An AP-1 Site in the Nerve Growth Factor Promoter Is Essential for 1,25-Dihydroxyvitamin D_3-Mediated Nerve Growth Factor Expression in Osteoblasts†

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ABSTRACT: 1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active metabolite of vitamin D, induces nerve growth factor (NGF) synthesis in a variety of different cell lines. The mechanism by which $1,25(OH)_2D_3$ induces NGF, however, is poorly understood. We used a series of full-length and truncated NGF promoter−human growth hormone (hGH) reporter gene plasmids to investigate the mechanism of $1,25(OH)_2D_3$-induced NGF expression in osteoblasts. Untransfected rat osteosarcoma cells (ROS 17/2.8) treated with $1,25(OH)_2D_3$ showed a 2-fold increase in NGF expression compared to control cells. ROS 17/2.8 osteosarcoma cells were transfected with the NGF−hGH reporter plasmids and treated with 10^{-8} M $1,25(OH)_2D_3$. The full-length NGF promoter (−1800 to +120)−hGH reporter construct showed an approximately 2-fold increase in hGH release. Plasmids with successive 5′-deletions showed enhanced hGH expression in treated cells and control cells. A similar series of NGF promoter−hGH reporter gene constructs, lacking the AP-1 site located within the first intron of the NGF gene, were also transiently transfected into ROS 17/2.8 cells. When these cells were treated with the same dose of $1,25(OH)_2D_3$, no increase in hGH expression was seen compared to control cells, demonstrating that this AP-1 site is essential for $1,25(OH)_2D_3$-mediated NGF up-regulation. Since $1,25(OH)_2D_3$ is known to activate the transcription of several genes through its interaction with the vitamin D receptor (VDR), we performed a series of gel electrophoretic mobility shift assays to determine if the VDR binds directly to the AP-1 sequence. No evidence of VDR binding, either as a homodimer or as a heterodimer, to the AP-1 sequence was observed. Treatment of ROS 17/2.8 cells with $1,25(OH)_2D_3$, however, resulted in an increase in AP-1 binding activity; however, no significant changes in *c-jun* and *c-fos* levels were observed. Our data show that in osteoblasts, $1,25(OH)_2D_3$ induces NGF expression indirectly by increasing AP-1 binding activity.

1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active metabolite of vitamin D, is known to activate the transcription of several genes (*1*−*7*). $1,25(OH)_2D_3$ induces transcription primarily through its interaction with the vitamin D receptor (VDR), a 47 kDa zinc-finger protein (*4*, *8*). The VDR binds to vitamin D response elements (VDREs) within the promoters of vitamin D responsive genes, either as a homodimer or as a heterodimer with the retinoic acid x receptor (RXR). Although the primary physiological role of $1,25(OH)_2D_3$ involves the maintenance of calcium and phosphorus homeostasis and bone mineralization, there is increasing evidence that the sterol plays a role in tissues unrelated to mineral metabolism. Epitopes for the VDR have been observed in dorsal root ganglia in vivo and in dorsal root ganglia cells maintained in culture (*9*), and work in our and other laboratories has detected the VDR or $1,25(OH)_2D_3$ binding in various areas of the central nervous system in the fetal and adult rat (*10*−*12*).

$1,25(OH)_2D_3$ has recently been shown to increase the expression of NGF in fibroblasts (*13*), osteoblasts (*14*), neuroblastomas (*15*), and glial cells (*7*) in vitro as well as in rat brain in vivo (*16*). The mechanism by which $1,25(OH)_2D_3$ induces NGF expression, however, is poorly understood. NGF is a member of a family of related neurotrophic factors which include brain-derived neurotrophic factor (BDNF) and neurotrophins 3, 4, and 6 (*17*). NGF is secreted in a target-derived, paracrine manner (*18*), and subsequent binding of NGF to cellular, cell surface receptors on sensory, sympathetic, and central nervous system neurons results in a pleiotropic response that has been shown to be important for neuronal development and survival (*19*). Synthesis of NGF mRNA in certain cell types can be induced by nerve lesion and damage (*20*, *21*). NGF expression has also been shown to be regulated by a variety of cytokines, growth factors, and secondary messenger activators (*22*−*24*). The NGF gene contains an AP-1 site

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within the first intron, 35 base pairs downstream from the transcription initiation site (*25*). This AP-1 site has been shown to be functionally required for basal level NGF transcription in fibroblasts (*26*, *27*). Members of the *fos* and *jun* family of transcription factors are known to bind to these AP-1 sequences. Indeed, lesion-induced increases in NGF expression have been shown to be mediated by *c-fos* (*20*). In addition, regulation of the NGF gene by β-adrenergic receptor activation may involve *c-fos* (*28*).

