0013-7227/96/$03.00/0
Endocrinology

Vol. 137, No. 5
Printed in U.S.A.

Antagonistic Effects of Transforming Growth Factor-β on Vitamin D_3 Enhancement of Osteocalcin and Osteopontin Transcription: Reduced Interactions of Vitamin D Receptor/Retinoid X Receptor Complexes with Vitamin D Response Elements

A. STAAL, A. J. VAN WIJNEN, R. K. DESAI, H. A. P. POLS, J. C. BIRKENHÄGER, H. F. DELUCA, D. T. DENHARDT, J. L. STEIN, J. P. T. M. VAN LEEUWEN, G. S. STEIN, AND J. B. LIAN

Department of Internal Medicine III (A.S., H.A.P.P., J.C.B., J.P.T.M.v.L.), Erasmus University Medical School, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands; Department of Cell Biology (A.S., A.J.v.W., R.K.D., J.L.S., G.S.S., J.B.L.), University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, Massachusetts 01655-0106; Department of Biochemistry (H.F.D.), University of Wisconsin-Madison, Madison, Wisconsin 53706; Department of Biological Science (D.T.D.), Rutgers University, Piscataway, New Jersey 08855-1059

ABSTRACT

Osteocalcin and osteopontin are noncollagenous proteins secreted by osteoblasts and regulated by a complex interplay of systemic and locally produced factors, including growth factors and steroid hormones. We investigated the mechanism by which transforming growth factor-β (TGFβ) inhibits 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$)-enhanced expression of the osteocalcin (OC) and osteopontin (OP) genes. ROS 17/2.8 cells, in which both genes are expressed, were transfected with reporter constructs driven by native (*i.e.* wild-type) rat OC and mouse OP promoters. TGFβ abrogated the 1,25-$(OH)_2D_3$ enhanced transcription of both the OC and OP genes. The inhibitory TGFβ response for each requires vitamin D response element (VDRE) sequences, although there are additional contributions from proximal basal regulatory elements. These transcriptional effects were further investigated for contribution of the *trans*-activating factors, which interact with OC and OP VDREs, involving the vitamin D receptor (VDR) and retinoid X receptor (RXR). Gel mobility shift assays show that TGFβ significantly reduces induction of the heterodimeric VDR/RXR complexes in 1,25-$(OH)_2D_3$-treated ROS 17/2.8 cells. However, Western blot and ligand binding analyses reveal that TGFβ does not affect nuclear availability of the VDR. We also show that activator protein-1 activity is up-regulated by TGFβ; thus, activator protein-1 binding sites in the OC promoter may potentially contribute to inhibitory effects of TGFβ on basal transcription. Our studies demonstrate that the inhibitory action of TGFβ on the 1,25-$(OH)_2D_3$ enhancement of OC and OP transcription in osteoblastic cells results from modulations of protein-DNA interactions at the OC and OP VDRE, which cannot be accounted for by changes in VDR protein levels. As OC and OP participate in bone turnover, our results provide insight into the contributions of TGFβ and 1,25-$(OH)_2D_3$ to VDR-mediated gene regulatory mechanisms operative in bone formation and/or resorption events. (*Endocrinology* **137:** 2001–2011, 1996)

OSTEOCALCIN (OC) and osteopontin (OP) are two major noncollagenous calcium binding proteins in bone (1). During development of the osteoblast phenotype, osteopontin is expressed during the period of active proliferation and decreases postproliferatively, and expression is up-regulated in mature osteoblasts. In contrast, osteocalcin is expressed only postproliferatively in mature osteoblasts with the onset of extracellular matrix mineralization (2). In ROS 17/2.8 osteosarcoma cells, however, both osteocalcin and osteopontin are constitutively expressed. Some evidence points to the involvement of both proteins in bone turnover; for example, both proteins bind to hydroxyapatite and are regulated by 1,25-$(OH)_2D_3$ and TGFβ. In addition, osteocalcin contributes to recruitment and/or differentiation of osteoclasts (3–5), and osteopontin appears to be important for attachment of the osteoclasts to the bone matrix (6, 7).

TGFβ and 1,25-$(OH)_2D_3$ are involved in regulation of both bone formation and resorption. The steroid hormone 1,25-$(OH)_2D_3$ plays a principle role in calcium homeostasis and skeletal metabolism (8), as well as influences expression of genes related to establishment and maintenance of the bone cell phenotype (9). 1,25-$(OH)_2D_3$ exerts its primary effect by binding to its nuclear receptor, the vitamin D receptor (VDR), which belongs to the steroid/retinoid/thyroid hormone receptor superfamily (10) and acts via binding to distinct vitamin D response elements (VDRE). VDREs have been identified in several genes related to osteoblastic differentiation and function: human osteocalcin (11, 12), rat osteocalcin (13–15), mouse osteopontin (16), rat 25-hydroxy- and 1,25-dihydroxyvitamin D_3 24-hydroxylase (17–20), calbindin D28k (21), and β_3 integrin (22).

TGFβ is locally produced in bone by osteoblasts and osteoclasts and represents a member of the TGFβ/BMP superfamily of polypeptide hormones, which play key roles in skeletal

Received October 6, 1995.
Address all correspondence and requests for reprints to the authors at: University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, Massachusetts 01655-0106.
* This work was supported by Grants AR-33920, AR-39588, and DK-46721 from NIH and the Netherlands Organization for Scientific Research (project no. 900-541-131). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of NIH.

development and bone remodeling (23, 24). In mammals, three isoforms of TGFβ have been identified: TGFβ$_1$, TGFβ$_2$, and TGFβ$_3$ (25, 26). TGFβ is present as a latent complex in bone and released from the extracellular matrix in an active form during bone resorption by osteoclasts (27). TGFβ modulates the expression of several markers of the osteoblast phenotype (28–35).

Recent studies suggest that regulation of bone metabolism by 1,25-(OH)$_2$D$_3$ and TGFβ is functionally coupled. For example, combined treatment with TGFβ and 1,25-(OH)$_2$D$_3$ of both human and rat osteoblast-like cells has been shown to affect parameters of osteoblastic differentiation and function (36–38). However, the mechanisms by which TGFβ and 1,25-(OH)$_2$D$_3$ together modulate the expression of osteoblast-related genes remains to be established. In this study, we examined the antagonistic effects of TGFβ$_1$ and TGFβ$_2$ on the 1,25-(OH)$_2$D$_3$ enhancement of osteocalcin and osteopontin gene transcription. We show that TGFβ inhibition of 1,25-(OH)$_2$D$_3$ stimulated transcription involves the selective down-regulation of VDR/RXR interactions with both the OC and OP VDREs.

Materials and Methods

Cell culture

ROS 17/2.8 osteosarcoma cells, kindly provided by Dr. G. Rodan and Dr. S. Rodan (Merck Sharp & Dohme Research Laboratories, West Point, PA) were maintained in Ham's F12 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 5% FCS at 37 C under 95% air/5% CO_2. For the experiments, cells were seeded in 100 × 20-mm plastic dishes at 0.7 × 10^6 cells/plate in Ham's F12 medium containing 5% FCS and cultured for 24 h. Subsequently, medium was replaced by Ham's F12 with 2% charcoal-treated FCS, and cells were cultured overnight and then incubated for 24 h with 10^{-8} M 1,25-(OH)$_2$D$_3$ and/or 2.5 ng/ml of either TGFβ$_1$ or TGFβ$_2$. 1,25-(OH)$_2$D$_3$ was kindly provided by Dr. M. Uskokovic, Hoffman-La Roche Inc. (Nutley, NJ). TGFβ$_1$ was purchased from R&D Systems (Minneapolis, MN). TGFβ$_2$ was kindly provided by Dr. J. Feyen, Sandoz Pharma Ltd. (Basel, Switzerland). Medium was analyzed for osteocalcin by RIA as previously described (39).

Gel mobility shift assay

Nuclear extracts were prepared as previously described (40). In brief, cells were scraped in PBS, and after centrifugation the cell pellet was reconstituted in a hypotonic lysis buffer (10 mM HEPES, pH 7.5, 10 mM KCl, and 0.2 mM EDTA) supplemented with 0.75 mM spermidine, 0.15 mM spermine, 1 mM EGTA, 1 mM dithiothreitol, and a broad spectrum of protease inhibitors: 0.2 mM PMSF, 70 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone, 10 μg/ml trypsin inhibitor, 0.5 μg/ml leupeptin, and 1.0 μg/ml pepstatin. Then 0.5% Nonidet P40 was added, and cells were lysed using a dounce homogenizer. After centrifugation, nuclear extracts were obtained by incubating nuclei in a hypertonic nuclear extraction buffer (20 mM HEPES, pH 7.5, 420 mM KCl, 25% glycerol and 0.2 mM EDTA) supplemented with the same reagents previously mentioned for the hypotonic lysis buffer. Desalting was performed by dilution with hypertonic nuclear extraction buffer without KCl.

For gel mobility shift assays, the following oligonucleotides were synthesized: rat osteocalcin VDRE (14): 5′-CTGCACT*GGGTGA*ATG*AGGACA*TTACTGA-3′; mouse osteopontin VDRE (16): 5′-ACAA*GGTTCA*CGAG*GTTCA*CGTCT-3′; metallothionein II/activator protein-1 (AP-1) consensus: 5′-AATTCG*TGACTCA*GCGCGCG-3′; transcription factor SP-1 consensus: 5′-CGGA*TGGGCGGGGCC*GGGGA*TGGGCGG*GCCCGG-3′.

