COMMITTEE CERTIFICATION OF APPROVED VERSION

The committee for Verónica Alejandra Tovar Sepúlveda certifies that this is the approved version of the following dissertation:

"DIFFERENTIAL REGULATION OF PTHRP GENE EXPRESSION BY 1,25$(OH)_2D_3$ IN PROSTATE CANCER CELL LINES"

Committee:

Miriam Falzon, Ph.D., Supervisor

Cheryl S. Watson, Ph.D.

Nancy L. Weigel, Ph.D.

Cary W. Cooper, Ph.D.

Melvyn S. Soloff, Ph.D.

Mary L. Thomas, Ph.D.

Dean, Graduate School

"DIFFERENTIAL REGULATION OF PTHRP GENE EXPRESSION BY 1,25$(OH)_2D_3$ IN PROSTATE CANCER CELL LINES"

by
Verónica Alejandra Tovar Sepúlveda, Diplom-Biologin

Dissertation
Presented to the Faculty of the University of Texas Graduate School of
Biomedical Sciences at Galveston
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

Approved by the Supervisory Committee

Miriam Falzon, Ph.D.
Cheryl S. Watson, Ph.D.
Nancy L. Weigel, Ph.D.
Cary W. Cooper, Ph.D.
Melvyn S. Soloff, Ph.D.
Mary L. Thomas, Ph.D.

August, 2005
Galveston, Texas

Key words: parathyroid hormone related protein, 1,25-dihydroxyvitamin D_3, negative vitamin D response element, vitamin D receptor, retinoid X receptor

Para mi abuelita, *Augusta Santander Herrera*, que en paz descanse.
Sin ti abuelita, nunca hubiese dedicado mi investigación al área oncológica.

ACKNOWLEDGEMENTS

This dissertation would not have been completed without the excellent mentoring of Dr. Miriam Falzon. I would like to express my great gratitude for her valuable guidance and patience. I would also like to sincerely thank Dr. Cary W. Cooper, Dr. Melvin S. Soloff, Dr. Mary L. Thomas, Dr. Cheryl S. Watson, and Dr. Nancy L. Weigel for serving on my advisory committee and for their valuable comments and guidance during this research and the development and finalization this dissertation project. I also appreciate the opportunity to have collaborated with Dr. Weigel in one of her projects and her collaboration in my dissertation project.

I would also like to thank Dr. Patricia K. Seitz for technical and caring advice in any of my different experiments. She is a wonderful trouble shutter. I wish to thank Dr. Gilbert R. Hillman for writing the program in Mathlab that we used to analyzed the fluorescence images.

Finally, I also wish to thank my beloved family members and my friends. Especially my parents, Ing. Pablo E. Tovar and Gloria Sepulveda de Tovar, and my wonderful husband, Dr. J. Gerardo Garcia Gallegos, for their love, patience and caring support.

"DIFFERENTIAL REGULATION OF PTHRP GENE EXPRESSION BY 1,25$(OH)_2D_3$ IN PROSTATE CANCER CELL LINES"

Publication No._____

Verónica Alejandra Tovar Sepúlveda, Ph.D.
The University of Texas Graduate School of Biomedical Sciences at Galveston, 2005

Supervisor: Miriam Falzon

Parathyroid hormone-related protein (PTHrP) is expressed by prostate cancer cells. Since PTHrP enhances the growth and osteolytic potential of prostate cancer cells, it is important to control PTHrP expression in these cells. 1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) downregulates PTHrP expression in a variety of human cell types, including prostate cancer cells. Therefore, downregulation of PTHrP gene expression by $1,25(OH)_2D_3$ may enhance the therapeutic benefits of $1,25(OH)_2D_3$ by neutralizing PTHrP's contribution to the pathogenesis and progression of prostate carcinoma and its tendency to metastasize to bone. In this study, we show that $1,25(OH)_2D_3$ and its non-hypercalcemic analog EB1089 decrease cell proliferation and suppress PTHrP mRNA and protein levels in the human prostate cancer cell lines LNCaP, C4-2, and PC-3. These cell lines represent early to advanced stages of prostate cancer, since LNCaP cells are weakly metastatic, androgen receptor (AR)-positive and androgen-responsive cells, C4-2 cells are AR-positive and androgen-independent LNCaP variants, and PC-3 cells are highly metastatic AR-negative cells. We identified a negative vitamin D response element ($nVDRE_{hPTHrP}$) within the human *PTHrP* gene; interaction of the vitamin D receptor (VDR) with this nVDRE is decreased by $1,25(OH)_2D_3$. However, transient transfection of $nVDRE_{hPTHrP}$ cloned upstream of the SV40 promoter downregulated promoter activity in response to $1,25(OH)_2D_3$ or EB1089 treatment in LNCaP and C4-2, but not in PC-3, cells. The retinoid X receptor (RXR) is a frequent heterodimeric partner of the VDR. We show that RXRα forms part of the nuclear protein complex that

interacts with $nVDRE_{hPTHrP}$ with the VDR in LNCaP, C4-2, and PC-3 cells. The RXR ligand 9-*cis*-retinoic acid (9-*cis*-RA) downregulates PTHrP mRNA levels in both LNCaP and C4-2 cells; this decrease is more pronounced in LNCaP than in C4-2 cells. 9-*cis*-RA also enhances the $1,25(OH)_2D_3$-mediated downregulation of PTHrP expression in both cell lines; this effect is more pronounced in LNCaP cells. Co-treatment with $1,25(OH)_2D_3$ or EB1089 and 9-*cis*-RA further decreased promoter activity driven via $nVDRE_{hPTHrP}$ in LNCaP, but not C4-2, cells. The proliferation of LNCaP, but not C4-2, cells is decreased by 9-*cis*-RA. These results indicate that PTHrP gene expression is regulated by $1,25(OH)_2D_3$ in a cell line-specific manner in prostate cancer cells.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

1α-(OH)ase	25-Hydroxyvitamin D_3-1α-hydroxylase
5α-red	5 Alpha-reductase
ANOVA	Analysis of variance
AR	Androgen receptor
ATP	Adenine-triphosphate
AU	Arbitrary unit
BPH	Benign prostatic hyperplasia
CAT	Chloramphenicol acetyltransferase
cRA or 9-*cis*-RA	9-*cis*-Retinoic acid
D_3 or $1,25(OH)_2D_3$	1,25-Dihydroxyvitamin D_3
dCTP	Deoxy-cytosine-triphosphate
DHT	Dihydrotestosterone
DR1	Two directly-repeating hexanucleotide motifs separated by one nucleotide
DR3	Two directly-repeating hexanucleotide motifs separated by three nucleotides
DTT	Dithiothreitol
EB	EB1089
EB1089	1,25-Dihydroxy-22,24-diene-24,26,27-trihomovitamin D_3
ECM	Extracellular matrix
EDTA	Ethylene glycol bis (β-aminoethylester)-N,N,N',N'-tetraacetic acid, or tetra (acetoxymethyl ester)
EGF	Epidermal growth factor
EMSA	Electrophoretic mobility shift assay
Ets	Erythroblast transformation specific
FBS	Fetal bovine serum
FSF	Follicle stimulating hormone
HHM	Humoral hypercalcemia of Malignancy

IGF	Insulin-like growth factor
IgG	Immunoglobulin G
LH	Luteinizing hormone
LH-RHa	Luteinizing hormone-releasing hormone analog
MAP kinase	Mitogen-activated protein kinase
NE	Neuroendrocrine
NLS	Nuclear localization signal
PBS	Phosphate buffered saline
PC	Prostate cancer
PCR	Polymerase chain reaction
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
PSA	Prostate specific antigen
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone related protein
RAR	Retinoid acid receptor
RT	Reverse transcription
RTase	Reverse transcriptase
RXR	Retinoid X receptor
S.E.M	Standard error of the mean
SDS	Sodium dodecyl sulfate
TBE	Tris, boric acid, and EDTA buffer
TGF-α	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
U	Unit
UNG	Uracil-n-glycosylase
VDR	Vitamin D receptor
VDRE	Vitamin D response element

CHAPTER ONE: INTRODUCTION

PROSTATE CANCER

Prostate cancer (PC) is the most commonly diagnosed life-threatening malignancy in men in developed countries, and seems ready to surpass lung cancer as the major cause of cancer death [1]. Huggins and Hodges first described PC as a testosterone-centric disease, because androgens seem to act by promoting growth and cell division [2,3]. The role of androgens, specifically dihydrotestosterone (DHT), in promoting PC is still unclear, but PC does not seem to occur in a group of pseudohermaphrodites in whom 5 alpha-reductase (5α-red) is absent [4]. Variability in the geographic incidence of PC may relate to different levels of DHT in different ethnic groups [4]. The role of androgens in induction and promotion of PC is not completely understood, but once the malignant transformation has become established, androgens certainly have a role in stimulating the malignant cell division and invasion [4].

Several recent clinical studies propose that neuroendocrine (NE) differentiation in the prostate gland predicts tumor relapse and progression after total prostatectomy and radiation therapy. There are several mechanisms by which NE differentiation can affect the natural history and prognosis of PC [5]. The absence of detectable nuclear androgen receptor (AR) strongly suggests that neoplastic NE cells are androgen-insensitive and escape hormonal control. NE cell types are defined by the most commonly used endocrine marker, chromogranin A [6]. Prostatic NE cells produce a variety of neurosecretory products with growth promoting activities, including bombesin/gastrin-related peptide, serotonin, EGF (epidermal growth factor), IGF (insulin-like growth factor), calcitonin, and parathyroid hormone related protein (PTHrP). These regulatory peptides also promote tumor progression and metastasis, and may act through lumencrine, endocrine, paracrine and autocrine mechanisms. They are probably involved in normal growth, differentiation and secretory functions of the prostate gland. In

summary, NE differentiation may have important implications in prostatic malignancies and promotion of tumor progression with significant endocrine features [6]. Furthermore, the absence of proliferation activity may endow NE tumor cells with relative resistance toward cytotoxic drugs and radiation therapy [6].

The knowledge that most PCs are androgen-dependent has been used in anti-androgens therapy and chemical castration or reversible medical castration using LH-RHa (luteinizing hormone-releasing hormone analog) to inhibit LH (luteinizing hormone) and FSF (follicle stimulating hormone) release and to subsequently suppress testosterone secretion. Also, oral or parenteral estrogens have been utilized to reduce growth by down-regulating the production of androgen itself, or by blocking the AR. In addition, multiple forms of combination therapies have been employed to obtain maximal androgen blockade [7]. The drawback of the hormonal therapies used at this time is the ability of many types of cancer to become androgen independent, which eliminates the main target of those therapies and limits their use to early stages of the diseases or in combination with prostatectomy and radiation therapy.

The fact that there are androgen independent types of PC and that in those the secretion of neuroendocrine peptides by NE cells promotes tumor growth, progression and metastases, could provide targets for new therapies in the future.

PARATHYROID HORMONE RELATED PROTEIN (PTHRP)

PTHrP was originally identified as the factor responsible for Humoral Hypercalcemia of Malignancy (HHM), one of the most frequent paraneoplastic syndromes [8-12], which is expressed by prostate, breast, lung, kidney, colon, and other tumors [13]. Several studies show that the protein is ubiquitously produced and secreted by most normal fetal and adult tissues [14,15]. However, PTHrP is not normally present in the circulation [16], suggesting an autocrine/paracrine, rather than endocrine, mechanism of action. PTHrP plays a physiological role in normal bone formation (where it is produced by the periarticular cells and acts on receptors located in the proliferating cartilage cells in a paracrine manner [17]); influences development of the mammary gland (where it is secreted

by epithelial cells; and acts in a paracrine manner on mesenchymal stromal cells [17]), skin and teeth; and regulates the contractility of smooth muscle (where it is secreted in response to mechanical stretch resulting in muscle relaxation, via paracrine/autocrine pathways [17]). These effects of PTHrP are mediated via the parathyroid hormone (PTH)/PTHrP type I (PTH1) receptor. In addition, PTHrP is also capable of entering the nucleus and/or the nucleolus to influence cellular events in an intracrine fashion [18-20]. The PTHrP molecule contains a midregion Nuclear Localization Signal (NLS) with multibasic clusters in the 88-106 region, similar to the NLS found in viral and mammalian transcription factors [18-21]. Paracrine/autocrine and intracrine effects of PTHrP are tissue specific and may have opposite outcomes. For example, overexpression of PTHrP in MCF-7 cells was associated with an increase in mitogenesis, whereas inhibiting endogenous PTHrP production resulted in decreased cell proliferation [22]. In contrast, PTHrP interaction with the cell surface PTH/PTHrP receptor resulted in decreased cell proliferation in the same cell line, effects that were abolished by neutralization of added peptide with an anti-PTHrP antiserum [22]. The net effect of autocrine/paracrine and intracrine effects of PTHrP in MCF-7 cells overproducing the protein was accelerated cell growth [22]. Similarly, PTHrP gene transfection into rat A10 vascular smooth cells resulted in a striking increase in mitogenesis [23]. Transfection of PTHrP lacking the NLS did not elicit the proliferative response, indicating that this is an intracrine effect [23]. This nuclear effect on proliferation is the diametric opposite of the effects of PTHrP when it is added to the rat vascular smooth muscle cells and interacts with cell surface receptors on these cells [23].

The human *PTHrP* gene is present as a single copy in chromosome 12; it has 9 exons and three distinctive promoters (P1, P2, and P3), with P3 being used most often in cancer cells [24] (Figure 1.1). All PTHrP transcripts have products from exons 5 and 6, these two exons encode the pre-pro region and the majority of the mature peptide [17]. Alternative splicing events give rise to three different isoforms (1-139, 1-141, 1-173 amino acids), which are identical in sequence in the first 139 amino acids [17] (Figure 1.1). These three isoforms are post-translationally processed to a family of mature secretory

forms of PTHrP (including 1-36, 38-94, 38-95 and 38-101, as well as the C-terminal fragments 107-138 and 109-138 [25,26]). PTHrP's amino-terminal part has PTH-like properties and plays a role in increased bone reabsorption [17]. The C-terminal fragments, on the contrary, are involved in the inhibition of bone reabsorption [27] and may also increase bone formation via induction of mitogenesis in the osteoblasts [28]. The mid-region fragments (amino acids 67-86) are involved in fetal calcium transport [29]. The signal sequence (-36 to -1) directs PTHrP to the endoplasmic reticulum where the pre-pro sequence is removed prior to secretion [17]. Each of these secretory forms is thought to have one or more unique receptors on the cell surface that mediates the paracrine and autocrine actions of PTHrP [24]. Only the PTH1 receptor, that binds to PTH, PTHrP, and their N-terminal analogs, has been cloned to date [30,31].

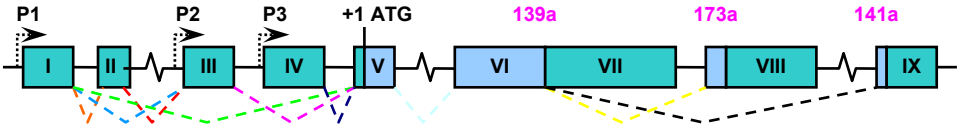


Figure 1.1. Diagrammatic representation of the genomic organization and alternative splicing pattern of the PTHrP gene. The coding regions and the untranslated sequences are indicated by the light blue and turquoise boxes, respectively. Indicated above the map (dashed arrows) are the three promoters, P1, P2, and P3, of which P1 and P3 are TATA-containing promoters (5′ to exons I and IV, respectively) and P2 is a GC-rich promoter (5′ to exon III). Known splicing events are indicated by the dashed lines below the map.

PTHrP and Prostate Cancer Cells

Recent studies provide direct evidence that PTHrP contributes to the pathogenesis and progression of prostate carcinoma and the tendency of this tumor to metastasize to bone [32,33]. While PTHrP-caused HHM is not a very common complication of disseminated prostate carcinoma, its occurrence is a significant contributor to the morbidity of the affected patients [34]. Normal prostate epithelial cells express low levels

of PTHrP, while in NE cells PTHrP is very abundant [35]. PTHrP is also widely distributed in prostate cancer cells [17] and the PTHrP 1-139 isoform is clearly detected in the prostatic cancer tissue [36].

