Identification of 1α,25-Dihydroxyvitamin D_3 Response Elements in the Human Transforming Growth Factor β2 Gene†

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ABSTRACT: Transforming growth factor-β (TGF-β) is one of the most abundant growth factors secreted by bone cells, and regulation of TGF-β expression is crucial for bone development and growth. Previous studies from our laboratory demonstrated that 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) inhibits human osteoblast and keratinocyte growth by increasing TGF-β2 secretion and synthesis. To examine the mechanism by which 1α,25$(OH)_2D_3$ regulates TGF-β2 transcription in osteoblasts, we ligated segments of the human TGF-β2 promoter 5′ of a growth hormone reporter gene in a growth hormone reporter plasmid and examined the effects of 1α,25$(OH)_2D_3$ administration on the expression of growth hormone in cells transfected with such chimeric promoter−reporter plasmids. The promoter region extending from −973 to +68 bp of the transcription start site elicited a 7-fold increase in reporter gene activity in transiently transfected osteoblasts after 1α,25$(OH)_2D_3$ treatment, whereas the region from −553 to +68 bp of the transcription start site showed no response following 1α,25$(OH)_2D_3$ treatment. Transfection of osteoblasts with reporter plasmids containing TGF-β2 promoter DNA from −869 to −658 bp revealed a 3.8-fold increase in reporter gene activity. DNA fragments from this region (−743 to −676 bp and −786 to −728 bp) ligated into reporter plasmids also showed increases in reporter activity. Gel retardation assay experiments showed that DNA fragments from −774 to −735 bp and −714 to −675 bp both formed a DNA−protein complex with bacterially expressed human retinoic acid X receptor α (RXRα) and vitamin D receptor (VDR) and with nuclear extracts from human bone cells. Addition of a vitamin D receptor antibody to reactions containing the aforesaid DNA fragments and bone cell nuclear extract resulted in further retardation of the mobility of the DNA−protein complex (super-shifting). Removal of two putative direct repeat DNA fragments in this region abolished VDR−RXRα−vitamin D response element complex formation. The TGF-β2 promoter contains two imperfect direct repeat DNA sequences: TGTAGAACAAGTAGA and AATGAAGTTGGTGGA that mediate the effect of 1α,25$(OH)_2D_3$.

Vitamin D_3, via its active metabolite, 1α,25$(OH)_2D_3$,[1] plays a significant role in the control of calcium and phosphate homeostasis, cellular differentiation, and the regulation of gene transcription (*1*−*3*). In osteoblast-like cells, cellular growth and the synthesis of collagen, alkaline phosphatase, and osteocalcin are influenced by 1α,25$(OH)_2D_3$ (*1*−*7*). In addition to systemic hormones, various cytokines and local growth factors such as TGF-β, have been shown to be indispensable to osteoblast differentiation and function such as matrix deposition and mineralization. TGF-β is involved in bone fracture repair, bone induction, and development (*8*−*10*). We have previously shown that 1α,25$(OH)_2D_3$ induces TGF-β2 synthesis in human fetal osteoblast cells and keratinocytes (*11*, *12*). In osteoblasts, TGF-β2 gene transcription is increased by 1α,25$(OH)_2D_3$ (*12*). The mechanism by which 1α,25$(OH)_2D_3$ induces TGF-β2 gene transcription, however, is not clear. The effects of 1α,25-$(OH)_2D_3$ are primarily mediated by its nuclear receptor (the vitamin D receptor or VDR) which is a member of the large family of nuclear ligand-activated receptors that function as transcription factors (*13*, *14*). The VDR is a 48 kDa zinc-finger protein (*5*, *15*) which activates transcription by binding to vitamin D response elements (VDREs) within the promoters of vitamin D responsive genes, either as a homodimer (*16*, *17*) or a heterodimer with the retinoic acid X receptor α (RXRα) (*18*−*21*), retinoic acid receptor (RAR) (*22*, *23*), or thyroid hormone receptors (T_3R) (*24*). Vitamin D response elements, to which the VDR binds, are comprised of direct repeats (DRs), palindromes (Ps), or inverted palindromes (IPs) of the hexameric core binding motif RRKNSA (R = A or G, K = G or T, S = C or G) (*17*, *24*, *25*).

To determine whether 1α,25$(OH)_2D_3$ induces TGF-β2 expression in osteoblasts via such repeat elements, we transiently transfected human fetal osteoblasts (hFOB cells) with a series of TGF-β2 promoter/human growth hormone

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[1] Abbreviations: TGF-β, transforming growth factor-β; 1α,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; hFOB, human fetal osteoblast; VDR, vitamin D receptor; RXR, retinoic acid X receptor; RAR, retinoic acid receptor; VDRE, vitamin D response element; hGH, human growth hormone; PCR, polymerase chain reaction; DIG, digoxigenin; mOP, mouse osteopontin; CSPD, disodium 3-(4-methoxyspiro(1,2-dioxetane-3,2′-(5′-chloro)tricyclo(3.3.1.1)decan)-4-yl)phenoyl phosphate.