Gel-purified oligonucleotides were radiolabeled with [γ^{32}P] ATP as described previously (41). Protein/DNA binding reactions were performed by combining 10 μl of a 5 μg protein mixture in a final KCl concentration of 100 mM with 10 μl of a DNA mixture containing 1 fmol/μl DNA, 0.2 μg/μl nonspecific competitor DNA, poly(dI-dC)*(dI-dC) and 2 mM dithiothreitol for 15 min. Protein/DNA complexes were separated in a 4% polyacrylamide gel (acrylamide:bisacrylamide = 80:1) in 0.5 × TBE electrophoresis buffer (89 mM Tris, 89 mM borate, 2 mM EDTA, pH 8.4). Gels were dried and exposed to x-ray film.

Transient transfection assay

The OC-chloramphenicol acetyltransferase (CAT) chimeric reporter gene constructs pOCZCAT and *Bgl*IICAT contain the osteocalcin promoter sequences −1097/+23 and −348/+23, respectively (42). The OP CAT chimeric reporter gene constructs pOP772CAT and pOP552CAT span nucleotides −772/+40 and −552/+41, respectively (16). The (VDRE)$_3$-tk-luciferase chimeric reporter gene construct, which was generously provided by Dr. L. J. Sturzenbecker (Hoffman-La Roche), contains three copies of the rat osteocalcin VDRE fused to thymidine kinase luciferase. Diethylaminoethyl-dextran-mediated transfections (43) were performed with cells plated at a density of 0.8 × 10^5 per well in six-well plates. CAT reporter gene constructs (5 μg) were cotransfected with 2 μg Rous sarcoma virus-luciferase plasmid or pSV2CAT (used as an internal control for transfection efficiency) 48 h after plating. After transfection, cells were fed with Ham's F12 medium supplemented with 5% FCS. Subsequently, 4 h after feeding, cells were incubated with TGFβ$_1$, TGFβ$_2$ and/or 1,25-(OH)$_2$D$_3$ in Ham's F12 with 2% charcoal-treated FCS. Cells were harvested after incubation for 24 h, and CAT activity was determined (43). CAT and luciferase assays were normalized on the basis of luciferase activity and CAT activity, respectively, as well as protein content by Bradford assay (Pierce). Quantitation was performed by using a β-emission analyzer (Betascope 603 Betagen, Waltham, MA).

Northern blot analysis

RNA isolation, electrophoresis of total RNA (10 μg) in formaldehyde gels, Northern blotting [Zetaprobe membranes (Bio-Rad, Melville, NY)], prehybridizations, and hybridizations were performed as described previously (44). The sources of the following probes used for hybridization are described by Shalhoub *et al.* (44): osteocalcin, osteopontin, histone H4, collagen type I, alkaline phosphatase, and vitamin D receptor probes. Values for mRNA were normalized to GAPDH and ribosomal 28S transcripts. For rehybridization, filters were washed for 2 h at 65 C with 5 mM Tris (pH 8.0), 0.2 mM EDTA (pH 8.0), 0.05% sodium pyrophosphate, and 0.1 × Denhardt's solution.

Western blot analysis

Nuclear proteins were separated in 12% SDS-PAGE gels (acrylamide: bisacrylamide = 30:1) (43) and transferred to Zetaprobe membrane followed by Western blot analysis (ECL, Amersham Life Sciences, Arlington Heights, IL). In brief, 5% blocking agent was used in PBS containing 0.1% Tween-20. The blot was subsequently incubated with VDR antibody (IVG8C11) in a 1:1000 dilution and incubated with the second antibody (peroxidase antimouse antibody at a 1:10,000 dilution).

Preparations of cell extracts and [^{3}H]1,25-(OH)$_2$D$_3$ binding assay

Vitamin D ligand binding assays were performed as described by Pols *et al.* (45). In brief, after incubation with TGFβ and/or 1,25-(OH)$_2$D$_3$, cells were washed with 2% BSA in serum-free medium for 2 h to remove residual unlabeled 1,25-(OH)$_2$D$_3$. Then cells were extracted on ice in a hypertonic buffer consisting of 300 mM KCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM dithiothreitol, 10 mM sodium molybdate, and 0.1% Triton X-100. High-speed supernatants were obtained, and 250 μl aliquots were incubated overnight at 4 C for single point assays with 0.25 nM [^{3}H]1,25-(OH)$_2$D$_3$ in the absence or presence of a 200-fold molar excess of unlabelled hormone. After removal of unbound ligand, receptor-bound vitamin D was quantitated by liquid scintillation counting.

Data analysis

To assess significance of the interaction between two agents, tested data were analyzed with analysis of variance (ANOVA) for two-way design (indicated by #). Other statistical analyses were performed using Student's *t* test (indicated by *).

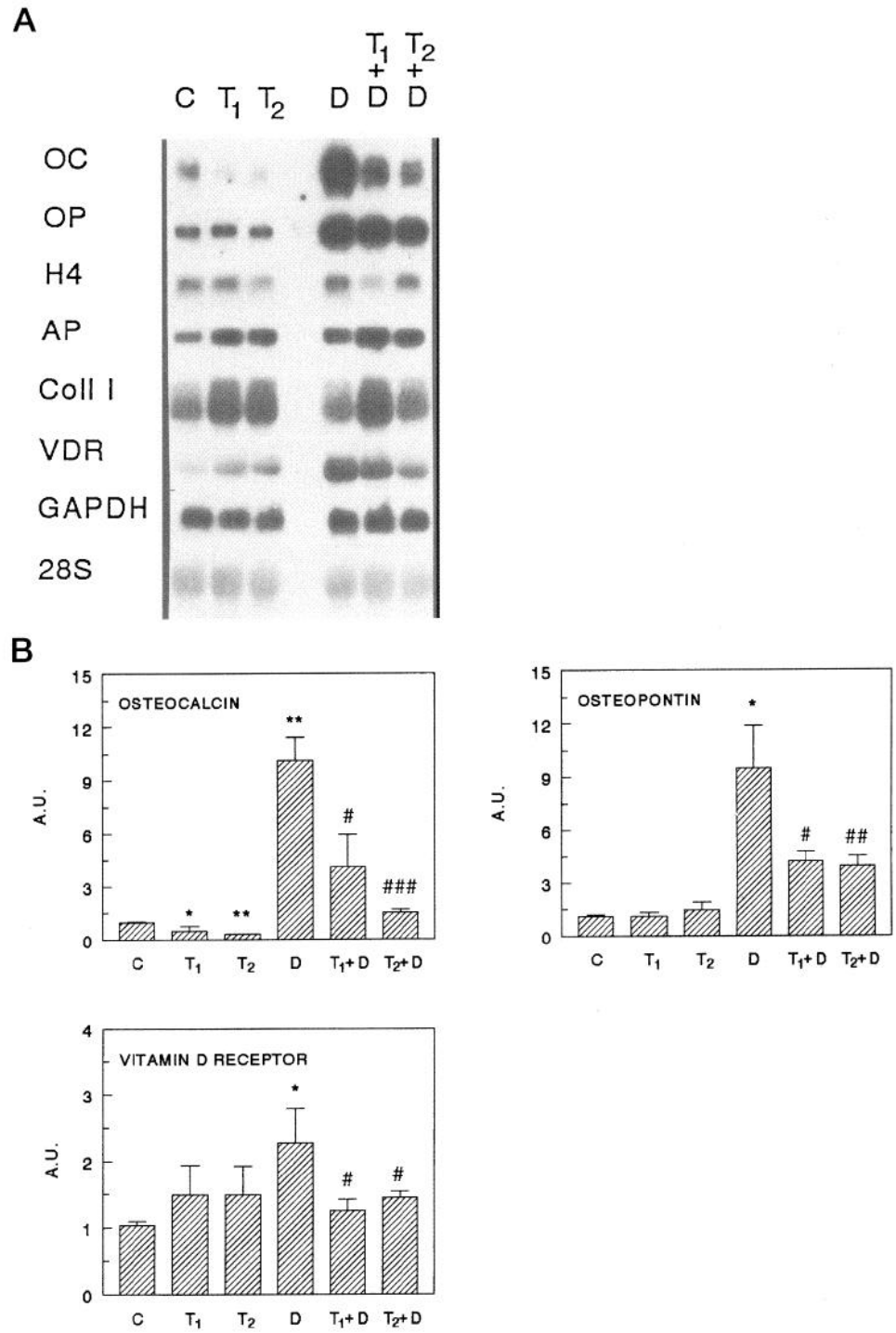

FIG. 1. TGFβ and/or 1,25-$(OH)_2D_3$ affect the expression of mRNAs related to osteoblast function and differentiation. Cells were cultured as described in *Materials and Methods* and incubated for 24 h with 2.5 ng/ml TGFβ_1 (T_1), 2.5 ng/ml TGFβ_2 (T_2), and/or 10^{-8} M 1,25-$(OH)_2D_3$ (D) or vehicle (C). Northern blots were hybridized with the following probes: osteocalcin (OC), osteopontin (OP), histone H4 (H4), alkaline phosphatase (AP), collagen type I (COLL I), vitamin D receptor (VDR), glyceraldehyde 6-phosphate dehydrogenase (GAPDH), and LS6 (28S ribosomal RNA). A, Composite autoradiograms from representative experiments. B, *Bar graphs* (arbitrary absorbance units, A.U.) of OC, OP, VDR and 28S represent quantitation of mRNA levels by densitometric scanning of the autoradiogram. OC, OP, and VDR mRNA levels are normalized for 28S. Similar results were obtained in multiple independent experiments. *, $P < 0.05$; **, $P < 0.001$ *vs.* control; #, $P < 0.05$; ##, $P < 0.01$; ##, $P < 0.005$ (by ANOVA). For details of P value calculation, see *Materials and Methods*.