In previous studies we demonstrated that PTHrP increases proliferation of the prostate cancer cell line PC-3 through both autocrine/paracrine and intracrine pathways[37]. PTHrP also decreases cell apoptosis in prostate cancer cells [38]. It also upregulates expression of the pro-invasive integrins α 1, α 6, and β 4, and increases adhesion to the extracellular matrix (ECM) components collagen type I, fibronectin, and laminin in the human AR-negative prostate cancer cell line, PC-3 [39,40]. Furthermore, PTHrP and its receptor are expressed in most prostate cancers and metastases, thus serving as a basis for both the autocrine/paracrine as well as the intracrine effects of this protein [17].

Asadi and Kukreja, 2005 [17] reviewed in deep detail the factors that regulate PTHrP gene expression in PC. PTHrP expression is regulated through its three promoters. PTHrP promoter usage is cell-and tissue-specific. Most cancer cells use primarily the P3 promoter, while P1 is used the least. Those promoters contain multiple positive and negative *cis*- and *trans*-acting regulatory sequences. Furthermore, the *PTHrP* gene is a downstream target for *ras* and *Src* oncogene activation. Some of these factors that regulate PTHrP gene expression, reviewed by Asadi and Kukreja 2005 [17], are androgens, growth factors [principally EGF, and transforming growth factor alpha and beta (TGF-α and TGF-β)], vitamin D, and the adenoviral protein ElA. Here, I will only discuss the effects of androgens and growth factors on PTHrP expression, and in the next section of this introduction, I will discuss the effects of vitamin D on PTHrP expression, which is the main focus of the present dissertation.

In the androgen-sensitive PC cell line, LNCaP, addition of androgens to the culture medium increases PTHrP mRNA levels, while in the androgen-independent PC-3 cells, no effect on PTHrP mRNA levels was seen [41,42]. Furthermore, transfection of functional androgen receptor into PC-3 cells led to a marked decrease in cell growth, and to an inhibition of PTHrP production after treatment with DHT [43]. Thus, in these AR-transfected PC-3 cells, cell growth correlates inversely with androgen sensitivity and

directly with PTHrP production *in vivo* and *in vitro* [43]. The effects of androgens on PTHrP production are mediated partially by transcriptional regulation via the AR [17].

EGF and TGF-α can stimulate the expression of PTHrP in various tissues. In normal prostate cells, benign prostatic hyperplasia (BPH), and prostate cancer cells, EGF stimulates PTHrP secretion [44]. Furthermore, establishment of tumor in the bone, and the associated increased bone reabsorption, results in the release of TGF-β, which is stored extensively in the bone matrix; this TGF-β release may stimulate PTHrP-production [45]. In fact, TGF-βl increased PTHrP mRNA and protein expression in canine prostate epithelial cells and prostate cancer cells. However, TGF-βl decreased PTHrP mRNA expression in prostate stromal cells [46]. The mechanisms of stimulation of PTHrP expression by TGF-β have been studied in normal keratinocytes, and squamous cell carcinoma and breast cancer cell lines [17,47]. TGF-β and its effector Smad3 activate the P3 promoter of the *PTHrP* gene through an AGAC box and an Ets binding site involving Etsl and to some extent Ets2 proteins [47,48]. In addition, TGF-β stimulates P3 promoter activity via Smad-independent pathways that involve the p38 MAP kinase [49].

The role of PTHrP in PC has been questioned because the protein is proteolytically cleaved by prostate specific antigen (PSA). PSA is a serine protease that is expressed by the secretory epithelial cells lining the human prostatic lumen [50]. PSA proteolytically cleaves PTHrP between amino acids 23 and 24 [51,52], the region where PTHrP interacts with the cell surface PTH1 receptor [30,31]. This cleavage eliminates PTHrP's ability to stimulate cAMP production [51,52]. However, PTHrP can still exert autocrine/paracrine effects though its other biologically active isoforms as well as through direct intracrine effects [18-22,37-40,53]. Thus, even though PSA cleaves PTHrP at the N-terminus, there are still other pathways via which the protein can exert its biological effects in the prostate. Therefore, cleavage by PSA does not diminish the importance of PTHrP in PC.

For these reasons, PTHrP production may be a key element supporting tumor growth and metastasis in prostate cancer. Therefore, downregulating its expression might provide therapeutic benefits.

1,25-DIHYDROXYVITAMIN D_3 ($1,25(OH)_2D_3$)

$1,25(OH)_2D_3$ or calcitriol, the biologically active form of vitamin D, is formed in the kidneys and other tissues after 1α-hydroxylation of $25\text{-}(OH)D_3$ (the major circulating metabolite of vitamin D) by 25-hydroxyvitamin D_3-1α-hydroxylase (1α-(OH)ase). This reaction is strongly stimulated by parathyroid hormone (PTH). The main function of $1,25(OH)_2D_3$ is the preservation of calcium homeostasis. $1,25(OH)_2D_3$ also affects several other cell systems, including the immune system (immunosuppression) and the regulation of growth (anti-proliferation) and differentiation (pro-differentiation) in various tissues and cell lines having vitamin D receptors (VDR)[54,55].

In prostate cancer, there is biologic evidence for an anti-cancer role of $25\text{-}(OH)D_3$; however, the epidemiologic data are non-supportive [56]. Studies on populations with low vitamin D deficiency report no association between higher $25\text{-}(OH)D_3$ levels and a reduced prostate cancer risk [57-61]. Only two studies [62,63], done in Nordic countries, report an inverse association for $25(OH)D_3$, however one of these studies found also a increased risk in men with the highest $25\text{-}(OH)D_3$ [63]. Additionally, there is one study that supports an anti-cancer role for $1,25(OH)_2D_3$ [57], while another study [58] suggest an inverse association for circulating $1,25(OH)_2D_3$ and aggressive prostate cancer, particularly in older men. Prostate cancer cells lose the ability to hydroxylate $25\text{-}(OH)D_3$ to $1,25(OH)_2D_3$, and thus may rely on the circulation as the main source of $1,25(OH)_2D_3$ [56]. Several studies suggest that higher circulating $1,25(OH)_2D_3$ is more important than $25\text{-}(OH)D_3$ for protection against aggressive poorly-differentiated prostate cancer [60,61]. The suppression of circulating $1,25(OH)_2D_3$ levels by calcium intake could explain why higher calcium and milk intakes appear to increase risk of advanced prostate cancer [56].

Several studies have shown that $1,25(OH)_2D_3$ inhibits the proliferation of many malignant cells, including prostate carcinoma cell lines [64-69] and primary cultures of normal and prostate cancers [70]. However, the extent to which $1,25(OH)_2D_3$ inhibits growth varies among different prostate cancer cell lines and is cell type-specific [71,72]. In clinical studies, $1,25(OH)_2D_3$ prolonged the PSA doubling time in patients with early recurrent PC who had no evidence of metastases [72]. However, $1,25(OH)_2D_3$ is

hypercalcemic *in vivo*, and therefore cannot be used clinically, suggesting the necessity of developing vitamin D analogs with increased antiproliferative potency and reduced hypercalcemic activity. Some analogs that have been developed include 1,25-dihydroxy-22,24-diene-24,26,27-trihomovitamin D_3 (EB1089 [73]), 22-ene-25-oxa-vitamin D (ZK156979), 1alpha-hydroxyvitamin D_2 (1alpha-OH-D_2), 22-oxa-1,25-(OH)2D3 (22-oxacalcitriol, OCT, maxacalcitol), 19-nor-1,25$(OH)_2D_2$ (paricalcitol), 1alpha$(OH)D_2$ (doxercalciferol), GS1590, CB1393 and MC1598.

1,25$(OH)_2D_3$ downregulates PTHrP expression in a variety of human cell types, including: in TT cells (a human C-cell line) [74], in primary cultures of normal human keratinocytes [75], in primary cultures of normal human mammary epithelial cells (HMEC) derived from nonlactating breast tissue [76], in a human lung squamous cancer cell line, NCI H520 [77,78], in human oral squamous carcinoma cell line (HSC-3) [79], in the human T cell line MT2 cells [80], and in both normal human melanocytes and a human amelanotic melanoma cell line (A375) [81]. In this series of studies, we report that 1,25$(OH)_2D_3$ and a non-hypercalcemic analog, EB1089, downregulate PTHrP gene expression and production in three human prostate cancer cell lines, the AR-positive LNCaP [82], the AR-negative PC-3 [82,83], and the AR-positive, but androgen independent LNCaP-derived C4-2 cell lines. Therefore, downregulating PTHrP expression via 1,25$(OH)_2D_3$ and its non-hypercalcemic analogs may provide extra therapeutic benefits in neutralizing PTHrP's contribution to the pathogenesis and progression of prostate carcinoma and the tendency of this tumor to metastasize to bone.

VITAMIN D RECEPTOR AND THE NEGATIVE VITAMIN D RESPONSE ELEMENT (nVDRE)

The majority of the genomic cellular effects of 1,25$(OH)_2D_3$ are mediated via the vitamin D receptor (VDR), a member of the superfamily of ligand-inducible nuclear transcription factors. The liganded VDR activates gene transcription through its binding to vitamin D response elements (VDREs), which consist of two direct (but imperfect) repeats (DR) separated by a three-nucleotide spacer (DR3). The VDR binds to the

VDRE in the target genes together with co-activators; this mechanism of action of the VDR is well understood (reviewed in Haussler *et al.*, 1998 [84]). Cheskis and Freedman [85] demonstrated that VDR is a monomer in solution; VDR binding to a specific DNA element leads to the formation of a homodimeric complex through a monomeric intermediate. They also found that $1,25(OH)_2D_3$, the ligand for VDR, decreases the amount of the DNA-bound VDR homodimer complex. It does so by significantly decreasing the rate of conversion of DNA-bound monomer to homodimer and at the same time enhancing the dissociation of the dimeric complex. This effectively stabilizes the bound monomeric species, which in turn serves to favor the formation of a VDR-RXR (retinoic X receptor) heterodimer. The ligand for RXR, 9-*cis*-retinoic acid, has the opposite effect of destabilizing the heterodimeric-DNA complex [85]. Koszewski *et al.* [86,87] showed similar findings using the murine osteopontin vitamin D response element (mOP VDRE [86]) and four other individual DNA fragments ranging in size from ca. 250–320 bp [87]. On the other hand, negative gene regulation by $1,25(OH)_2 D_3$ is not as well defined. $1,25(OH)_2D_3$ inhibits the expression of numerous genes, including the PTH [88,89] and PTHrP genes [74-83]. The identified nVDRE within the human PTH gene possesses just one of the two hexameric motifs that form the core of the consensus positive VDRE [90]. The human PTHrP gene, which is derived from a common ancestral gene with the PTH gene [11], also includes a single heptameric DNA sequence that is closely homologous to the nVDRE within the PTH gene [80].

In Chapter Two of the present study [82], we investigated whether this single heptameric motif within the human PTHrP gene ($nVDRE_{hPTHrP}$ [80]) is responsible for the vitamin D-mediated downregulation of PTHrP gene expression in the cell lines LNCaP and PC-3. Our results indicate that $nVDRE_{hPTHrP}$ mediates the PTHrP gene repression in response to $1,25(OH)_2D_3$ and EB1089 in LNCaP, but not in PC-3, cells. However, $1,25(OH)_2D_3$ does downregulate PTHrP gene expression via a transcriptional pathway in PC-3 cells [83]. In Chapter Three, we examined the $nVDRE_{hPTHrP}$-mediated response to $1,25(OH)_2D_3$ in prostate cancer cell lines that represent different stages of prostate cancer progression. We compared this response in the androgen-responsive LNCaP cells vs. the

androgen-independent C4-2 cells, which are more metastatic variants of LNCaP cells[91,92]. Now we report that 1,25$(OH)_2D_3$ and EB1089 downregulate $nVDRE_{hPTHrP}$-driven promoter activity in both LNCaP and C4-2 cells.

9-*CIS*-RETINOIC ACID AND THE RETINOIC X RECEPTOR (RXR)

9-*cis*-Retinoic acid (9-*cis*-RA), a metabolite of vitamin A, can bind with high affinity and activate both the retinoic X receptor (RXR-α,-β and -γ) and the retinoic acid receptor (RAR-α,-β and -γ). Both receptors are members of the superfamily of ligand-activated transcription factors and can mediate the biological activity of 9-*cis*-RA mainly as RAR-RXR heterodimers [93,94]. In the presence of 9-*cis*-RA, RXR binds to its cognate DNA regulatory elements to activate transcription predominantly as a homodimer [95,96]. In the absence of ligand, RXR can form stable tetramers or heterodimerize with other members of ligand-activated transcription factors including the VDR [97-99]. RXR mediates many biological processes, including cell growth (e.g. RXR-selective ligands are very active in inhibiting PC-3 and LNCaP prostate cancer clonal growth [100]), differentiation, metabolism, morphogenesis, and homeostasis during embryonic development and post-natal life [101,102].

VDR BINDING PARTNERS

The VDR normally forms a heterodimer with the RXR to induce 1,25$(OH)_2D_3$-mediated transcriptional activation through heterodimeric binding to the VDRE, as is the case of 1,25$(OH)_2D_3$-mediated gene regulation via $VDRE_{mop}$ (which consists of the DR3-motif [99]) in different cells. In contrast, it was demonstrated that the $nVDRE_{hPTHrP}$ specifically interacts with the Ku-antigen, but not with RXR, along with the VDR in the human T-cell line MT-2 (human adult T cell lymphoma/leukemia virus-infected T cells[80]. However, studies about the effects of 1,25$(OH)_2D_3$ and 9-*cis*-RA on PTHrP expression in human oral squamous carcinoma cells showed that both 1,25$(OH)_2D_3$ and 9-*cis*-RA suppress PTHrP expression [79]. The inhibitory effect of 1,25$(OH)_2D_3$ and 9-*cis*-RA on PTHrP mRNA levels was additive, suggesting that this inhibition might be through the

binding of the heterodimer (RXR-VDR) to the nVDRE(s) within the PTHrP gene, or that the VDR and RXR may be exerting their effects through different sequence elements; however, these sequences were not mapped. In Chapter Three, we demonstrated that RXR, and not Ku, forms part of the nuclear protein complex that interacts with the $nVDRE_{hPTHrP}$ along with the VDR in both LNCaP and C4-2 cells. These results suggest that the accessory proteins which interact with the VDR to promote PTHrP downregulation are cell-line specific. These specific interactions might depend on the presence of cell type-specific transcription factors and/or on cell line-specific promoter utilization.

In Chapter Three, we also report that 9-*cis*-RA downregulates PTHrP mRNA levels in both LNCaP and C4-2 cells, though this effect is more pronounced in LNCaP cells. Furthermore, our experiments using the $nVDRE_{hPTHrP}$ cloned upstream of the SV40 promoter show that treatment with 9-*cis*-RA decreased promoter activity driven by this construct in LNCaP, but not in C4-2 cells. These results suggest that the down-regulation of PTHrP mRNA levels by 9-*cis*-RA in C4-2 cells might be mediated either via a different vitamin D response element than the $nVDRE_{hPTHrP}$ or via activation by 9-*cis*-RA of either RAR or RXR promoting RAR-RXR heterodimer, RXR homodimer, or PPAR-RXR (peroxisome proliferator-activated receptor) heterodimer formation.

We also show that the combination of $1,25(OH)_2D_3$ and 9-*cis*-RA allows the usage of lower concentrations of each compound to reach significant downregulation of PTHrP gene expression in LNCaP and C4-2 cells. Thus, understanding the mechanisms for downregulation of PTHrP expression by $1,25(OH)_2D_3$ may lead to the development of combination therapies for the treatment of prostate cancer that are more effective and result in lower adverse side effects.

