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Identification of a Vitamin D_3 Response Element in the Fibronectin Gene That Is Bound by a Vitamin D_3 Receptor Homodimer

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Abstract Fibronectin (FN) is an important adhesive noncollagenous glycoprotein involved in maintenance of the extracellular matrix and cell adhesiveness, loss of which has been implicated in the metastatic potential of cells. Regulation of FN occurs at the transcriptional level by the active metabolite of vitamin D_3, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$). Transient transfection of homologous and heterologous promoter reporter constructs into ROS 17/2.8 (rat osteosarcoma), NIH 3T3 (mouse fibroblast), and MCF-7 (human mammary carcinoma) cell lines showed a consistent two- to threefold induction of transcription when stimulated with 1,25-$(OH)_2D_3$. These heterologous promoter transfection studies with gel shift analysis locate a third, natural DR6-type vitamin D responsive element (VDRE) at nucleotide positions −171 to −154 in the murine FN promoter. Interestingly, this VDRE is also present in rat and human FN promoters. This study shows that 1,25-$(OH)_2D_3$ induces FN transcription from an existing elevated basal transcriptional activity by acting through two putative hexameric core binding motifs which bind VDR homodimers. Furthermore, the FN VDRE is the first homodimer-type VDRE that is not overlaid by a DR3-type structure.

Key words: fibronectin, VDR, homodimer, vitamin D regulation, transcription

Fibronectin (FN) in mammalian cells is a key constituent noncollagenous glycoprotein in the extracellular matrix (ECM), the globular network of proteins that provides a scaffold on which integrin molecules and other proteins assemble [Alexander and Werb, 1989; Dean et al., 1988; Dufour et al., 1988; McCarthy et al., 1985]. FN participates in establishing cell architecture, having an integral role in morphogenesis and development. In bone development, expression of FN occurs after the phase of cell proliferation providing an essential foundation for early matrix-induced bone differentiation [Reddi, 1984; Stein et al., 1990]. Depletion of extracellular FN in the extracellular matrix has been reported to result in changed cell-to-substrate and cell-to-cell interactions [Hynes, 1990; Tryggvason et al., 1987; Vlodavsky et al., 1990], resulting in loss of cellular adhesiveness and the aquisition of migratory or metastatic behaviour in malignant cells [McDonnell and Matrisian, 1990; Ostrowski et al., 1988; Sanchez-Lopez et al., 1988]. Understanding the molecular mechanisms which affect cell growth and differentiation and regulate the production of key components in the ECM such as FN could enable therapeutic control of this metastatic feature [Yoon et al., 1988].

The hormonal action of 1,25-$(OH)_2D_3$ is mediated through the vitamin D receptor (VDR) which is widely distributed [Manolagas, 1987], in part explaining its diverse biological effects. 1,25-$(OH)_2D_3$ has similar effects on normal and malignant cells in regulation of proliferation rate [Eisman et al., 1979; Frampton et al., 1983]. FN production is induced by 1,25-$(OH)_2D_3$ in a range of bone, fibroblast, and cancer cell lines [Manolagas, 1987; Jones and Calverly, 1993; Franceschi et al., 1985], indicating that the hor-

Abbreviations used: bp(s), base pair(s); CAT, chloramphenicol acetyltransferase; ECM, extracellular matrix; FN, fibronectin; nt(s), nucleotide(s); OC, osteocalcin; oligo, oligonucleotide; VDR, vitamin D receptor; VDRE, vitamin D response element; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3.

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mone may be operating in an antiproliferative fashion on metastatic cells. New therapeutic strategies based on 1,25-$(OH)_2D_3$ analogues show promise as anticancer agents [Reichel et al., 1989].

Franceschi et al. [1987] showed that 1,25-$(OH)_2D_3$ altered cell growth in a human osteosarcoma cell line, MG-63, and led to increased concentrations of FN. Similarly, induction of FN by 1,25-$(OH)_2D_3$ and consequent cellular morphological changes have been reported [Bonewald et al., 1992; Brackman et al., 1992; Dokoh et al., 1984; Silbermann et al., 1987]. Finally, FN mRNA levels are induced by 1,25-$(OH)_2D_3$ [Franceschi et al., 1985], suggesting transcriptional regulation. The vitamin D receptor (VDR), retinoic acid receptor (RAR), and retinoid X receptor (RXR) belong to a family of steroid hormone receptors which mediate gene regulation by binding *cis*-acting elements, which are comprised of varied core binding motif arrangements termed hormone response elements (HREs) [Green and Chambon, 1988; Evans, 1988; O'Malley, 1990]. Several response element models have been proposed which allow classification of natural and synthetic response elements. Umesono et al. [1991] proposed the 3-4-5 spacing rule to define the preference of VDR, T_3R (thyroid hormone receptor), and RAR for three, four, and five intervening nucleotides between two hexameric core motifs arranged as direct repeats, respectively. Studies on receptor heterodimerization [Yu et al., 1991; Leid et al., 1992; Marks et al., 1992; Kliewer et al., 1992] have shown that VDR, T_3R, and RAR require RXR as a heterodimeric partner to bind two core DNA binding motifs. However, potential for greater biological diversity exists in models proposed by Carlberg et al. [1993] and Schrader et al. [1993]. It has been shown that VDRs do not only form heterodimers with RXR but also homodimers [Carlberg et al., 1993] and heterodimers with RAR [Schrader et al., 1993] and T_3R [Schrader et al., 1994b]. These four VDR complexes do not bind only to natural VDREs formed by direct repeats spaced by three and six nucleotides (DR3- and DR6-type VDREs) [Carlberg et al., 1993] but also to inverted palindromes spaced by nine nucleotides (IP9-type VDRE) [Schrader et al., 1995]. In addition, the polarity of a heterodimeric complex can affect the magnitude of induction response and the dose response of the hormone, increasing the flexibility of nuclear signalling by 1,25-$(OH)_2D_3$ [Schrader et al., 1994, 1995] by permitting a hierarchy of gene response for a given hormone concentration.

In this report, we provide evidence that FN is a primary 1,25-$(OH)_2D_3$ responding gene. We have identified a natural, functional VDRE present in the mouse, human, and rat FN promoters. The FN VDRE has DR6-type structure that is bound specifically by VDR homodimers.

