

Antagonistic Effects of Transforming Growth Factor- β on Vitamin D₃ Enhancement of Osteocalcin and Osteopontin Transcription: Reduced Interactions of Vitamin D Receptor/Retinoid X Receptor Complexes with Vitamin D Response Elements

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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 (TGF β) inhibits 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃)-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. TGF β abrogated the 1,25-(OH)₂D₃ enhanced transcription of both the OC and OP genes. The inhibitory TGF β 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 TGF β significantly reduces induction of the heterodimeric VDR/RXR complexes in 1,25-(OH)₂D₃-treated ROS 17/2.8 cells. However, Western blot and ligand binding analyses reveal that TGF β does not affect nuclear availability of the VDR. We also show that activator protein-1 activity is up-regulated by TGF β ; thus, activator protein-1 binding sites in the OC promoter may potentially contribute to inhibitory effects of TGF β on basal transcription. Our studies demonstrate that the inhibitory action of TGF β on the 1,25-(OH)₂D₃ 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 TGF β and 1,25-(OH)₂D₃ 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)₂D₃ and TGF β . 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).

TGF β and 1,25-(OH)₂D₃ are involved in regulation of both bone formation and resorption. The steroid hormone 1,25-(OH)₂D₃ 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)₂D₃ 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 D₃ 24-hydroxylase (17–20), calbindin D28k (21), and β_3 integrin (22).

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

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development and bone remodeling (23, 24). In mammals, three isoforms of TGF β have been identified: TGF β_1 , TGF β_2 , and TGF β_3 (25, 26). TGF β is present as a latent complex in bone and released from the extracellular matrix in an active form during bone resorption by osteoclasts (27). TGF β 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 TGF β is functionally coupled. For example, combined treatment with TGF β 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 TGF β 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 TGF β_1 and TGF β_2 on the 1,25-(OH) $_2$ D $_3$ enhancement of osteocalcin and osteopontin gene transcription. We show that TGF β 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 μ g/ml streptomycin, and 5% FCS at 37 C under 95% air/5% CO $_2$. For the experiments, cells were seeded in 100 \times 20-mm plastic dishes at 0.7 \times 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 TGF β_1 or TGF β_2 . 1,25-(OH) $_2$ D $_3$ was kindly provided by Dr. M. Uskokovic, Hoffman-La Roche Inc. (Nutley, NJ). TGF β_1 was purchased from R&D Systems (Minneapolis, MN). TGF β_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 PMSE, 70 μ g/ml N-tosyl-L-phenylalanine chloromethyl ketone, 10 μ g/ml trypsin inhibitor, 0.5 μ g/ml leupeptin, and 1.0 μ g/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'-CTGCACTGGGTGAATGAG-GACATTACTGA-3'; mouse osteopontin VDRE (16): 5'-ACAAGGT-TCACGAGGTTACCGTCT-3'; metallothionein II/activator protein-1 (AP-1) consensus: 5'-AATTCGTGACTCAGCGCGG-3'; transcription factor SP-1 consensus: 5'-CGGATGGCGGGCCGGGATGGGCGG-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 μ l of a 5 μ g protein mixture in a final KCl concentration of 100 mM with 10 μ l of a DNA mixture containing 1 fmol/ μ l DNA, 0.2 μ g/ μ l nonspecific competitor DNA, poly(dI-dC)(dI-dC) and 2 mM di-

thiothreitol for 15 min. Protein/DNA complexes were separated in a 4% polyacrylamide gel (acrylamide:bisacrylamide = 80:1) in 0.5 \times 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 β gIIICAT 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 \times 10 5 per well in six-well plates. CAT reporter gene constructs (5 μ g) were cotransfected with 2 μ g 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 TGF β_1 , TGF β_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 (Betascop 603 Betagen, Waltham, MA).

Northern blot analysis

RNA isolation, electrophoresis of total RNA (10 μ g) 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 \times 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 TGF β 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 μ l 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 *).