OBJECTIVES AND EXPERIMENTAL DESIGN

The overall goal of this project was to study the effects of $1,25(OH)_2D_3$ and a non-hypercalcemic analog, EB1089 on PTHrP gene expression in prostate cancer cells

and the underlying mechanisms involved. The major hypotheses were that (1) down-regulation of PTHrP gene expression by $1,25(OH)_2D_3$ and its non-hypercalcemic analogs contributes to the therapeutic benefits of $1,25(OH)_2D_3$ in prostate cancer; and (2) the mechanisms underlying this response are cell type-specific and correlate with the different stages of prostate cancer progression. In order to test this hypothesis, we used the AR-negative, highly metastatic, PC-3 cell line, which was initiated from bone metastasis of prostatic adenocarcinoma; the AR-positive, weakly metastatic, LNCaP cell line, which was initiated from lymph node metastasis of prostate carcinoma; and the AR-positive, but androgen independent, and more metastatic C4-2 cell line, which was established by co-inoculating LNCaP cells with nontumorigenic mouse stromal fibroblasts derived from an osteosarcoma, as models to investigate the following issues:

Objective 1: To investigate the effects of $1,25(OH)_2D_3$ and a non-hypercalcemic analog, EB1089, on cell proliferation, and PTHrP expression and production by human prostate cell lines

There have been conflicting reports about the effects of $1,25(OH)_2D_3$ on PC-3 cell proliferation. In Chapter Two, we therefore measured the effects of $1,25(OH)_2D_3$ and EB1089 on the proliferation of the PC-3 cell line. The LNCaP cell line was used as a positive reference for growth inhibition, since all published studies have reported that the proliferation of this cell line is inhibited by $1,25(OH)_2D_3$ and EB1089 [64-69,71,72]. We also examined the effects of $1,25(OH)_2D_3$ and EB1089 on PTHrP mRNA and protein levels, using Northern blot analysis, immunoassays for secreted PTHrP, and immuno-fluorescence labeling for cellular PTHrP levels. In Chapter Three, we compared the effects of $1,25(OH)_2D_3$ and EB1089 on PTHrP gene expression in LNCaP cells and their more metastatic variants, C4-2 cells.

Objective 2: To determine the mechanisms via which $1,25(OH)_2D_3$ and non-hypercalcemic analog downregulate PTHrP gene expression

To explore the mechanisms involved in the downregulation of PTHrP gene expression, we investigated first whether the single heptameric motif within the promoter 1 region of the human PTHrP gene ($nVDRE_{hPTHrP}$) is responsible for the $1,25(OH)_2D_3$-mediated downregulation of PTHrP gene expression in PC-3, LNCaP (addressed in Chapter Two), and C4-2 (addressed in Chapter Three) cells using the chloramphenicol acetyl transferase (CAT) gene as reporter and the electrophoretic mobility shift assay (EMSA). We also asked whether the VDR is involved in negative regulation of the PTHrP gene by $1,25(OH)_2D_3$ and EB1089 and identified the VDR binding partner in this negative regulation (addressed in Chapter Three). As part of this objective, we also studied 1) the effects of 9-*cis*-RA, alone and as a combination treatment with $1,25(OH)_2D_3$ or EB1089, on PTHrP gene expression, and 2) the role of 9-*cis*-RA in negative regulation of the PTHrP gene by $1,25(OH)_2D_3$ and EB1089 via the $nVDRE_{hPTHrP}$ (also addressed in Chapter Three).

Model Systems:

Chapter Two:

In this part of the study, we chose the AR-negative highly metastatic PC-3 cell line, which was initiated from a bone metastasis of prostatic adenocarcinoma; and the AR-positive weakly metastatic LNCaP cell line, which was initiated from a lymph node metastasis of prostate carcinoma. The reasons for choosing these two cell lines are summarized in Table I. In summary: these two cell lines differ in their AR content, and also vary greatly in their metastatic potential. Since both cell lines express detectable levels of PTHrP and VDR, the differential PTHrP regulation as it reflects to different stages of PC progression can be tested.

Table I. Summary of properties of PC-3 and LNCaP cell lines which makes them an appropriate model system

	PC-3	LNCaP
PTHrP levels	++	+
VDR	+	+
AR	-	+
Metastatic potential	+++	-

Chapter Three:

In this part of the study, we chose the AR-positive weakly metastatic LNCaP cell line, which was initiated from a lymph node metastasis of prostate carcinoma; and the AR-positive, but androgen independent more metastatic LNCaP-variant, the C4-2 cell line, which was established by inoculating castrated mice with LNCaP cells together with nontumorigenic mouse stromal fibroblasts derived from an osteosarcoma. The reasons for choosing these two cell lines are summarized in Table II. In summary: both cell lines express similar levels of AR, however, while LNCaP cell growth is androgen-dependent, C4-2 cell growth is AR-dependent and androgen-independent. The two cell lines also represent different stages of prostate carcinoma.

Our principal goal in comparing the effects of $1,25(OH)_2D_3$ and EB1089 on PTHrP gene expression and cell growth in these two closely related cell lines was to mimic the effect of these compounds on the natural progression of lethal phenotypes of human prostate cancer to androgen independence and their propensity to form osseous metastases (represented by the C4-2 cell line [103]).

Table II. Summary of properties of LNCaP and C4-2 cell lines which makes them an appropriate model system

	LNCaP	C4-2
AR	+	+
Androgen dependent	+	-
Metastatic potential	-	+

- Karyotypic analysis shows that LNCaP and C4-2 cells share common marker chromosomes.
- Cluster designation (CD) phenotyping show similarities between both cell lines.

CHAPTER TWO: PROSTATE CANCER CELL TYPE-SPECIFIC REGULATION OF THE HUMAN PTHRP GENE VIA A NEGATIVE VDRE*

ABSTRACT

Parathyroid hormone-related protein (PTHrP) is expressed by prostate cancer cells. Since PTHrP increases the growth and enhances the osteolytic effects of prostate cancer cells, it is important to control the level of PTHrP expression in these cells. We show that 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and its non-hypercalcemic analog, EB1089, suppress PTHrP mRNA and protein levels in the human prostate cancer cell lines PC-3 and LNCaP. The human PTHrP gene contains a sequence element homologous to the negative vitamin D response element within the parathyroid hormone gene. This DNA sequence ($nVDRE_{hPTHrP}$) bound the vitamin D receptor present in nuclear extracts from both PC-3 and LNCaP cells. However, when cloned upstream of the SV40 promoter and transiently transfected into PC-3 and LNCaP cells, $nVDRE_{hPTHrP}$ downregulated promoter activity in response to $1,25(OH)_2D_3$ or EB1089 treatment in LNCaP, but not in PC-3, cells. These results may help to explain why some prostate cancers appear to be refractory to treatment with vitamin D analogs.

INTRODUCTION

The primary role of the biologically active form of vitamin D, 1,25-dihydroxy-vitamin D_3 ($1,25(OH)_2D_3$), is to maintain calcium homeostasis [104]. $1,25(OH)_2D_3$ is also involved in the regulation of cellular proliferation and differentiation in various tissues and cell lines having vitamin D receptors (VDR) [104]. Generally, $1,25(OH)_2D_3$ acts as a

* "Reprinted from Molecular and Cellular Endocrinology, Vol 204, Tovar Sepulveda V.A. and Falzon M., Prostate cancer cell type-specific regulation of the human PTHrP gene via a negative VDRE, Pages No. 51-64, Copyright (2003), with permission from Elsevier"

growth inhibitor in many of these cell types, including prostate carcinoma cell lines [64,65,68] and primary cultures of normal and prostate cancers [70]. However, the degree to which $1,25(OH)_2D_3$ inhibits the growth of different prostate cancer cell lines varies, and is cell type-specific [71,72].

Many of the cellular effects of $1,25(OH)_2D_3$ are mediated via the vitamin D receptor (VDR), a member of the superfamily of ligand-activated transcription factors [105]. The mechanisms via which these receptors activate gene transcription have been extensively studied (reviewed in [106]). In contrast, the mechanisms via which the VDR and other members of this superfamily mediate transcriptional repression are not as well understood. $1,25(OH)_2D_3$ has been shown to inhibit transcription of several genes, including parathyroid hormone (PTH) [90,107] and parathyroid hormone-related protein (PTHrP) [77,78,80,83,108]. In the human PTH gene, the negative VDRE (nVDRE) contains only one of the two heptameric motifs that form the core of the consensus positive VDRE [90]. The human PTHrP gene, thought to be derived from an ancestral gene in common with the PTH gene [11], also contains a single heptameric DNA sequence which is highly homologous to the nVDRE within the PTH gene [80].

PTHrP was initially identified through its role in Humoral Hypercalcemia of Malignancy, and is found in prostate, breast, lung and kidney tumors, among others [12]. More recently, the protein was shown to be distributed in most normal fetal and adult tissues [14,109]. PTHrP affects both cell proliferation and apoptosis in prostate cancer cells [37,38]. In PC-3 human prostate cancer cells, PTHrP also upregulates expression of the pro-invasive integrins α1, α6, and β4, and increases adhesion to the extracellular matrix (ECM) components collagen type I, fibronectin, and laminin [39,40]. Furthermore, recent studies provide direct evidence for a role of PTHrP expression in the development of bone metastasis in patients with prostate carcinoma [32,33]. Therefore, PTHrP production by prostate cancer cells may be one of the key elements supporting tumor growth and metastasis.

Prostate cancer is a common malignancy in the United States, Western Europe, and Australia, and is the most common cancer and the second leading cause of cancer-

related deaths in men in the United States [54]. Therefore, downregulating PTHrP expression via $1,25(OH)_2D_3$ and its non-hypercalcemic analogs may be therapeutically important. Here we examined the effects of $1,25(OH)_2D_3$ and a non-hypercalcemic analog, EB1089 (1α,25-dihydroxy-22,24-diene-24,26,27-trihomovitamin D_3 [73]), on PTHrP gene expression and production in two human prostate cancer cell lines, the androgen receptor (AR)-positive LNCaP and the AR-negative PC-3 cell lines. We also investigated whether the single heptameric motif within the human PTHrP gene ($nVDRE_{hPTHrP}$ [80]) is responsible for the vitamin D-mediated downregulation of PTHrP gene expression in these two cell lines. We report that $nVDRE_{hPTHrP}$ mediates PTHrP gene repression in response to $1,25(OH)_2D_3$ and EB1089 in LNCaP, but not in PC-3, cells. However, $1,25(OH)_2D_3$ does downregulate PTHrP gene expression via a transcriptional pathway in PC-3 cells [83].

MATERIALS AND METHODS

Materials

$1,25(OH)_2D_3$ and EB1089, kindly provided by Dr. M. Uskokovic (Hoffmann La-Roche, Inc., Nutley, New Jersey) and Dr. Lise Binderup (Leo Pharmaceuticals, Ballerup, Denmark), respectively, were dissolved in ethanol at 10^{-4} M. Fetal bovine serum (FBS) and dialyzed FBS were obtained from Atlanta Biologicals (Norcross, GA) and Sigma (St. Louis, MO), respectively. Tissue culture supplies were purchased from Life Technologies, Inc. (Gaithersburg, MD). Anti-human PTHrP mouse monoclonal antibody was obtained from Oncogene Research Products (Cambridge, MA). The fluorescent goat anti-mouse antibody (Alexa Fluor™ 488) was purchased from Molecular Probes, Inc. (Eugene, OR). Anti-VDR antibody and normal rat polyclonal IgG were obtained from Affinity Bioreagents (Golden, CO) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

Cell Culture

PC-3 and LNCaP cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). PC-3 cells were grown at 37 ºC in a humidified 95% O_2-5% CO_2 atmosphere in Ham's F-12 medium supplemented with 7% FBS and L-glutamine. LNCaP cells were grown under the same conditions in RPMI 1640 medium supplemented with 10% FBS and L-glutamine. In some of the experiments, as indicated below, conventional FBS was replaced with dialyzed FBS in order to reduce the amount of endogenous vitamin D compounds present in the serum.

Cell proliferation

To measure the effects of $1,25(OH)_2D_3$ or EB1089 on PC-3 and LNCaP cell proliferation, cells were plated in 24-well dishes at 10^4 cells/well in medium containing 7% (PC-3) or 10% (LNCaP) FBS. After 24 h, the medium was replaced with dialyzed FBS-containing medium. After a further 24 h, the cells were treated with the indicated concentrations of $1,25(OH)_2D_3$ or EB1089 (10^{-9} to 10^{-7} M). Control cells received an equivalent volume of vehicle (ethanol; 0.01% final concentration). Cells were counted after 0, 3 and 7 days of treatment. After 3 days, the growth medium was replaced with fresh $1,25(OH)_2D_3$- or EB1089-containing medium. The cells were then trypsinized and counted using a Coulter Counter (Hialeah, FL).

Plasmids and Oligonucleotides

The 231 bp DNA fragment used as a probe to detect PTHrP mRNA in Northern blot analysis was generated by the reverse transcription/polymerase chain reaction (RT/PCR) using a pair of oligonucleotides (upstream, 5'-CTGGTTCAGCAGTGGAGCGTC-3' and downstream, 5'-GTTAGGGGACACCTCCGAGGT-3') spanning exons 5 and 6 of the human PTHrP gene, and was cloned into the vector pCR II (Invitrogen) as previously described [77]. *Eco*RI was used to release the fragment from the recombinant plasmid. For Northern blot analysis, the isolated fragment was labeled by asymmetric PCR, using the downstream primer and [α-^{32}P]dCTP (6,000 Ci/mmol; Amersham). To normalize for equal RNA loading and transfer, a DNA fragment containing cyclophilin sequences [110]

was labeled by the random primer extension method with a multiprime labeling kit (Amersham) and [α-^{32}P]dCTP (6,000 Ci/mmol).

Oligonucleotides for electrophoretic mobility shift assays (EMSA) and for construction of the chloramphenicol acetyltransferase (CAT) constructs for transfection were synthesized as single-stranded molecules that were annealed before use as probes and competitors. For use as probes, these oligonucleotides were 5' end-labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase. The following oligonucleotide sequences for the negative VDRE from the human PTHrP gene ($nVDRE_{hPTHrP}$ [80]) and the mouse osteopon-tin gene ($VDRE_{mop}$, positive control [111]) were used as probes. The sequence of the upper strand is shown.

$nVDRE_{hPTHrP}$ -546 TAAAGTGCTAT<u>AGaTTCA</u>TATTTGGTTTAT -517

$VDRE_{mop}$ -760 AGAGCAACA<u>AGGTTC</u>ACG<u>AGGTTC</u>ACGTCTC -730

In $nVDRE_{hPTHrP}$, the position of the heptanucleotide motif comprising the putative nVDRE is underlined. The lower case **a** represents the sole difference between the nVDRE from the human PTH gene (in this case present on the lower or anti-strand [90]) and the $nVDRE_{hPTHrP}$. In $VDRE_{mop}$, the underlined sequences represent the two hexanucleotide motifs from the mouse osteopontin gene [111]. In this case, each of these motifs is identical to the nVDRE motif within the human PTH gene [90].

The oligonucleotides corresponding to the $nVDRE_{hPTHrP}$ and $VDRE_{mop}$ were cloned upstream of the SV40 promoter in the vector pCAT promoter (Promega, Madison, WI). pCAT promoter was used as the empty vector control. These constructs were used in transient transfection assays, as described below.

Northern blot analysis

Total RNA was isolated using RNA STAT-60 (Tel-Test "B", Friendswood, TX), and poly(A)$^+$ RNA was prepared using the mRNA Isolator kit (Clontech Laboratories, Inc., Palo Alto, CA). RNA gel electrophoresis was performed under standard conditions,

using 25 μg of total RNA or 2 μg of poly(A^+) RNA. The RNA was then blotted onto nitrocellulose (Schleicher and Schuell, Keene, NH) by capillary action. The probe for detection of PTHrP was prepared by asymmetric PCR, as described above. The blots were prehybridized for 30 min and hybridized for 2 h in ExpressHyb (Clontech, Palo Alto, CA) at 65 °C. After hybridization, the blots were washed twice in 2 x SSC (1 x SSC is 0.15 M NaCl plus 0.15 M sodium citrate), 0.05% SDS for 15 min at room temperature, and then twice in 0.1 x SSC, 0.1% SDS at 50°C for 30 min. The washed membranes were exposed to Kodak X-Omat film (Eastman Kodak, Rochester, NY) at -70 °C with intensifying screens.

To normalize for any differences in RNA loading and transfer, the membranes were also probed with a DNA fragment containing cyclophilin sequences [110], as described above. After autoradiography, the intensities of the bands representing PTHrP and cyclophilin were quantified using the Sigmagel program (Jandel Scientific, San Rafael, CA). Densitometry was carried out using exposure times producing signals in the linear range of film sensitivity. Darker exposures are presented in the figures for illustration purposes.