MATERIALS AND METHODS

Cell Culture and Northern Analysis

NIH 3T3 cells, a Swiss mouse fibroblast cell line (American Type Culture Collection, Rockville, MD), were cultured and maintained in Dulbecco's modified Eagle's medium (pH 7.3), supplemented with 10% (v/v) fetal calf serum, 4 mM L-glutamine, 20 mM HEPES, 1.28 mM gentamicin, insulin (100 U/500 mL) and penicillin/streptomycin (12,500 IU/500 mL). Confluent NIH 3T3 cells treated with 1,25-$(OH)_2D_3$ (at 1 nM) (a gift of M. Uskokovic, Hoffmann LaRoche, Nutley, NJ), 9-*cis* RA, all-*trans* RA (retinoid derivatives of vitamin A, both at 100 nM) (Hoffmann LaRoche, Basel, Switzerland), L-3,5,3′-triiodothyronine (T_3) (at 1 μM) (Sigma Chemical Company, Sydney, Australia), and forskolin (10 μM) (Sigma). Cells were costimulated with 1,25-$(OH)_2D_3$ and each of the above-mentioned hormone treatments for 16 h at 37°C. Total RNA (20 μg) was isolated using the acid–guanidine thiocyanate–phenol-choloform method [Chomczynski and Sacchi, 1986], electrophoresed through a 1% agarose-formaldehyde gel, and blotted onto nitrocellulose (Hybond-N^+; Amersham, Sydney, Australia). A 2.2 kb rat FN cDNA [Schwarzbauer et al., 1983] fragment was radiolabelled with [α-^{32}P]-dCTP (specific activity 10^8 cpm/μg) and hybridised to Northern blotted RNA at 50°C. Filters were washed for 2 min at room temperature (R/T), followed by 2 × 15 min at R/T in 2× SSC, 0.1% SDS, followed by 2 × 15 min at 50°C in 0.2× SSC, 0.1% SDS, and exposed to Kodak BioMax MR X-ray film (Integrated Sciences, Sydney, Australia) overnight with intensifying screens. FN mRNA levels were quantified by densitometry on a Personal Densitometer SI (Molecular Dynamics) and reported as induction over control FN mRNA levels once corrected for volumetric optical density of the 18S rRNA band.

Plasmid Construction

Reporter plasmid constructs containing the human osteocalcin (OC) gene promoter fused to the chloramphenicol acetyltransferase (CAT) reporter gene have been described earlier [Morrison et al., 1989]. The murine FN gene promoter region with adjacent polylinker sequence, 528 bp in length [Polly and Nicholson, 1993], was subcloned directly into *Hind* III/*Bgl* II sites of pTKCAT (a gift of R.J. Miksicek, German Cancer Research Center, Heidelberg, Germany) in exchange for TK and represents nucleotides −227 to +301 with respect to the transcription start site [Polly and Nicholson, 1993]. This intermediary plasmid contained some FN exon sequence which was out of frame with the CAT gene. This was corrected to produce pFNCAT by excising a *Bal* I to *Nco* I region (nts −46/+744), which originated in the FN gene leader sequence and continued into the CAT reporter gene sequence. To repair the deleted CAT gene downstream of the FN promoter, a blunt *Nco* I fragment representing the CAT gene was excised from pTKCAT and inserted into the deleted intermediary plasmid to give rise to pFNCAT. A series of heterologous promoter constructs was designed to include or exclude putative VDRE sequences. An 82 bp fragment was cloned as a single copy in both forward (pFN82F.OSCAT1) and reverse (pFN82R.OSCAT1) orientations into the *Sac* I site of pOSCAT1 (nts −344/+34, which is nonresponsive to 1,25-$(OH)_2D_3$ [Morrison et al., 1989]). Complementary 65 bp oligonucleotides representing nucleotides −206 to −145 in the FN promoter were designed with *Hind* III ends to facilitate cloning into the *Hind* III site of pOSCAT1. Reporter constructs included pFN65F.OSCAT1 (single copy of 65 bp fragment in the forward orientation) and pFN65.3R.OSCAT1 (three copy multimer of 65 bp fragment in reverse orientation).

Complementary oligonucleotides were then designed to contain directly repeated hexameric core binding motifs with varied intervening nucleotide arrangements. Each complementary pair of oligonucleotides was annealed and represented truncated FN fragments of the 65 bp fragment described above. Oligonucleotides used in this study were: fibronectin promoter VDRE region 65-mer (FN65), 5′-ATCAGCATCTCTTTTGTTCGCGGGAACCCACCGTACCCCGTGACGTCACCCGGACTCTGGG-3′; fibronectin promoter VDRE region 32-mer (FN32), 5′-ACCCACCGTACCCCGTGACGTCACCCGGACTC-3′; human osteocalcin VDRE (OC VDRE), 5′-TGGTGACTCACCGGGTGAACGGGGCA-3″. Fibronectin VDRE derivatives were: Am, 5′-CCGGGTGACGTCACGGGGGTA-3′; Ah, 5′-CCGGGTGACGTCACGGGGGA-3′; Bm, 5′-ACGGGGTACGGTGGGTTCG-3′; Bh, 5′-ACGGGGGACTGTGGGTTCG-3′; Cm, 5′-TGACGTCACGGGGTA-3′. **Am** (nts −152/−171) and **Ah** (nts −161/−180) represented directly repeated half-site response elements with six intervening nucleotides (DR6) for the mouse and human FN VDRE, respectively. **Bm** (nts −164/−182) and **Bh** (−173/−191) represented directly repeated half-site response elements with five intervening nucleotides (DR5) for the mouse and human FN VDRE, respectively. **Cm** (nts −157/−171) represented directly repeated half-site response elements with one intervening nucleotide (DR1) for the mouse FN VDRE. **Am, Ah, Bm, Bh,** and **Cm** were subcloned into the *Xba* I site upstream of the thymidine kinase promoter (*tk*) in pBLCAT2 [Luckow and Schutz, 1987] as previously described by Schrader et al. [1994a].