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(hGH) reporter constructs. Cells transfected in this manner were treated with 1α,25(OH)$_2$D$_3$, and growth hormone concentrations in the supernatant media were assayed. Using such methods we identified two direct repeat vitamin D response elements in the human TGF-β2 promoter. We show that heterodimers of the VDR and RXRα bind to sequences in the promoter of the TGF-β2 gene. Two direct repeat elements in the TGF-β2 gene mediate this effect.

MATERIALS AND METHODS

General. Concentrations of 1α,25(OH)$_2$D$_3$ (a gift from Dr. M. Uskokovic, Hoffmann-LaRoche, Nutley, NJ) in ethanol ($\epsilon_{265\ nm}$ = 18 200 M^{-1} cm^{-1}) and oligonucleotides and nucleic acids in water or aqueous buffer were determined by ultraviolet spectroscopy with a Beckman DU-70 spectrophotomer (Beckman Instruments, Fullerton, CA). Oligonucleotides were synthesized on an Applied Biosystems Oligonucleotide synthesizer (Applied Biosystems, Foster City, CA) using phosphoramidate chemistry (*26*). Polymerase chain reaction (PCR) methods were used to synthesize segments of the TGF-β2 promoter (*12*). Initial subcloning of PCR DNA fragments was carried out using an Invitrogen TA cloning kit (Invitrogen Corporation, San Diego, CA). DNA sequencing was performed by Sanger dideoxy sequencing methods (*27*) on an Applied Biosystems 373A DNA sequencer (Applied Biosystems, Foster City, CA). Polyacrylamide gel electrophoresis was carried out using precast gels (Jule Inc. Biotechnology, New Haven, CT) and a Bio-Rad gel system (Bio-Rad, Hercules, CA).

Synthesis of TGF-β2 Promoter Constructs. Series of TGF-β2 promoter constructs were synthesized by PCR amplification of the TGF-β2 promoter region and ligated into the pOGH plasmid or into the pTKGH plasmid (Nichols Institute Diagnostics, San Juan Capistrano, CA). In pOGH plasmid constructs, the TGF-β2 native promoter was used for gene expression. In the pTKGH plasmid constructs, a heterologous herpes simplex virus thymidine kinase (TK) promoter was used for gene expression in addition to small fragments of the TGF-β2 promoter DNA.

Cell Culture and Transfection of hFOB Cells. Human fetal osteoblast cells (hFOB) were a gift from Dr. T. C. Spelsberg (*28*). The cells were maintained in DMEM/F12 medium supplemented with 10% fetal bovine serum and 300 μg/mL G418. For transfection, 1 $\times$ 10^5 hFOB cells per well were seeded in 6-well plates and grown for 24 h in DMEM/F12 medium supplemented with 10% FBS and 300 μg/mL G418. FuGENE transfection reagent (6 μL) (Boehringer Mannheim, Indianapolis, IN) and 2 μg of DNA (1 μg of plasmids with various lengths of the TGF-β2 promoter ligated in pOGH or pTKGH plasmids plus 1 μg of pPGKlacZ as an internal control of the transfection efficiency) were added into each well. After incubation for 20 h at 37 °C, 2 mL of serum free medium was replaced with growth medium containing 10% FBS, and the cells were treated with 10^{-8} M 1α,25(OH)$_2$D$_3$ or an equal volume of vehicle (ethanol). After 40 h, hGH concentration was determined in supernatant medium from each well, and β-galactosidase activity was assayed in cell lysates.

Human Growth Hormone Enzyme-Linked Immunosorbent Assay (ELISA). The assay was performed using an hGH ELISA kit (Boehringer Mannheim, Indianapolis, IN). In brief, cell culture supernatants were collected and centrifuged to remove cellular debris. Cell supernatants (200 μL) were added to microtiter plate wells coated with a polyclonal hGH antibody, and the plates were incubated at 37 °C for 1 h. Following removal of the supernatants, the wells were washed 5 times with PBS. Anti-hGH-digoxigenin (200 μL) (DIG) (1 μg/mL) was added to each well. The plates were incubated for 1 h at 37 °C after which solutions were removed and the wells were washed five times with PBS. Anti-DIG-peroxidase (200 μL) (POD) (200 mU/ml) was added to each well, and the plates were incubated for an additional 1 h at 37 °C. After the solution was removed and the wells were washed 5 times, POD substrate was added and the plates were incubated at room temperature for 30 min. The absorbance of each well was measured at 405 nm, with a reference wavelength of 490 nm, using a microtiter plate reader.