To determine the mechanism by which $1,25(OH)_2D_3$ induces NGF expression, we transfected rat osteoblastic osteosarcoma ROS 17/2.8 cells with a series of NGF promoter/human growth hormone (hGH) reporter constructs including a set of constructs lacking the AP-1 site located within the first intron of the NGF gene (*26*). As mentioned previously, $1,25(OH)_2D_3$ has been shown to induce NGF mRNA expression in this cell line (*14*) and NGF expression has been detected within bone marrow (*29*). The role of NGF in bone cells is not fully understood, although a recent study has shown that topical application of NGF improves fracture healing in rats (*30*). Cells transfected with the NGF promoter/hGH reporter plasmids containing the AP-1 sequence showed an increase in hGH expression when treated with $1,25(OH)_2D_3$ while those transfected with plasmids lacking the AP-1 sequence did not show an increase in hGH expression. $1,25(OH)_2D_3$-treated cells also showed an increase in AP-1 binding activity; however, no significant increase in the expression of *c-jun* or *c-fos* was observed. The results suggest that $1,25(OH)_2D_3$ induces NGF expression via the AP-1 site in the NGF gene by increasing AP-1 binding activity.

MATERIALS AND METHODS

Nerve Growth Factor ELISA. ROS 17/2.8 cells were plated into 6-well plates at a density of 50 000 cells per well and allowed to proliferate in growth medium (Ham's F12 with 10% FBS) for 2 days with daily changes of medium. The cells were then treated with 10^{-6}, 10^{-8}, 10^{-10}, and 10^{-12} M $1,25(OH)_2D_3$ (Hoffman LaRoche, Nutley, NJ) or an equivalent volume of vehicle (ethanol). After 6 h, the medium was removed from the wells and frozen at −20 °C. The cells in each well were lifted and counted using a Coulter counter (Coutler Electronics Inc., Hialeah, FL). NGF expression was measured by coating the appropriate number of wells of a 96-well microtiter plate with 80 ng/mL murine monoclonal anti-NGF antibody (Boehringer Mannheim, Indianopolis, IN) dissolved in coating buffer [50 mM Na_2CO_3/$NaHCO_3$, 0.1% (w/v) NaN_3, pH 9.6], followed by incubation at 37 °C for 2 h. The coating buffer was replaced with 200 μL of blocking solution [0.5% (w/v) BSA in coating buffer], and the plate was incubated at 37 °C for 30 min. The wells were then washed 5 times with wash buffer [50 mM Tris-HCl, 200 mM NaCl, 10 mM $CaCl_2$, 0.1% (w/v) Triton X-100, 0.05% (w/v) NaN_3, pH 7.0]. After washing, 200 μL of the treated medium samples was applied to the appropriate wells and the plate incubated at 4 °C overnight. The wells were then washed 5 times with wash buffer. Murine monoclonal anti-NGF β-galactosidase-conjugated antibody (100 μL, 0.4 unit/mL) (Boehringer Mannheim) was added to each well followed by incubation at 37 °C for 4 h. After five washings, 200 μL of substrate solution [40 mg of chlorophenolred β-D-galactopyranoside dissolved in 20 mL of 100 mM Hepes, 150 mM NaCl, 2 mM $MgCl_2$, 1% (w/v) BSA, 0.1% (w/v) NaN_3, pH 7.0] was added to each well. The plate was incubated at 37 °C for 30 min to allow for color development. The absorbance of each well was measured at 570 nm.

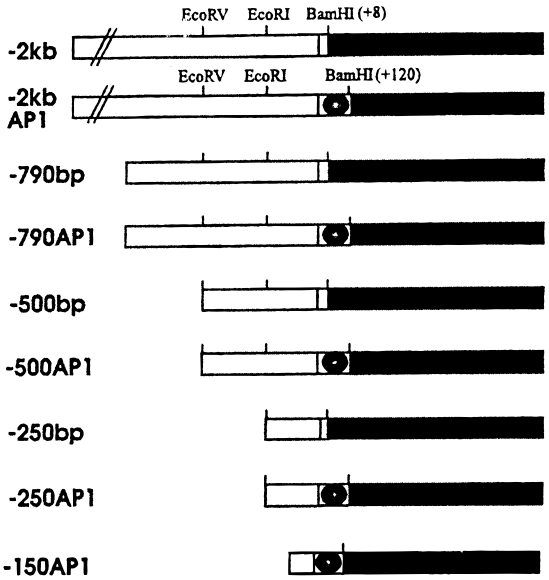

FIGURE 1: Schematic representation of NGF promoter/hGH chimeric plasmid constructs (modified from Cowie et al., ref *26*). The NGF promoter from −2000 to +120 bp was cloned into plasmid pOhGH as described by Cowie et al. (ref *26*). The constructs lacking the AP-1 site had the region from +8 to +120 deleted. The region of the NGF promoter is represented by an open bar while the hGH reporter sequence is represented by a solid bar.