Cell Culture, Transfections, and CAT Assays

Osteoblast-like rat osteosarcoma cells, ROS 17/2.8 (a gift of S. Rodan, Merck Sharp and Dohme, West Point, PA), were cultured and maintained in Ham F-12 medium (ICN, Sydney, Australia) [as described by Rodan and Rodan, 1984; Morrison and Eisman, 1993]. NIH 3T3 cells, a Swiss mouse fibroblast cell line (American Type Culture Collection), were cultured and maintained in Dulbecco's modified Eagle's medium (described in cell culture and Northern analysis section). ROS 17/2.8 and NIH 3T3 cells were transfected by calcium-phosphate precipitation with slight modification of the method of Wigler et al., [1979], as previously described [Morrison et al., 1989; Morrison and Eisman, 1993]. Cells were cotransfected with an internal reference control plasmid, pRSVβ-gal [Gorman et al., 1982]. The human breast cancer cell line MCF-7 was cultured and transfected as described by Carlberg et al. [1993] and Schrader et al. [1993]. MCF-7 cells were cotransfected with pCH110 as an internal control for transfection efficiency. Transfected ROS 17/2.8, NIH 3T3, and MCF-7 cells were treated with 1,25-$(OH)_2D_3$ (at a final concentration of 12–100 nM in ethanol or ethanol vehicle). MCF-7 cells were treated with 9-*cis* RA alone or in combination with

1,25-$(OH)_2D_3$ (Hoffmann LaRoche, Basel, Switzerland) (at a final concentration of 100 nM) as described by Schrader et al. [1994a]. The cells were harvested 16 h after treatment and assayed for CAT activity in ROS 17/2.8 and NIH 3T3 cells with a nonchromatographic method [Sleigh, 1986]. MCF-7 cells were assayed with a chromatographic method as described by Pothier et al. [1992]. CAT activities were corrected for β-galactosidase activity and determined as the ratios of the CAT activity of ligand-stimulated cells to that of mock-induced controls [Morrison et al., 1989; Schrader et al., 1994a].

Gel Shift Analysis

Nuclear extracts from untreated NIH 3T3 and ROS 17/2.8 cells were isolated by the method of Dignam et al. [1983]. Oligonucleotides were synthesised to represent the human OC VDRE and surrounding DNA sequences (Fig. 4ii) (nts −521/−457) [Morrison et al., 1989], a 32 bp fragment of the FN promoter (Fig. 4i) (nts −180/−148) [Polly and Nicholson, 1993], and a 65 bp fragment of the FN promoter (Fig. 4i) (nts −206/−145) [Polly and Nicholson, 1993]. Complementary oligonucleotides were annealed and labelled to 10^8 cpm/μg by Klenow fill-in. Probes were gel-purified through a 15% acrylamide gel. DNA probes (5,000–30,000 cpm/reaction) were incubated with 2.5–7.5 μg of nuclear extract (determined by Bio-Rad Protein Assay; Bio-Rad Laboratories, Sydney, Australia) in 10 mM Tris-HCl/HEPES, pH 7.9, 50 mM KCl, 0.5 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT, and 6 μg poly dI-dC for 20 min at room temperature [Sagami et al., 1986]. Antibody interference was performed with antibodies against the DNA- and ligand-binding domains (50 μg) [Tuohimaa et al., 1992]. DNA-protein reactions described above underwent additional postincubations with antibodies for 15 min at room temperature. Gel preelectrophoresis was performed for 15 min at 4°C. The reaction mixtures were electrophoresed at 35 mA through a 5% nondenaturing polyacrylamide gel containing 1.6% glycerol prepared in 0.5× strength TBE buffer (89 mM Tris Base (Sigma-Aldrich, Sydney, Australia), 89 mM Boric acid (BDH-Analar Merck, Sydney, Australia), 4 mM EDTA (Boehringer-Mannheim, Sydney), pH 7.4) [Baldwin, 1990; Garner and Revzin, 1981]. Gels were dried under vacuum at 80°C and exposed to X-ray film overnight with intensifying screens.

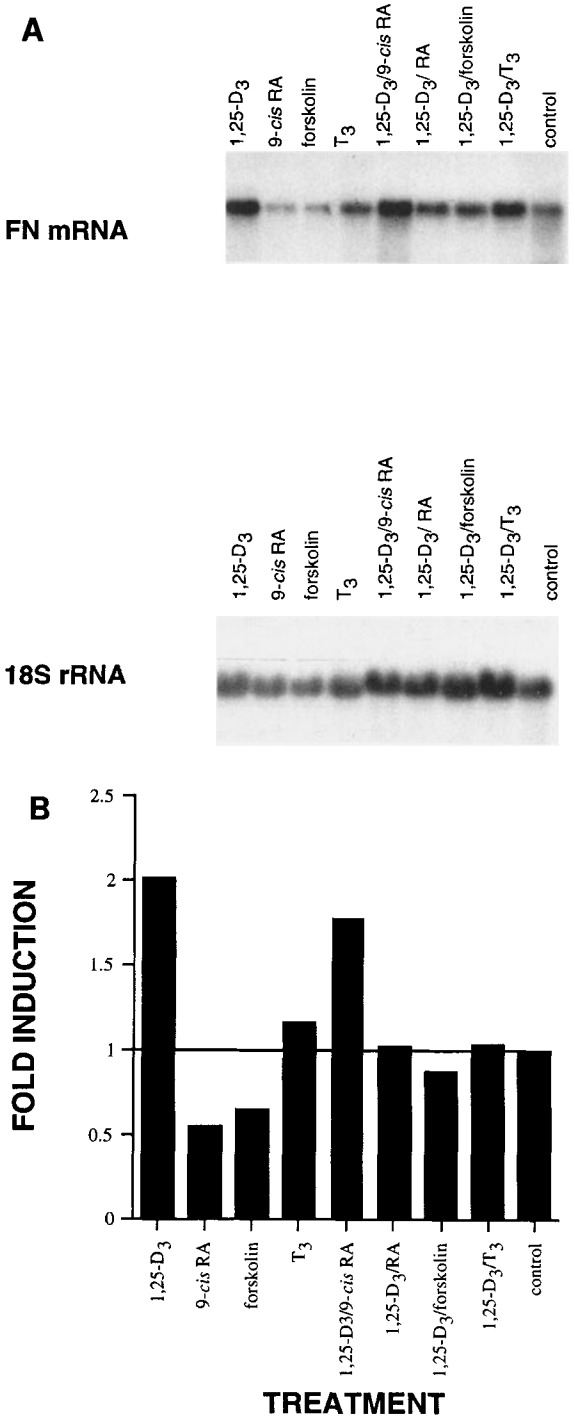

Fig. 1. **A:** Northern analysis of NIH 3T3 cells treated with 1,25-$(OH)_2D_3$, 9-*cis* RA, all-*trans* RA, forskolin, and T_3 for 16 h at 37°C showed that FN gene expression was regulated by 1,25-$(OH)_2D_3$ twofold that of control levels. **B:** *Densitometry* analysis corrected for ribosomal 18S expression levels showed that 1,25-$(OH)_2D_3$/9-*cis* RA treatment did not result in levels of expression above that for 1,25-$(OH)_2D_3$ alone.