β-Galactosidase Assay. To measure transfection efficiency, cells were pelleted and lysed in 0.5 mL of lysis buffer (0.25 M Tris, pH 8.0). The suspension was frozen on dry ice and thawed in a 37 °C water bath. Freezing and thawing cycles were repeated 2 times. Insoluble cell material was pelleted by centrifugation, and the supernatant was used to assay β-galactosidase (Invitrogen Corporation, San Diego, CA).

Human Bone Cell Nuclear Extracts. Human bone cell (hFOB) nuclear extracts were isolated as previously described (*29*).

Expression of VDR and RXRα. The cDNA for human VDR was provided by J. W. Pike (Ligand Pharmaceuticals, San Diego, CA), and the cDNA for human retinoid X receptor α (RXRα) was provided by R. Evans (The Salk Institute for Biological Studies, La Jolla, CA). PCR and synthesis of hVDR and RXRα expression vectors was carried out as previously described for the ligand-binding domain and DNA-binding domain of the human vitamin D receptor (*31, 32*). Full-length hVDR and human RXRα were expressed at low temperature (20 °C) as GST fusion proteins in the pGEX-4T-2 vector using *Escherichia coli* BL21 cells (Pharmacia Biotech, Piscataway, NJ). Fusion protein eluted from glutathione sepharose was cleaved with thrombin and further purified by ion exchange chromatography. The full length hVDR was purified by Mono Q chromatography (Mono Q HR 10/10) (Pharmacia Biotech, Piscataway, NJ) using running buffer (50 mM Tris, 10 mM DTT, pH 8.0). Protein was eluted by a gradient of 0−1 M NaCl in running buffer. Characterization of purified proteins was carried out by SDS−PAGE performed as previously described (*32*). Purified RXRα consisted of residues 26−462 in human RXRα (26−462 RXRα), residues 1−25 having been cleaved by thrombin at an internal thrombin site (Gly24-Arg25-Gly26). Purified VDR consisted of the full length protein (residues 1−427) with an N-terminal Gly-Ser extension derived from the thrombin cleavage site in the GST fusion protein. Amino acid analyses were consistent with the expected protein constructs. The molecular masses as determined by electrospray ionization mass spectrometry were consistent with the other characterization data (*33*). For gel shift assays, protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Electrophoretic Gel Mobility Shift Assay. The following oligonucleotides were used in electrophoretic gel mobility shift analyses. TGF-β2 promoter sequence: (−774 to −735

bp) 5′AAGTTATGAGTAGTGTAGAACAAGTAGACATCAAACACTT 3′ and (−714 to −675 bp) 5′ TATGCTATGGAAAGAATGAAGTTGGTGGATAATGTTTAGC 3′. Mouse osteopontin vitamin D response element (mOP VDRE): 5′ GCTCGGGTAGGGTTCACGAGGTTCACTCGACTCG 3′, used as positive control. Another two oligonucleotides contain TGF-β2 promoter sequences: (between −785 and −731 bp) 5′ TTGAAAATGTCAAGTTATGAGTAGCATCAAACACTTAAAA 3′ and (between −725 and −671 bp) 5′ CTTCCTGGATTATGCTATGGAAAGTAATGTTTAGCCTAGC 3′, but without the putative VDREs. In all cases, the complementary strand was also synthesized, and annealing to form double-stranded DNA was performed as described below. Oligonucleotides were purified through NAP-25 columns (Pharmacia Biotech, Piscataway, NJ). Annealing was carried out by mixing complementary single-stranded oligonucleotides in a molar ratio of 1:1 in TEN buffer (10 mM Tris-HCl, 1 mM EDTA, 0.1 M NaCl, pH 8.0) and incubating the mixture at 95 °C for 5 min. The mixtures were then cooled slowly to room temperature. The annealed double-stranded oligonucleotides (4 pmol) were labeled at 37 °C for 15 min with 1 μL DIG-11-ddUTP (1 mM) (Boehringer Mannheim, Indianapolis, IN), 50 units of terminal transferase, and labeling buffer (0.2 M potassium cacodylate, 25 mM Tris-HCl, pH 6.6, 0.25 mg/mL BSA, 5 mM $CoCl_2$). Unincorporated oligonucleotides were removed by ethanol precipitation. Labeled oligonucleotides, with or without cold oligonucleotides, were mixed with recombinant RXRα and VDR at a 1:3 ratio of DNA/protein in the binding buffer (20 mM Hepes, pH 7.6, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, Tween 20, 0.2% (w/v), 30 mM KCl, 1 μg/μL poly dI-dC). Mixtures were incubated at 30 °C for 30 min. The reaction mixtures were electrophoresed on a 5% polyacrylamide gel in 0.25X TBE (IX TBE is 0.09 M Tris-borate, 0.002 M EDTA) buffer at 150 V. The DNA−protein complexes were transferred onto positively charged nylon membranes (Boehringer Mannheim, Indianapolis, IN) and cross-linked to the membrane using UV light (UV Stratalinker 2400, Stratagene, La Jolla, CA). The membrane was briefly washed in washing buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5, Tween 20, 0.3% v/v), blocked for 30 min, and then incubated with anti-digoxigenin-AP, Fab fragment (0.075 units/mL) for 30 min. After two 15 min washes, the membrane was incubated for 10 min in substrate solution (0.1 mg/mL CSPD, 0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5) in a hybridization bag. After incubating at 37 °C for 10 min, the membrane was exposed to Bio Max MR film with an intensifying screen for 25 min at room temperature. For experiments with bone cell nuclear extracts, oligonucleotides from −774 to −735 and −714 to −675 were annealed to their complementary strands as for digoxigenin gel shifts and radiolabeled as described previously (*31*). Gel mobility shift assays in the presence or absence of VDR antibody were carried out as described previously except that 4% acrylamide gels and 0.25X TBE gel buffer were used with 5 μg of human osteoblast cell nuclear extract in binding buffer (20 mM Hepes, 1 mM EDTA, 1 mM DTT, 30 mM KCl, 10% glycerol, pH 7.6) and 1.6 × 10^6 cpm ^{32}P-labeled probe incubated for 30 min at 30 °C (*31*).