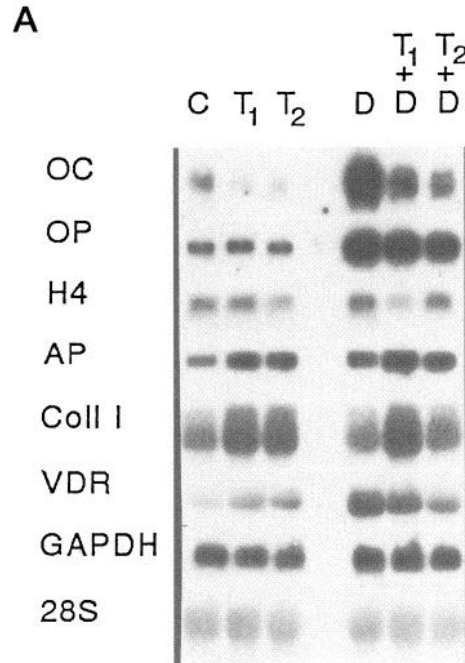
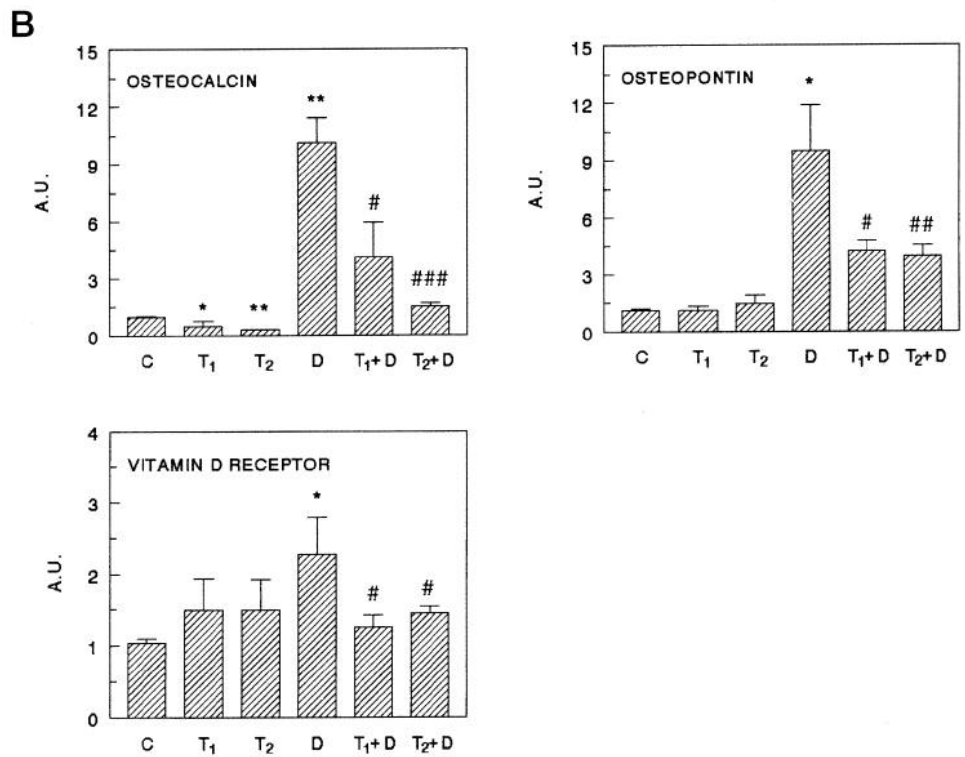


FIG. 1. TGFβ and/or 1,25-(OH)₂D₃ affect the expression of mRNAs related to osteoblast function and differentiation. Cells were cultured as described in *Materials and Methods* and incubated for 24 h with 2.5 ng/ml TGFβ₁ (T₁), 2.5 ng/ml TGFβ₂ (T₂), and/or 10⁻⁸ M 1,25-(OH)₂D₃ (D) or vehicle (C). Northern 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 TGFβ₁, TGFβ₂, and 1,25-(OH)₂D₃ influence expression of genes related to osteoblast function and differentiation

As osteoblasts are responsive to TGFβ₁ and TGFβ₂ 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)₂D₃ on OC and OP gene expression. We have previously established that under our experimental conditions, 1,25-(OH)₂D₃ enhancement of OC gene expression is maximal at 24 h after administration of the

ligand (9). Also, although TGF β modulates parameters of 1,25-(OH) $_2$ D $_3$ -mediated gene expression as a component of an earlier response (*i.e.* 6 h), significant effects of TGF β on OC gene expression are not observed until the 24-h time point (37).

The combined effects of TGF β and 1,25-(OH) $_2$ D $_3$ 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) $_2$ D $_3$ treatment. Both TGF β_1 and TGF β_2 inhibited the 1,25-(OH) $_2$ D $_3$ -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 TGF β on basal level of OC gene expression. For 1,25-(OH) $_2$ D $_3$ -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, TGF β and 1,25-(OH) $_2$ D $_3$ 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 TGF β and 1,25-(OH) $_2$ D $_3$ treatment, demonstrating the selective responsiveness of the OC and OP genes to these factors.

Effects of combined treatment with TGF β and 1,25-(OH) $_2$ D $_3$ on OC and OP transcription

1,25-(OH) $_2$ D $_3$ 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 TGF β inhibits 1,25-(OH) $_2$ D $_3$ 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 TGF β and/or 1,25-(OH) $_2$ D $_3$. 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 TGF β_1 and TGF β_2 . 1,25-(OH) $_2$ D $_3$ increased basal CAT activity 8-fold in 24 h. This increase was inhibited 20- to 30-fold after incubation with either TGF β_1 or TGF β_2 . Thus, in addition to a significant inhibition of basal transcription, both TGF β subtypes abrogated 1,25-(OH) $_2$ D $_3$ enhanced OC transcription.