In Vitro Translation of Nuclear Receptors and DNA Binding Assays

Linearized cDNAs for VDR and RXRα were used for in vitro transcription (Promega, Wallisellen, Switzerland). Each RNA (5 μg) was mixed with 175 μl rabbit reticulocyte lysate, 100 U RNasin, and 20 μM complete amino acid mixture (Promega) in a total volume of 250 μl and incubated at 30°C for 180 min. DNA probes were prepared and radiolabelled and used with in vitro translated receptors in gel shift experiments as outlined previously [Schrader et al., 1994a].

Statistical Analysis

Data from transfection experiments were tested for significant differences by Student's *t*-test. Significant *P* values for transfection experiments were taken as less than 0.01.

RESULTS

1,25-$(OH)_2D_3$ Modulates FN Gene Expression

To test for regulation of the FN gene, NIH 3T3 cells were treated with 1,25-$(OH)_2D_3$ (vitamin D_3), 9-*cis* retinoic acid (9-*cis* RA), retinoic acid (all-*trans* RA), L-3,5,3'-triiodothyronine (T_3), and the adenylate cyclase stimulator, forskolin. Combinations of 1,25-$(OH)_2D_3$ with each of these hormones were used to assess possible interactions in 1,25-$(OH)_2D_3$ signalling (Fig. 1A). 1,25-$(OH)_2D_3$ increased FN gene expression in NIH 3T3 cells twofold that of untreated FN mRNA levels. In contrast, 9-*cis* RA treatment resulted in downregulation of basal and 1,25-$(OH)_2D_3$ stimulated FN expression in NIH 3T3 cells. Treatment with T_3 and forskolin did not further increase FN mRNA above control levels. Cotreatment of cells with 1,25-$(OH)_2D_3$ with T_3 and 1,25-$(OH)_2D_3$ with 9-*cis* RA did not result in a synergistic effect above levels of FN expression when stimulated with 1,25-$(OH)_2D_3$ alone. Forskolin treatment of NIH 3T3 cells resulted in downregulated FN expression with marked repression of 1,25-$(OH)_2D_3$–stimulated FN gene expression compared to basal and 1,25-$(OH)_2D_3$–treated control levels (Fig. 1B).

Regulation of the pFNCAT Construct by 1,25-$(OH)_2D_3$

pFNCAT, pOSCAT2, and pOSCAT1 (Fig. 2A) were transfected into ROS 17/2.8 and NIH 3T3 cells and tested for 1,25-$(OH)_2D_3$ regulation. pFNCAT activity was induced by 1,25-$(OH)_2D_3$

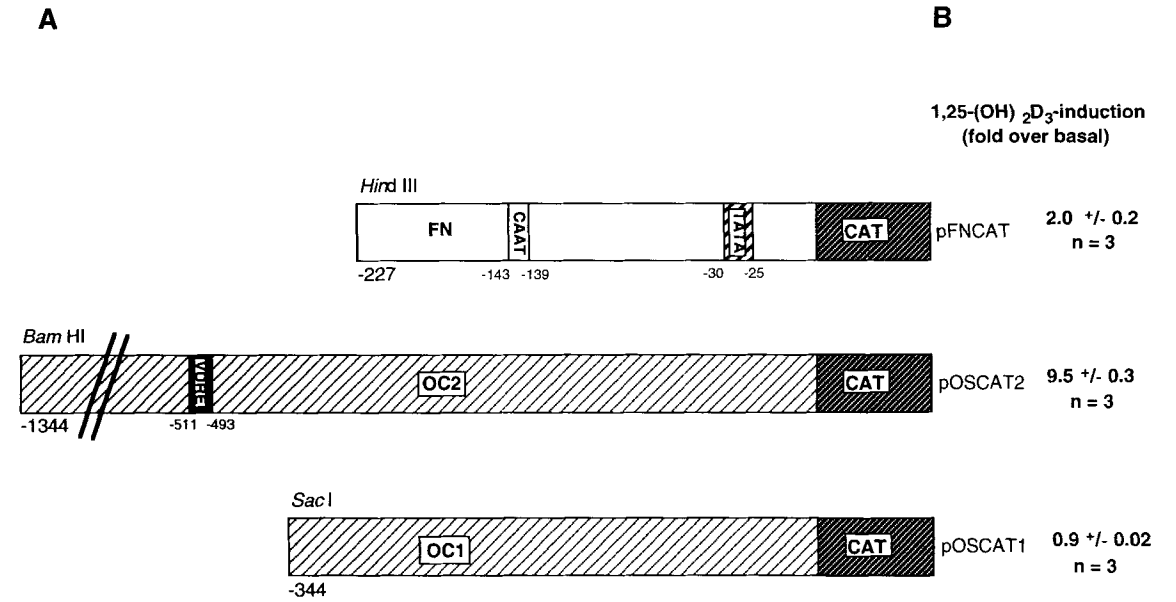

Fig. 2. Schematic representation and transcriptional activity of pFNCAT, pOSCAT2, and pOSCAT1 promoter constructs assessed by CAT assay in NIH 3T3 cells. **A:** The region −1,344/+34 of the OC gene promoter was fused in front of the CAT gene in pTKCAT in exchange for *tk* to generate the 1,25-$(OH)_2D_3$ responsive reporter construct pOSCAT2 [Morrison et al., 1989]. Similarly, areas spanning nucleotides −227/+46 of the FN gene promoter and sequences −344/+34 from the OC promoter were inserted in front CAT to generate pFNCAT and pOSCAT1 (nonresponsive to 1,25-$(OH)_2D_3$ [Morrison et al., 1989]), respectively. **B:** Basal and vitamin D–induced CAT activity (16 h at 12 nM) of pOSCAT2, pFNCAT, and pOSCAT1 is shown.

in NIH 3T3 twofold from a high basal level (Fig. 2B). pOSCAT2 basal activity was lower than basal pFNCAT activity, but 1,25-$(OH)_2D_3$ induced activity (9.5-fold) to levels comparable to pFNCAT 1,25-$(OH)_2D_3$–induced activity. Basal transcriptional activity of pOSCAT1 was low and was not induced by 1,25-$(OH)_2D_3$. Transfection of pFNCAT into ROS 17/2.8 cells resulted in a basal activity two-thirds of that seen in NIH 3T3 but with a comparable twofold increase with 1,25-$(OH)_2D_3$ treatment (data not shown).

