activation. J Biol Chem 269:7297–7303.

Luisi BF, Xu WX, Otwinowski Z, Freedman LP, Yamamoto KR, Sigler PB (1991): Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature 352:497–505.

MacDonald PN, Haussler CA, Terpening CM, Galligan MA, Reeder MC, Whitfield GK, Haussler MR (1991): Baculovirus-mediated expression of the human vitamin D receptor. Functional characterization, vitamin D response element interactions, and evidence for a receptor auxiliary factor. J Biol Chem 266:18808–18813.

MacDonald PN, Dowd DR, Haussler MR (1994): New insight into the structure and functions of the vitamin D receptor. Sem Nephrol 14:101–118.

Maina CV, Riggs PD, Grandea AG, Slatko BE, Moran LS, Tagliamonte JA, McReynolds LA, Guan CD (1988): An *Escherichia coli* vector to express and purify foreign proteins by fusion to and separation from maltose-binding protein. Gene 74:365–373.

Manglesdorf DJ, Umesono K, Kliewer SA, Borgmeyer U, Ong ES, Evans RM (1991): A direct repeat in the cellular retinol-binding protein type II gene confers differential regulation by RXR and RAR. Cell 66:555–561.

McDonnell DP, Scott RA, Kerner SA, O'Malley BW, Pike JW (1989): Functional domains of the human vitamin D3 receptor regulate osteocalcin gene expression. Mol Endocrinol 3:635–644.

Morrison N, Eisman J (1993): Role of the negative glucocorticoid regulatory element in glucocorticoid repression of the human osteocalcin promoter. J Bone Miner Res 8:969–975.

Nakajima S, Hsieh JC, MacDonald PN, Haussler CA, Galligan MA, Jurutka PW, Haussler MR (1993): Purified human vitamin D receptor overexpressed in *E. coli* and baculovirus systems does not bind 1,25-dihydroxyvitamin D3 hormone efficiently unless supplemented with a rat liver nuclear extract. Biochem Biophys Res Commun 197: 478–485.

Nakajima S, Hsieh JC, MacDonald PN, Galligan MA, Haussler CA, Whitfield GK, Haussler MR (1994): The C-terminal region of the vitamin D receptor is essential to form a complex with a receptor auxiliary factor required for high affinity binding to the vitamin D–responsive element. Mol Endocrinol 8:159–172.

Nishikawa J, Kitaura M, Matsumoto M, Imagawa M, Nishihara T (1994): Difference and similarity of DNA sequence recognized by VDR homodimer and VDR/RXR heterodimer. Nucleic Acids Res 22:2902–2907.

Pike JW, Donaldson CA, Marion SL, Haussler MR (1982): Development of hybridomas secreting monoclonal antibodies to the chicken intestinal 1α, 25-dihydroxyvitamin D_3 receptor. Proc Natl Acad Sci USA 79:7719–7723.

Polly P, Nicholson RC (1993): Sequences of the mouse fibronectin-encoding gene promoter region. Gene 137:353–354.

Polly P, Carlberg C, Eisman JA, Morrison NA: Identification of a vitamin D_3 response element in the fibronectin gene that is bound by a vitamin D_3 receptor homodimer. J Cell Biochem, 60: 322–333.

Pols HA, Birkenhager JC, Schilte JP, Bos MP, van Leeuwen JP (1991): The effects of MC903 on 1,25-(OH)2D3 receptor binding, 24-hydroxylase activity and in vitro bone resorption. Bone Mineral 14:103–111.

Pols HA, Birkenhager JC, van Leeuwen JP (1994): Vitamin D analogues: From molecule to clinical application. Clin Endocrinol 40:285–292.

Ross TK, Prahl JM, DeLuca HF (1991): Overproduction of rat 1,25-dihydroxyvitamin D3 receptor in insect cells using the baculovirus expression system. Proc Natl Acad Sci USA 88:6555–6559.

Sagami I, Tsai H, Wang M, Tsai B, O'Malley B (1986): Identification of two factors required for transcription of the ovalbumin gene. Mol Cell Biol 6:4259–4267.

Schrader M, Muller KM, Carlberg C (1994a): Specificity and flexibility of vitamin D signaling. Modulation of the activation of natural vitamin D response elements by thyroid hormone. J Biol Chem 269:5501–5504.

Schrader M, Muller KM, Nayeri S, Kahlen JP, Carlberg C (1994b): Vitamin D_3–thyroid hormone receptor heterodimer polarity directs ligand sensitivity of transactivation. Nature 370:382–386.

Schwabe JW, Chapman L, Finch JT, Rhodes D (1993): The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: How receptors discriminate between their response elements. Cell 75:567–578.

Smith CL, Hager GL, Pike JW, Marx SJ (1991): Overexpression of the human vitamin D3 receptor in mammalian cells using recombinant adenovirus vectors. Mol Endocrinol 5:867–878.

Sone T, McDonnell DP, O'Malley BW, Pike JW (1990): Expression of human vitamin D receptor in *Saccharomyces cerevisiae.* Purification, properties, and generation of polyclonal antibodies. J Biol Chem 265:21997–22003.

Studzinski GP, McLane JA, Uskokovic MR (1993): Signaling pathways for vitamin D–induced differentiation: Implications for therapy of proliferative and neoplastic diseases. Crit Rev Eukaryotic Gene Expression 3:279–312.

Towers TL, Luisi BF, Asianov A, Freedman LP (1993): DNA target selectivity by the vitamin D3 receptor: Mechanism of dimer binding to an asymmetric repeat element. Proc Natl Acad Sci USA 90:6310–6314.

Walters MR (1992): Newly identified actions of the vitamin D endocrine system. Endocr Rev 13:719–764.

Yu VC, Delsert C, Andersen B, Holloway JM, Devary OV, Nääar AM, Kim SY, Boutin J-M, Glass CK, Rosenfeld MG (1991): RXRβ: A coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. Cell 67:1251–1266.