Results

Effects of TGFβ_1, TGFβ_2, and 1,25-$(OH)_2D_3$ influence expression of genes related to osteoblast function and differentiation

As osteoblasts are responsive to TGFβ_1 and TGFβ_2 and display three classes of TGFβ receptors (46), we explored possible differential effects of these two major isoforms present in bone. Northern blot analysis reveals inhibitory effects of both TGFβ1 and TGFβ2 on basal osteocalcin gene expression (Fig. 1). Steady-state mRNA levels of osteopontin were not affected by either growth factor after 24 h of treatment. The 24-h time point was selected for our studies as this treatment interval facilitates assessment of the combined effects of TGF-β and 1,25-$(OH)_2D_3$ on OC and OP gene expression. We have previously established that under our experimental conditions, 1,25-$(OH)_2D_3$ enhancement of OC gene expression is maximal at 24 h after administration of the

ligand (9). Also, although TGFβ modulates parameters of 1,25-$(OH)_2D_3$- mediated gene expression as a component of an earlier response (*i.e.* 6 h), significant effects of TGFβ on OC gene expression are not observed until the 24-h time point (37).

The combined effects of TGFβ and 1,25-$(OH)_2D_3$ on OC and OP gene expression are also shown in Fig. 1. Both OC and OP mRNA levels were increased 6- to 10-fold after 24 h of 1,25-$(OH)_2D_3$ treatment. Both TGFβ_1 and TGFβ_2 inhibited the 1,25-$(OH)_2D_3$-induced increases in OC or OP expression. However, the magnitude of the decrease was reproducibly greater for osteocalcin in multiple experiments, consistent with the influence of TGFβ on basal level of OC gene expression. For 1,25-$(OH)_2D_3$-treated cells, the level of secreted OC in the medium, which reflects OC protein synthesis, parallels the changes in OC gene expression (Table 1). For comparison, TGFβ and 1,25-$(OH)_2D_3$ effects on other bone-related proteins (*e.g.* alkaline phosphatase, collagen type 1, and vitamin D receptor) and cell proliferation marker (histone H4) were determined. These genes display different patterns of expression after TGFβ and 1,25-$(OH)_2D_3$ treatment, demonstrating the selective responsiveness of the OC and OP genes to these factors.

Effects of combined treatment with TGFβ and 1,25-$(OH)_2D_3$ on OC and OP transcription

1,25-$(OH)_2D_3$ regulates OC and OP gene expression by specific interactions of VDR containing transcription factor complexes with defined VDREs in the promoters of both genes. To gain insight into the mechanism by which TGFβ inhibits 1,25-$(OH)_2D_3$ enhancement of OC and OP gene expression, we performed transient transfections assays in ROS 17/2.8 cells with OC and OP promoter CAT fusion constructs in the presence of TGFβ and/or 1,25-$(OH)_2D_3$. After transfection with an OC promoter construct of 1100 nucleotides, which contains the VDRE (pOCZCAT; Fig. 2A), we observed a 4- to 6-fold decrease in basal CAT activity in response to both TGFβ_1 and TGFβ_2. 1,25-$(OH)_2D_3$ increased basal CAT activity 8-fold in 24 h. This increase was inhibited 20- to 30-fold after coincubation with either TGFβ_1 or TGFβ_2. Thus, in addition to a significant inhibition of basal transcription, both TGFβ subtypes abrogated 1,25-$(OH)_2D_3$ enhanced OC transcription.

To ascertain the extent to which TGFβ effects on OC depend on VDRE sequences, ROS 17/2.8 cells were transfected with a construct in which the VDRE was deleted (p*Bgl*IICAT; Fig. 2B). As expected, the construct did not respond to 1,25-$(OH)_2D_3$. TGFβ_1 or TGFβ_2 either alone or in the presence of 1,25-$(OH)_2D_3$ caused a 4- to 6-fold decrease in CAT activity. This inhibitory effect of TGFβ on basal OC gene transcription is consistent with the presence of a TGFβ response element in the proximal promoter (47). Notably, the fold decrease resulting from coincubation of TGFβ and 1,25-$(OH)_2D_3$ was significantly less for the OC promoter CAT construct lacking the VDRE (4- to 6-fold; p*Bgl*IICAT) as compared with the OC CAT construct containing the VDRE (20- to 30-fold; pOCZCAT). To assess the promoter selectivity of TGFβ effects on transcription of bone-related genes, we performed similar experiments with CAT reporter gene constructs fused to the SV40 promoter (pSV2CAT) or lacking a promoter (pSV0CAT). TGFβ has only modest inhibitory effects on the virally derived SV40 promoter (1.3-fold; data not shown).

TABLE 1. Effect of either TGFβ_1 or TGFβ_2 and 1,25$(OH)_2D_3$ on osteocalcin secretion in medium of ROS 17/2.8 cells (ng/ml)

	Control	TGFβ_1	TGFβ_2
control	2.76 ± 0.23	2.13 ± 0.44	2.26 ± 0.40
1,25$(OH)_2D_3$	15.45 ± 2.15	3.44 ± 0.35	3.14 ± 0.08

Cells were cultured as described (*Materials and Methods*) and incubated for 24 h with 2.5 ng/ml TGFβ_1, 2.5 ng/ml TGFβ_2, and/or 10^{-8} M 1,25$(OH)_2D_3$. Data are expressed as mean ± SD.

Similar to results with the OC promoter, transfection with the osteopontin promoter construct pOP772CAT (Fig. 3A), containing 772 nucleotides of 5′ flanking sequences, showed a decrease in basal CAT activity after incubation with either TGFβ_1 or TGFβ_2 (2-fold). 1,25-$(OH)_2D_3$ caused an 11-fold increase in CAT activity, which was inhibited 7-fold by TGFβ_1 and TGFβ_2. The OP construct lacking the VDRE (pOP552CAT; Fig. 3B) does not respond to 1,25-$(OH)_2D_3$. We noted that a modest level of TGFβ inhibition (2-fold) of OP basal promoter activity persists. Taken together, our results suggest that TGFβ exerts a major effect on 1,25-$(OH)_2D_3$-mediated OC and OP transcription and requires a VDRE containing promoter segment.

To investigate directly the effect of TGFβ and 1,25-$(OH)_2D_3$ on VDRE-mediated transcription, we transfected cells with the $(VDRE)_3$-tk-luciferase reporter gene construct, which contains three copies of the rat OC VDRE (Fig. 4). Treatment with 1,25-$(OH)_2D_3$ increased basal luciferase activity approximately 4-fold in 24 h. This increase was inhibited 50% and 30% by TGFβ_1 and TGFβ_2, respectively, indicating that the inhibitory effect of TGFβ on 1,25-$(OH)_2D_3$ enhancement of OC transcription is at least partly mediated by the VDRE.

TGFβ and 1,25-$(OH)_2D_3$ modulate protein/DNA interactions at VDREs

To address directly the mechanism by which TGFβ abrogates 1,25-$(OH)_2D_3$-dependent enhancement of OC and OP transcription, we examined protein/DNA interactions at VDREs. Gel shift assays were performed with nuclear proteins from ROS 17/2.8 cells treated with TGFβ and/or 1,25-$(OH)_2D_3$, using labeled OC and OP VDRE oligonucleotides. Fig. 5 shows the well established induction of the VDR containing complexes at both OC and OP VDREs after treatment of ROS 17/2.8 cells with 1,25-$(OH)_2D_3$ (16, 40, 48–50). Nuclear proteins from control or TGFβ_2-treated cells do not show VDRE protein/DNA interactions. However, combined treatment of 1,25-$(OH)_2D_3$ with TGFβ caused a significant reduction of the VDR containing complex at both the OC and OP VDREs. Similar reductions in VDRE complexes were observed in TGFβ_1-treated cells (data not shown). This indicates that the inhibitory effects by TGFβ_1 and TGFβ_2 on the 1,25-$(OH)_2D_3$-induced increase in OC and OP transcription are at least in part due to decreased binding of transcription factors to the VDRE.