Identification of a VDRE

Regions of the FN promoter were assessed and the VDRE mapped by promoter construct deletion analysis. Transfection of pFN82F.OSCAT1 and pFN82R.OSCAT1 (containing an 82 bp FN fragment, nts −227/−145) into ROS 17/2.8 and NIH 3T3 cell lines resulted in twofold 1,25-$(OH)_2D_3$–induced transcription conferred onto pOSCAT1 by sequences in both forward (pFN82F.OSCAT1; 2.01 ± 0.31) and reverse (pFN82R.OSCAT1; 1.95 ± 0.05) orientations in both cell lines (Fig. 3). A fragment 65 bp in length, representing the VDRE-like region within the FN promoter (nts −206/−145) was used in heterologous promoter plasmid constructs pFN65F.OSCAT1 (as a single copy) and pFN65.3R.OSCAT1 (as a trimer in reverse orientation) (Fig. 3) and transfected into NIH 3T3 cells to identify regions responsible for 1,25-$(OH)_2D_3$ induction. The trimer repeat in the reverse orientation showed twofold induction (2.03 ± 0.5) with 1,25-$(OH)_2D_3$ in NIH 3T3 cells, while the monomer in the forward orientation was less active (1.24 ± 0.16).

Screening of putative VDRE regions in the mouse, rat, and human FN promoters with the hexameric motif RRKNSA (R = A or G, K = G or T, S = C or G) [Carlberg, 1995] identified

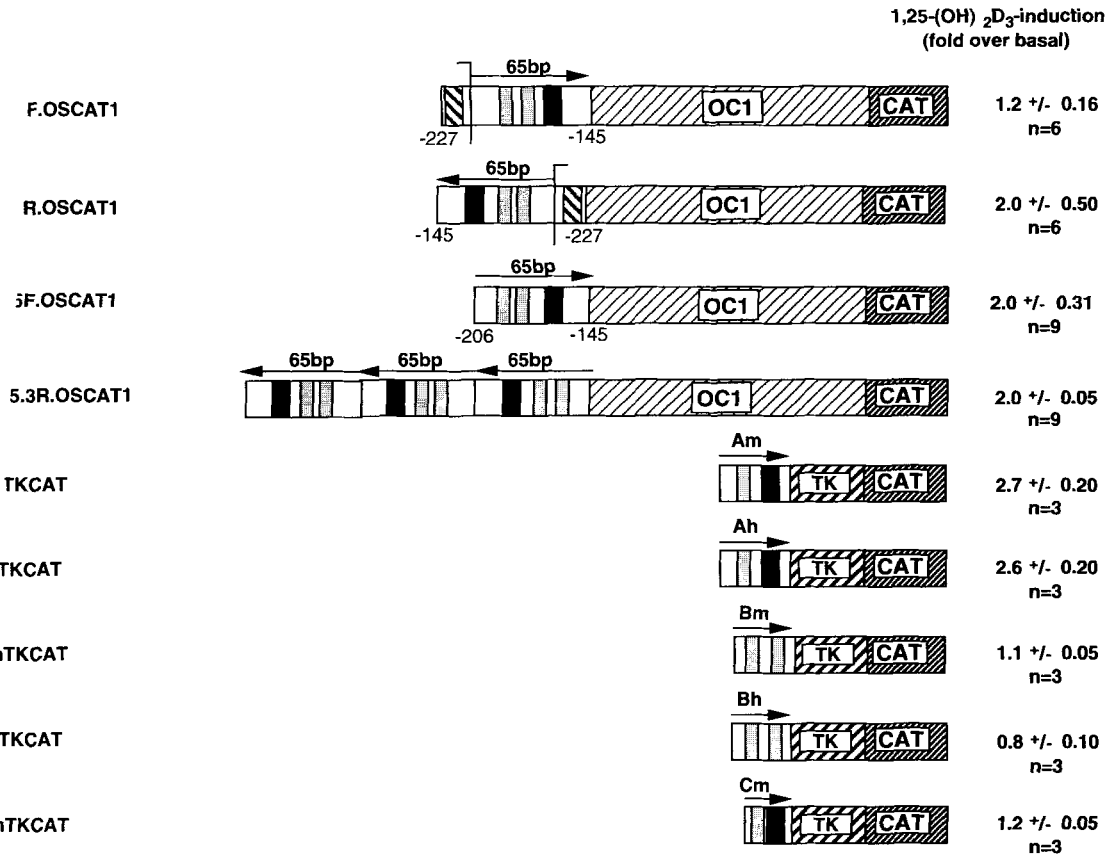

Fig. 3. Diagramatic representation and transcriptional assessment of heterologous promoter constructs in NIH 3T3 cells. FN fragment arrangements used in heterologous promoter constructs are illustrated with 1,25-$(OH)_2D_3$ induction alongside each construct. Closed rectangles represent well-characterised VDRE half-sites; bold striped rectangles in pFN82F.OSCAT1 and pFN82R.OSCAT1 represent an estrogen response element half-site. Open rectangles depict putative VDRE half-sites unique to the FN promoter.