Immunoassay for secreted PTHrP

The amount of PTHrP secreted into the culture medium was measured using an immunoradiometric "sandwich" assay (Nichols Institute, San Juan Capistrano, CA) employing two affinity-purified antisera to human PTHrP. One antiserum, labeled with ^{125}I, recognizes PTHrP amino acid residues 1-40, while the second, labeled with biotin, recognizes PTHrP residues 60-72. This kit provides a standard, human PTHrP (1-86), as a positive control. The detection limit of this kit is 0.7 pmol/liter [112]. PC-3 and LNCaP cells were grown to ~ 50% confluence and then treated with the indicated concentrations of $1,25(OH)_2D_3$ or EB1089. After 48 h, the conditioned medium was collected and frozen at -80 ºC for future use, and the cell number was determined using a Coulter Counter (Coulter electronics, Inc., Hialeah, FL). Before assay, aliquots of the conditioned media (range 0.4 to 1 ml, calculated to represent the same number of cells) were concentrated to 0.2 ml by acetone precipitation. Unconditioned medium (that never

was exposed to cells), similarly concentrated, served as the negative control. The assay was carried out per the manufacturer's specifications.

Immunofluorescence labeling

To detect PTHrP, cells grown on Lab-Tek chamber slides (Nalge Nunc, Napierville, IL) were treated with $1,25(OH)_2D_3$ or EB1089 (10^{-8} M) for 72 h. After a wash with cold phosphate-buffered saline (PBS), they were fixed in acetone for 10 min at -20 °C, washed again with PBS, incubated in 50 mM ammonium chloride in PBS for 15 min at room temperature, and then washed with PBS. After blocking non-specific protein binding sites by incubation with 5% milk (Novagen, Madison, WI) for 1 h at room temperature and washing with PBS, the cells were incubated with primary antibody (anti-human PTHrP mouse monoclonal IgG; Oncogene Research Products) for 60 min at room temperature. Control cells received no primary antibody. After another wash with cold PBS, the cells were treated with goat anti-mouse secondary antibody (Alexa^{TM} 488; Molecular Probes) for 1 h at room temperature, washed again in PBS, mounted using a Prolong Antifade Kit (Molecular Probes), and analyzed using an Olympus fluorescence microscope (Olympus Corp., Melville, NY) with a wide-band green filter.

Electrophoretic mobility shift assay

Nuclear extracts were prepared from untreated cells or from cells pretreated with 10^{-7} or 10^{-8} M $1,25(OH)_2D_3$ or EB1089 for 48 h, as described by Liu *et al.* (2000) [113]. EMSAs were performed as previously described [114]. Binding reactions contained 20 mM Tris–HCl (pH 7.5), 60 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 4% glycerol, 100 µg/ml bovine serum albumin (BSA), 25 ng poly(dI-dC) · poly(dI-dC) (Amersham Biotech., Inc., Piscataway, NJ) as a non-specific competitor, 10 000 cpm of 5' end-labeled DNA (0.003-0.01 ng) and 1.5 to 3 µl of nuclear extract (concentration 4–6 mg/ml; each reaction contained an equal amount of protein), in a final volume of 25 µl. After a 40 min incubation at 25 °C, the binding reactions were fractionated through a native 5% polyacrylamide gel (29% acrylamide, 1% bis-acrylamide in 0.5 X TBE), which was autoradiographed with an intensifying screen at -70 °C. Competition experiments were

carried out under the same conditions, using a 100- to 200-fold molar excess of competitor oligomers. In some experiments, nuclear extracts were pretreated with 10^{-8} M or 10^{-7} M $1,25(OH)_2D_3$ or EB1089 for 30 min before addition of competitor and probe DNA. In binding reactions carried out in the presence of antibody, anti-VDR antibody (Affinity Bioreagents; concentration 1 µg/ml) or normal rat polyclonal IgG (Santa Cruz Biotechnology, concentration 1 µg/ml) was preincubated with nuclear extract in the presence of binding buffer for 20 min at 25 ºC. Non-specific competitor and probe DNA were then added, and incubation was carried out for a further 40 min.

Transfection and CAT assays

For transfection, PC-3 or LNCaP cells were plated in 6-well dishes (1×10^5 cells/ well) in medium containing 7% (PC-3) or 10% (LNCaP) FBS. When the cells had reached ~ 70% confluence, transfection was carried out in the absence of FBS, using ~ 1.5 µg of plasmid DNA (constructs described in Plasmids and Oligonucleotides section) in the presence of 4 µl Lipofectamine Plus™ and 6 µl Plus Reagent (Life Technologies). After 4 h, dialyzed FBS was added to the cells (final concentration 10%). After 12 h, the cells were washed with PBS, and $1,25(OH)_2D_3$ or EB1089 (10^{-8} or 10^{-7} M) in 1% dialyzed FBS was added; control cells received an equal volume of ethanol (vehicle control). Cells were harvested after 48 h, washed with PBS, and lysed by incubation with 400 µl/well of Reporter Lysis buffer (Promega) for 15 min at room temperature. The cells were then scraped, transferred to microcentrifuge tubes, and heated for 10 min at 60 ºC to inactivate endogenous deacetylase activity. After a 2 min centrifugation, the supernatant was collected and stored at -80 ºC for analysis of CAT activity. To correct for variations in cell number, CAT assays were performed using the same amount of cellular protein per sample, determined by the Bio-Rad assay (Bio-Rad, Hercules, CA). CAT assays were carried out using the CAT enzyme assay system (Promega), per the manufacturer's specifications. The standard reaction contained cell extract (25 - 100 µl), 5 µl of n-butyryl-CoA (cofactor), and 4 µl of [^{14}C] chloramphenicol (50 - 62 mCi/mmol; Amersham Biosciences) in a final volume of 125 µl. After incubation at 37 ºC for 30 to 60 min, the n-butyrylated chloramphenicol products were

extracted with 400 µl xylene (Sigma-Aldrich). After washing the upper xylene phase twice with 100 µl of 0.25 M Tris-HCl, pH 8.0, 200 µl of this organic phase was processed for liquid scintillation counting. As a negative control, reactions were carried out in the absence of cell extract; this value was subtracted from the values obtained in the presence of cell extract.

Statistics

Numerical data are presented as the mean ± SEM. The data were analyzed by ANOVA followed by a Bonferroni post test to determine the statistical significance of differences. All statistical analyses were performed using Instat Software (GraphPad Software, Inc., San Diego, CA).

RESULTS

1,25(OH)$_2$D$_3$ and EB1089 decrease PC-3 and LNCaP cell proliferation

We measured the effects of 1,25(OH)$_2$D$_3$ and EB1089 on the proliferation of PC-3 and LNCaP cells. Treating these cells with 1,25(OH)$_2$D$_3$ or EB1089 caused a concentration-dependent decrease in cell growth that was more profound in LNCaP than in PC-3 cells. Thus, a 10^{-7} M concentration of either compound for 7 days decreased cell number by ~ 2- to 2.5-fold in PC-3 cells vs. ~ 3.5- to 4-fold in LNCaP cells (Fig. 2.1). LNCaP cell number was decreased ~ 2-fold after a 3 day treatment with 10^{-7} M 1,25(OH)$_2$D$_3$ or EB1089 (Fig. 2.1). At the same concentration, only 1,25(OH)$_2$D$_3$ produced a significant decrease in cell number after a 3 day treatment (Fig. 2.1). Concentrations < 10^{-8} M produced proportionately smaller effects (Fig. 2.1). These effects of 1,25(OH)$_2$D$_3$ and EB1089 on LNCaP cell proliferation are in agreement with previous studies [72,115]. However, the effects of 1,25(OH)$_2$D$_3$ and EB1089 on PC-3 cell proliferation have been less clearly defined, as both growth inhibition and a lack of effect have been reported [115,116]. Here we show that these compounds decrease both PC-3 and LNCaP cell proliferation, though LNCaP cells appear to be more sensitive.

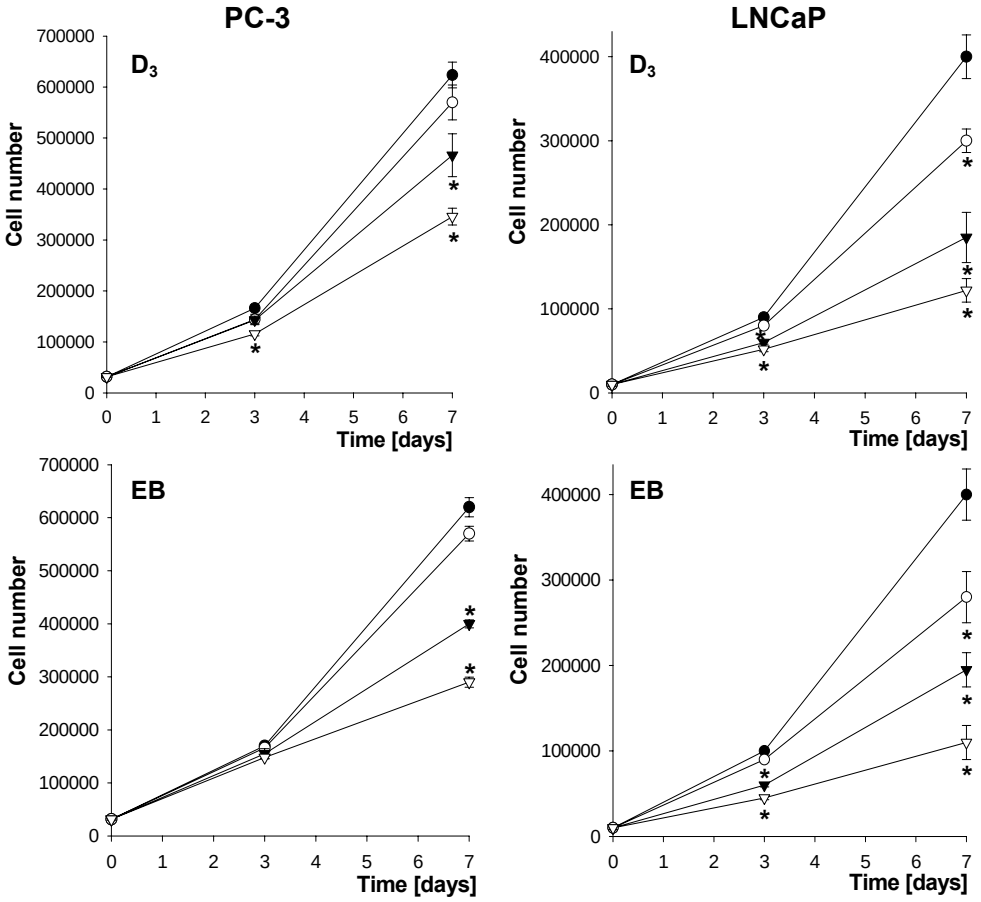


Figure 2.1. Proliferation of PC-3 and LNCaP cells treated with 1,25(OH)$_2$D$_3$ or EB1089. Cells were plated in medium containing 7% (PC-3 cells) or 10% (LNCaP cells) dialyzed FBS at a density of 10^4 cells/well in 24-well dishes. After 24 h, the cells were treated with 10^{-9} to 10^{-7} M 1,25(OH)$_2$D$_3$ (D$_3$) or EB1089 (EB). Control cells were treated with ethanol (vehicle). At the indicated time intervals, the cells were trypsinized, and cell numbers were determined using a Coulter Counter. Each point is the mean ± S.E.M. of three independent experiments (four wells per experiment). Where no error bar is shown, the S.E.M. is smaller than the symbol. ●, vehicle control; ○, 10^{-9} M; ▼, 10^{-8} M; ▽, 10^{-7} M. *, significantly different from control (vehicle only) at $P < 0.01$.

1,25(OH)$_2$D$_3$ and EB1089 decrease steady-state PTHrP mRNA and protein levels in PC-3 and LNCaP cells

Both PC-3 and LNCaP cells have been shown to express functional VDRs [72,115] and therefore offer appropriate model systems to study the effects of vitamin D analogs on PTHrP mRNA and protein levels. We have previously shown that 1,25(OH)$_2$D$_3$ causes a concentration-dependent decrease in PTHrP mRNA levels in PC-3 cells ([83], Fig. 2.2, A). Here we show that treatment with 10^{-10} M to 10^{-8} M EB1089 for 24 h also caused a concentration-dependent decrease in PC-3 cell PTHrP mRNA levels (Fig. 2.2, A). Densitometric scanning of the autoradiographs revealed a ~ 5-fold decrease in mRNA by 10^{-8} M EB1089. Concentrations of 10^{-10} and 10^{-9} M produced proportionally smaller effects (Fig. 2.2, A). The peak effect was observed after 24 h, was essentially of the same magnitude after 48 h, and was decreased by 72 h. Since PC-3 cells maintained in 7% FBS express relatively low levels of PTHrP mRNA [83], these experiments were carried out using poly(A^+) RNA.

PTHrP mRNA levels were also decreased in a concentration-dependent manner by 1,25(OH)$_2$D$_3$ and EB1089 in LNCaP cells (Fig. 2.2, B). Essentially the same profile was obtained as with PC-3 cells; a maximal effect was observed after treatment with 10^{-8} M 1,25(OH)$_2$D$_3$ or EB1089 for 24 h (Fig. 2.2, B). Since LNCaP cells express even lower PTHrP mRNA levels than PC-3 cells, these experiments were also carried out using poly(A^+) RNA.

We have also previously shown that 1,25(OH)$_2$D$_3$ decreases PTHrP secretion in a concentration-dependent manner in PC-3 cells ([83], Fig. 2.3, A). Here we show that EB1089 produces similar effects in this cell line (Fig. 2.3, A), and that both 1,25(OH)$_2$D$_3$ and EB1089 decrease PTHrP secretion in a concentration-dependent manner in LNCaP cells (Fig. 2.3, B). Thus, we observed a ~ 50 to 70% decrease in secreted PTHrP after treatment with 10^{-8} M 1,25(OH)$_2$D$_3$ or EB1089 for 48 h (treatment was carried out for 48 h to allow accumulation of measurable levels of PTHrP in the culture medium). Lower concentrations of these vitamin D analogs produced proportionally smaller effects ([83], Fig. 2.3, A and B).

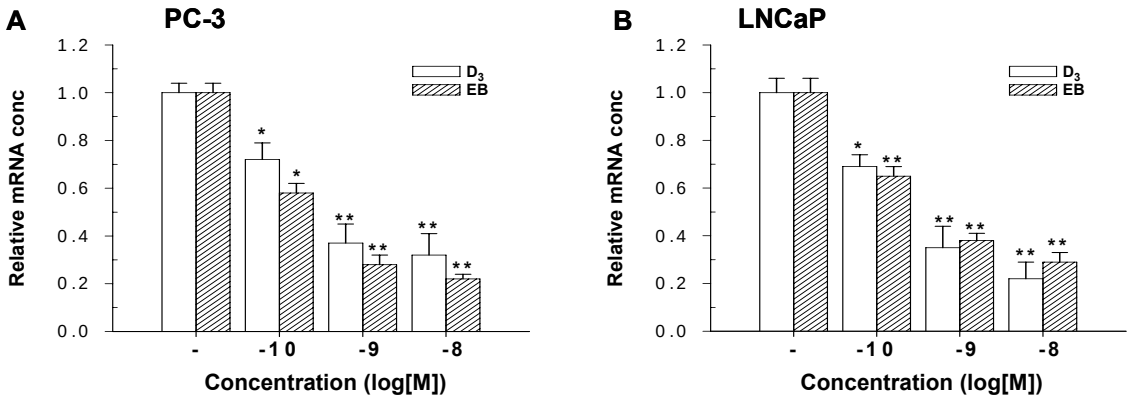


Figure 2.2. Effects of 1,25(OH)$_2$D$_3$ or EB1089 on steady-state PTHrP mRNA levels in PC-3 and LNCaP cells. Cells were cultured in the absence (-) or presence of the indicated concentrations (log of molarity) of 1,25(OH)$_2$D$_3$ (D$_3$) or EB1089 (EB) for 24 h. Poly (A+) RNA was then prepared and analyzed by Northern blot analysis. Each bar represents the mean ± S.E.M. of three independent experiments. Significantly different from the control (no 1,25(OH)$_2$D$_3$ or EB1089 treatment) at *, $P < 0.05$; ** $P < 0.01$.