Because both TGFβ and 1,25-$(OH)_2D_3$ modulate expression of the fos and jun family of proto-oncogenes in osteoblasts (34, 51–53), we also examined the effects of these agents

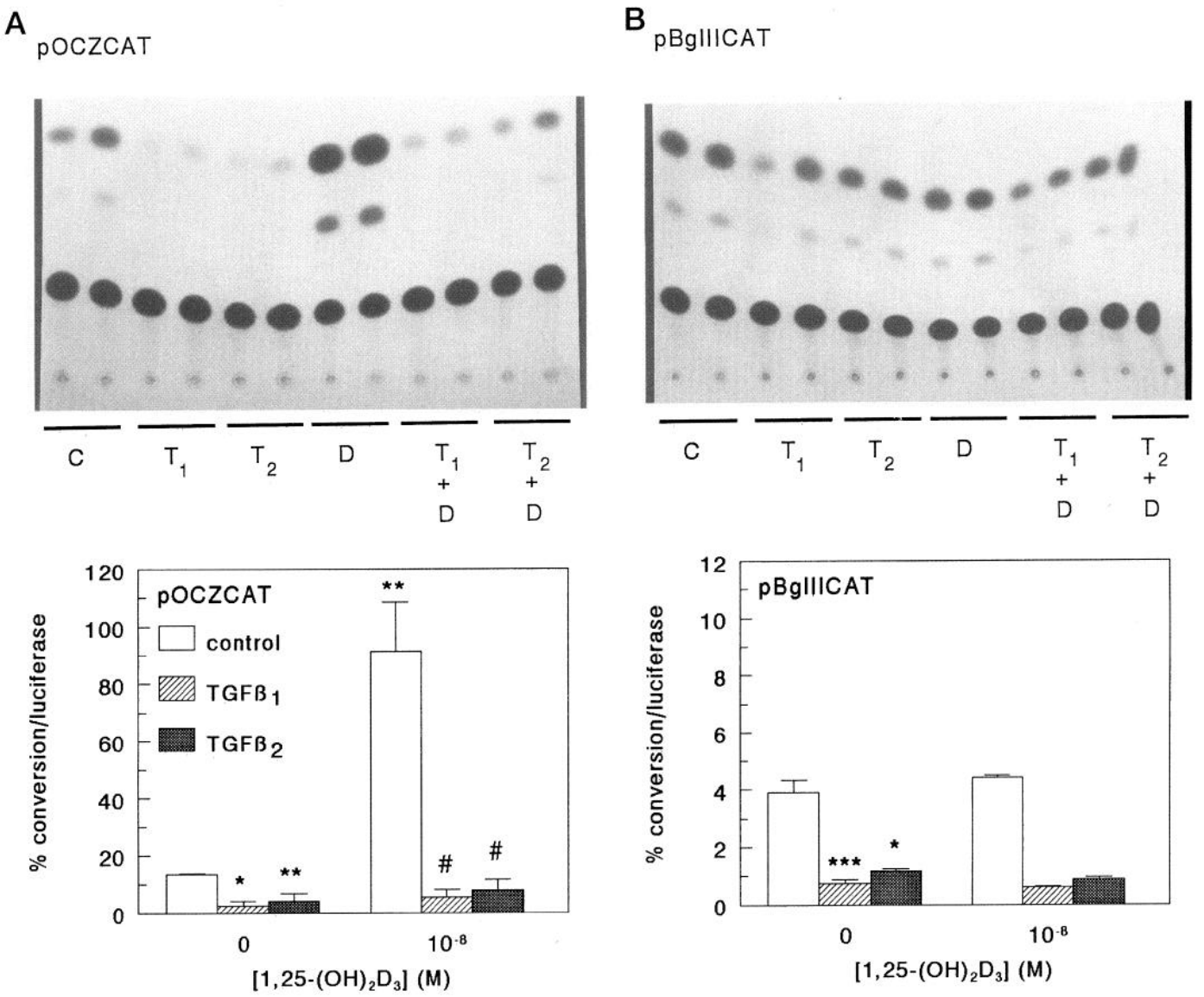

FIG. 2. TGFβ_1 and TGFβ_2 antagonize basal and 1,25-$(OH)_2D_3$ stimulated OC transcription. ROS 17/2.8 cells were transiently transfected with the following osteocalcin promoter-CAT fusion constructs: A) pOCZCAT, −1097/+23, and B) p*Bgl*IICAT −348/+23. Cells were transfected and 4 h later incubated for 24 h in the absence (control) or presence of 2.5 ng/ml TGFβ1 (T_1), on TGFβ2 (T_2). These treatment groups were coincubated with either vehicle (C, O) or 1,25-$(OH)_2D_3$ (D, 10^{-8} M). Upper portions of each panel show one representative autoradiogram of a CAT assay. The lower portion of each panel are *bar graphs* representing mean ± SD of duplicates and triplicates from three independent transfection experiments after quantitation using a β-emission analyzer. CAT activity was corrected for transfection efficiency by luciferase activity. *, $P < 0.001$; ** $P < 0.005$; ***, $P < 0.01$ *vs.* control; #, $P < 0.01$, calculated by ANOVA. For details of P value calculation, see *Materials and Methods*.

on AP-1 activity in ROS 17/2.8 osteosarcoma cells. Gel shift assays were performed using AP-1 and SP-1 consensus sequences as the probes (Fig. 6). The AP-1 complex was increased after incubation with either TGFβ or 1,25-$(OH)_2D_3$, although the increase observed with TGFβ was more pronounced than with 1,25-$(OH)_2D_3$. Combined treatment caused a further enhancement of the AP-1 complex. No modulation of SP-1 binding activity was observed, which represents an internal control for protein quantitation. These findings indicate selectivity of the TGFβ- and 1,25-$(OH)_2D_3$-mediated signaling mechanisms that modulate nuclear transcription factor levels.

VDR protein levels are not rate-limiting for VDRE interactions in the presence of TGFβ and 1,25-$(OH)_2D_3$

Because changes in VDR mRNA levels were observed after treatment with TGFβ_1, TGFβ_2, and 1,25-$(OH)_2D_3$ (Fig. 1), we studied the effects of TGFβ_1 and TGFβ_2 in the absence or presence of 1,25-$(OH)_2D_3$ on VDR protein levels, as a possible mechanism for the inhibitory effects of TGFβ on VDR/RXR interactions at the OC and OP VDREs. We investigated the effect of TGFβ on homologous up-regulation of the VDR by Western blot analysis. The results indicate that nuclear VDR levels are only detected after treatment with 1,25-$(OH)_2D_3$, and that neither TGFβ_1 nor TGFβ_2 affects the 1,25-$(OH)_2D_3$-