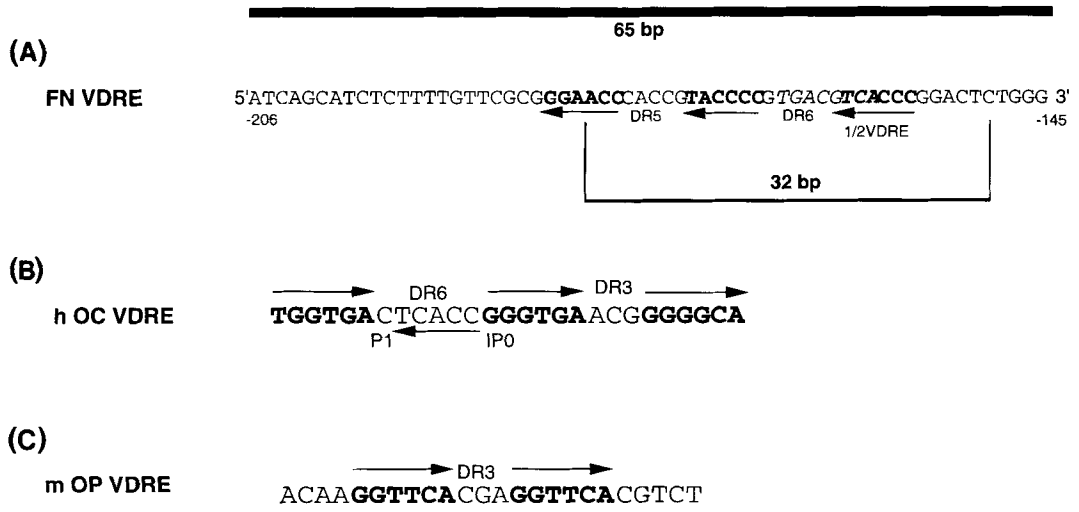

Fig. 4. Sequences of known VDREs. Schematic representation of the 65 bp FN fragment is shown **(A)**. Under- and overlined regions indicate 32 bp and 65 bp fragments (used in gel shift), respectively. Putative 1,25-$(OH)_2D_3$ responsive half-sites located at −171/−166 and −159/−154 within the 65 bp fragment are indicated by arrows. Half-sites are demarcated in human osteocalcin and osteopontin VDREs [Morrison et al., 1989; Noda et al., 1990] **(B, C)**. Cyclic AMP response element consensus sequences in the FN VDRE-containing region are highlighted in italics (i).

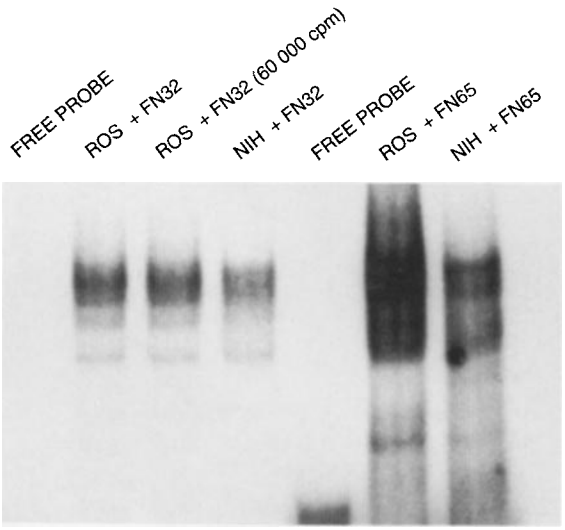

Fig. 5. Gel mobility shift assays show protein-DNA complexes identified in the FN gene promoter responsible for conferring 1,25-$(OH)_2D_3$ response. Affinity differences, based on band intensity, between the 65 bp (30,000 cpm) and 32 bp (30,000 cpm) FN fragments for endogenous VDR from ROS 17/2.8 nuclear extracts (7.5 μg) are representative of three separate experiments.

candidate half-sites. Comparison of this sequence to known VDREs in a range of promoters recognised a short sequence *GGGTGA*, identical to that in the human and rat osteocalcin (OC) VDREs (Fig. 4). The other putative half-site *GGGGTA*, located at −171/−166, is similar to the human osteocalcin VDRE half-site *GGGGCA*. The 65 bp fragment contains a DR6 VDRE containing two hexameric half-sites, *GGGTGA* and *GGGGTA*, directly repeated with six intervening nucleotides. This core DR6 region is also present in the human FN promoter.

Characterisation of DNA/Protein Complexes

FN fragments used in heterologous promoter constructs were tested by gel shift assay. DNA-protein complexes formed in gel shift assays were consistent with binding between the 65 bp fragment (nts −206/−145) and endogenous VDR from ROS 17/2.8 and NIH 3T3 cells (Fig. 5), as judged by comparison with hOC VDRE-protein interactions. A shorter 32 bp fragment (nts −180/−148) containing both hexameric motifs was used to assess specificity of binding. Competition gel shift experiments with 10×, 50×, and 100× (molar excesses) unlabelled oligonucleotides demonstrated that the FN VDRE and OC VDRE competitively bind factors of similar molecular size (Fig. 6A), indicating specific interactions between these two elements and possibly the same protein factors. Inhibition of gel shift complex formation was observed with the monoclonal antibody (mAb 9A7) [MacDonald et al., 1991] (not shown) and the polyclonal antibody (αhVDR-103) [Tuohimaa et al., 1992] (Fig. 6B). The 9A7 monoclonal antibody recognises the hinge region between the DNA-recognition and ligand-binding domains of the VDR [Pike et al., 1982], while the polyclonal antibody (αhVDR-