Cell Transfection. ROS 17/2.8 cells were cultured in Ham's F12 medium supplemented with 10% FBS. ROS 17/2.8 cells were plated into 6-well plates at a density of 50 000 cells per well. After 24 h, cells were switched to serum-free Ham's F12 medium, and lipofectamine (15 μL) was used to transfect cells with the NGF promoter−hGH reporter plasmids. The plasmids consisted of various lengths of the NGF promoter ligated into an hGH reporter vector (*26*). The name and length of NGF promoter in each construct (Figure 1) are as follows: −2kbAP1 (−1800 to +120); −790AP1 (−790 to +120); −500AP1 (−500 to +120); −250AP1 (−250 to +120); and −150AP1 (−150 to +120). A second series of constructs, referred to as −2kb, −790, −500, and −250, were prepared by truncating the promoter at +8, thereby deleting the AP-1 sequence located within the first intron of the NGF gene (*26*). A total of 2 μg of the promoter construct DNA (*26*) was added to each dish along with 1 μg of pPGKlacZ as an internal control to measure transfection efficiency. After 5 h, serum-free medium was replaced with growth medium containing 10% FBS. The cells were grown for 48 h, following which they were treated with 10^{-8} M $1,25(OH)_2D_3$ or an equal volume of vehicle (ethanol). After 4 h, the media were assayed for hGH activity using an ELISA kit (Boehringer Mannheim). The cells were pelleted and lysed by resuspending the cell pellet in 0.5 mL of 10 mM Tris, pH 7.9, 0.15 M NaCl, 1 mM EDTA, 0.5% NP40 and placing the suspension on ice for 1 h. The cell nuclei were pelleted by spinning in a microcentrifuge, and 30 μL of the supernatant was used for the β-galactosidase assay (*31*) to measure tranfection efficiency.

Electrophoretic Gel Mobility Shift Assays. The following oligonucleotides were used in the electrophoretic gel mobility shift analyses (*32*, *33*): the NGF AP-1 sequence, 5′ AGCGCATCGGTGAGTCAGGCTTCTCTGA GCCGA 3′; and the mouse osteopontin vitamin D response element (mOP VDRE), 5′ GCTCGGGTAGGGTTCACGAGGTTCACTCGACTCG 3′. In both cases, the complementary strand was also synthesized. Double-stranded DNA was prepared by heating single-stranded oligonucleotides in equimolar concentrations at 100 °C followed by cooling to room temperature over 60 min. DNA end-labeling was performed using 10 ng of double-stranded DNA, T4 polynucleotide kinase buffer, [γ-^{32}P]ATP (6000 Ci/mmol), and 10 units of T4 polynucleotide kinase and incubating at 37 °C for 60 min. Unincorporated nucleotides were removed by ethanol precipitation (*31*).

Gel shift samples were prepared by adding the double-stranded DNA probes to incubation buffer [20 mM Hepes, 20% (w/v) glycerol, 0.1 M KCl, 0.2 mM EDTA, 20 μM $ZnCl_2$, 2.5 mM DTT, pH 7.9]. To 1−3 pmol of the double-stranded DNA were added various combinations of the DNA binding domain of the VDR (*34*), HeLa cell nuclear extract, and rabbit polyclonal antibody made against bacterially expressed full-length human VDR (*35*, *36*). Following incubation on ice for 30 min, the samples were separated on an 8% polyacrylamide gel made with running buffer [16% (w/v) glycine, 270 mM Tris base, pH 7.5, 15 mM EDTA] at a constant current of 25 mA at 4 °C. The gel was then dried for 1 h and exposed to BIO-Max X-ray film for 16 h.

To test for AP-1 binding activity, cell nuclear extracts from ROS 17/2.8 cells treated with 1,25$(OH)_2D_3$ or vehicle for 1 and 6 h were prepared. The cell nuclear extracts were combined with the ^{32}P-labeled double-stranded AP-1 DNA prepared as described above, and incubated on ice for 30 min. The samples were processed as described above.

c-fos and c-jun Expression. ROS 17/2.8 cells were plated at a density of 500 000 cells per T-75 flask and allowed to grow to 75−80% confluency. The cells were then treated with 10^{-8} M 1,25$(OH)_2D_3$ or vehicle, and total RNA was harvested from the cells at various time points using a Qiagen RNeasy kit (Qiagen, Santa Clarita, CA). To measure *c-fos* and *c-jun* RNA expression, 5 μg of total RNA was applied to a nitrocellulose membrane using a vacuum manifold and vacuum-baked for 2 h. The membrane was prehybridized for 4 h at 42 °C in 50% deionized formamide, 2× SSC, 5× Denhardt's solution, 0.5% SDS, 100 μg/mL sheared salmon sperm DNA and hybridized in the same solution containing ^{32}P end-labeled *c-jun* or *c-fos* specific oligonucleotide DNA probes (R & D Research, Inc.) at 42 °C for 16 h. The membrane was washed 4 times in 2× SSC, 0.5% SDS for 20 min at room temperature, followed by two washes at 42 °C. Following washing, the membranes were exposed to BioMax MR film with an intensifying screen at −70 °C. To ensure equal loading of RNA in all the wells, the blots were stripped by boiling in 0.5% SDS for 5 min, and reprobed using a 600 bp ^{32}P-radiolabeled cyclophilin probe. The autoradiographs were scanned on a Macintosh PowerMac 8500 computer employing a UMAX PowerLook II scanner driven by Adobe Photoshop 4.0 software (Adobe Systems Inc., San Jose, CA). The individual bands were quantitated using the public domain NIH Image program developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/.