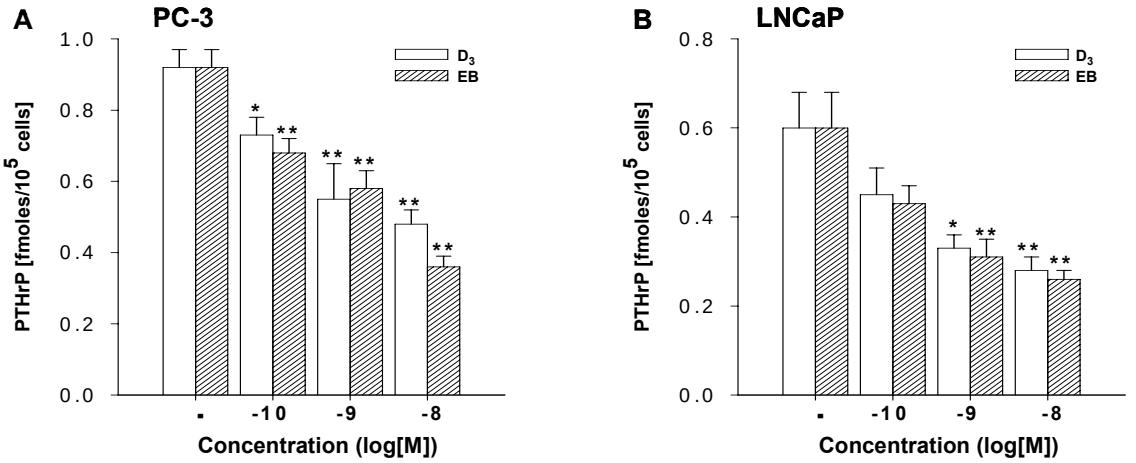


Figure 2.3. Effects of 1,25(OH)$_2$D$_3$ or EB1089 on secreted PTHrP levels in PC-3 and LNCaP cells. Cells were cultured in the absence (-) or presence of the indicated concentrations (log of molarity) of 1,25(OH)$_2$D$_3$ (D$_3$) or EB1089 (EB) for 48 h. Conditioned medium was collected and concentrated, and secreted PTHrP was measured by an immunoradiometric assay. Each bar is the mean ± S.E.M. of three independent experiments, obtained after subtracting the background value (unconditioned medium). Significantly different from the control (no 1,25(OH)$_2$D$_3$ or EB1089 treatment) at *, $P < 0.05$; ** $P < 0.01$.

Cytoplasmic and nuclear PTHrP staining was assessed by fluorescence immuno-cytochemistry (Fig. 2.4, A and B). The level of cytoplasmic staining was higher in PC-3 cells than in LNCaP cells (Fig. 2.4, A and B); in fact, to allow clear visualization of signal in LNCaP cells, these cells were exposed for a longer period than were PC-3 cells. The level of nuclear staining was also higher in PC-3 cells than in LNCaP cells (Fig. 2.4, A and B). Treating these cell lines with 10^{-8} M $1,25(OH)_2D_3$ or EB1089 for 72 h decreased both whole cell and nuclear PTHrP staining ([83]; Fig. 2.4, A and B). For LNCaP cells, this decrease is more evident at higher magnifications (Fig. 2.4, B, bottom panels). Treatment with the two vitamin D analogs also altered the morphology of both PC-3 and LNCaP cells. This effect was more pronounced with LNCaP cells, where the normal extended morphology was replaced by a more rounded shape (Fig. 2.4, B).

The nVDRE$_{hPTHrP}$ confers vitamin D responsiveness on LNCaP, but not PC-3, cells

To determine whether the nVDRE$_{hPTHrP}$ sequence, located between nucleotides -546 and -517 of the human PTHrP gene, confers responsiveness to the vitamin D_3 analogs $1,25(OH)_2D_3$ and EB1089, this sequence was cloned upstream of the SV40 promoter in the vector pCAT promoter. This construct was used in transient transfection assays in PC-3 or LNCaP cells, which were then treated with $1,25(OH)_2D_3$ or EB1089. In order to determine whether these cells do respond to these vitamin D compounds in transient transfection assays, the VDRE from the mouse osteopontin gene (VDRE$_{mop}$ [111]), also cloned in the vector pCAT promoter, was used as a positive control.

Treatment of LNCaP cells with 10^{-7} or 10^{-8} M $1,25(OH)_2D_3$ or EB1089 for 48 h inhibited CAT activity driven by the nVDRE$_{hPTHrP}$ sequence, such that CAT activity in the treated cells was ~ 30 to 60 % that of cells transfected with the empty vector (Fig. 2.5).

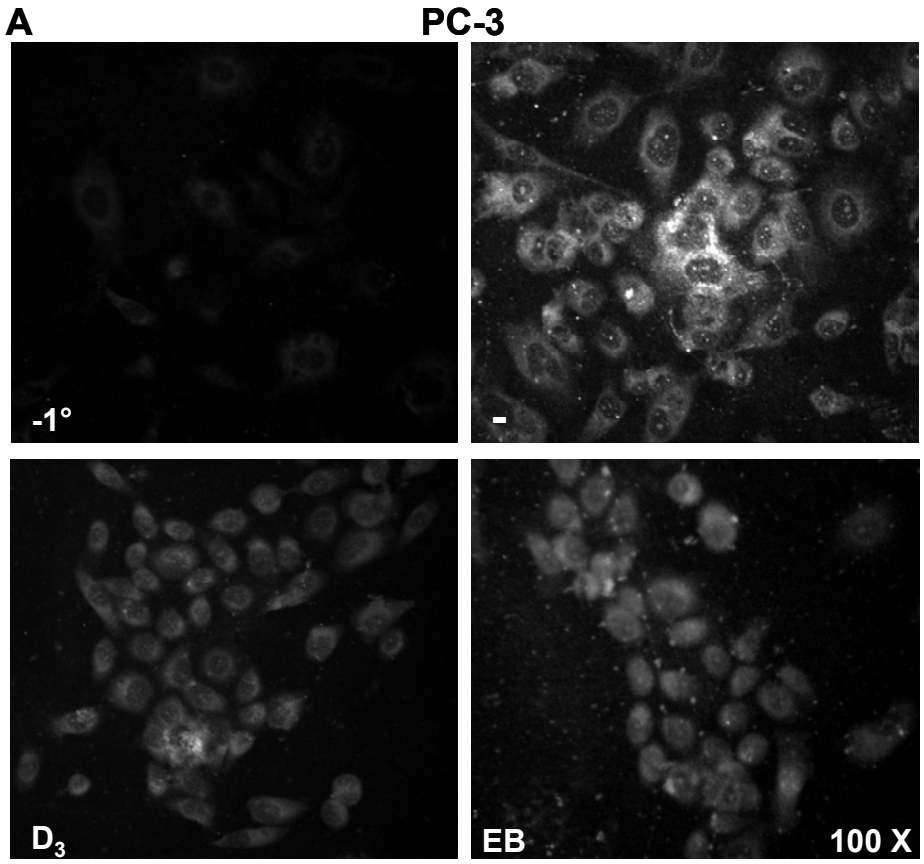
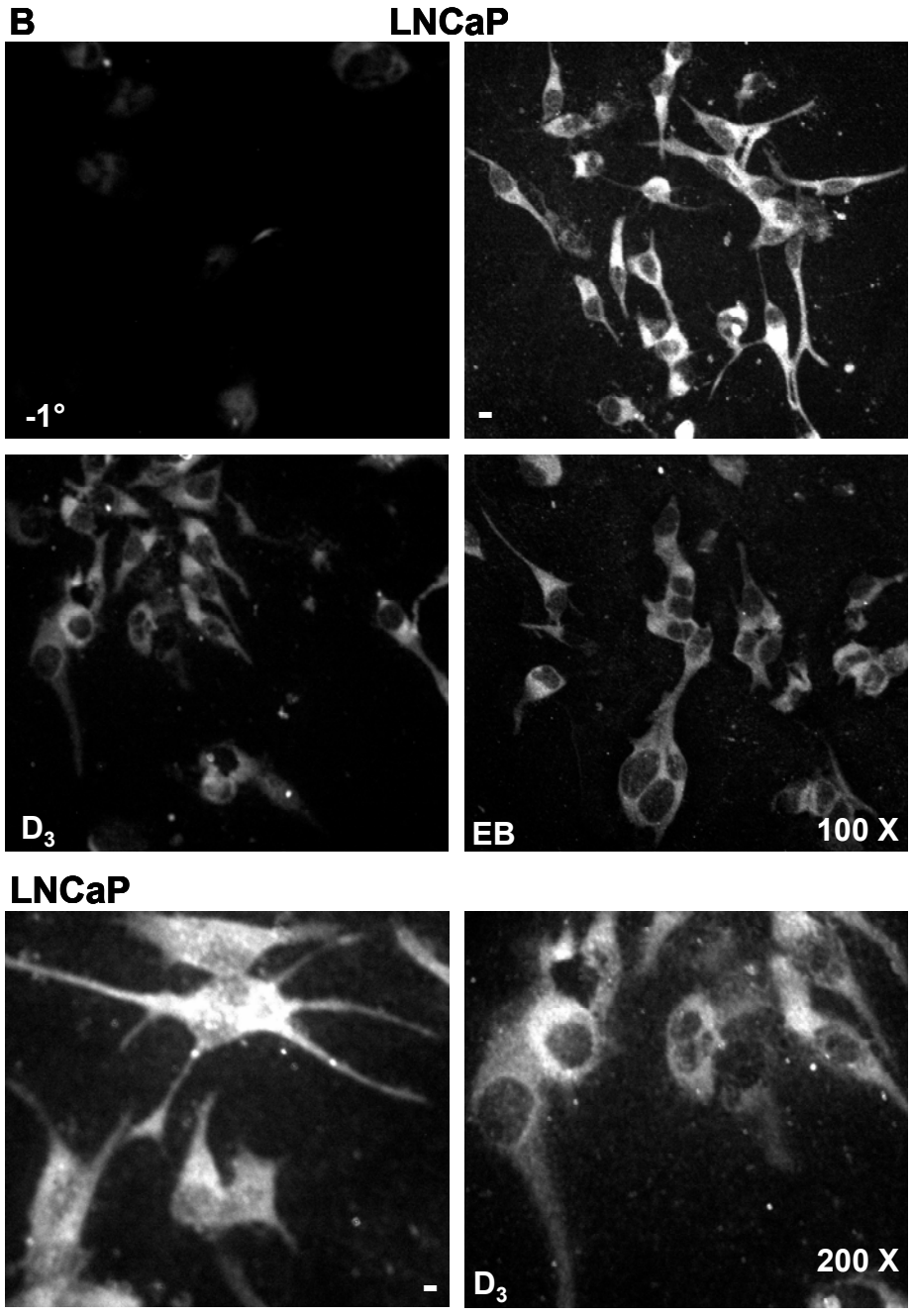


Figure 2.4. Effects of $1,25(OH)_2D_3$ or EB1089 in PC-3 (A) and in LNCaP (B) cells on cellular PTHrP levels. Cells were cultured in the presence of 10^{-8} M $1,25(OH)_2D_3$ or EB1089 for 72 h. Immunofluorescence was carried out as described in Materials and Methods. -1°, cells incubated in the absence of primary antibody; EB, cells cultured in the presence of EB1089; D_3, cells cultured in the presence of $1,25(OH)_2D_3$; -, vehicle (ethanol) control.


B
LNCaP
-1°
-
D3
EB
100 X
LNCaP
-
D3
200 X

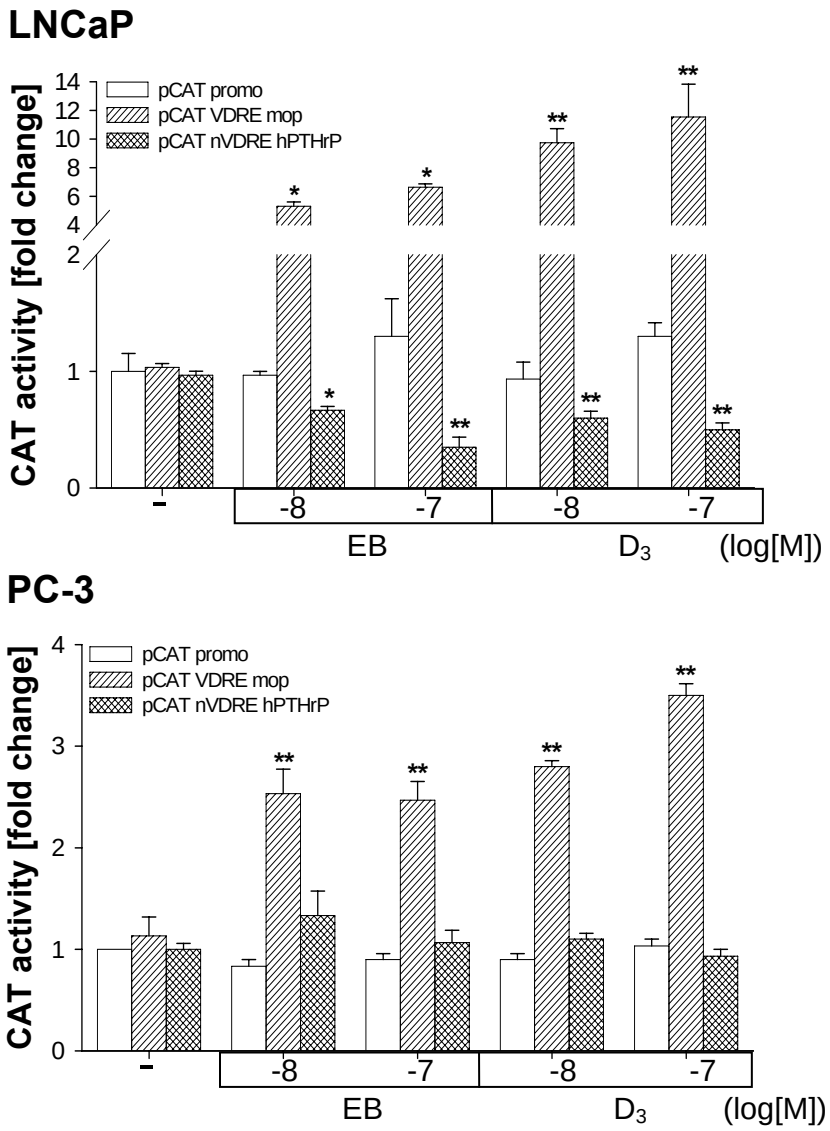


Figure 2.5. Effects of 1,25(OH)$_2$D$_3$ and EB1089 on promoter activity mediated via nVDRE$_{hPTHrP}$ in LNCaP or PC-3 cells. The nVDRE$_{hPTHrP}$ sequence was cloned upstream of the SV40 promoter/CAT gene in the vector pCAT promoter (pCAT nVDRE$_{hPTHrP}$), and transfected into LNCaP or PC-3 cells. pCAT VDRE$_{mop}$ and pCAT promo (empty vector) served as positive and negative controls, respectively. After transfection, cells were treated with the indicated concentrations (log of molarity) of 1,25(OH)$_2$D$_3$ (D$_3$) or EB1089 (EB) for 48 h. Control transfections (-) received ethanol (vehicle). Promoter activity was measured after 48 h by the CAT assay. Each bar represents the mean ± S.E.M. of three independent experiments. Significantly different from the control at *, $P < 0.05$; ** $P < 0.01$.

In contrast, the same concentration of $1,25(OH)_2D_3$ or EB1089 had no effect on CAT activity driven by the $nVDRE_{hPTHrP}$ sequence in PC-3 cells (Fig. 2.5). Transfection of the pCAT/$VDRE_{mop}$ construct produced a significant increase in CAT activity in both LNCaP and PC-3 cells in response to $1,25(OH)_2D_3$ or EB1089 (Fig. 2.5), confirming the functionality of the VDR in these cells, as well as the ability of these cells to respond to these vitamin D analogs. However, the $VDRE_{mop}$-mediated increase in promoter activity in response to the vitamin D analogs was ~ 3-fold greater in LNCaP than in PC-3 cells (Fig. 2.5). Ethanol (vehicle control) had no significant effect on CAT activity in either PC-3 or LNCaP cells.

$1,25(OH)_2D_3$ and EB1089 interact with the VDR in vitro in both LNCaP and PC-3 cells

We examined the interaction between the $nVDRE_{hPTHrP}$ or $VDRE_{mop}$ sequences and nuclear proteins from PC-3 and LNCaP cells. As shown in Fig. 2.6, nuclear extracts from both cell lines formed protein-DNA complexes with these oligonucleotides. The migratory position of the major complex appeared similar for the $nVDRE_{hPTHrP}$ and $VDRE_{mop}$ oligonucleotides (Fig. 2.6). The involvement of the VDR in these complexes was established by carrying out EMSA in the presence of an anti-VDR antibody, which blocks interaction of the VDR with its response element [117]. This antibody did inhibit complex formation with both the $nVDRE_{hPTHrP}$ and $VDRE_{mop}$ oligonucleotides, as shown in Fig. 2.6.