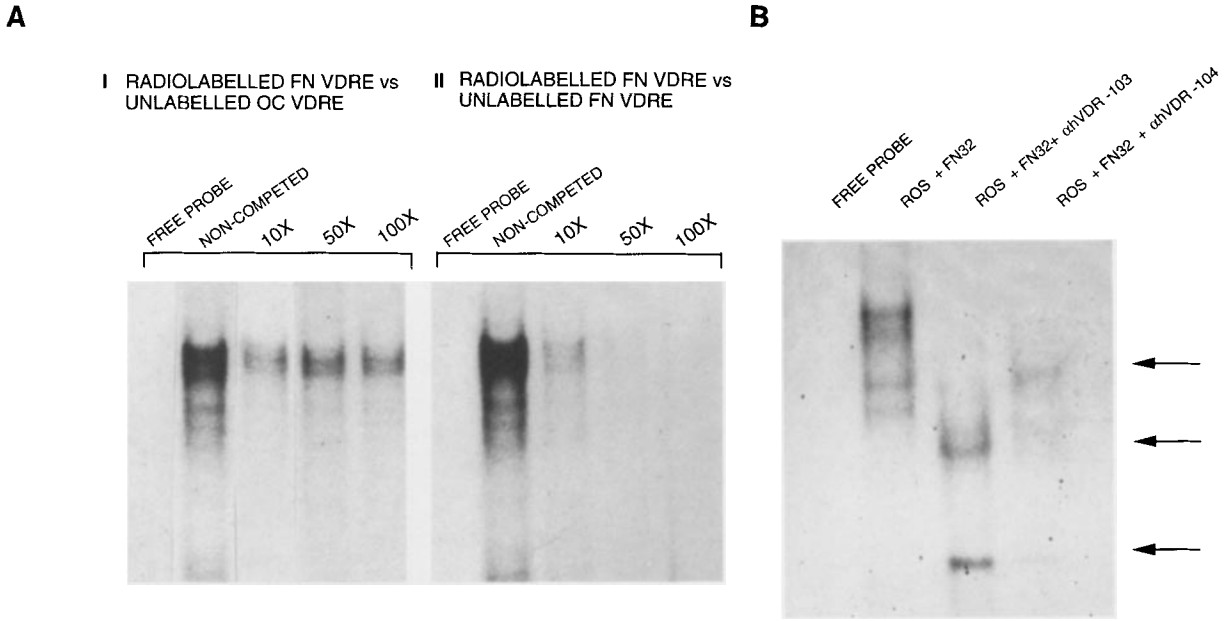

Fig. 6. Competition band shift analysis illustrates specificity of VDR protein for VDRE DNA. **A:** Panel *I* shows competition of FN VDRE (5,000 cpm) vs. unlabelled OC VDRE in increasing molar excess for VDR from NIH 3T3 nuclear extract (2.5 μg). Panel II shows labelled FN VDRE competed against unlabelled FN VDRE. **B:** Specificity of VDR protein for VDRE-like sequences was further validated by incubating ROS 17/2.8 nuclear extract (7.5 μg) with FN VDRE (20,000 cpm) with additional incubations including antibodies αhVDR-103 (50 μg) and αhVDR-104 (50 μg) against the DNA- and ligand-binding domains of the VDR. The slow mobility complex (*upper arrow*) is dissociated, allowing labelled VDRE to bind to other proteins with a faster mobility (*lower two arrows*). The data shown are representative of three independent experiments for each section (A,B).

103) [Tuohimaa et al., 1992] interacts with the N-terminal DNA-binding domain of VDR. Lower molecular weight DNA-protein complexes were identified (indicated by arrows, Fig. 6B) when the FN VDRE was dissociated from VDR by monoclonal and polyclonal antibodies, suggesting that additional proteins are able to bind these sequences.

Vitamin D Signalling is Independent of Other Steroid Hormones

Am (mouse DR6), **Ah** (human DR6), **Bm** (mouse DR5), **Bh** (human DR5), and **Cm** (mouse DR1) (Fig. 7A) representing the FN VDRE with various intervening nucleotide spacing were subcloned as single copy elements upstream of *tk* in pBLCAT2 and assessed for transcriptional activation by various ligands in MCF-7 cells (Fig. 3, 7B). The response elements **Am** and **Ah** showed $1,25\text{-}(OH)_2D_3$–induced transcription which was 2.7-fold that of controls. **Bm, Bh,** and **Cm** elements did not respond to $1,25\text{-}(OH)_2D_3$, 9-*cis* RA, or combined $1,25\text{-}(OH)_2D_3$ and 9-*cis* RA treatment above control levels of transcription (Fig. 7B). These data show that VDRE activity contained in this region of the FN promoter confers responsiveness to a heterologous promoter both as a single copy and as a multicopy element.

FN VDRE Binds VDR as a Homodimer

Am, Ah, Bm, Bh, and **Cm** representing shorter FN fragments within the 65 bp FN fragment (Fig. 7A) were used in gel shift experiments with in vitro translated VDR and RXRα to clarify the regions and mechanisms involved in VDR binding to FN VDRE and consequent $1,25\text{-}(OH)_2D_3$ signalling. **Am** and **Ah** containing the DR6 sequence configuration bound VDR as a homodimer (Fig. 7C). These data were consistent with functional observations in transient transfection (Fig. 7B). **Bm, Bh** (DR5), and **Cm** (DR1) did not bind VDR-VDR or VDR-RXRα in gel shift assay (Fig. 7C).

DISCUSSION

The data presented in this paper describe a region of the murine FN promoter which is responsive to $1,25\text{-}(OH)_2D_3$. The 20 bp region −171/−152 (**Am**) contained two sequence elements with homology to known vitamin D response element half-sites. This region is also conserved in rat and human FN promoters. The