To ascertain the extent to which TGF β effects on OC depend on VDRE sequences, ROS 17/2.8 cells were transfected with a construct in which the VDRE was deleted (pBgIIICAT; Fig. 2B). As expected, the construct did not respond to 1,25-(OH) $_2$ D $_3$. TGF β_1 or TGF β_2 either alone or in the presence of 1,25-(OH) $_2$ D $_3$ caused a 4- to 6-fold decrease in CAT activity. This inhibitory effect of TGF β on basal OC gene transcription

TABLE 1. Effect of either TGF β_1 or TGF β_2 and 1,25(OH) $_2$ D $_3$ on osteocalcin secretion in medium of ROS 17/2.8 cells (ng/ml)

	Control	TGF β_1	TGF β_2
control	2.76 \pm 0.23	2.13 \pm 0.44	2.26 \pm 0.40
1,25(OH) $_2$ D $_3$	15.45 \pm 2.15	3.44 \pm 0.35	3.14 \pm 0.08

Cells were cultured as described (*Materials and Methods*) and incubated for 24 h with 2.5 ng/ml TGF β_1 , 2.5 ng/ml TGF β_2 , and/or 10 $^{-8}$ M 1,25(OH) $_2$ D $_3$. Data are expressed as mean \pm SD.

is consistent with the presence of a TGF β response element in the proximal promoter (47). Notably, the fold decrease resulting from coinubation of TGF β and 1,25-(OH) $_2$ D $_3$ was significantly less for the OC promoter CAT construct lacking the VDRE (4- to 6-fold; pBgIIICAT) as compared with the OC CAT construct containing the VDRE (20- to 30-fold; pOCZCAT). To assess the promoter selectivity of TGF β 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). TGF β has only modest inhibitory effects on the virally derived SV40 promoter (1.3-fold; data not shown).

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 TGF β_1 or TGF β_2 (2-fold). 1,25-(OH) $_2$ D $_3$ caused an 11-fold increase in CAT activity, which was inhibited 7-fold by TGF β_1 and TGF β_2 . The OP construct lacking the VDRE (pOP552CAT; Fig. 3B) does not respond to 1,25-(OH) $_2$ D $_3$. We noted that a modest level of TGF β inhibition (2-fold) of OP basal promoter activity persists. Taken together, our results suggest that TGF β exerts a major effect on 1,25-(OH) $_2$ D $_3$ -mediated OC and OP transcription and requires a VDRE containing promoter segment.

To investigate directly the effect of TGF β and 1,25-(OH) $_2$ D $_3$ 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) $_2$ D $_3$ increased basal luciferase activity approximately 4-fold in 24 h. This increase was inhibited 50% and 30% by TGF β_1 and TGF β_2 , respectively, indicating that the inhibitory effect of TGF β on 1,25-(OH) $_2$ D $_3$ enhancement of OC transcription is at least partly mediated by the VDRE.

TGF β and 1,25-(OH) $_2$ D $_3$ modulate protein/DNA interactions at VDREs

To address directly the mechanism by which TGF β abrogates 1,25-(OH) $_2$ D $_3$ -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 TGF β and/or 1,25-(OH) $_2$ D $_3$, 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) $_2$ D $_3$ (16, 40, 48–50). Nuclear proteins from control or TGF β_2 -treated cells do not show VDRE protein/DNA interactions. However, combined treatment of 1,25-(OH) $_2$ D $_3$ with TGF β caused a significant reduction of the VDR containing complex at both the OC and OP VDREs. Similar reductions in VDRE complexes were observed in TGF β_1 -treated cells (data not shown). This indicates that the inhibitory effects by TGF β_1 and TGF β_2 on the 1,25-(OH) $_2$ D $_3$ -induced increase in OC and OP transcription are at least in part due to decreased binding of transcription factors to the VDRE.