Since the major protein-DNA complex obtained with the $nVDRE_{hPTHrP}$ and $VDRE_{mop}$ oligonucleotides showed similar migratory positions in both PC-3 and LNCaP cells, we carried out cross-competition experiments to determine whether the same accessory proteins, in combination with the VDR, were involved in the interactions with these two oligonucleotide sequences. Data for LNCaP and PC-3 nuclear extracts are shown in Fig. 2.7 and Fig. 2.8, respectively.

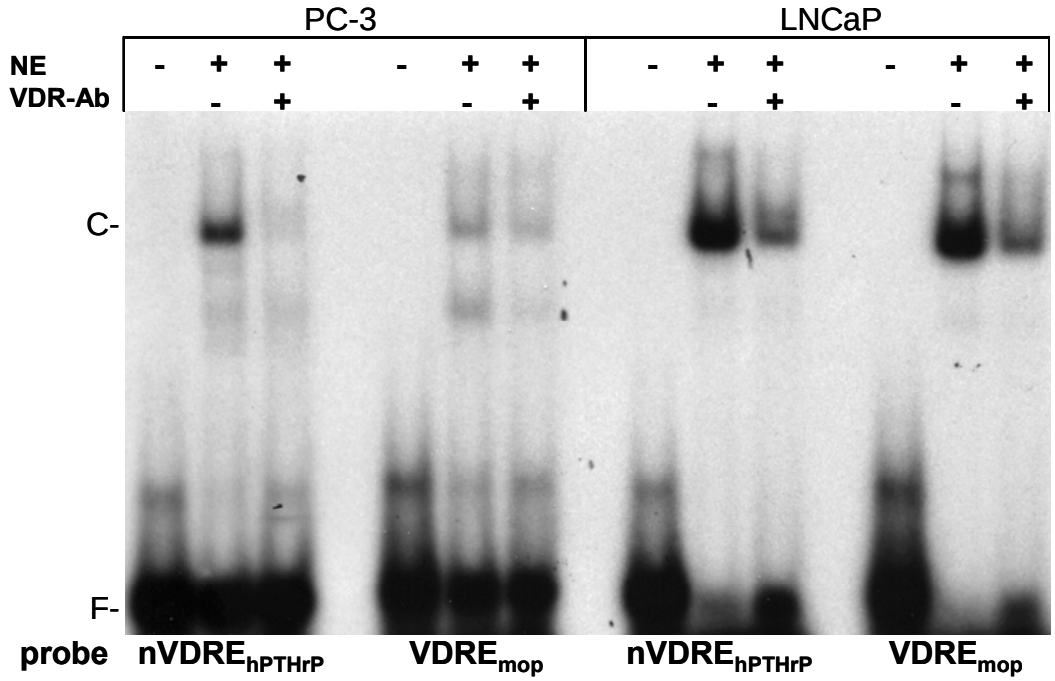


Figure 2.6. Effects of anti-VDR antibody on the interaction of nVDRE$_{hPTHrP}$ with nuclear proteins from PC-3 and LNCaP cells. VDRE$_{mop}$ was used as positive control. Anti-VDR antibody (VDR-Ab) was incubated with 1.5 µl of nuclear extract (NE) from PC-3 or LNCaP cells before addition of poly (dI-dC)·poly (dI-dC) (nonspecific competitor) and the indicated probe. C, DNA/nuclear protein complex; F, free probe.

When nVDRE$_{hPTHrP}$ was used as probe, both nVDRE$_{hPTHrP}$ and VDRE$_{mop}$ competed effectively at 100- and 200-fold molar excess (Fig. 2.7,A and 2.8,A). In contrast, when VDRE$_{mop}$ was used as probe, nVDRE$_{hPTHrP}$ was a very inefficient competitor, even at 200-fold molar excess, while VDRE$_{mop}$ was an effective competitor (Fig. 2.7,B and 2.8,B). These results indicate that (1) the nVDRE$_{hPTHrP}$ and VDRE$_{mop}$ have different affinities for their respective nuclear proteins, (2) there may be differences in the composition of the protein-DNA complexes formed with the nVDRE$_{hPTHrP}$ and VDRE$_{mop}$ oligonucleotides, and (3) the VDRE$_{mop}$-nuclear protein interaction is stronger than the nVDRE$_{hPTHrP}$-nuclear protein interaction.

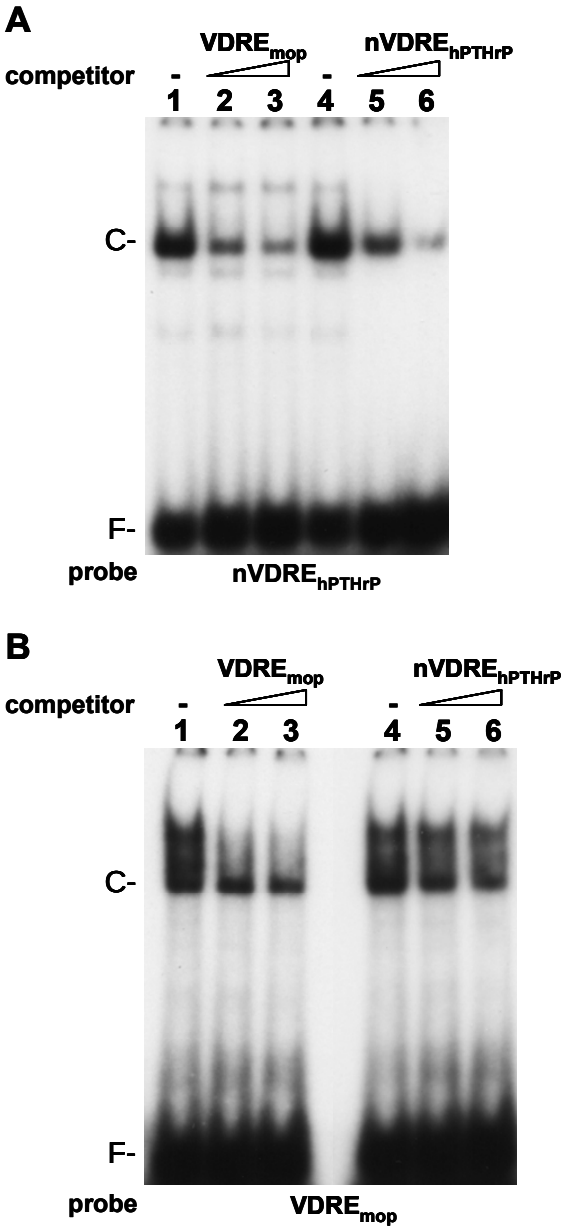


Figure 2.7. Cross-competition experiments using nuclear extracts from LNCaP cells and nVDRE$_{hPTHrP}$ (A) or VDRE$_{mop}$ (B) as probes. The specific competitors are indicated at the top. Poly (dI-dC)·poly (dI-dC) (non-specific competitor) and 1.5 µl of nuclear extract were used in each binding reaction. The binding reactions in lanes 1 and 4 were carried out in the absence of specific competitor (-), and in lanes 2 and 5, 3 and 6, in the presence of a 100- or 200-fold molar excess of the indicated specific competitor, respectively. C, DNA/nuclear protein complex; F, free probe.

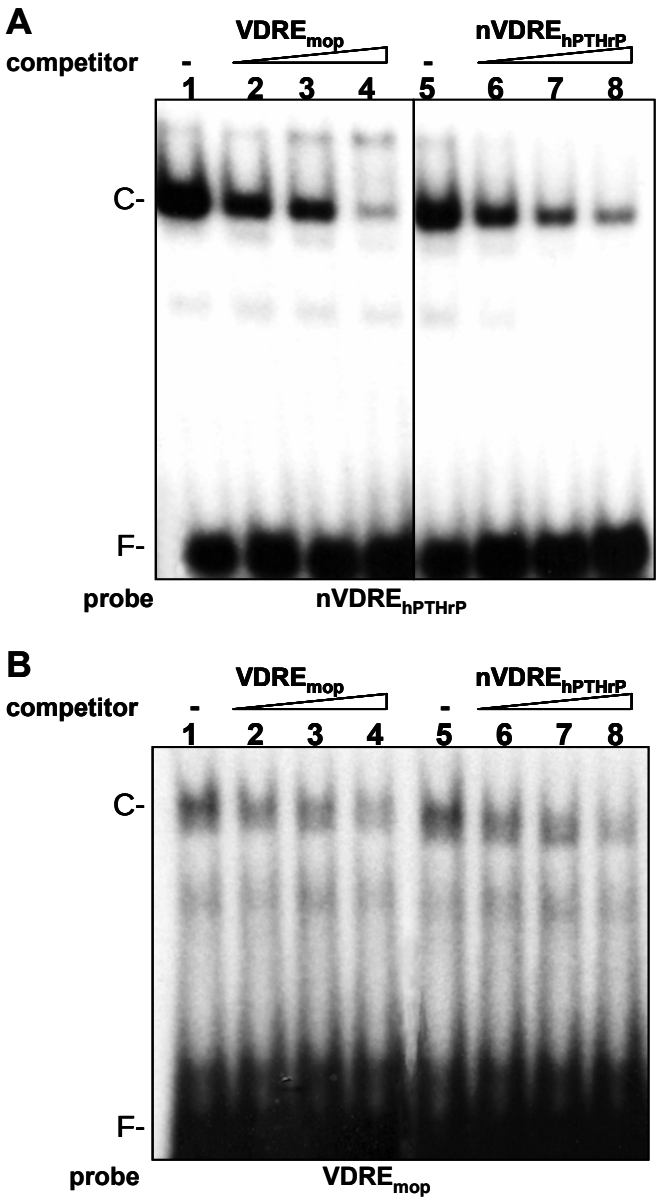


Figure 2.8. Cross-competition experiments using nuclear extracts from PC-3 cells and nVDRE$_{hPTHrP}$ (A) or VDRE$_{mop}$ (B) as probes. The specific competitors are indicated at the top. Poly (dI-dC)·poly (dI-dC) (non-specific competitor) and 1.5 µl of nuclear extract were used in each binding reaction. The binding reactions in lanes 1 and 5 were carried out in the absence of specific competitor (-), and in lanes 2 and 6, 3 and 7, 4 and 8 in the presence of a 50-, 100- or 200-fold molar excess of the indicated specific competitor, respectively. C, DNA/nuclear protein complex; F, free probe.

1,25(OH)$_2$D$_3$ and EB1089 attenuate the binding between nVDRE$_{hPTHrP}$ and the VDR

The effects of 1,25(OH)$_2$D$_3$ and EB1089 on the nVDRE$_{hPTHrP}$-VDR interaction were investigated using nuclear extracts from cells pretreated with these compounds. Treating LNCaP cells with 1,25(OH)$_2$D$_3$ or EB1089 for 48 h weakened the binding between nVDRE$_{hPTHrP}$ and LNCaP nuclear proteins in a dose-dependent manner (Fig. 2.9,A). Treating PC-3 cells under the same conditions also weakened the protein-DNA interactions (Fig. 2.10,A).

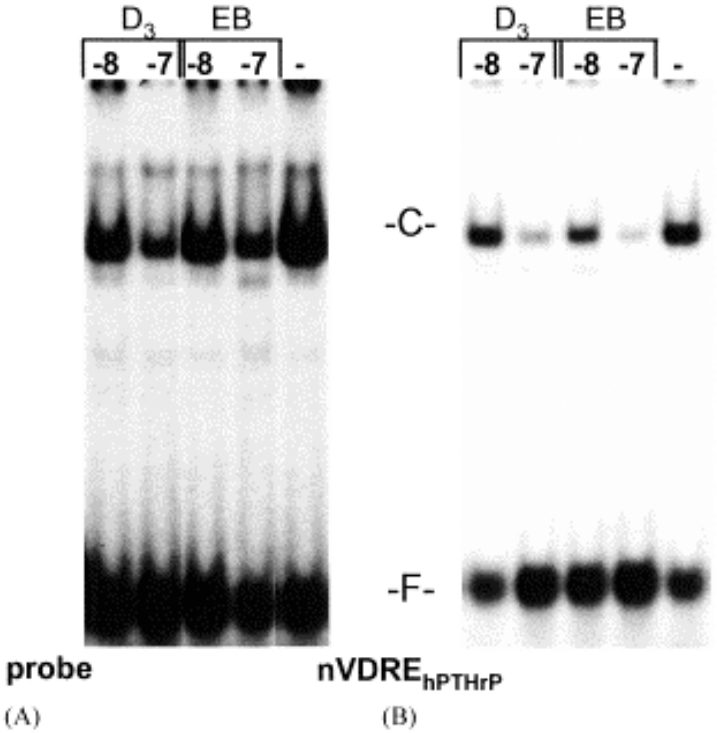


Figure 2.9. Effects of 1,25(OH)$_2$D$_3$ and EB1089 on the interaction between the nVDRE$_{hPTHrP}$ sequence and the VDR in nuclear extracts from LNCaP cells. Poly (dI-dC)·poly (dI-dC) (non-specific competitor) and 1.5 µl of nuclear extract from LNCaP cells were used in each binding reaction. D$_3$ = 1,25(OH)$_2$D$_3$; EB = EB1089;- = vehicle (ethanol) control. (A) Nuclear extracts were prepared from LNCaP cells pre-treated with the indicated concentrations (log of molarity) of 1,25(OH)$_2$D$_3$ or EB1089 for 48 h. (B) Nuclear extracts from LNCaP cells were treated with the indicated concentrations (log of molarity) of 1,25(OH)$_2$D$_3$ or EB1089 for 30 min before addition of probe DNA. C, DNA/nuclear protein complex; F, free probe.

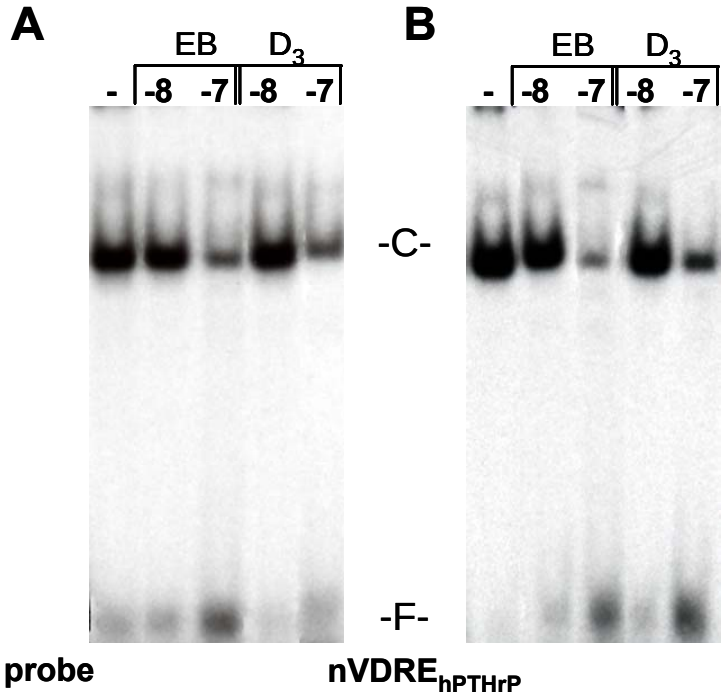


Figure 2.10. Effects of $1,25(OH)_2D_3$ and EB1089 on the interaction between the $nVDRE_{hPTHrP}$ sequence and the VDR in nuclear extracts from PC-3 cells. Poly (dI-dC)·poly (dI-dC) (non-specific competitor) and 1.5 µl of nuclear extract from PC-3 cells were used in each binding reaction. $D_3 = 1,25(OH)_2D_3$; EB = EB1089;- = vehicle (ethanol) control. (A) Nuclear extracts were prepared from PC-3 cells pre-treated with the indicated concentrations (log of molarity) of $1,25(OH)_2D_3$ or EB1089 for 48 h. (B) Nuclear extracts from PC-3 cells were treated with the indicated concentrations (log of molarity) of $1,25(OH)_2D_3$ or EB1089 for 30 min before addition of probe DNA. C, DNA/nuclear protein complex; F, free probe.