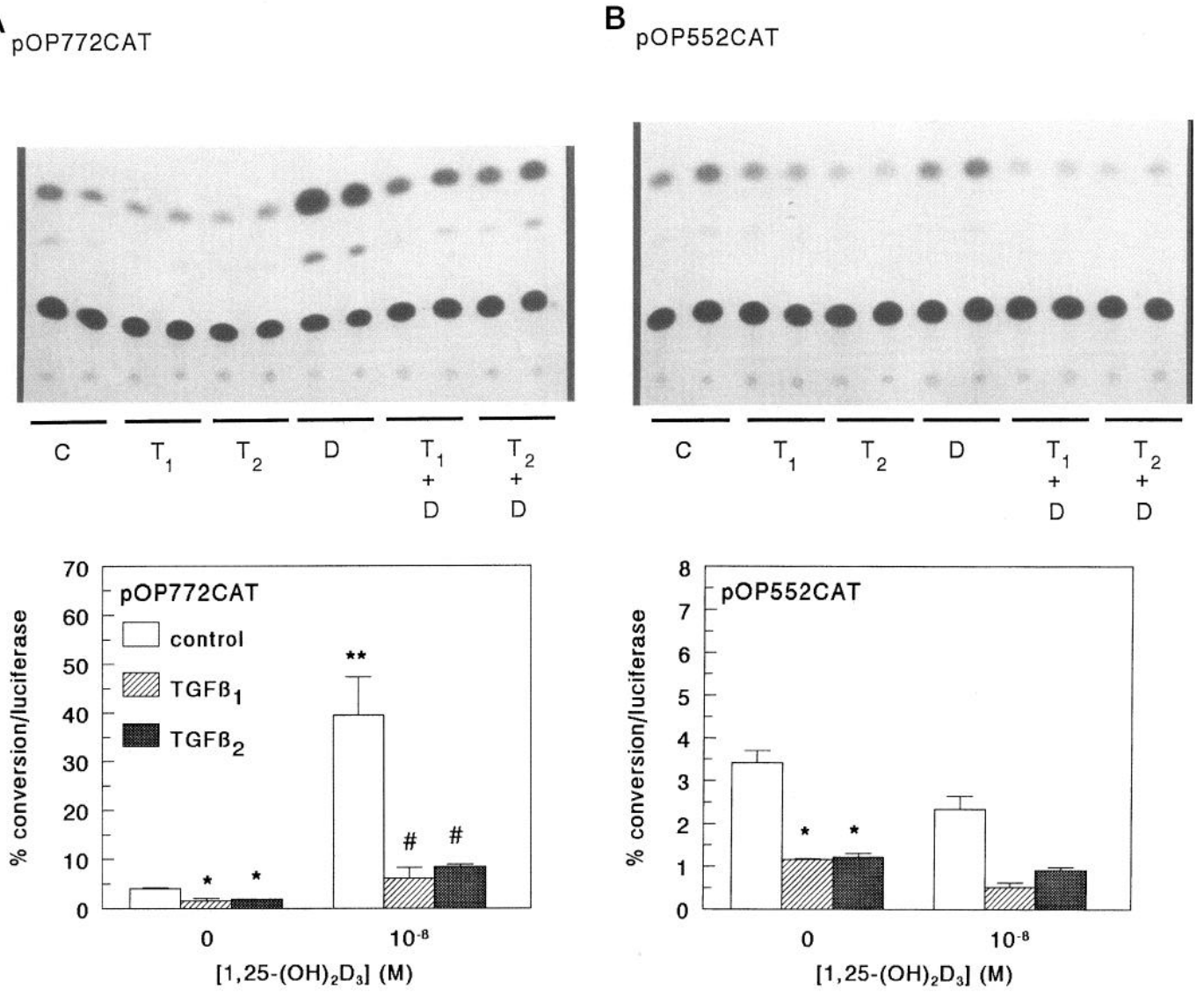

FIG. 3. Effects of TGFβ_1 and TGFβ_2 on 1,25-$(OH)_2D_3$-stimulated OP transcription. ROS 17/2.8 cells were transiently transfected with the following osteopontin promoter-CAT fusion constructs: A, pOP772CAT, −772/+40; B, pOP552CAT −552/+40. Cells were transfected and 4 h later incubated for 24 h in the absence (control) or presence of 2.5 ng/ml TGFβ1 (T_1), or TGFβ2 (T_2). These treatment groups were coincubated with either vehicle (C, O) or 1,25-$(OH)_2D_3$ (D, 10^{-8} M). *Upper portion* of each panel shows one representative autoradiogram of a CAT assay. The *lower portion* of each panel are *bar graphs* representing mean ± SD of duplicates and triplicates from three independent transfection experiments after quantitation using a β-emission analyzer. CAT activity was corrected for transfection efficiency by luciferase activity. *, $P < 0.001$; **, $P < 0.005$ *vs.* control; #, $P < 0.01$ (by ANOVA). For details of P value calculation, see *Materials and Methods.*

dependent increase in VDR levels (Fig. 7A). Subtle variations in the 1,25-$(OH)_2D_3$-treated lanes appear to reflect similar variations in the nonspecific band migrating slower than the VDR. The lack of an effect by TGFβ on VDR protein levels in the presence of 1,25-$(OH)_2D_3$ is further corroborated by quantitative analysis of VDR protein using immunoradiometric assays; homologous up-regulation of VDR number in nuclear extracts after 1,25-$(OH)_2D_3$ treatment was not affected by cotreatment with either TGFβ_1 or TGFβ_2 (data not shown). Ligand binding assays in whole cell extracts using isotopically labeled 1,25-$(OH)_2D_3$ (Fig. 7B) also demonstrate homologous up-regulation of 1,25-$(OH)_2D_3$-receptor number as reflected by a 5-fold increase in ligand binding after 1,25-$(OH)_2D_3$ treatment. Cotreatment with TGFβ (24 h) does not affect the homologous up-regulation of 1,25-$(OH)_2D_3$-receptor number in the presence of 1,25-$(OH)_2D_3$. Thus, the evidence presented in Fig. 7 indicates that TGFβ inhibition of protein/DNA interactions at the VDRE is not the consequence of a reduction in the level of VDR protein.

We have found that TGFβ alone causes a subtle enhancement of VDR levels in whole cell extracts after 24 h of treatment ($P < 0.05$). We have previously reported that the magnitude of this response is higher at 6 h after TGFβ administration (37). The modestly elevated levels of VDR protein after 24 h of TGFβ treatment therefore may represent a residual, and perhaps persistent, component of TGFβ responsiveness. Our previous observation that TGFβ enhances VDR levels in whole cell extracts as a component of an earlier response (*i.e.* 6 h) (37) and our

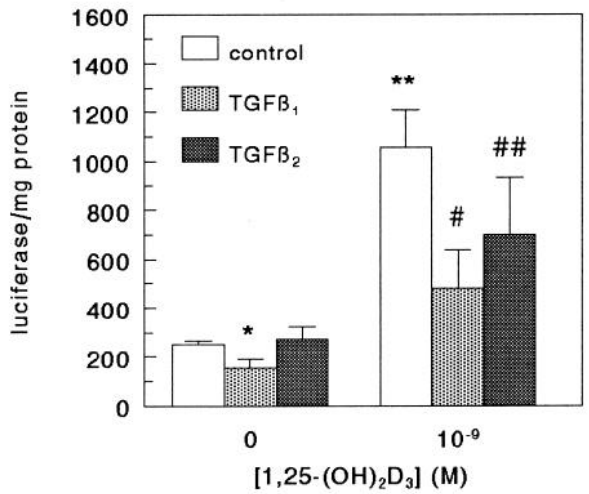

FIG. 4. TGFβ_1 and TGFβ_2 inhibit 1,25-$(OH)_2D_3$ enhancement of OC VDRE-mediated transcription. ROS 17/2.8 cells were transiently transfected with the $(VDRE)_3$-tk-luciferase construct. Cells were transfected and 4 h later incubated for 24 h in the absence or presence of 2.5 ng/ml TGFβ_1 and TGFβ_2. These treatment groups were coincubated with either vehicle or 1,25-$(OH)_2D_3$ (10^{-8} M). *Bar graphs* represent mean ± SD of luciferase activity. *, $P < 0.05$; ** $P < 0.005$ *vs.* control; #; $P < 0.01$; ##; $P < 0.05$ calculated as significance of interaction (by ANOVA). For details of P value calculation, see *Materials and Methods*.

current findings that show that TGFβ does not significantly influence homologously up-regulated levels of the VDR protein at 24 h are both consistent with a complex temporal regulatory mechanism for modulation of the VDR levels that may in part be compartmentalized.

Discussion

In this study we focused on gene regulatory mechanisms that control OC and OP expression, which are affected by the combined activities of 1,25-$(OH)_2D_3$ and TGFβ. The basis of our studies is supported by previous studies (36, 37) and our present findings, which show that TGFβ inhibits the 1,25-$(OH)_2D_3$-induced increase of both OC and OP mRNA levels. The physiological relevance of TGFβ inhibition of the 1,25-$(OH)_2D_3$ enhanced expression of osteoblast-related genes is exemplified by decreased secretion of the osteocalcin protein, a major functional product of osteoblasts. In addition, by directly comparing the actions of TGFβ_1 and TGFβ_2, we show that these agents exert identical effects on regulation of OC and OP gene expression. This finding is consistent with the observation that TGFβ_1- and TGFβ_2-mediated cell signaling pathways operate via shared TGFβ receptor classes (46).

Using transfection assays with osseous cells and reporter gene constructs containing the native OC and OP gene promoters, we establish that 1,25-$(OH)_2D_3$ related effects of TGFβ on expression of the OC and OP genes are mediated by transcriptional mechanisms. TGFβ abrogates 1,25-$(OH)_2D_3$ enhancement of OC transcription, which requires the presence of the OC VDRE within the promoter. However, TGFβ also inhibits basal OC transcription in the absence of 1,25-$(OH)_2D_3$, which occurs irrespective of the presence of VDRE sequences. TGFβ repression of basal OC gene transcription is mediated by a TGFβ response element (TGRE) in the proximal promoter of the OC gene (47). Transcriptional repression involves binding of AP-1 family members to the TGRE (47). Our results show that TGFβ increases AP-1 binding activity both in the presence or absence of 1,25-$(OH)_2D_3$ in ROS 17/2.8 cells. Thus, increased levels of AP-1 binding may contribute to inhibition of OC gene transcription.

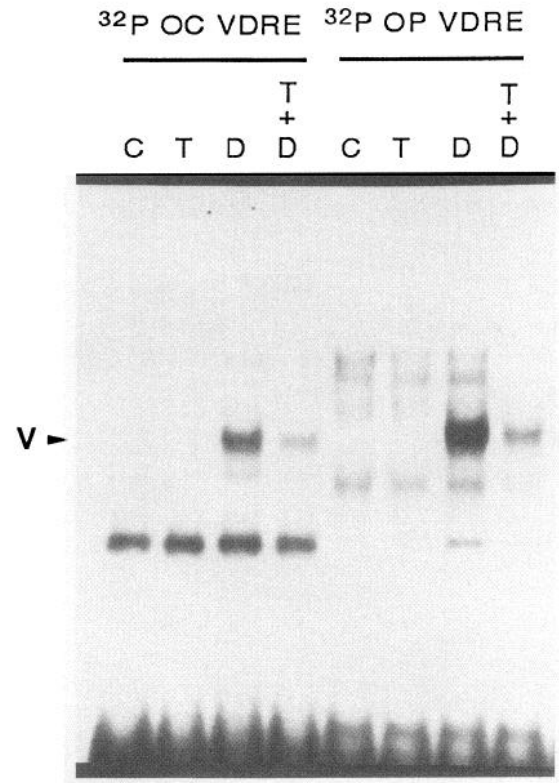

FIG. 5. TGFβ modulates interactions of endogenous 1,25-$(OH)_2D_3$-inducible VDR/RXR complexes from ROS 17/2.8 cells. Binding of nuclear proteins to the OC VDRE and OP VDRE was analyzed in gel mobility shift assays. A, Cells were incubated for 24 h with vehicle (C), 2.5 ng/ml TGFβ_2 (T), 10^{-8} M 1,25-$(OH)_2D_3$ (D) or both (T + D). Nuclear protein preparations were incubated with either radiolabeled OC- or OP-VDRE. The vitamin D responsive complexes (V) for the OC- and OP-VDRE are indicated by the *arrowhead*.