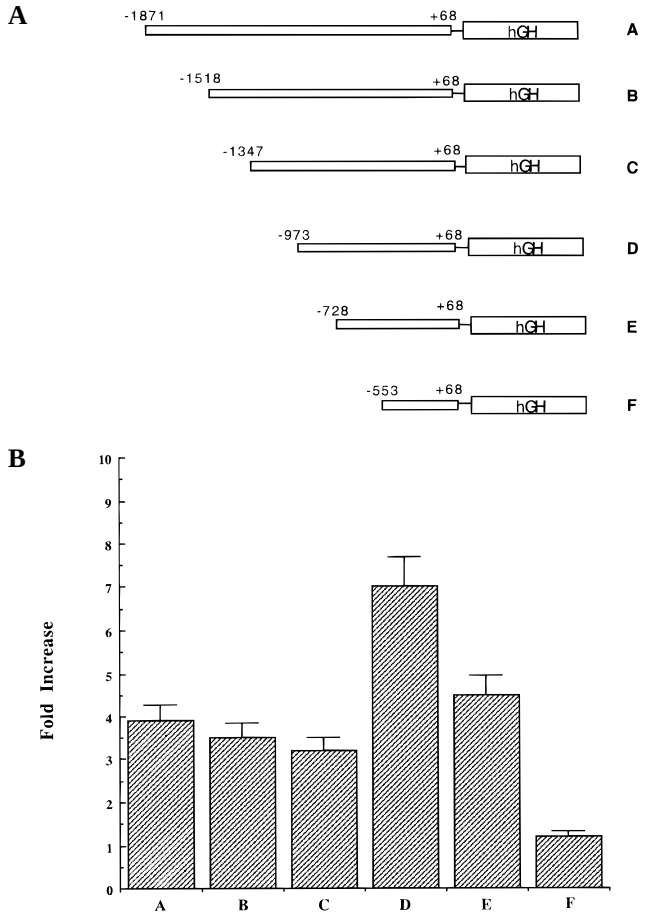

FIGURE 1: (A) Schematic representation of TGF-β2 promoter/hGH chimeric constructs. The constructs were cloned into pOGH. The region of TGF-β2 promoter is represented by an open bar while the hGH reporter sequence is marked as hGH in the open bar. (B) ELISA measuring the expression of human growth hormone in the supernatant of hFOB cells. The hFOB cells were transfected with a series of TGF-β2 promoter/hGH chimeric constructs and treated with 10^{-8} M 1α,25$(OH)_2D_3$ for 40 h. The fold increase is relative to cells treated with same volume of ethanol. Data are means ± SE of three experiments.