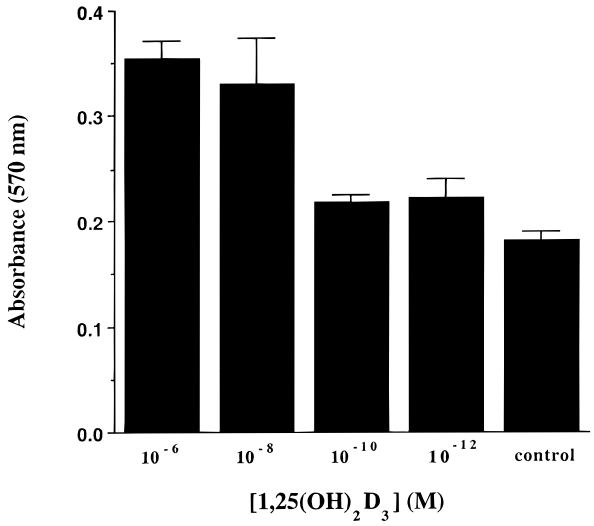

FIGURE 2: Sandwich ELISA measuring the expression of NGF in ROS 17/2.8 cells treated with various doses of 1,25$(OH)_2D_3$. ROS 17/2.8 cells were treated with the indicated concentrations of 1,25$(OH)_2D_3$ or ethanol (control), and after 6 h, the medium was removed and tested for the presence of NGF (see text for details). Data are the mean ± SEM values of four separate experiments.

RESULTS

As shown in Figure 2, treatment of the rat osteosarcoma cell line ROS 17/2.8 with 1,25$(OH)_2D_3$ for 6 h results in a dose-dependent increase in NGF expression. There was a 1.95- and 1.81-fold increase in NGF expression in cells treated with 10^{-6} and 10^{-8} M 1,25$(OH)_2D_3$, respectively. The increase in NGF expression was more modest at lower concentrations of 1,25$(OH)_2D_3$ (i.e., 10^{-10} and 10^{-12} M). These results are similar to those reported for the induction of NGF mRNA by 1,25$(OH)_2D_3$ using the same cell line (*14*).

To detect regions within the NGF promoter critical for 1,25$(OH)_2D_3$-induced expression of NGF, we transfected full-length (−2kbAP1) and 5′-truncated NGF promoter/hGH reporter plasmids (−790AP1, −500AP1, −250AP1, and −150AP1) into the ROS 17/2.8 cells. The cells were treated for 4 h with either 10^{-8} M 1,25$(OH)_2D_3$ or vehicle, and the expression of hGH was measured. Treatment of the cells with 1,25$(OH)_2D_3$ resulted in an approximately 4-fold increase in hGH expression for the full-length plasmid and a 2-fold increase in hGH expression for the truncated plasmids compared to control cells (Figure 3). Plasmids with successive 5′-deletions showed enhanced hGH expression in treated cells and control cells. This transcriptional enhancement may be due to a nonspecific effect of plasmid sequences (*26*); however, this effect could also be a result of the deletion of negative regulatory elements within the 5′-region of the NGF promoter.

A second series of full-length and truncated NGF promoter/hGH reporter constructs, lacking the AP-1 binding site located within the first intron of the NGF gene, were transfected into ROS 17/2.8 cells. These transfected cells were treated with either 10^{-8} M 1,25$(OH)_2D_3$ or vehicle for 4 h, and the expression of hGH was measured. As shown in Figure 4, no enhancement of the expression of hGH was

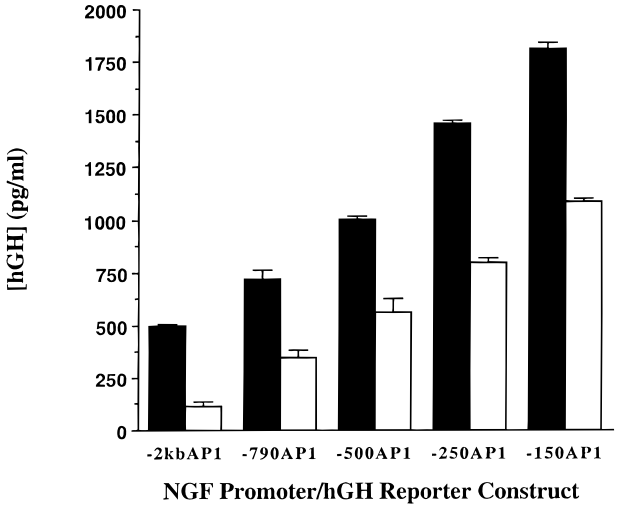

FIGURE 3: Expression of hGH in ROS 17/2.8 cells transfected with NGF promoter/hGH reporter constructs and treated with 10^{-8} M $1,25(OH)_2D_3$. ROS 17/2.8 cells were transfected with a series of truncated NGF promoter/hGH reporter constructs containing the AP-1 site (for a complete description, see text) and treated with 10^{-8} M $1,25(OH)_2D_3$ (■) or vehicle (□). Data are the mean ± SEM values of four separate experiments.