We note that TGFβ also inhibits basal OP gene transcription. This inhibition of OP gene transcription, as monitored by reporter gene expression after treatment with TGFβ for 24 h, is not reflected by changes in OP mRNA levels. It appears that the TGFβ dependent inhibition of OP transcription may be compensated by posttranscriptional modulatory events, including mRNA processing, transport, and/or stability. Alternatively, the observed differences between TGFβ effects on OC transcription and OP mRNA level may reflect differences in the regulation of the endogenous gene and the OP CAT chimeric reporter gene construct. Noda *et al.* (16) observed that TGFβ increases OP mRNA levels, concomitant with stimulation of OP gene transcription, based on results from nuclear run-on assays after a 72 h treatment with TGFβ. This apparent discrepancy in the observed effects of TGFβ on OP gene expression may relate to differences in experimental design, as well as perhaps reflect chronic effects of TGFβ on cell growth and differentiation of osteoblasts.

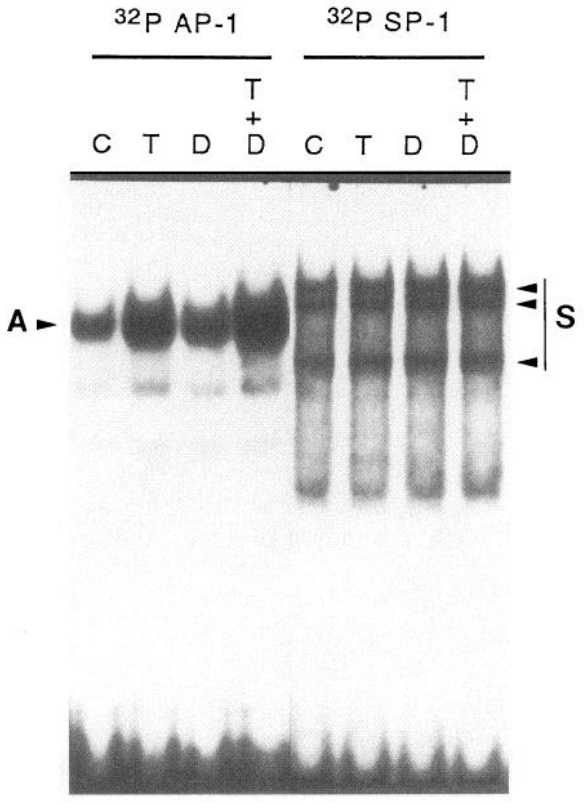

FIG. 6. Selective enhancement of AP-1 activity by TGFβ and 1,25-$(OH)_2D_3$. Gel mobility shift assays were performed with AP-1 and SP-1 consensus oligonucleotides. Nuclear extracts were identical to those in Fig. 5. The same abbreviations and symbols are as indicated in Fig. 5. The AP-1 complexes (A) represent a composite of heterodimers of the fos and jun family members and are indicated by the arrowhead. The *triple arrowhead* (S) indicates three specific complexes mediated by the SP-1 family of transcription factors; the *upper complex* represents SP-1, and the two *lower complexes* are SP-3 related proteins.

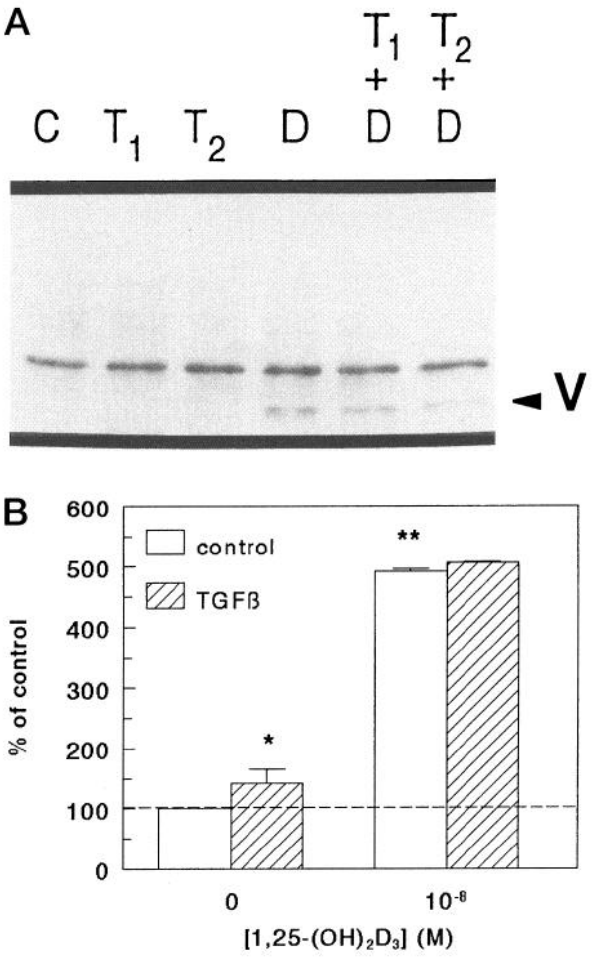

FIG. 7. Homologous up-regulation of VDR protein levels by 1,25-$(OH)_2D_3$ is not affected by TGFβ. Western blot analysis (A) was performed with nuclear proteins from ROS 17/2.8 cells, which were separated in a 12% SDS-PAGE gel and transferred to a solid support. Detection of VDR levels was performed using the VDR antibody IVG8C11. The abbreviations used are the same as indicated in Figs. 4 and 5, and the VDR protein (V) is indicated by the *arrowhead*. The subtle variations in the 1,25-$(OH)_2D_3$-treated lanes appear to reflect similar variations in the nonspecific band migrating slower than the VDR. Ligand binding assays (B) using [^{3}H]1,25-$(OH)_2D_3$ were carried out with cytosolic preparations from ROS 17/2.8 cells treated with 1,25-$(OH)_2D_3$ and/or TGF-β_2. The values on the ordinate were calculated by dividing fmol [^{3}H] 1,25-$(OH)_2D_3$ bound per mg protein by the values obtained from untreated cells. *Bar graphs* represent mean ± SD of duplicates from two independent 1,25-$(OH)_2D_3$ ligand binding experiments. *, $P < 0.05$; **, $P < 0.0001$ *vs.* control.

We tested the hypothesis that transcriptional repression of 1,25-$(OH)_2D_3$-enhanced OC and OP gene expression may be achieved by modulating protein/DNA interactions at the OC and OP VDREs. Transcriptional up-regulation of the OC gene by 1,25-$(OH)_2D_3$ requires the positive cis-acting VDRE, which interacts with VDR/RXR heterodimers *in vitro* (49, 54–57) and *in vivo* (58). Our results show that TGFβ inhibits both 1,25-$(OH)_2D_3$ enhancement of OC VDRE-mediated transcription and the 1,25-$(OH)_2D_3$-dependent induction of VDR containing transcription factor complexes interacting with the OC- and OP-VDREs. Thus, we propose that TGFβ may directly influence OC and OP gene transcription by modulating 1,25-$(OH)_2D_3$-dependent protein/DNA interactions at the OC and OP VDREs. This concept is supported by previous findings that another polypeptide regulator of OC transcription, TNF-α (59), influences 1,25-$(OH)_2D_3$-regulated transcription by modifications of protein/DNA interactions at the VDRE.

Regulation of the nuclear representation of VDR/RXR heterodimers may occur at multiple levels of control. For example, our results show that VDR mRNA levels are increased in a 1,25-$(OH)_2D_3$-dependent manner, which is reflected by increased VDR protein levels as monitored by Western blot analysis and 1,25-$(OH)_2D_3$ ligand binding assays, as well as induction of VDR/RXR heterodimer interactions with the VDRE. In coincubation experiments with 1,25-$(OH)_2D_3$, TGFβ reduces the 1,25-$(OH)_2D_3$-dependent increase in VDR mRNA, but this decrease is not paralleled by a reduction in VDR protein levels. This finding is consistent with the observation that stabilization of VDR protein by 1,25-$(OH)_2D_3$ ligand binding, contributes to the homologous up-regulation of the VDR (60). Taken together, these data suggest that VDR mRNA levels are not rate-limiting for synthesis of VDR protein, and VDR protein levels are not rate-limiting for formation of VDR/RXR heterodimers in

cells treated with both TGFβ and 1,25-$(OH)_2D_3$. Thus, the mechanism by which TGFβ reduces VDR/RXR heterodimer interactions at VDREs appears to involve posttranslational events, *e.g.* phosphorylation and/or protein/protein interactions, including effects of TGFβ on nuclear availability of RXR proteins.

Phosphorylation of the VDR influences VDRE binding activity and transcriptional activation function of VDR/RXR heterodimers (40, 50, 61, 62). Hyperphosphorylation of the VDR correlates with decreased VDRE binding activity (50, 61), and phosphorylation pathways involving protein kinase C may posttranslationally modify VDR *in vivo*. Because TGFβ-mediated signal transduction occurs via protein kinase C (63) and 1,25-$(OH)_2D_3$ action also involves protein kinase C (61, 64), the possibility arises that TGFβ may alter phosphorylation of the VDR with consequential repression of VDR/RXR binding activity and transcriptional activation.