Because both TGF β and 1,25-(OH) $_2$ D $_3$ modulate expression of the fos and jun family of proto-oncogenes in osteoblasts (34, 51–53), we also examined the effects of these agents

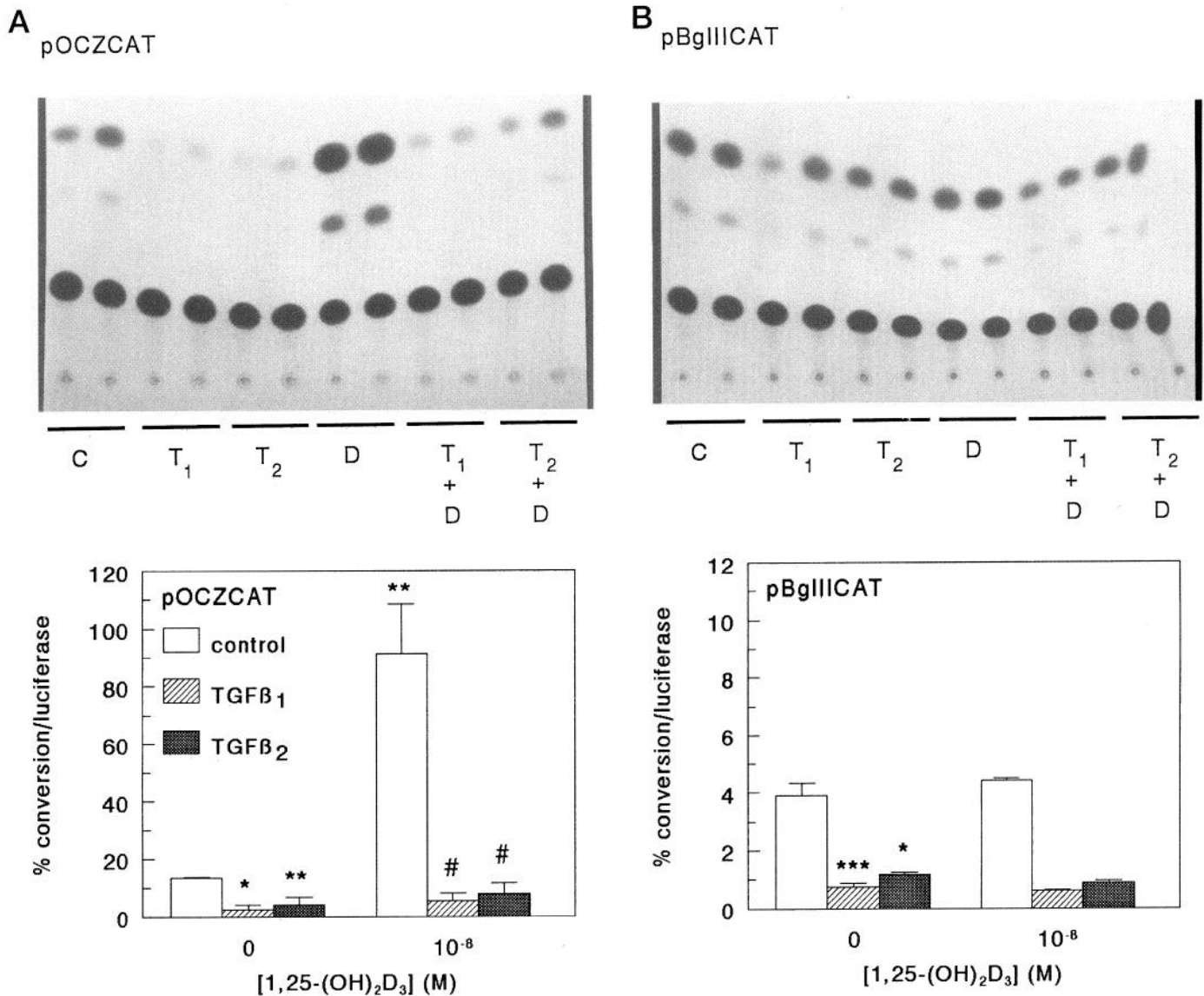


FIG. 2. TGF β ₁ and TGF β ₂ antagonize basal and 1,25-(OH)₂D₃ stimulated OC transcription. ROS 17/2.8 cells were transiently transfected with the following osteocalcin promoter-CAT fusion constructs: A) pOCZCAT, -1097/+23, and B) pBgIIICAT -348/+23. Cells were transfected and 4 h later incubated for 24 h in the absence (control) or presence of 2.5 ng/ml TGF β ₁ (T₁), on TGF β ₂ (T₂). These treatment groups were coincubated with either vehicle (C, O) or 1,25-(OH)₂D₃ (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 \pm 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 TGF β or 1,25-(OH)₂D₃, although the increase observed with TGF β was more pronounced than with 1,25-(OH)₂D₃. 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 TGF β - and 1,25-(OH)₂D₃-mediated signaling mechanisms that modulate nuclear transcription factor levels.