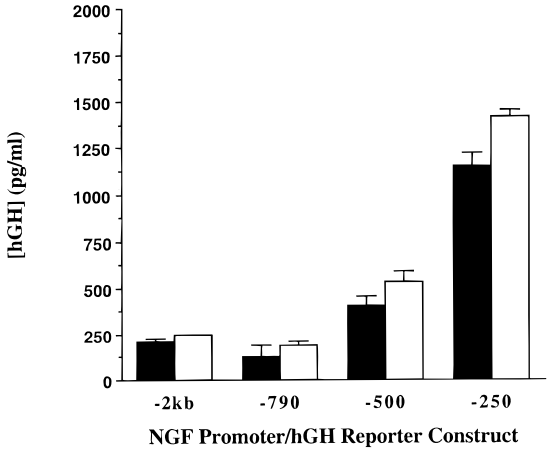

FIGURE 4: Expression of hGH in ROS 17/2.8 cells transfected with NGF promoter/hGH reporter constructs treated with 10^{-8} M $1,25(OH)_2D_3$. ROS 17/2.8 cells were transfected with a series of truncated NGF promoter/hGH reporter constructs lacking the AP-1 site (for a complete description see text) and treated with 10^{-8} M $1,25(OH)_2D_3$ (■) or vehicle (□). Data are the mean ± SEM values of four separate experiments.

observed in transfected cells treated with $1,25(OH)_2D_3$ when compared to control cells. This result suggests that this AP-1 site is critical for the increased expression of NGF caused by $1,25(OH)_2D_3$.

$1,25(OH)_2D_3$ 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 as a heterodimer with RXR. To determine whether the VDR binds directly to the AP-1 site in the NGF gene thereby increasing transcription of the NGF gene, we performed a series of electrophoretic mobility gel-shift and "super-shift" experiments examining the binding of the DNA binding domain of the VDR (VDR-DBD) to this AP-1 sequence. No detectable binding was seen between the VDR-DBD and the AP-1 sequence (Figure 5, lane 3). A shifted band was seen in the presence of HeLa cell nuclear extract (lane 2); however, addition of a polyclonal VDR antibody to the mixture did not result in a super-shifted band, suggesting the VDR was not part of the complex binding to the AP-1 sequence (Figure 5, lane 6). These experiments were repeated under different ionic strength conditions (27 mM Tris base, pH 7.5, 13.2 mM sodium acetate, 0.5 mM EDTA) as well as with the full-length VDR, and no evidence of VDR binding to the AP-1 sequence was observed (data not shown).

As a positive control, the binding of the VDR-DBD to the mouse osteopontin (OP) VDRE was examined. The VDR-DBD has been shown to bind to the OP VDRE under low ionic strength conditions (*37*). Under low ionic strength conditions, the binding of the VDR-DBD to the OP VDRE was observed (data not shown). We have shown previously that the VDR polyclonal antibody does not bind the OP VDRE (*37*). Under high ionic strength conditions [16% (w/v) glycine, 270 mM Tris base, pH 7.5, 15 mM EDTA], no binding of the VDR-DBD to the OP VDRE was observed; however, addition of the VDR antibody resulted in a super-shifted band between the VDR-DBD and the OP VDRE (Figure 5, lane 11). Presumably, the addition of the antibody has a stabilizing effect on the VDR-DBD/OP VDRE complex. A super-shifted band was also seen in the mixture containing the VDR-DBD, the HeLa cell nuclear extract, and the VDR antibody (Figure 5, lane 12). Similar results were obtained using the full-length VDR in place of the VDR-DBD. Thus, if the VDR were able to bind to the AP-1 sequence, our gel shift or super-shift experiments should have detected it.

We performed a gel shift assay measuring changes in AP-1 binding activity in ROS 17/2.8 cells treated with 10^{-8} M $1,25(OH)_2D_3$ using the AP-1 binding sequence from the NGF gene. As shown in Figure 6, a significant increase in AP-1 binding activity was observed within 1 h of treatment and was increased after 6 h of treatment, suggesting that $1,25-(OH)_2D_3$ treatment of the cells increases AP-1 binding.

Since *c-fos* and *c-jun* are known to form the protein complex which binds to AP-1 sequences, changes in *c-fos* and *c-jun* mRNA were measured in ROS 17/2.8 cells treated with $1,25(OH)_2D_3$. Total RNA was harvested from ROS 17/2.8 cells treated with 10^{-8} M $1,25(OH)_2D_3$ at various time points ranging from 15 min to 24 h. The levels of *c-fos* and *c-jun* expression were measured using antisense oligonucleotide probes to each species. No significant change in the expression of *c-fos* or *c-jun* was observed in cells treated with $1,25(OH)_2D_3$ compared to control cells (Figure 7). This suggests that the increase in AP-1 binding is not the result of an increase in the expression of *c-fos* or *c-jun*.