RESULTS

To detect and localize the DNA sequences in the TGF-β2 promoter responsible for 1α,25$(OH)_2D_3$-mediated stimulation of transcription, we synthesized a series of TGF-β2 promoter/hGH reporter constructs which included various lengths of the TGF-β2 promoter sequence fused to the human growth hormone reporter gene in the pOGH plasmid. The constructs are as follows: A (−1871 to +68 bp/hGH), B (−1518 to +68 bp/hGH), C (−1347 to +68 bp/hGH), D (−973 to +68 bp/hGH), E (−728 to +68 bp/hGH), and F (−553 to +68 bp/hGH) (Figure 1A). After transfection of hFOB cells with these constructs, the cells were treated with 10^{-8} M 1α,25-$(OH)_2D_3$ for 40 h. Following 1α,25$(OH)_2D_3$ treatment, constructs A−E showed increases in hGH expression (Figure 1B), whereas ethanol (vehicle)-treated cells showed no changes in hGH expression. Construct F did not show any increase in hGH expression. Among these constructs, construct D stimulated hGH expression the most after 1α,-25$(OH)_2D_3$ treatment (7-fold). These results suggested that the TGF-β2 promoter sequence responsible for the stimulation of transcription by 1α,25$(OH)_2D_3$ is located between −973 to −553 bp.

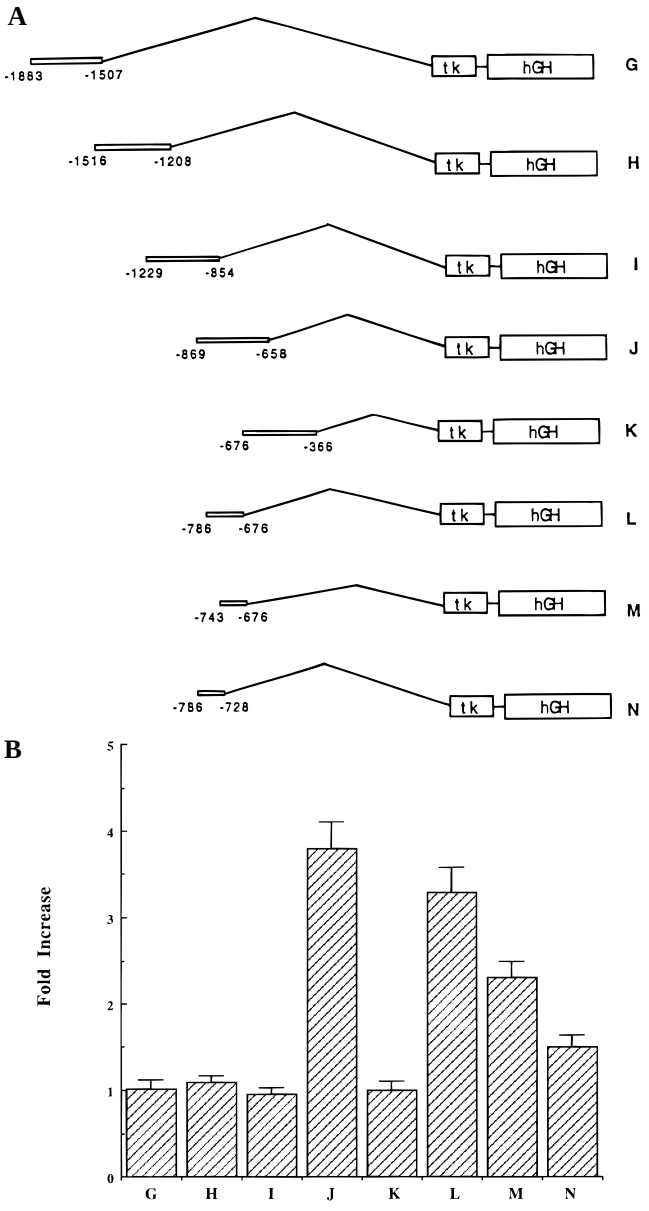

FIGURE 2: (A) Schematic representation of TGF-β2 promoter fragment/TK promoter/hGH chimeric constructs. The constructs were cloned into pTKGH. The region of TGF-β2 promoter is represented by an open bar while the hGH reporter sequence is marked as hGH in the open bar. The lines represent the truncated part of TGF-β2 promoter sequence. (B) ELISA measuring the expression of human growth hormone in the supernatant of hFOB cells. The hFOB cells were transfected with a series of TGF-β2 promoter fragment/TK promoter/hGH chimeric constructs and treated with 10^{-8} M 1α,25(OH)$_2$D$_3$ for 40 h. The fold increase is relative to cells treated with the same volume of ethanol. Data are means $\pm$ SE of three experiments.