VDR protein levels are not rate-limiting for VDRE interactions in the presence of TGF β and 1,25-(OH)₂D₃

Because changes in VDR mRNA levels were observed after treatment with TGF β ₁, TGF β ₂, and 1,25-(OH)₂D₃ (Fig. 1), we studied the effects of TGF β ₁ and TGF β ₂ in the absence or presence of 1,25-(OH)₂D₃ on VDR protein levels, as a possible mechanism for the inhibitory effects of TGF β on VDR/RXR interactions at the OC and OP VDREs. We investigated the effect of TGF β 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)₂D₃, and that neither TGF β ₁ nor TGF β ₂ affects the 1,25-(OH)₂D₃-

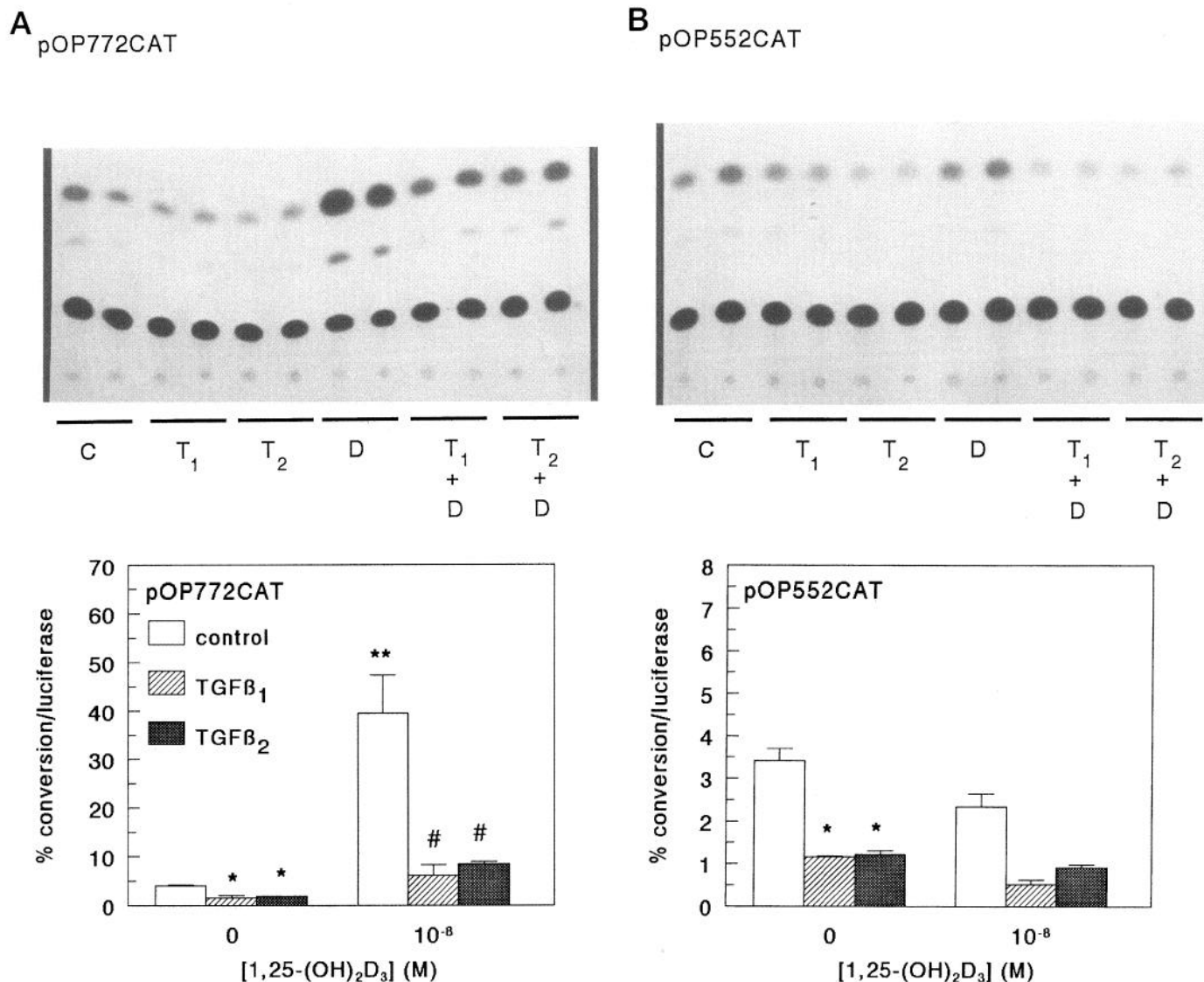


FIG. 3. Effects of TGF β ₁ and TGF β ₂ on 1,25-(OH)₂D₃-stimulated OP transcription. ROS 17/2.8 cells were transiently transfected with the following osteopontin promoter-CAT fusion constructs: A, pOP772CAT, -772/+40; B, pOP552CAT -552/+40. Cells were transfected and 4 h later incubated for 24 h in the absence (control) or presence of 2.5 ng/ml TGF β ₁ (T₁), or TGF β ₂ (T₂). These treatment groups were coincubated with either vehicle (C, O) or 1,25-(OH)₂D₃ (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 \pm 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)₂D₃-treated lanes appear to reflect similar variations in the nonspecific band migrating slower than the VDR. The lack of an effect by TGF β on VDR protein levels in the presence of 1,25-(OH)₂D₃ 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)₂D₃ treatment was not affected by cotreatment with either TGF β ₁ or TGF β ₂ (data not shown). Ligand binding assays in whole cell extracts using isotopically labeled 1,25-(OH)₂D₃ (Fig. 7B) also demonstrate homologous up-regulation of 1,25-(OH)₂D₃-receptor number as reflected by a 5-fold increase in ligand binding after 1,25-(OH)₂D₃ treatment. Cotreatment with TGF β (24 h) does