Incubating LNCaP nuclear extracts *in vitro* with $1,25(OH)_2D_3$ or EB1089 for 30 min before addition of probe DNA ($nVDRE_{hPTHrP}$) also decreased protein-DNA complex formation in a dose-dependent manner (Fig. 2.9,B). Similar results were obtained with nuclear extracts from PC-3 cells, with decreased protein/DNA complex formation after treatment with 10^{-7} M or 10^{-8} M $1,25(OH)_2D_3$ or EB1089 for 30 min (Fig. 2.10,B). Taken together, these results indicate that treatment with either $1,25(OH)_2D_3$ or EB1089

weakens the binding between the VDR and $nVDRE_{hPTHrP}$, but not $VDRE_{mop}$, in PC-3 and LNCaP cells.

DISCUSSION

The role of vitamin D in prostate cancer has been extensively studied, and vitamin D deficiency has been linked to an increased incidence of this disease (reviewed in [54]). The clinical usefulness of $1,25(OH)_2D_3$ is limited by its effect on calcium metabolism and the associated risks of hypercalcemia and soft tissue calcifications. Like $1,25(OH)_2D_3$, the vitamin D analog EB1089 exerts strong antiproliferative effects on cancer cells. However, unlike $1,25(OH)_2D_3$, EB1089 has relatively minor effects on calcium metabolism [118-120]. Another advantage of EB1089 is its relative metabolic stability *in vivo* (vs. $1,25(OH)_2D_3$) as a result of different routes of side-chain metabolism for the two compounds [121]. EB1089 has been undergoing clinical evaluation in patients with proliferative disorders, including cancer and psoriasis [121].

In this study, we report that both $1,25(OH)_2D_3$ and EB1089 decrease steady-state PTHrP mRNA as well as secreted and intracellular PTHrP levels in the human prostate cancer cell lines, PC-3 and LNCaP. These data are in agreement with and extend our previous study that showed downregulation of PTHrP gene expression and protein levels by $1,25(OH)_2D_3$ in PC-3 cells [83]. PTHrP plays an important role in the growth and metastasis of prostate cancers *in vivo* [32,38,122]. In addition, PTHrP overexpression in PC-3 cells enhances cell-surface expression of the pro-invasive integrins α1, α6, and β4, and increases adhesion to the ECM proteins collagen type I and laminin [39]. Conversely, $1,25(OH)_2D_3$ has been shown to decrease the expression of the α6 and β4 integrin subunits in PC-3, DU145 [123] and SK-MEL-28 (human melanoma) [124] cells. $1,25(OH)_2D_3$ also inhibits tumor invasion [125,126], and more specifically, PC-3 and DU145 cell adhesion, migration, and invasion [123]. These effects may be mediated, at least partially, via the $1,25(OH)_2D_3$-mediated downregulation of PTHrP expression. Hence, downregulation of PTHrP expression by vitamin D analogs may be clinically significant.

In this study, we have also shown that promoter activity driven by a putative nVDRE from the human PTHrP gene ($nVDRE_{hPTHrP}$) was downregulated in a cell line-specific manner in response to $1,25(OH)_2D_3$ or EB1089. This putative $nVDRE_{hPTHrP}$ shows a high degree of homology to the negative vitamin D response element present in the human PTH gene ($nVDRE_{hPTH}$ [90]). Sequence comparison between the two nVDREs shows that 11 of 13 nucleotides are identical, with the $nVDRE_{hPTHrP}$ being in reverse orientation to the $nVDRE_{hPTH}$. The $nVDRE_{hPTHrP}$ also shows a high degree of homology to the nVDRE within the chicken TGFβ2 gene [127]. Of 13 nucleotides, 12 are identical; the only difference lies in the heptameric motif, with an AGATTCA within $nVDRE_{hPTHrP}$ and an AGGTTCA within the TGFβ2 gene [127]. The heptameric AG(G/A)TTCA in the $nVDRE_{hPTHrP}$, $nVDRE_{hPTH}$, and $nVDRE_{TGF\beta2}$ genes also shows significant homology to the heptameric motif which makes up the core element to which the vitamin D, thyroid hormone, and retinoic acid (retinoic acid and RXR) receptors bind [128]. However, unlike these classical VDREs, which consist of two such motifs separated by a spacer of three nucleotides (DR3 [111,128]), only one such motif is present in the nVDRE genes [80,90].

In this study, the $nVDRE_{hPTHrP}$ cloned upstream of the SV40 promoter was used to study vitamin D responsiveness in PC-3 and LNCaP cells. Treatment with $1,25(OH)_2D_3$ or EB1089 decreased promoter activity driven by this construct in LNCaP, but not in PC-3 cells. The VDRE from the mouse osteopontin gene ($VDRE_{mop}$), also cloned upstream of the SV40 promoter, was used to confirm that PC-3 cells can respond to vitamin D treatment. In this case, treatment with $1,25(OH)_2D_3$ or EB1089 increased promoter activity driven by $VDRE_{mop}$ in both cell lines. Therefore, the effects of $1,25(OH)_2D_3$ and its analogs on PTHrP expression mediated through the $nVDRE_{hPTHrP}$ appear to be cell-type specific. In this and previous studies [83], we have shown that $1,25(OH)_2D_3$ does downregulate PTHrP gene expression in PC-3 cells, indicating the presence of a second nVDRE in the PTHrP gene. It is also possible that different negative regulatory proteins mediate downregulation of PTHrP gene expression in PC-3 and LNCaP cells via the $nVDRE_{hPTHrP}$, and that the 30 bp $nVDRE_{hPTHrP}$ sequence used here is not long enough to allow binding of these negative regulators in PC-3 cells. We

and others have shown that the rat PTHrP gene contains at least two DNA sequence elements responsible for negative regulation by vitamin D [129,130]; one sequence comprises a single heptameric motif, and is oriented similar to the nVDRE within the human PTHrP gene. The second sequence resembles the prototypical (DR3) VDRE, with two heptameric motifs separated by a three nucleotide spacer [129,130]. Negative regulation of the chicken PTH gene by vitamin D is also mediated via a DR3 sequence [107]. Therefore, it appears that both the single heptameric and the DR3 nVDREs are involved in downregulation of PTHrP gene expression.

Our EMSA experiments show that nuclear proteins from both PC-3 and LNCaP cells interact with the $nVDRE_{hPTHrP}$, as well as with the $VDRE_{mop}$ (positive control) sequences. As demonstrated by including an anti-VDR antibody in the binding reactions, the VDR forms part of the protein-DNA complex formed with nuclear extracts from both cells lines. The migratory positions of the major complexes obtained with $nVDRE_{hPTHrP}$ and $VDRE_{mop}$ appear similar by EMSA. Since we have not yet identified any other components of the $nVDRE_{hPTHrP}$-nuclear protein complex apart from the VDR, the reason for this apparently similar migration of the protein complexes with these two VDREs is at present unknown. However, the cross-competition EMSA experiments, using $nVDRE_{hPTHrP}$ or $VDRE_{mop}$ as probes and reciprocal competitors, indicate that binding of nuclear proteins to the DR3 sequence, represented by $VDRE_{mop}$, is stronger than that to the single heptamer motif, represented by $nVDRE_{hPTHrP}$, in that there was inefficient competition of $VDRE_{mop}$ by $nVDRE_{hPTHrP}$, while the reverse did not occur.

The mechanism via which $1,25(OH)_2D_3$ represses PTHrP gene expression is not well understood. Abe *et al.* [79] have shown that both $1,25(OH)_2D_3$ and 9-*cis*-RA suppressed PTHrP gene expression in HSC-3 human oral squamous carcinoma cells, indicating involvement of a VDR/RXR heterodimer. However, the sequence element via which this effect was mediated has not been identified. In contrast, Nishishita *et al.* [80] have shown that, in MT-2 cells (human adult T-cell lymphoma/ leukemia virus-infected T cells), the $nVDRE_{hPTHrP}$ does not bind the RXR in combination with VDR, and transcription from this site is not influenced by 9-*cis*-RA. Rather, Ku antigen has been

shown to be part of the protein-DNA complex formed between MT-2 nuclear extracts and the $nVDRE_{hPTHrP}$ sequence. The Ku antigen, a heterodimeric nuclear protein with strong affinity for the ends of linear double-stranded DNA and for nicked circular DNAs, has been identified as a subunit of the DNA-activated protein kinase (DNA-PK) that mediates DNA activation by linear DNA fragments or double-stranded oligonucleotides [131,132]. The Ku antigen also functions as a sequence-specific transcription factor. For example, the p70 and p86 subunits of Ku have been shown to interact with the negative calcium-responsive elements in association with redox factor 1 [133], and with the mouse mammary tumor virus promoter in association with the glucocorticoid receptor [134]. In the human PTHrP gene, binding of VDR and Ku in close proximity on the $nVDRE_{hPTHrP}$ sequence may induce vitamin D-mediated phosphorylation of the VDR by Ku antigen, thereby weakening the ability of VDR to bind to its cognate DNA sequence [80]. The net result in MT-2 cells [80], and possibly LNCaP cells (this study), is decreased promoter activity driven by $nVDRE_{hPTHrP}$ in response to vitamin D treatment. However, this vitamin D-mediated effect on promoter activity is not evident in PC-3 cells. This lack of effect in PC-3 cells may be associated with a defect in the ability of Ku to phosphorylate the VDR in response to vitamin D treatment, defects in other components of the transcription machinery that preclude incorporation of Ku, or a mutation in the VDR which precludes its phosphorylation by Ku antigen. By analogy, in a recent study, Malloy *et al.* [135] report that a mutation in helix 12 of the VDR impairs its interaction with the steroid coactivator-1 (SRC-1) protein, as well as with DRIP205, a subunit of the VDR-interacting protein (DRIP) coactivator complex. While this mutant VDR exhibited many normal properties, including ligand binding, heterodimerization with the RXR, and binding to the VDRE, it was unable to elicit $1,25(OH)_2D_3$-dependent transactivation [135]. The clinical manifestation of this mutation is hereditary vitamin D-resistant rickets [135]. Thus, it is possible that a similar mechanism may explain why different cell lines show different responses to vitamin D analogs, as well as why some prostate cancers appear to be refractory to treatment with vitamin D analogs.

In conclusion, the association between PTHrP expression and prostate cancer growth and metastasis clearly demonstrates the importance of controlling PTHrP production by prostate cancer cells. The current study shows that $1,25(OH)_2D_3$ and EB1089 decrease steady-state PTHrP mRNA and protein levels in PC-3 and LNCaP cells. In LNCaP, but not in PC-3 cells, this effect appears to be partially mediated via the $nVDRE_{hPTHrP}$, indicating that the human PTHrP gene may contain a second, independent nVDRE sequence. The effects of $1,25(OH)_2D_3$ and EB1089 on PTHrP levels also provides the rationale for the use of non-hypercalcemic vitamin D analogs to control PTHrP production in prostate cancer.

CHAPTER THREE: PROSTATE CANCER CELL TYPE-SPECIFIC INVOLVEMENT OF THE RXR IN REGULATION OF THE HUMAN PTHRP GENE VIA A NEGATIVE VDRE

ABSTRACT

Parathyroid hormone-related protein (PTHrP) increases the growth and osteolytic potential of prostate cancer cells, making it important to control PTHrP expression in these cells. We show that 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and its non-hypercalcemic analog, EB1089, decrease PTHrP mRNA and cellular protein levels in the androgen-dependent human prostate cancer cell line LNCaP and its androgen-independent derivative, the C4-2 cell line. This effect is mediated via a negative vitamin D response element ($nVDRE_{hPTHrP}$) within the human PTHrP gene, and involves an interaction between $nVDRE_{hPTHrP}$ and the vitamin D receptor (VDR). The retinoid X receptor (RXR) is a frequent heterodimeric partner of the VDR. We show that RXRα forms part of the nuclear protein complex that interacts with $nVDRE_{hPTHrP}$ along with the VDR in LNCaP and C4-2 cells. We also show that the RXR ligand, 9-*cis*-retinoic acid (9-*cis*-RA), downregulates PTHrP mRNA levels; this decrease is more pronounced in LNCaP than in C4-2 cells. In addition, 9-*cis*-RA enhances the $1,25(OH)_2D_3$-mediated downregulation of PTHrP expression in both cell lines; this effect also is more pronounced in LNCaP cells. Proliferation of LNCaP, but not C4-2, cells is decreased by 9-*cis*-RA. Promoter activity driven by $nVDRE_{hPTHrP}$ cloned upstream of the SV40 promoter and transiently transfected into LNCaP and C4-2 cells was downregulated in response to $1,25(OH)_2D_3$ and EB1089 in both cell lines. Co-treatment with these compounds and 9-*cis*-RA further decreased CAT activity in LNCaP, but not C4-2, cells.

These results indicate that PTHrP gene expression is regulated by $1,25(OH)_2D_3$ in a cell line-specific manner in prostate cancer cells.

INTRODUCTION

Prostate cancer is the most common cancer and the second leading cause of cancer-related deaths among males in the United States [1]. Several studies have demonstrated that parathyroid hormone-related protein (PTHrP) contributes to the pathogenesis and progression of prostate carcinoma and its tendency to metastasize to the bone [32,33]. It is therefore clinically important to develop agents that decrease PTHrP production by prostate cancer cells.

Numerous studies link vitamin D deficiency with an increased incidence of prostate cancer, making dietary vitamin D an important agent in the prevention of this cancer (reviewed in [54]). 1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the hormonally active form of vitamin D, inhibits the proliferation of many types of cancer cells, including prostate carcinoma cells. The clinical effectiveness of $1,25(OH)_2D_3$ is limited by its action on calcium metabolism and the increased risk of hypercalcemia. This effect is diminished for a number of $1,25(OH)_2D_3$ analogs, such as EB1089 (1 ,25-dihydroxy-22,24-diene-24,26,27-trihomovitamin D_3 [73]), making these analogs more therapeutically useful.

$1,25(OH)_2D_3$ also downregulates PTHrP expression in a number of human cell types, including the prostate cancer cell lines PC-3 and LNCaP [82,83]. In a previous study, we reported that a negative vitamin D response element (nVDRE) located between nucleotides -546 and -517 of the human PTHrP gene ($nVDRE_{hPTHrP}$) confers responsiveness to $1,25(OH)_2D_3$ and EB1089 in the weakly metastatic androgen receptor (AR)-positive LNCaP cells, but not in the highly metastatic AR-negative PC-3 cells [82]. In order to determine whether the $nVDRE_{hPTHrP}$-mediated response to $1,25(OH)_2D_3$ differs in prostate cancer cells representing an early to more advanced stage of prostate cancer, we compared this response in the androgen-responsive LNCaP cells to that in the androgen-independent C4-2 cells [91,92]. The C4-2 cell line is a second-generation LNCaP

subline which expresses lower AR protein and mRNA levels than the LNCaP cell line. C4-2 cells are androgen-independent, have higher anchorage-independent cell growth, and express 5- to 10-fold higher prostate-specific antigen (PSA) levels [91,92]. In contrast to LNCaP cells, C4-2 cells metastasize to the lymph nodes and bone when injected orthotopically or subcutaneously into nude mice [91,92]. Previously we also demonstrated that downregulation of PTHrP gene expression by $1,25(OH)_2D_3$ and EB1089 involves an interaction between the $nVDRE_{hPTHrP}$ sequence and the vitamin D receptor (VDR [82]), a member of the superfamily of ligand-activated transcription factors [105]. The mechanisms via which this receptor activates gene transcription and mediates many of the cellular effects of $1,25(OH)_2D_3$ have been widely investigated (reviewed in [136]). Regulation of gene expression via the VDR frequently involves other binding partners. The most predominant interaction is with the retinoid X receptor (RXR), another member of the ligand-activated transcription factor superfamily [99]. The RXR/VDR complex binds to its cognate vitamin D response element (VDRE) to induce $1,25(OH)_2D_3$-mediated transcriptional activation in different cells [99].

The mechanisms via which the VDR mediates transcriptional repression are not as well understood. Two candidate proteins shown to be involved with the VDR in the negative regulation of the PTHrP gene expression by $1,25(OH)_2D_3$ are the RXR and Ku-antigen, the regulatory component of the DNA-PK (DNA-dependent protein kinase) holoenzyme [79,80]. The RXR/VDR heterodimer has been shown to mediate downregulation of PTHrP gene expression in the human oral squamous carcinoma cell line HSC-3 [79]. Thus, treatment with $1,25(OH)_2D_3$ or the RXR ligand 9-*cis*-retinoic acid (9-*cis*-RA) decreased PTHrP mRNA levels [79]. Co-treatment with $1,25(OH)_2D_3$ and 9-*cis*-RA produced an additive downregulatory effect on PTHrP mRNA levels, suggesting that this inhibition might be mediated through the binding of the RXR/VDR heterodimer to the nVDRE(s) within the PTHrP gene, even though these nVDRE sequence(s) were not mapped. In contrast, in the leukemia virus-infected human adult T cell lymphoma cell line MT-2, downregulation of PTHrP gene expression through the $nVDRE_{hPTHrP}$ involves an interaction of the VDR with Ku-antigen, and not with the RXR [80]. Thus, it may be

concluded that the proteins that interact with the VDR to promote downregulation of PTHrP gene expression may be cell type-specific. Regulation of PTHrP gene expression may depend on the presence of cell type-specific transcription factors, or on cell type-specific promoter utilization.