DISCUSSION

This study provides new insights into the mechanism by which $1,25(OH)_2D_3$ up-regulates NGF expression. The observation that the removal of the AP-1 sequence within the first intron of the NGF gene abolishes $1,25(OH)_2D_3$-induced NGF expression in osteoblast cells clearly points to this as a critical region in the up-regulation of NGF expression by $1,25(OH)_2D_3$. This AP-1 element has been shown to be involved in lesion-induced increases in NGF mRNA as well as being functionally required for basal level transcription in transiently and stably transformed fibroblasts (*20*, *26*, *27*). Our results show that $1,25(OH)_2D_3$ induces

Lane	1	2	3	4	5	6	7	8		9	10	11	12
DNA probe	+	+	+	+	+	+	+	+		+	+	+	+
VDR DBD	-	-	+	+	-	-	+	+		+	+	+	+
CNE	-	+	-	+	-	+	-	+		-	+	-	+
VDR Ab	-	-	-	-	+	+	+	+		-	-	+	+

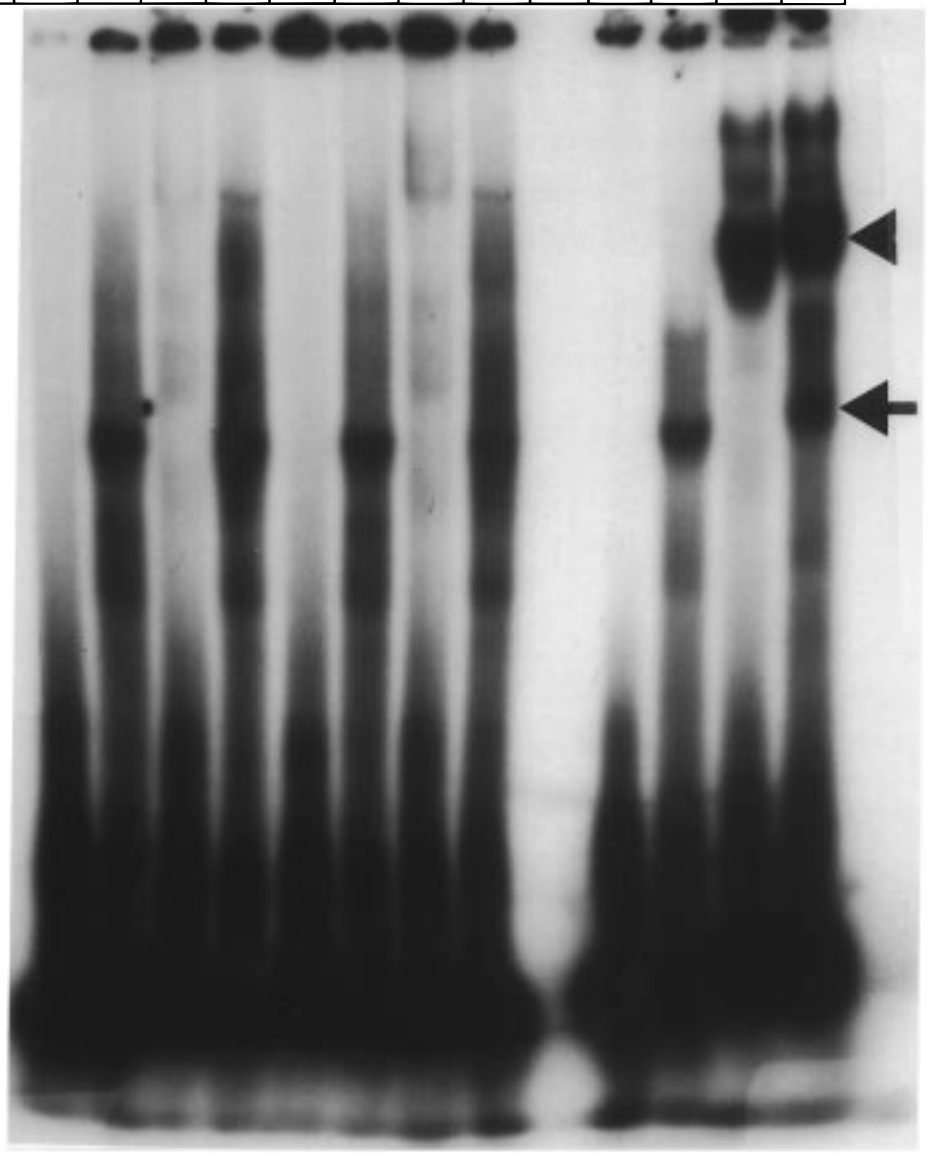

FIGURE 5: Gel shift and super-shift assay examining the binding of the VDR DNA-binding domain (DBD) to the AP-1 consensus sequence located within the NGF promoter (lanes 1−8) or to the osteopontin (OP) response element. Lane 1, AP-1 probe; lane 2, HeLa cell nuclear extract (CNE)/AP-1 probe; lane 3, VDR-DBD/AP-1 probe; lane 4, VDR-DBD/HeLa CNE/AP-1 probe; lane 5, VDR antibody (Ab)/AP-1 probe; lane 6, HeLa CNE/VDR Ab/AP-1 probe; lane 7, VDR-DBD/VDR Ab/AP-1 probe; lane 8, VDR-DBD/HeLa CNE/VDR Ab/AP-1 probe. As a positive control, the binding of the VDR-DBD to the VDRE from the mouse osteopontin (OP) promoter region was examined. Lane 9, VDR-DBD/OP probe; lane 10, VDR-DBD/HeLa CNE/OP probe; lane 11, VDR-DBD/VDR Ab/OP probe; lane 12, VDR-DBD/HeLa CNE/VDR Ab/OP probe. The arrowhead represents the super-shifted complex containing the labeled OP VDRE, the VDR-DBD, and the VDR Ab. The arrow represents the shifted complex containing the OP VDRE, DNA DBD, and other proteins in the CNE.