not affect the homologous up-regulation of 1,25-(OH)₂D₃-receptor number in the presence of 1,25-(OH)₂D₃. Thus, the evidence presented in Fig. 7 indicates that TGF β 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 TGF β 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 TGF β administration (37). The modestly elevated levels of VDR protein after 24 h of TGF β treatment therefore may represent a residual, and perhaps persistent, component of TGF β responsiveness. Our previous observation that TGF β enhances VDR levels in whole cell extracts as a component of an earlier response (*i.e.* 6 h) (37) and our

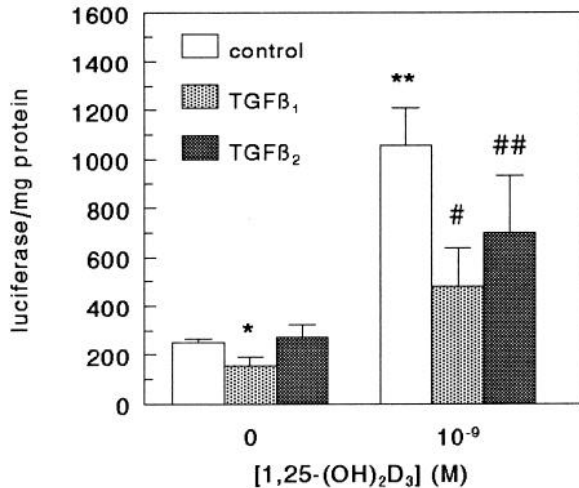


FIG. 4. TGF β_1 and TGF β_2 inhibit 1,25-(OH)₂D₃ enhancement of OC VDRE-mediated transcription. ROS 17/2.8 cells were transiently transfected with the (VDRE)₃-tk-luciferase construct. Cells were transfected and 4 h later incubated for 24 h in the absence or presence of 2.5 ng/ml TGF β_1 and TGF β_2 . These treatment groups were incubated with either vehicle or 1,25-(OH)₂D₃ (10⁻⁸ M). Bar graphs represent mean \pm 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 TGF β 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)₂D₃ and TGF β . The basis of our studies is supported by previous studies (36, 37) and our present findings, which show that TGF β inhibits the 1,25-(OH)₂D₃-induced increase of both OC and OP mRNA levels. The physiological relevance of TGF β inhibition of the 1,25-(OH)₂D₃ 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 TGF β_1 and TGF β_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 TGF β_1 - and TGF β_2 -mediated cell signaling pathways operate via shared TGF β 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)₂D₃ related effects of TGF β on expression of the OC and OP genes are mediated by transcriptional mechanisms. TGF β abrogates 1,25-(OH)₂D₃ enhancement of OC transcription, which requires the presence of the OC VDRE within the promoter. However, TGF β also inhibits basal OC transcription in the absence of 1,25-(OH)₂D₃, which occurs irrespective of the presence of VDRE sequences. TGF β repression of basal OC gene tran-