A second series of constructs was made using the pTKGH plasmid (Figure 2A). Eight different TGF-β2 promoter fragments were amplified by PCR and ligated into pTKGH 5′ of the TK promoter. hFOB cells were transfected with these constructs. These transfected cells were treated with 10^{-8} M 1α,25(OH)$_2$D$_3$ in ethanol or the same volume of ethanol alone for 40 h, and expression of hGH was measured. As shown in Figure 2B, fragment J which contains the TGF-β2 promoter sequence -869 to -658 bp increased hGH expression 3.8-fold after the 10^{-8} M 1α,25(OH)$_2$D$_3$ treatment. Also, fragment L, which contains the promoter sequence from -786 to -676 bp, showed a 3.3-fold

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-786
ATTGAAAATGTCAAGTTATGAGTAG TGTAGA ACA AGTAGA CATCAAACACTT
AAAATTCCAGCTTCCTGGATTATGCTATGGAAAG AATGAA GTT GGTGGA TAA
TGTTTAG
   -676
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FIGURE 3: Sequence of two vitamin D response elements in the human TGF-β2 gene promoter region. The imperfect RRKNSA hexameric repeats are underlined and shown in bold and italicized type.

stimulation of hGH expression that was comparable to, and statistically indistinguishable from, that seen with construct J ($p > 0.05$). No enhancement of the expression of hGH was observed in cells transfected constructs G, H, I, or K after treatment with 10^{-8} M 1α,25(OH)$_2$D$_3$. This suggests that the TGF-β2 promoter fragment from -786 to -676 bp is critical for the increased expression of TGF-β2 caused by 1α,25(OH)$_2$D$_3$.

1α,25(OH)$_2$D$_3$ is known to activate the transcription of several genes by activating the VDR which has been shown to bind to specific DNA sequences known as vitamin D response elements (VDRE) either as a homodimer or heterodimer with RXR. VDREs are usually composed of direct repeats (DRs), palindromes (Ps), or inverted palindromes (IPs) of hexameric core binding motif RRKNSA (R = A or G, K = G or T, S = C or G). In the TGF-β2 promoter extending from -786 to -676 bp, we found two imperfect DR3 motifs. To test if these two DR3 motifs were functional as VDREs, two smaller constructs which included the TGF-β2 promoter sequence -743 to -676 bp (construct M) and -786 to -728 bp (construct N) (Figures 2A and 3) in the pTKGH plasmid were transfected into hFOB cells. Concentrations of hGH increased 2.3-fold in construct M transfected cells (M vs G, $p < 0.05$) and 1.5-fold in construct N transfected cells (N vs G, $p < 0.05$, Figure 2B). Concentrations of hGH were higher in supernatants of construct M transfected cells compared to construct N transfected cells ($p < 0.05$). These results suggest that the TGF-β2 promoter contains two vitamin D response elements which give a combined response that is greater than that of either one alone.

To further localize a VDRE in the TGF-β2 promoter, we performed gel mobility shift assays with human recombinant VDR and RXRα and fragments of the TGF-β2 promoter. The mouse osteopontin (mOP) VDRE was used as a positive control. The two 40-mer annealed oligonucleotides which contain one of the two putative VDRE, respectively, showed binding to bacterially expressed human VDR and RXRα (Figure 4, lanes 1 and 2). The shifted band migrated in a manner similar to the mOP VDRE$-$VDR$-$RXRα complex (Figure 4, lane 7). The two 40-mer annealed oligonucleotides lacking a putative VDRE did not show binding to the VDR/RXRα (Figure 4, lanes 3 and 4). A 250-fold excess of cold TGF-β2 oligonucleotides inhibited the binding between TGF-β2 oligonucleotides containing the VDREs and VDR/RXRα (Figure 4, lanes 5 and 6). A 100-fold excess of mouse osteopontin oligonucleotide inhibited the binding between TGF-β2 oligonucleotides containing the VDREs and VDR/RXRα (Figure 4, lanes 8 and 9). Cell nuclear extracts from human osteoblasts bound to both VDRE-containing oligonucleotides, and the addition of an antibody to the VDR

Table 1: Minimal VDREs Present in Vitamin D Regulated Genes

human TGF-β2	−700 to −686	**aatgaa** gtt **ggtgga**	this work
	−761 to −747	**tgtaga** aca **agtaga**	this work
rat osteocalcin		**aggtca** agg **aggtca**	(*47*)
human osteocalcin		**gggtga** atg **aggaca**	(*40*)
mouse osteopontin		**ggttca** cga **ggttca**	(*39*)
rat 24-hydroxylase	-proximal	**aggtga** gtg **aggggcg**	
	-distal	**ggttca** gcg **ggtgcg**	(*36*)
human calbindin D_{9K}		**tgccct** tccttatgg **ggttca**	(*41*)
mouse calbindin D_{28K}		**gggggga** tgtg **aggaga**	(*48*)
mouse fibronectin		**gggtga** cgtcac **ggggta**	(*49*)