Accumulating evidence suggests that the functions of 1,25-$(OH)_2D_3$ and TGFβ in the regulation of bone metabolism and parameters of osteoblast differentiation are strongly interrelated (34, 36–38). TGFβ is capable of increasing 1,25-$(OH)_2D_3$ receptor number (65, 37). 1,25-$(OH)_2D_3$ stimulates homologous up-regulation of the VDR, which is required for the induction of VDR/RXR heterodimers, resulting in the transcriptional activation of 1,25-$(OH)_2D_3$ responsive genes. 1,25-$(OH)_2D_3$ also stimulates TGFβ synthesis and secretion by bone cells, as well as enhances TGFβ incorporation into bone (66, 67). The inhibitory effect of TGFβ on 1,25-$(OH)_2D_3$-enhancement may provide a negative regulatory loop in the action of 1,25-$(OH)_2D_3$.

The results presented here suggest that TGFβ influences OC and OP gene transcription. The effect of TGFβ on OC gene transcription may occur via a two-pronged mechanism. First, TGFβ abrogates 1,25-$(OH)_2D_3$ enhancement of OC transcription by down-regulating binding of VDR/RXR heterodimers to the OC VDRE. Secondly, TGFβ also inhibits OC transcription in the presence or absence of 1,25-$(OH)_2D_3$, which occurs via AP-1 binding sites (47, 53), perhaps mediated by TGFβ enhanced levels of AP-1 proteins. Similarities in the inhibitory effect of TGFβ on 1,25-$(OH)_2D_3$-enhanced expression of two genes, OC and OP, encoding proteins with distinct properties, are consistent with functional linkage of these proteins in bone turnover.

Acknowledgments

We thank Rosa Mastrototaro and Elizabeth Buffone for technical assistance, Gert-Jan van den Bemd for assistance with immunoradiometric assays, and Chaitali Banerjee and Jean Prahl for their experimental advice. We also thank Judy Rask for editorial assistance.

References

1. **Boskey AL** 1992 Mineral-matrix interactions in bone and cartilage. *Clin Ortho Rel Res* 281:244–274
2. **Stein GS, Lian JB** 1993 Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype. Endocr Rev 14:424–442
3. **Lian JB, Tassinari M, Glowacki J** 1984 Resorption of implanted bone prepared from normal and warfarin-treated rats. J Clin Invest 73:1223–1226
4. **Chenu C, Colucci S, Grano M, Zigrino P, Barattolo R, Zambonin G, Baldini N, Vergnaud P, Delmas PD, Zallone AZ** 1994 Osteocalcin induces chemotaxis, secretion of matrix proteins, and calcium-mediated intracellular signaling in human osteoclast-like cells. J Cell Biol 127:1149–1158
5. **Glowacki J, Lian JB** 1987 Impaired recruitment and differentiation of osteoclast progenitors by osteocalcin-deplete bone implants. Cell Diff 21:247–254
6. **Reinholt FP, Hultenby K, Oldberg A, Heinegard D** 1990 Osteopontin - a possible anchor of osteoclasts to bone. Proc Natl Acad Sci USA 87:4473–4475
7. **Flores ME, Norgard M, Heinegard D, Reinholt FP, Andersson G** 1992 RGD-directed attachment of isolated rat osteoclasts to osteopontin, bone sialoprotein, and fibronectin. Exp Cell Res 201:526–530
8. **DeLuca HF** 1988 The vitamin D story: a collaborative effort of basic science and clinical medicine. FASEB J 2:224–236
9. **Owen TA, Aronow MA, Barone LM, Bettencourt B, Stein G, Lian JB** 1991 Pleiotropic effects of vitamin D on osteoblast gene expression are related to the proliferative and differentiated state of the bone cell phenotype: dependency upon basal levels of gene expression, duration of exposure and bone matrix competency in normal rat osteoblast cultures. Endocrinology 128:1496–1504
10. **Tsai MJ, O'Malley BW** 1994 Molecular mechanisms of action of steroid/thyroid superfamily members. Annu Rev Biochem 63:451–486
11. **Kerner SA, Scott RA, Pike JW** 1989 Sequence elements in the human osteocalcin gene confer basal activation and inducible response to vitamin D_3. Proc Natl Acad Sci USA 86:4455–4459
12. **Ozono K, Liao J, Keener SA, Scott RA, Pike JW** 1990 The vitamin D-responsive element in the human osteocalcin gene. J Biol Chem 265:21881–21888
13. **Demay MB, Gerardi JM, DeLuca HF, Kronenberg HM** 1990 DNA sequences in the rat osteocalcin gene that bind the 1,25-dihydroxyvitamin D_3 receptor and confer responsiveness to 1,25-dihydroxyvitamin D_3. Proc Natl Acad Sci USA 87:369–373
14. **Markose ER, Stein JL, Stein GS, Lian JB** 1990 Vitamin D-mediated modifications in protein-DNA interactions at two promoter elements of the osteocalcin gene. Proc Natl Acad Sci USA 87:1701–1705
15. **Terpening CM, Haussler CA, Jurutka PW, Galligan MA, Komm BS, Haussler MR** 1991 The vitamin D-responsive element in the rat bone Gla protein gene is an imperfect direct repeat that cooperates with other *cis*-acting elements in 1,25-dihydroxyvitamin D_3-mediated transcriptional activation. Mol Endocrinol 6:557–562
16. **Noda M, Vogel RL, Craig AM, Prahl J, DeLuca HF, Denhardt DT** 1990 Identification of a DNA sequence responsible for binding of the 1,25-dihydroxyvitamin D_3 receptor and 1,25-dihydroxyvitamin D_3 enhancement of mouse secreted phosphoprotein 1 (*Spp-1* or osteopontin) gene expression. Proc Natl Acad Sci USA 87:9995–9999
17. **Ohyama Y, Ozono K, Uchida M, Shinki T, Kato S, Suda T, Yamamoto O, Noshiro M, Kato Y** 1994 Identification of a Vitamin D-responsive element in the 5'-flanking region of the rat 25-hydroxyvitamin D_3 24-hydroxylase gene. J Biol Chem 269:10545–10550
18. **Hahn CN, Kerry DM, Omdahl JL, May BK** 1994 Identification of a vitamin D responsive element in the promoter of the rat cytochrome $P450_{24}$ gene. Nucleic Acids Res 22:2410–2416
19. **Zierold C, Darwish HM, DeLuca HF** 1995 Two vitamin D response elements function in the rat 1,25-dihydroxyvitamin D 24-hydroxylase promoter. J Biol Chem 270:1675–1678
20. **Kahlen JP, Carlberg C** 1994 Identification of a vitamin D receptor homodimer-type response element in the rat calcitriol 24-hydroxylase gene promoter. Biochem Biophys Res Commun 202:1366–1372
21. **Gill RK, Christakos S** 1993 Identification of sequence elements in mouse calbindin-D28k gene that confer 1,25-dihydroxyvitamin D3- and butyrate-inducible responses. Proc Natl Acad Sci USA 90:2984–2988
22. **Cao X, Ross FP, Zhang L, MacDonald PN, Chappel J, Teitelbaum SL** 1993 Cloning of the promoter for the avian integrin beta 3 subunit gene and its regulation by 1,25-dihydroxyvitamin D3. J Biol Chem 268:27371–27380
23. **Robey PG, Young MF, Flanders KC, Roche NS, Kondaiah P, Reddi AH, Termine JD, Sporn MB, Roberts AB** 1987 Osteoblasts synthe-