NGF expression by increasing AP-1 binding activity to the AP-1 element. $1,25(OH)_2D_3$ treatment also increases AP-1 binding activity in myelogenous leukemia cells (*38*) and proliferating osteoblasts; however, treatment of post-proliferative osteoblasts with $1,25(OH)_2D_3$ does not affect AP-1 binding activity (*39*, *40*).

This work represents the first report of the involvement of the AP-1 site in hormone-induced NGF up-regulation. The requirement for the AP-1 site suggests three possible mechanisms for $1,25(OH)_2D_3$-induced NGF expression. First, $1,25(OH)_2D_3$ could up-regulate NGF gene transcription through direct binding of the VDR to the AP-1 site. An AP-1 binding site is present in the VDRE of the osteocalcin (OC) gene which, in addition to the VDR, also binds *c-fos* and *c-jun* (*41*, *42*). Cotransfection of *c-fos* and *c-jun* expression plasmids with an OC VDRE promoter/chloramphenicol (CAT) reporter construct reduces the basal and $1,25(OH)_2D_3$-stimulated expression of the CAT gene, suggesting that *c-fos* and *c-jun* bind to the AP-1 site and reduce the ability of the VDR to bind or stimulate the OC VDRE. Our gel shift experiments clearly show that $1,25(OH)_2D_3$ does not directly activate NGF expression through the binding of

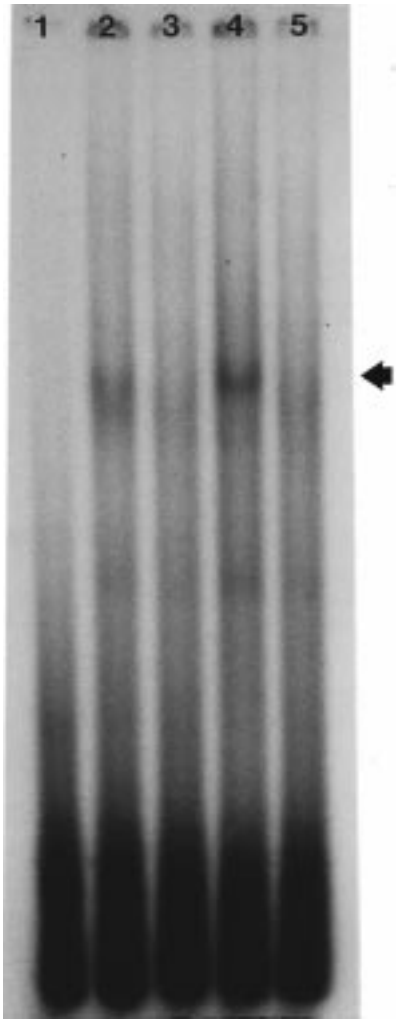

FIGURE 6: Effect of $1,25(OH)_2D_3$ on AP-1 binding activity. ROS 17/2.8 cells were treated with 10^{-8} M $1,25(OH)_2D_3$ or vehicle, and cell nuclear extract (CNE) was isolated at 1 and 6 h. The AP-1 binding activity was measured by gel shift assay using the double-stranded AP-1 DNA described under Materials and Methods. Lane 1, AP-1 probe; lane 2, 1 h 10^{-8} M $1,25(OH)_2D_3$-treated CNE/AP-1 probe; lane 3, 1 h vehicle-treated CNE/AP-1 probe; lane 4, 6 h 10^{-8} M $1,25(OH)_2D_3$-treated CNE/AP-1 probe; lane 5, 6 h vehicle-treated CNE/AP-1 probe.