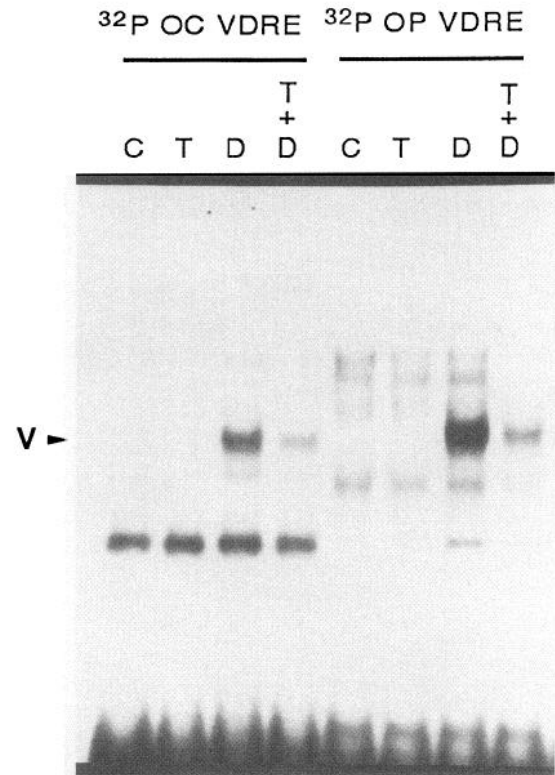


FIG. 5. TGF β modulates interactions of endogenous 1,25-(OH)₂D₃-inducible VDR/RXR complexes from ROS 17/2.8 cells. Binding of nuclear proteins to the OC VDRE and OP VDRE was analyzed in gel mobility shift assays. A, Cells were incubated for 24 h with vehicle (C), 2.5 ng/ml TGF β_2 (T), 10⁻⁸ M 1,25-(OH)₂D₃ (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.

scription is mediated by a TGF β 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 TGF β increases AP-1 binding activity both in the presence or absence of 1,25-(OH)₂D₃ in ROS 17/2.8 cells. Thus, increased levels of AP-1 binding may contribute to inhibition of OC gene transcription.

We note that TGF β also inhibits basal OP gene transcription. This inhibition of OP gene transcription, as monitored by reporter gene expression after treatment with TGF β for 24 h, is not reflected by changes in OP mRNA levels. It appears that the TGF β dependent inhibition of OP transcription may be compensated by posttranscriptional modulatory events, including mRNA processing, transport, and/or stability. Alternatively, the observed differences between TGF β 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 TGF β 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 TGF β . This apparent discrepancy in the observed effects of TGF β on OP gene expression may relate to differences in experimental design, as well as perhaps reflect chronic effects of TGF β on cell growth and differentiation of osteoblasts.

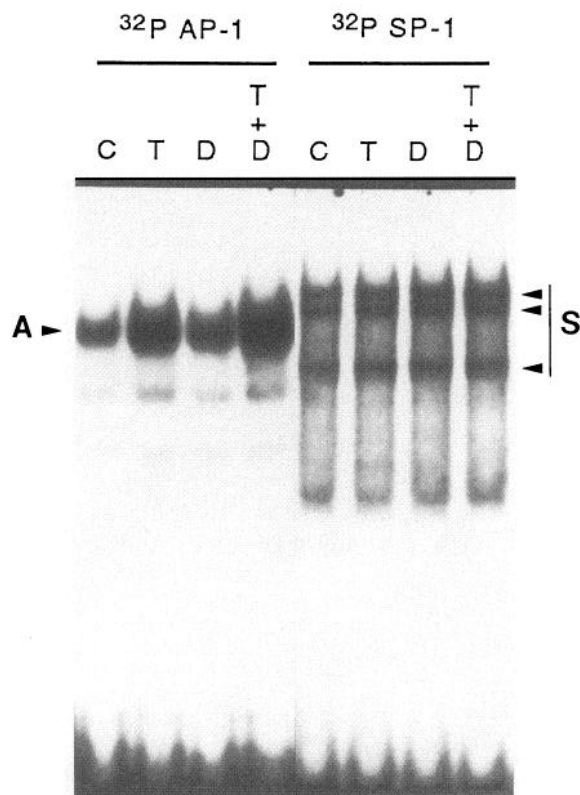


FIG. 6. Selective enhancement of AP-1 activity by TGF β and 1,25-(OH) $_2$ D $_3$. Gel mobility shift assays were performed with AP-1 and SP-1 consensus oligonucleotides. Nuclear extracts were identical to those in Fig. 5. The same abbreviations and symbols are as indicated in Fig. 5. The AP-1 complexes (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.

We tested the hypothesis that transcriptional repression of 1,25-(OH) $_2$ D $_3$ -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) $_2$ D $_3$ 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 TGF β inhibits both 1,25-(OH) $_2$ D $_3$ enhancement of OC VDRE-mediated transcription and the 1,25-(OH) $_2$ D $_3$ -dependent induction of VDR containing transcription factor complexes interacting with the OC- and OP-VDREs. Thus, we propose that TGF β may directly influence OC and OP gene transcription by modulating 1,25-(OH) $_2$ D $_3$ -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) $_2$ D $_3$ -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) $_2$ D $_3$ -dependent manner, which is reflected by increased VDR protein levels as monitored by