size and respond to transforming growth factor-type beta (TGF-beta) *in vitro*. J Cell Biol 105:457–463
24. **Bonewald LF, Dallas SL** 1994 Role of active and latent transforming growth factor β in bone formation. J Cell Biochem 55:350–357
25. **Sporn MB, Roberts AB, Wakefield LM, de Crombrugghe B** 1987 Some recent advances in the chemistry and biology of transforming growth factor-beta. J Cell Biol 105:1039–1045
26. **Massague J** 1990 The transforming growth factor-β family. Annu Rev Cell Biol 6:597–641
27. **Oursler MJ** 1994 Osteoclast synthesis and secretion and activation of latent transforming growth factor beta. J Bone Miner Res 9:443–452
28. **Canalis E, Pash J, Varghese S** 1993 Skeletal growth factors. Crit Rev Eukaryot Gene Expr 3:155–166
29. **Rosen DM, Stempien SA, Thompson AY, Seyedin SM** 1988 Transforming growth factor-beta modulates the expression of osteoblast and chondroblast phenotypes *in vitro*. J Cell Physiol 134:337–346
30. **Wrana JL, Maeno M, Hawrylyshyn B, Yao Kl Domenicucci C, Sodek J** 1988 Differential effects of transforming growth factor-beta on the synthesis of extracellular matrix proteins by normal fetal rat calvarial bone cell populations. J Cell Biol 106:915–924
31. **Rodan GA, Noda M** 1991 Gene expression in osteoblastic cells. Crit Rev Eukaryot Gene Exp 1:85–98
32. **Katagiri R, Lee T, Takeshima H, Suda T, Tanaka H, Omura S** 1990 Transforming growth factor-beta modulates proliferation and differentiation of mouse clonal osteoblastic MC3T3-E1 cells depending on their maturation stages. Bone Miner 11:285–293
33. **Ber R, Kubota T, Sodek J, Aubin JE** 1991 Effects of transforming growth factor-beta on normal clonal bone cell populations. Biochem Cell Biol 69:132–140
34. **Breen EC, Ignotz RA, McCabe LM, Stein JL, Stein GS, Lian JB** 1994 TGFβ alters growth and differentiation related gene expression in proliferating osteoblasts *in vitro*, preventing development of the mature bone phenotype. J Cell Physiol 160:323–335
35. **Harris SE, Bonewald LF, Harris MA, Sabatini M, Dallas S Feng JQ, Ghosh-Choudhury N, Wozney J, Mundy GR** 1994 Effects of transforming growth factor beta on bone nodule formation and expression of bone morphogenetic protein 2, osteocalcin, osteopontin, alkaline phosphatase, and type I collagen mRNA in long-term cultures of fetal rat calvarial osteoblasts. J Bone Miner Res 9:855–863
36. **Bonewald LF, Kester MB, Schwartz Z, Swain LD, Khare A, Johnson TL, Leach RJ, Boyan BD** 1992 Effects of combining transforming growth factor β and 1,25-dihydroxyvitamin D_3 on differentiation of a human osteosarcoma (MG-63). J Biol Chem 267:8943–8949
37. **Staal A, Birkenhäger JC, Pols HAP, Buurman CJ, Vink-van Wijngaarden T, Kleinekoort WMC, van den Bemd GJCM, van Leeuwen JPTM** 1994 Transforming growth factor β-induced dissociation between vitamin D receptor level and 1,25-dihydroxyvitamin D_3 action in osteoblast-like cells. Bone Miner 26:27–42
38. **Ingram RT, Bonde SK, Riggs L, Fitzpatrick LA** 1994 Effects of transforming growth factor beta (TGFβ) and 1,25-dihydroxyvitamin D_3 on the function, cytochemistry and morphology of normal human osteoblast-like cells. Differentiation 55:153–163
39. **Gundberg CM, Hauschka PV, Lian JB, Gallop PM** 1984 Osteocalcin: isolation, characterization, and detection. Methods Enzymol 107:516–544
40. **Shakoori AR, van Wijnen AJ, Bortell R, Owen TA, Stein JL, Lian JB, Stein GS** 1994 Variations in Vitamin D Receptor transcription factor complexes associated with the osteocalcin gene vitamin D responsive element in osteoblasts and osteosarcoma cells. J Cell Biochem 55:1–12
41. **van Wijnen AJ, van den Ent FMI, Lian JB, Stein JL, Stein GS** 1992 Overlapping and CpG methylation-sensitive protein-DNA interactions at the histone H4 transcriptional cell cycle domain: distinctions between two human H4 gene promoters. Mol Cell Biol 12:3273–3287
42. **Aslam F, Shalhoub V, van Wijnen AJ, Banerjee C, Bortell R, Shakoori AR, Litwack G, Stein JL, Stein GS, Lian JB** Contributions of distal, proximal promoter elements to glucocorticoid regulation of osteocalcin gene transcription. Mol Endocrinol 9:679–690
43. Ausubel FM, Brent R, Kingston R, Moore DD, Seidman JG, Smith JA, Struhl K (eds) 1989 in Current Protocols in Molecular Biology, Wiley Press, New York
44. **Shalhoub V, Jackson ME, Lian JB, Stein GS, Marks Jr SC** 1991 Gene expression during skeletal development in three osteopetrotic rat mutations: evidence for osteoblast abnormalities. J Biol Chem 266: 9847–9856
45. **Pols HAP, Birkenhäger JC, Schilte JP, Visser TJ** 1988 Evidence that the self-induced metabolism of 1,25-dihydroxyvitamin D-3 limits the homologous up-regulation of its receptor in rat osteosarcoma cells. Biochim Biophys Acta 970:122–129
46. **Kingsley DM** 1994 The TGFβ superfamily: new members, new receptors and new genetic tests of function in different organisms. Genes Dev 8:133–146
47. **Banerjee C, Stein JL, van Wijnen AJ, Frenkel B, Lian JB, Stein GS** 1996 Transforming growth factor-β1 responsiveness of the rat osteocalcin gene is mediated by an activator protein-1 binding site. Endocrinology 137:1991–2000
48. **Owen TA, Bortell R, Shalhoub V, Heinrichs A, Stein JL, Stein GS, Lian JB** 1993 Postproliferative transcription of the rat osteocalcin gene is reflected by vitamin D-responsive developmental modifications in protein-DNA interactions at basal and enhancer promoter elements. Proc Natl Acad Sci USA 90:1503–1507
49. **Bortell R, Owen TA, Bidwell JP, Gavazzo P, Breen E, van Wijnen AJ, DeLuca HF, Stein JL, Lian JB, Stein GS** 1992 Vitamin D-responsive protein-DNA interactions at multiple promoter regulatory elements that contribute to the level of rat osteocalcin gene expression. Proc Natl Acad Sci USA 89:6119–6123
50. **Desai RK, van Wijnen AJ, Stein JL, Stein GS, Lian JB,**Control of 1,25-dihydroxyvitamin D3 receptor mediated enhancement of osteocalcin gene transcription: effects of perturbing phosphorylation pathways by okadaic acid and staurosporine. Endocrinology 136: 5685–5693
51. **Pertovaara L, Sistonen L, Bos TJ, Vogt PK, Keski-Oja J, Alitalo K** 1989 Enhanced jun gene expression is an early genomic response to transforming growth factor β stimulation. Mol Cell Biol 9:1255–1262
52. **Candeliere GA, Prud'homme J, St.-Arnaud R** 1991 Differential stimulation of fos and jun family members by calcitriol in osteoblastic cells. Mol Endocrinol 12:1780–1788
53. **Owen TA, Bortell R, Yocum SA, Smock SL, Zhang M, Abate C, Shalhoub V, Aronin N, Wright KL, van Wijnen AJ, Stein JL, Curran T, Lian JB, Stein GS** 1990 Coordinate occupancy of AP-1 sites in the vitamin D responsive and CCAAT box elements by fos-jun in the osteocalcin gene: a model for phenotype suppression of transcription. Proc Natl Acad Sci USA 87:9990–9994
54. **Yu VC, Delsert C, Andersen B, Holloway JM, Devary OV, Näär AM, Kim SY, Boutin JM, Glass CK, Rosenfeld MG** 1991 RXR beta: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. Cell 67:1251–1266
55. **Kliewer SA, Umesono K, Mangelsdorf DJ, Evans RM** 1992 Retinoid × receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling. Nature 355:446–449
56. **Leid M, Kastner P, Lyons R, Nakshatri H, Saunders M, Zacharewski T, Chen JY, Staub A, Garnier JM, Mader S, Chambon P** 1992 Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. Cell 68:377–395
57. **Marks MS, Hallenbeck PL, Nagata T, Segars JH, Appella E, Nikodem VM, Ozato K** 1992 H-2RIIBP (RXR beta) heterodimerization provides a mechanism for combinatorial diversity in the regulation of retinoic acid and thyroid hormone responsive genes. EMBO J 11:1419–1435
58. **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
59. **Kuno H, Kurian SM, Hendy GN, White J, DeLuca HF, Evans CO, Nanes MS** 1994 Inhibition of 1,25-dihydroxyvitamin D3 stimulated osteocalcin gene transcription by tumor necrosis factor-alpha: structural determinants within the vitamin D response element. Endocrinology 134:2524–2531
60. **Arbour NC, Prahl JM, DeLuca HF** 1993 Stabilization of the vitamin D receptor in rat osteosarcoma cells through the action of 1,25-dihydroxyvitamin D3. Mol Endocrinol 7:1307–1312
61. **Hsieh JC, Jurutka PW, Nakajima S, Galligan MS, Haussler CA,**

Shimizu Y, Shimizu N, Whitfield GK, Haussler MR 1993 Phosphorylation of the human vitamin D receptor by protein kinase C. Biochemical and functional evaluation of the serine 51 recognition site. J Biol Chem 268:15118–15126

62. **Darwish HM, Burmester JK, Moss VE, DeLuca HF** 1993 Phosphorylation is involved in transcriptional activation by the 1,25-dihydroxyvitamin D_3 receptor. Biochim Biophys Acta 1167:29–36
63. **Ohtsuki M, Massague J** 1992 Evidence for the involvement of protein kinase activity in Transforming Growth Factor-β signal transduction. Mol Cell Biol 12:261–265
64. **van Leeuwen JPTM, Birkenhäger JC, van den Bemd GJCM, Buurman CJ, Staal A, Bos MP, Pols HAP** 1992 Evidence for the functional involvement of protein kinase C in the action of 1,25-dihydroxyvitamin D3 in bone. J Biol Chem 267:112562–12569
65. **Schneider HG, Michelangeli VP, Frampton RJ, Grogan JL, Ikeda K, Martin TJ, Findlay DM** 1992 Transforming growth factor-β modulates receptor binding of calciotropic hormones and G protein-mediated adenylate cyclase responses in osteoblast-like cells. Endocrinology 131:1383–1389
66. **Finkelman RD, Linkhart TA, Mohan S, Lau Kh Baylink DJ, Bell NH** 1991 Vitamin D deficiency causes a selective reduction in deposition of transforming growth factor beta in rat bone: possible mechanism for impaired osteoinduction. Proc Natl Acad Sci USA 88:3657–3660
67. **Petkovich PM, Wrana JL, Grigoriadis AE, Heersche JN, Sodek J** 1987 1,25-dihydroxyvitamin D3 increases epidermal growth factor receptors and transforming growth factor beta-like activity in a bone-derived cell line. J Biol Chem 262:13424–13428