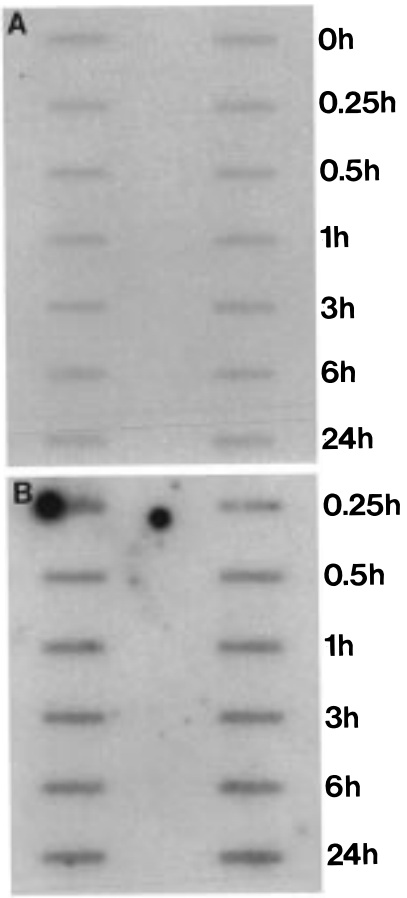

FIGURE 7: Effect of $1,25(OH)_2D_3$ on *c-fos* and *c-jun* expression. ROS 17/2.8 cells were treated with 10^{-8} M $1,25(OH)_2D_3$ or vehicle, and total RNA was isolated at the time points shown on the figure. The expression of (A) c-fos and (B) c-jun was assayed by slot blot hybridization using ^{32}P end-labeled *c-fos* and *c-jun* specific DNA probes (R & D Research, Inc.).

the VDR to the AP-1 site. Furthermore, based on sequences of previously defined VDREs (*43*), analysis of the sequence surrounding the AP-1 site does not show the presence of an apparent VDRE.

The second possible mechanism for $1,25(OH)_2D_3$-induced activation of NGF expression via the AP-1 site involves an increase in expression of *c-fos* and *c-jun*, which form the AP-1 binding complex (*40*). An increase in *c-fos* mRNA expression has been shown to mediate lesion-induced increases in NGF (*20*). A putative VDRE has also been detected within the *c-fos* promoter, suggesting $1,25(OH)_2D_3$ is capable of mediating *c-fos* expression (*44*). ROS 17/2.8 cells treated with $1,25(OH)_2D_3$, however, did not show a significant increase in *c-fos* or *c-jun* expression. These results suggest that the increase in NGF expression caused by $1,25(OH)_2D_3$ involves an increase in AP-1 binding activity without a concurrent increase in *c-fos* or *c-jun* expression.

A third possible mechanism is the regulation of AP-1 transcriptional activity through a protein−protein interaction involving the AP-1 complex and the VDR. Similar to the results presented here with the VDR, the glucocorticoid receptor (GR) is able to influence AP-1 activity without actually binding to DNA (*45*). It has also been shown that the AP-1 complex affects GR activity without actually binding to an AP-1 DNA binding site within or near the GR response element. These findings suggest that members of the nuclear hormone receptor family may interact with members of the AP-1 transcription factor family through protein−protein interactions possibly mediated by other as yet unidentified protein(s). In a study examining patients with glucocorticoid-resistant asthma (*46*), a significant reduction in the interaction between the GR and AP-1 was observed; however, no changes in the expression of *c-fos* or *c-jun* mRNA were detected, when compared to normal patients. This suggests the GR can influence AP-1 activity without altering the levels of *c-fos* or *c-jun*. We believe a similar situation may occur in the $1,25(OH)_2D_3$-induced NGF expression reported above, where AP-1 activity is regulated through a protein−protein interaction involving the VDR and the AP-1 complex, without directly altering the expression of *c-fos* and *c-jun*. In fibroblasts, $1,25(OH)_2D_3$ has been shown to induce NGF expression without increasing *c-fos* mRNA levels, whereas serum and phorbol 12-myristate increase both NGF and *c-fos* expression (*24*). It is possible, however, that the VDR could bind to DNA at another site, regulating the expression of another protein which results

in *c-fos* or *c-jun* phosphorylation or some other type of posttranslational modification, leading to an increase in AP-1 binding activity. It is possible that $1,25(OH)_2D_3$ might modulate nerve growth factor expression by yet other mechanisms that do not involve the AP-1 site.

In conclusion, our results show that the AP-1 site located within the first intron of the NGF gene is necessary for $1,25(OH)_2D_3$-mediated up-regulation of the NGF gene. The mechanism involves an increase in AP-1 binding activity in cells treated with $1,25(OH)_2D_3$. This increase in AP-1 binding activity, however, is not mediated by increases in *c-fos* or *c-jun* expression and does not involve direct binding of the VDR to the AP-1 site in the NGF gene. AP-1-mediated $1,25(OH)_2D_3$-induced NGF expression, shown here in osteoblasts, may be relevant to other cell types that respond to $1,25(OH)_2D_3$.

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