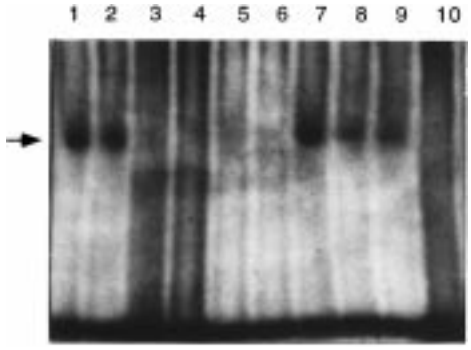

FIGURE 4: Gel mobility shift assay examining the binding of the bacterially expressed human VDR and RXRα to the TGF-β VDREs or to the mOP VDRE. Lane 1: TGF-β2 promoter fragment −774 to −735 bp incubated with purified human VDR and RXRα. Lane 2: TGF-β2 promoter fragment −714 to −675 bp incubated with purified human VDR and RXRα. Lane 3: TGF-β2 promoter fragment containing sequence between −785 and −731 bp but with DR3 repeat sequence (TGTAGAACAAGTAGA) deleted, incubated with purified human VDR and RXRα. Lane 4: TGF-β2 promoter fragment containing the sequence between −725 and −671 bp but with DR3 repeat sequence (AATGAAGTTGGTGGA) deleted, incubated with purified human VDR and RXRα. Lane 5: TGF-β2 promoter fragment −774 to −735 bp incubated with purified human VDR and RXRα plus 250-fold excess unlabeled annealed TGF-β2 oligonucleotides. Lane 6: TGF-β2 promoter fragment −714 to −675 bp incubated with purified human VDR and RXRα plus 250-fold excess unlabeled annealed TGF-β2 oligonucleotides. Lane 7: mouse osteopontin (mOP) VDRE incubated with purified human VDR and RXRα. Lane 8: TGF-β2 promoter fragment −774 to −735 bp incubated with purified human VDR and RXRα plus 100-fold excess unlabeled mOP VDRE. Lane 9: TGF-β2 promoter fragment −714 to −675 bp incubated with purified human VDR and RXRα plus 100-fold excess unlabeled mOP VDRE. Lane 10: random annealed oligonucleotides from TGF-β2 promoter incubated with purified human VDR and RXRα. The arrow points to the shifted complex bands.

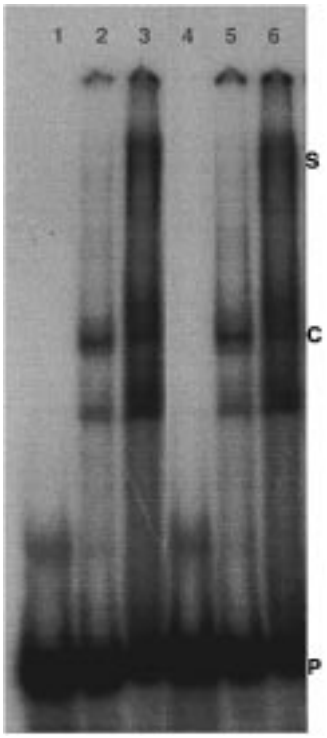

FIGURE 5: Gel mobility shift assay examining the binding of human osteoblast cell nuclear extract to the TGF-β2 VDREs. Lane 1: ^{32}P-labeled TGF-β2 promoter fragment −774 to −735 bp alone. Lane 2: ^{32}P-labeled TGF-β2 promoter fragment −774 to −735 bp incubated with human osteoblast cell nuclear extract. Lane 3: ^{32}P-labeled TGF-β2 promoter fragment −774 to −735 bp incubated with human osteoblast cell nuclear extract and VDR antibody. Lane 4: ^{32}P-labeled TGF-β2 promoter fragment −714 to −675 bp alone. Lane 5: ^{32}P-labeled TGF-β2 −714 to −675 bp incubated with human osteoblast cell nuclear extract. Lane 6: ^{32}P-labeled TGF-β2 −714 to −675 bp incubated with human osteoblast cell nuclear extract and VDR antibody. Key: S, supershifted band in the presence of the VDR antibody; C, gel shifted complex; P, unbound probe.

further retarded the mobility of these complexes (Figure 5).

DISCUSSION

Vitamin D, through its physiologically active metabolite 1α,25$(OH)_2D_3$, affects bone cell growth differentiation, and this action is primarily mediated by its nuclear receptor (*3*, *34*). The vitamin D receptor is a member of the nuclear receptor superfamily (*13*, *14*). Nuclear receptors activate transcription by binding to response elements that consist of hexameric core binding motifs in promoters of their target genes. The specific identification of hormone response elements is critical for understanding how nuclear receptors regulate target gene transcriptional activity (*35*).