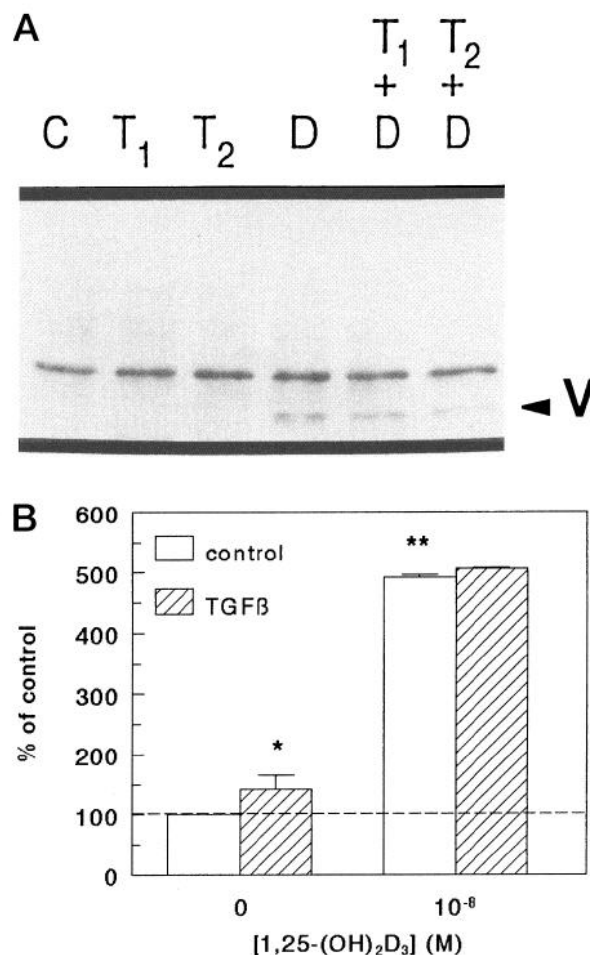


FIG. 7. Homologous up-regulation of VDR protein levels by 1,25-(OH) $_2$ D $_3$ is not affected by TGF β . Western blot analysis (A) was performed with nuclear proteins from ROS 17/2.8 cells, which were separated in a 12% SDS-PAGE gel and transferred to a solid support. Detection of VDR levels was performed 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) $_2$ D $_3$ -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) $_2$ D $_3$ were carried out with cytosolic preparations from ROS 17/2.8 cells treated with 1,25-(OH) $_2$ D $_3$ and/or TGF- β_2 . The values on the ordinate were calculated by dividing fmol [3 H] 1,25-(OH) $_2$ D $_3$ bound per mg protein by the values obtained from untreated cells. Bar graphs represent mean \pm SD of duplicates from two independent 1,25-(OH) $_2$ D $_3$ ligand binding experiments. *, $P < 0.05$; **, $P < 0.0001$ vs. control.

Western blot analysis and 1,25-(OH) $_2$ D $_3$ ligand binding assays, as well as induction of VDR/RXR heterodimer interactions with the VDRE. In coinubation experiments with 1,25-(OH) $_2$ D $_3$, TGF β reduces the 1,25-(OH) $_2$ D $_3$ -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) $_2$ D $_3$ 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 TGF β and 1,25-(OH) $_2$ D $_3$. Thus, the mechanism by which TGF β reduces VDR/RXR heterodimer interactions at VDREs appears to involve posttranslational events, e.g. phosphorylation and/or protein/protein interactions, including effects of TGF β 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 TGF β -mediated signal transduction occurs via protein kinase C (63) and 1,25-(OH) $_2$ D $_3$ action also involves protein kinase C (61, 64), the possibility arises that TGF β 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) $_2$ D $_3$ and TGF β in the regulation of bone metabolism and parameters of osteoblast differentiation are strongly interrelated (34, 36–38). TGF β is capable of increasing 1,25-(OH) $_2$ D $_3$ receptor number (65, 37). 1,25-(OH) $_2$ D $_3$ 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) $_2$ D $_3$ responsive genes. 1,25-(OH) $_2$ D $_3$ also stimulates TGF β synthesis and secretion by bone cells, as well as enhances TGF β incorporation into bone (66, 67). The inhibitory effect of TGF β on 1,25-(OH) $_2$ D $_3$ -enhancement may provide a negative regulatory loop in the action of 1,25-(OH) $_2$ D $_3$.

The results presented here suggest that TGF β influences OC and OP gene transcription. The effect of TGF β on OC gene transcription may occur via a two-pronged mechanism. First, TGF β abrogates 1,25-(OH) $_2$ D $_3$ enhancement of OC transcription by down-regulating binding of VDR/RXR heterodimers to the OC VDRE. Secondly, TGF β also inhibits OC transcription in the presence or absence of 1,25-(OH) $_2$ D $_3$, which occurs via AP-1 binding sites (47, 53), perhaps mediated by TGF β enhanced levels of AP-1 proteins. Similarities in the inhibitory effect of TGF β on 1,25-(OH) $_2$ D $_3$ -enhanced expression of two genes, OC and OP, encoding proteins with distinct properties, are consistent with functional linkage of these proteins in bone turnover.

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