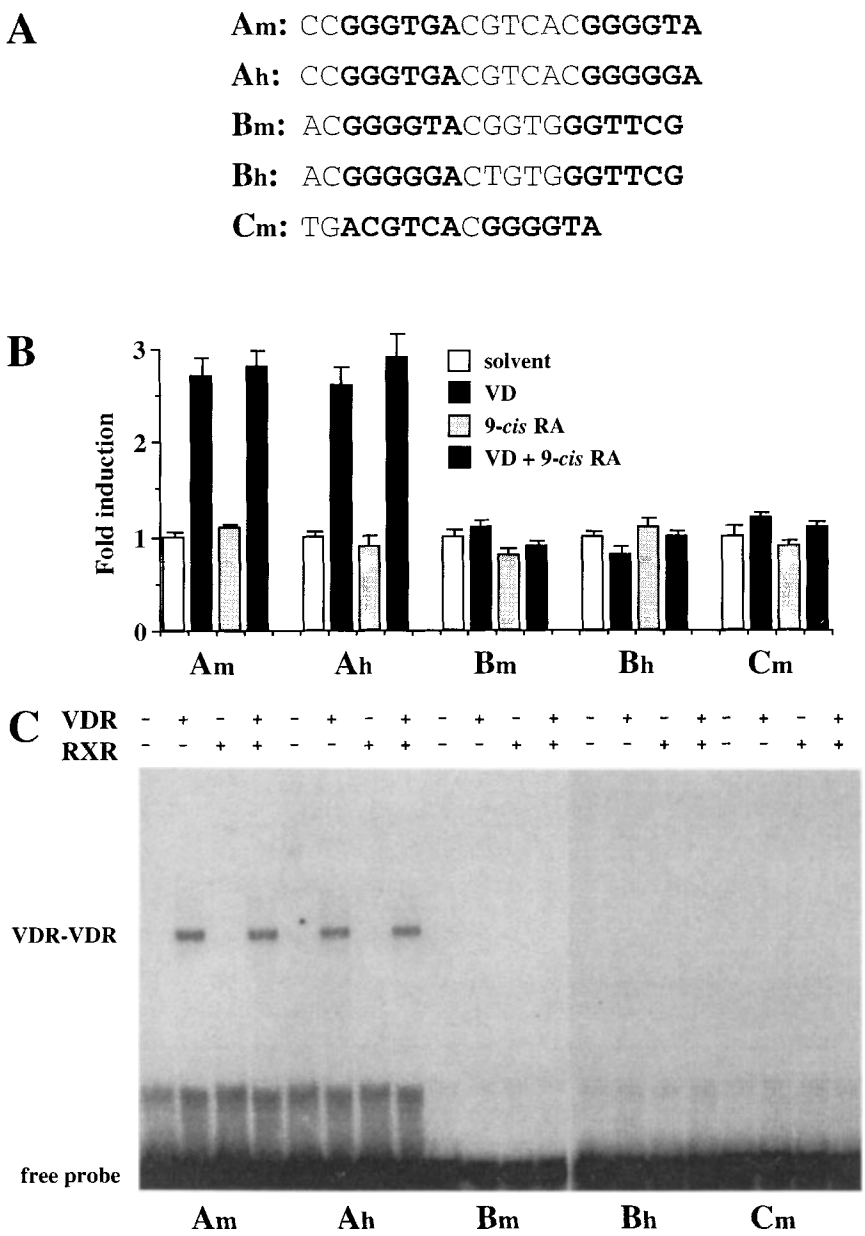

Fig. 7. Truncated fragments of the 65 bp FN fragment which contained core hexameric VDRE half-sites were assessed in CAT and gel shift assays. **A: Am, Ah** (DR6-type), **Bm, Bh** (DR5-type), and **Cm** (DR1-type) response elements are shown. **B:** 1,25-$(OH)_2D_3$–stimulated transcription of **Am** and **Ah** was 2.7-fold that of controls. **Bm, Bh,** and **Cm** did not show 1,25-$(OH)_2D_3$–or 9-cis RA-induced transcription. **C:** Gel shift experiments with in vitro translated VDR and RXRα receptors are shown below corresponding CAT assay data (B). **Am** and **Ah** bound VDR as a homodimer; these fragments were unable to bind VDR-RXRα heterodimers. **Bm, Bh,** and **Cm** did not bind VDR-VDR homodimers or VDR-RXRα heterodimers.

observed 2–2.7-fold 1,25-$(OH)_2D_3$ induction of transcription (Figs. 2B, 7B) is comparable with the twofold upregulation of FN at the mRNA level observed by us (Fig. 1) and also reported by Franceschi et al. [1987]. Synergistic 1,25-$(OH)_2D_3$/9-*cis* RA ligand-induced transcriptional effects did not result (Fig. 7B). These results may reflect cell-specific differences in RXR content which often determine 9-*cis* transcriptional effects [MacDonald et al., 1993]. Similarly, a single copy of the human osteocalcin VDRE confers a twofold induction when cloned in a heterologous promoter [Morrison et al., 1989]. The inability of the 65 bp FN fragment to function in the single copy constructs may reflect promoter context or the need for accessory se-

quence elements. The 82 bp fragment (nts −227/−145) in pFN82F.OSCAT1 and pFN82R.OSCAT1 (twofold 1,25-$(OH)_2D_3$ induction) contained a distal 5′ estrogen half-site *AGGTCA* identical to that found 1.3 kb upstream in the rat FN promoter [Patel et al., 1987]. We searched the available sequence of the human fibronectin promoter and rat fibronectin promoter (which extends 1.3 kb 5′) and noted numerous steroid element-like sequences. Interestingly, the core DR6 VDRE region in the mouse fibronectin promoter we propose as responsible for vitamin D_3–induced activity is conserved in the human fibronectin promoter. Regardless, the 65 bp FN fragment is capable of binding the VDR with high affinity and is able to confer responsiveness to a heterologous promoter, meeting the requirement of a VDRE.

Gel shift experiments with nuclear extracts from ROS 17/2.8 and NIH 3T3 demonstrated the presence of two major DNA-protein complexes which were consistent with VDR protein binding patterns observed for other VDREs [Noda et al., 1990; Ozono et al., 1990; Kerner et al., 1989]. Core VDRE regions in human and murine FN, **Am, Ah** (DR6), **Bm, Bh** (DR5), and **Cm** (DR1) used in gel shift with in vitro translated VDR and RXRα, showed that the FN VDRE is a DR6 element which binds VDR as a homodimer (Fig. 7C). Spacing differences by intervening nucleotides of hexameric half-sites determines the binding preference of VDR homodimers (VDR-VDR) or VDR-retinoid heterodimers (VDR-RAR, VDR-RXR). Obvious differences are shown where VDR homodimers preferably bind FN DR6-type (in the absence of RXR), not FN DR5- and FN DR1-type response elements (Fig. 7C). These observations complement functional data which provide a mechanism of 1,25-$(OH)_2D_3$ transcriptional effects (Fig. 7B). Unlabelled oligonucleotide competition and antibody gel shift experiments showed that VDR exhibited sequence-specific binding to the FN promoter.

The high basal activity of pFNCAT suggests that certain elements other than the VDRE (possibly the CRE found at nucleotide positions −164 to −157) may be contributing to FN expression in NIH 3T3 cells (Fig. 4). The CRE described by Miao et al. [1993] underlies the VDRE identified in this paper, suggesting the possibility of interaction between these two regulatory mechanisms and the probability of an overlapping contiguous element. Moreover, cooperative VDR and nonreceptor transcription factor binding to DNA has been reported by Liu and Freedman [1994], presenting evidence for novel protein-protein mechanistic interactions.

The identification of novel natural VDREs should allow a better understanding of the range of the possible sequences that confer biological responsiveness to 1,25-$(OH)_2D_3$ in a variety of genes. Clearly, naturally occuring VDREs can range from simple direct repeats as in the mouse osteopontin VDRE [Noda et al., 1990] to complex direct and palindromic half-site arrangements such as in the human OC VDRE [Morrison et al., 1989; Schrader et al., 1993]. These produce a range of VDREs according to different sequence combinations and permutations which provide for numerous pathways of 1,25-$(OH)_2D_3$ signalling via heterodimeric or homodimeric receptor-DNA interactions [Carlberg, 1995].

Considering the high basal activity of FN promoter, induction by 1,25-$(OH)_2D_3$ could result in a substantial change in FN protein produced. Transcriptional regulation of the FN gene promoter through an authentic VDRE by 1,25-$(OH)_2D_3$ and its analogues may be a method of controlling cellular proliferation and differentiation. Studies on 1,25-$(OH)_2D_3$ analogue effects on transcription can now be extended to other models of vitamin D signalling pathways. This mode of steroidal gene regulation we have described may have important implications in cancer therapy and control in metastatic disease.

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