This study provides new insights into the mechanism by which 1α,25$(OH)_2D_3$ regulates TGF-β2 expression in human osteoblast cells. We had previously shown that TGF-β2 gene transcription was increased in osteoblasts after 1α,25$(OH)_2D_3$ treatment (*12*). We have now identified DNA sequence elements in the human TGF-β2 gene promoter that mediate the response to 1α,25$(OH)_2D_3$. Two separate response elements, each one of which contains two half-sites separated by three nonspecific bases, form a complex tandem VDRE. A 43-base pair fragment separates these two VDREs. Both elements are required for maximum transcriptional responses to 1α,25$(OH)_2D_3$. Each VDRE is capable of submaximally responding to 1α,25$(OH)_2D_3$ in the reporter gene system.

The presence of two hormone responsive elements in the same promoter has been reported before. In the rat 24-hydroxylase promoter, two vitamin D response elements separated by a 93-base pair fragment have been reported. This 93-base pair intervening fragment can be deleted

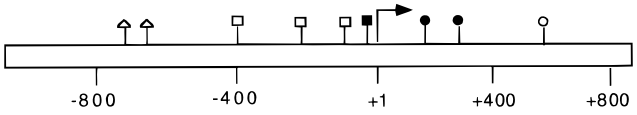

FIGURE 6: Major regulatory elements in 5′ region of TGF-β2 gene. Transcription starts at the arrow (+1). Key: ●, AP-2 binding sites; ■, TATAA box; ○, Sp-1 binding site; □, cAMP responsive elements; △, vitamin D response elements.

without significantly reducing the transcription response potential of the system (*36*). Similar situations also occur with other steroid response elements. For example, the vitellogenin gene has two estrogen response elements (*37*); the mouse mammary tumor virus long terminal repeat contains multiple copies of the glucocorticoid response element (*38*). Multiple response elements existing in promoter sequences may be needed to enhance steroid-regulated gene expression.

The identification of novel VDREs defines the range of possible sequences that confer biological responsiveness to 1α,25(OH)$_2$D$_3$ in a variety of genes. Among all the hormone response elements, VDREs show the highest variability of core binding motifs. In addition to DR3 repeat DNA elements, DR4-, DR6-, IP7-, and IP9-type structures have been recognized as candidate VDREs. VDREs range from simple direct repeats as are found in the mouse osteopontin VDRE and the human osteocalcin VDRE (*39*, *40*) to complex inverted palindrome half-site arrangements seen in the human calbindin D$_{9k}$ gene (*41*) (Table 1). The presence of VDREs with different sequence combinations suggests that there are numerous pathways of 1α,25(OH)$_2$D$_3$ signaling via heterodimeric or homodimeric receptor−DNA interactions (*42*). It is of interest to note that the increase in TGF-β2 synthesis in human osteoblast cultures brought about by 1α,25(OH)$_2$D$_3$ is comparable to the increase in reporter gene activity noted in this report (12). While this does not eliminate the possibility of a role for posttranslational mechanisms in the control of TGF-β2 synthesis in osteoblasts, it does suggest that transcriptional mechanisms are important in this regard.

1α,25(OH)$_2$D$_3$ also alters gene transcription by mechanisms other than those described above. For example, we have demonstrated that an AP-1 element in the NGF gene promoter is required for 1α,25(OH)$_2$D$_3$ regulation of NGF synthesis (*43*). An AP-1 element also plays a role in the 1α,25(OH)$_2$D$_3$-mediated osteocalcin gene transcription (*44*). Functional analysis of the TGF-β2 promoter shows that the human TGF-β2 promoter sequence contains Sp1 and AP-2 sites, a TATAA box and cAMP-responsive elements, whereas an AP-1 response element cannot be identified (Figure 6) (*45*, *46*). These analyses suggest that TGF-β2 gene transcription might be influenced by other calciotropic hormones such as parathyroid hormone (PTH), which acts by stimulating cAMP production in cells. 1α,25(OH)$_2$D$_3$ and PTH might act synergistically in modulating TGF-β2 transcription. The regulation of the TGF-β2 promoter is likely to be quite complex, however, since studies have shown that the TGF-β2 promoter sequence between −778 to −508 bp modulates gene transcription in a manner which is dependent upon the cell type (*46*).

In conclusion, we have identified two vitamin D response elements in the human TGF-β2 gene promoter region, which are separated by a 43-base pair fragment. Transcriptional regulation of the TGF-β2 gene promoter by 1α,25(OH)$_2$D$_3$ through specific and unique VDREs is likely to be an important method by which 1α,25(OH)$_2$D$_3$ controls cellular proliferation and differentiation.

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