In the present study, we report that 9-*cis*-RA downregulates PTHrP mRNA levels in LNCaP and C4-2 cells. The $1,25(OH)_2D_3$-mediated downregulation of PTHrP expression is enhanced by 9-c*is*-RA in both LNCaP and C4-2 cells at low concentrations of either compound, but only in LNCaP cells at higher concentration of these compounds. $1,25(OH)_2D_3$ downregulates $nVDRE_{hPTHrP}$-driven promoter activity in LNCaP and C4-2 cells. Co-treatment with $1,25(OH)_2D_3$ and 9-*cis*-RA causes a further decrease in CAT activity in LNCaP, but not in C4-2 cells. In both cell lines, the VDR and RXRα form part of the nuclear protein complex that interacts with $nVDRE_{hPTHrP}$. Furthermore, 9-cis-RA decreases cell proliferation of LNCaP, but not C4-2 cells. Similar effects were observed with EB1089. Understanding the mechanisms for downregulation of PTHrP expression and decreased proliferation in response to $1,25(OH)_2D_3$ may lead to the development of combination therapies for the treatment of prostate cancer that are more effective and result in lower side effects than those currently available.

MATERIALS AND METHODS

Materials

$1,25(OH)_2D_3$ and EB1089, kindly provided by Dr. M. Uskokovic (Hoffmann La-Roche, Inc., Nutley, New Jersey) and Dr. Lise Binderup (Leo Pharmaceuticals, Ballerup, Denmark), respectively, were dissolved in ethanol at 10^{-4} M. 9-*cis*-Retinoic acid was obtained from Sigma-Aldrich Co. (St. Louis, MO) and was also dissolved in ethanol at 10^{-4} M. Fetal bovine serum (FBS) and dialyzed FBS were obtained from Atlanta Biologicals (Norcross, GA) and Sigma (St. Louis, MO), respectively. Tissue culture supplies were purchased from Life Technologies, Inc. (Gaithersburg, MD). Anti-human PTHrP mouse monoclonal antibody was obtained from Oncogene Research Products (Cambridge, MA). The fluorescent goat anti-mouse antibody (labeled with Alexa

Fluor™ 488) was purchased from Molecular Probes, Inc. (Eugene, OR). Anti-VDR monoclonal antibody (MA1-710) was obtained from Affinity Bioreagents (Golden, CO), and the anti-RXRα polyclonal antibody (D-20), anti-Ku-70 polyclonal antibody (C-19), and normal rat polyclonal IgG were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Cell culture

LNCaP cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). C4-2 cells were purchased from UroCor, Inc. (Oklahoma City, OK). LNCaP and C4-2 cells were grown at 37 °C in a humidified 95% O_2 - 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% FBS and L-glutamine. In some of the experiments, as indicated below, conventional FBS was replaced with dialyzed FBS in order to minimize the exposure of the cells to endogenous steroids present in the serum.

Cell proliferation

To measure the effects of $1,25(OH)_2D_3$, EB1089 and/or 9-*cis*-RA on LNCaP and C4-2 cell proliferation, cells were plated in 24-well dishes at 10^4 cells/well in medium containing 10% FBS. After 24 h, the medium was replaced with dialyzed FBS-containing medium (final FBS concentration, 10%). After a further 24 h, the cells were treated with 10^{-7} M $1,25(OH)_2D_3$, EB1089, and/or 9-*cis*-RA. Control cells received an equivalent volume of vehicle (ethanol; 0.01% final concentration). Cells were counted after 0, 3, 7 and 11 days of treatment. The growth medium was replaced every 3 days with fresh $1,25(OH)_2D_3$-, EB1089-, and/or 9-*cis*-RA-containing medium. The cells were then trypsinized and counted using a Coulter Counter (Hialeah, FL).

Immunofluorescence labeling

To detect PTHrP, cells grown on Lab-Tek chamber slides (Nalge Nunc, Napierville, IL) were treated with 10^{-7} M $1,25(OH)_2D_3$, EB1089, and/or 9-*cis*-RA for 72 h. Cellular PTHrP was detected as previously described [82]. In brief, the cells were fixed in acetone, incubated in 50 mM ammonium chloride in PBS, and the non-specific

protein binding sites were blocked by incubation with 5% milk protein (Novagen, Madison, WI) for 1 h at room temperature. The cells were then incubated overnight at 4 ºC with primary antibody (anti-human PTHrP mouse monoclonal IgG; Oncogene Research Products). Control cells did not receive primary antibody. After another wash with cold PBS, the cells were treated with goat anti-mouse secondary antibody (Alexa^{TM} 488; Molecular Probes) for 1 h at room temperature, mounted using Aqua Poly/Mount (Polysciences, Inc.; Warrington, PA), and analyzed using an Olympus fluorescence microscope (Olympus Corp., Melville, NY) with a wide-band green filter.

Fluorescence images, acquired as described above, were stored as tif files and were transferred to a laboratory computer. Images were analyzed using a program written in the Mathlab system (The Mathworks Inc., Natick, MA) by Dr. Gilbert R. Hillman. The images showed several fluorescent-labeled cells, with most of the image area consisting of unlabeled, or faintly nonspecifically labeled, background. An intensity histogram was computed for the entire image; the shape of the histogram was a skewed normal Gaussian curve, representing an approximately normal distribution of pixel intensity within the background upon which was superimposed a smaller number of higher-intensity labeled pixels. The portion of the histogram greater than 0.3 times its observed peak value was used as input to a nonlinear regression procedure that fitted a normal distribution to the data; substantial deviation of the actual data from the fitted curve occurred only at the higher-intensity region of the histogram, and represented the distribution of fluorescent pixels. The difference between the fitted normal curve and the observed histogram was used to calculate FL, the weighted average fluorescent labeling intensity per pixel within labeled cells, according to the following formula:

$$FL = \frac{\sum_{i=t}^{255} (P_{Oi} - P_{Fi})(i - t)}{\sum_{i=t}^{255} (P_{Oi} - P_{Fi})}$$

Where P_{Oi} is the number of pixels at intensity i in the observed histogram, P_{Fi} is the number of pixels at intensity i in the fitted normal curve, and t is a threshold equal to 1.96 times the fitted s of the normal curve.

This distribution was extracted by subtracting the fitted from the observed curve. A threshold was set at an intensity equal to 1.96 standard deviations above the mean of the fitted normal distribution, and the area of the difference curve was determined as the sum of the number of pixels at each intensity level, weighted by the difference between that intensity and the threshold. This number was divided by the number of labeled pixels observed, and was a measure of the average intensity of fluorescent labeling within labeled cells.

Reverse Transcriptase-Real-time Polymerase Chain Reaction Analysis of PTHrP Gene Expression.

To determine if PTHrP expression is responsive to $1,25(OH)_2D_3$, EB1089 and/or 9-*cis*-RA treatment, we performed reverse transcriptase (RT)-real-time Polymerase Chain Reaction (PCR) analysis. LNCaP and C4-2 cells were plated in 6-well dishes at 10^5 cells/well in medium containing 10% FBS. At ~70% confluence, the medium was replaced with medium containing dialyzed FBS. After 12 h, the cells were washed and incubated with the indicated concentration of $1,25(OH)_2D_3$, EB1089 and/or 9-*cis*-RA for 48 h as indicated. Cells from two different wells were harvested and then combined, and total RNA was extracted using the RNAqueous® isolation kit per the manufacturer's instructions (Ambion Inc., Austin, TX). RNA concentrations were determined by spectrophotometry.

RT reactions: RT reactions were performed with 2 μg of total cellular RNA using random hexamer primers and TaqMan® Reverse Transcription Reagents (ABI, Applied Biosystems, Foster City, CA). A typical reaction contained 2 μg RNA, 1X RT Buffer, 2.5 μM random hexamer primers, 2.5 U/μl MultiScribe™ Reverse Transcriptase (RTase), 2 mM dNTP Mixture (500 μM each), 5.5 mM $MgCl_2$, 0.4 U/μl RNase Inhibitor, and diethylpyrocarbonate (DEPC)-treated water in a final volume of 25 μl. The reaction mixture was incubated for 10 min at 25 ºC, 30 min at 48 ºC and 5 min at 95 ºC using a GeneAmp PCR-System (PerkinElmer Life and Analytical Sciences, Inc.; Boston, MA). An aliquot of the RT reaction mixture (6 μl) was used as the template for real-time PCR to detect PTHrP mRNA and 18S rRNA (for normalization purposes) transcripts. PTHrP

primers (Assays-on-Demand™, P/N 4331182, 20× assay mix of primers), the PTHrP TaqMan MGB probe (FAM™ dye-labeled, PTHLH (parathyroid hormone-like hormone), NM 002820, Hs00174969 m1: CGCCGCCTCAAAAGAGCTGTGTCTG) and the pre-developed 18S rRNA primers as endogenous control (VIC™-dye labeled probe, TaqMan® assay reagent, P/N 4319413E) were obtained from ABI (Applied Biosystems, Foster City, CA).

Relative quantification of gene expression: Separate tubes (singleplex) real-time PCR was performed using 40 ng cDNA for both target gene and endogenous control. The universal PCR master mix reagent kit was used as a source for the reagents (P/N 4304437). The cycling parameters for real-time PCR were: UNG activation at 50 °C for 2 min, AmpliTaq activation at 95 °C for 10 min, denaturation at 95 °C for 15 sec and annealing/extension at 60 °C for 1 min (repeated 40 times) on an ABI7000 real time PCR machine. Duplicate C_T values were analyzed in Microsoft Excel using the comparative C_T ($\Delta\Delta C_T$) method as described by the manufacturer (Applied Biosystems, Foster City, CA). The amount of target ($2^{-\Delta\Delta CT}$) was obtained by normalizing to the endogenous reference (18S) and relative to vehicle-treated control as the baseline (using the facilities of UTMB's Sealy Center for Cancer Cell Biology's Real Time PCR core facility, http://www.utmb.edu/scccb/pcr/index.htm). As negative controls, we carried out PCR reactions without DNA and RT-reactions without RTase.

Plasmids and oligonucleotides

Oligonucleotides for electrophoretic mobility shift assays (EMSA) and for construction of the chloramphenicol acetyltransferase (CAT) constructs for transfection were synthesized as single-stranded molecules that were annealed before use as probes and competitors. For use as probes, these oligonucleotides were 5′ end-labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase. The following oligonucleotide sequences for the nVDRE from the human PTHrP gene ($nVDRE_{hPTHrP}$ [80]) and the mouse osteopontin gene ($VDRE_{mop}$, positive control [111]) were used as probes. The sequence of the upper strand is shown.

$nVDRE_{hPTHrP}$ -546 TAAAGTGCTATAGaTTCATATTTGGTTTAT -517

$VDRE_{mop}$ -760 AGAGCAACAAGGTTCACGAGGTTCACGTCTC -730

In $nVDRE_{hPTHrP}$, the position of the heptanucleotide motif comprising the putative nVDRE is underlined. The lower case *a* represents the sole difference between the nVDRE from the human PTH gene (in this case present on the lower or antisense-strand [90]) and the $nVDRE_{hPTHrP}$. In $VDRE_{mop}$, the underlined sequences represent the two hexanucleotide motifs from the mouse osteopontin gene [111]. In this case, each of these motifs is identical to the nVDRE motif within the human PTH gene [90].

The oligonucleotides corresponding to the $nVDRE_{hPTHrP}$ and $VDRE_{mop}$ were cloned upstream of the SV40 promoter in the vector pCAT promoter (Promega, Madison, WI), as previously described [82]. pCAT promoter was used as the empty vector control. These constructs were used in transient transfection assays, as described below.

Electrophoretic mobility shift assay

Nuclear extracts were prepared from untreated LNCaP and C4-2 cells grown in RPMI 1640 medium supplemented with 10% FBS and L-glutamine, as described by Liu et al. (2000) [113]. Binding reactions for EMSAs contained 20 mM Tris-HCl (pH 7.5), 60 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 4 % glycerol, 100 µg/ml bovine serum albumin (BSA), 25 ng of poly(dI-dC)·poly(dI-dC) (Amersham Biotech., Inc., Piscataway, NJ) as a non-specific competitor, probe DNA (10 000 cpm; 0.003-0.01 ng), and nuclear extract (1 µl; protein concentration 4-6 mg/ml; each reaction contained an equal amount of protein) in a final volume of 25 µl. After incubation for 40 min at 25 °C, one of the following antibodies was added: anti-VDR antibody (1 mg/ml), anti-RXRα antibody (1 mg/ml), anti-Ku-70 antibody (1 mg/ml) or the normal polyclonal IgG antibody (1 mg/ml; as negative control for unspecific antibody binding). After a further 30 min incubation at 25 °C, the reactions were fractionated through a native 5% polyacrylamide gel (29% acrylamide, 1% bis-acrylamide in 0.5 X TBE), which was dried and autoradiographed with an intensifying screen at -70 °C.

Transfection and CAT assays

Transfection and CAT assays were carried out as previously described [82]. Briefly, LNCaP or C4-2 cells were plated in 6-well dishes at 10^5 cells/well in medium containing 10% FBS. At ~70% confluence, the cells were transferred to serum-free medium and transfected with 1.5 µg of plasmid DNA (constructs described under the plasmids' section) in the presence of 4 µl Lipofectamine Plus™ and 6 µl Plus Reagent (Gibco BRL, Life Technologies). After 4 h, dialyzed FBS was added to the cells (final concentration 10%). After a further 12 h recovery, the cells were washed with PBS, transferred to 1% dialyzed FBS, and treated with 10^{-7} M $1,25(OH)_2D_3$, EB1089 and/or 9-*cis*-RA for 48 h. Control cells received an equal volume of ethanol (vehicle control). After 48 h, the cells were harvested, washed with PBS, and lysed using the Reporter Lysis buffer (Promega) for 15 min at room temperature. The cells were then scraped, transferred to microcentrifuge tubes, and heated for 10 min at 60 ºC to inactivate endogenous deacetylase activity. After a 2 min centrifugation, the supernatant was collected and stored at -80 °C for analysis of CAT activity. To correct for variations in cell number, CAT assays were performed using the same amount of cellular protein per sample, determined using the Bio-Rad assay (Bio-Rad, Hercules, CA). CAT assays were carried out using the CAT enzyme assay system (Promega), as per the manufacturer's specifications. The standard reaction contained: cell extract (10-25 µl), 5 µl of n-butyryl-CoA (cofactor), and 3 µl of [^{14}C]chloramphenicol (50-62 mCi/mmol; Amersham Biosciences) in a final volume of 125 µl. After incubation at 37 ºC for 30 min, the n-butyrylated chloramphenicol products were extracted with 400 µl xylene (Sigma-Aldrich). After washing the upper xylene phase twice with 100 µl of 0.25 M Tris-HCl, pH 8.0, 200 µl of this organic phase was processed for liquid scintillation counting. As a negative control, reactions were carried out in the absence of cell extract; this value was subtracted from the values obtained in the presence of cell extract. The CAT activity values obtained were plotted as fold-change, using the vehicle control-treated cells as the CAT activity reference (represented as 1.0).

Statistics

Numerical data are presented as the mean ± standard error of the mean (S.E.M). The data were analyzed by one-way analysis of variance (ANOVA) followed by a Tukey-Kramer multiple comparisons post-test to determine the statistical significance of differences. All statistical analyses were performed using INSTAT Software (GraphPad Software, Inc., San Diego, CA).

RESULTS

The RXR forms part of the complex that interacts with nVDRE$_{hPTHrP}$ in both LNCaP and C4-2 cells

We previously showed by EMSA that nuclear proteins from LNCaP cells interact with the nVDRE$_{hPTHrP}$ sequence [82]. Complex formation was inhibited by inclusion of an anti-VDR antibody in the binding reaction, confirming that the VDR forms part of the nuclear protein complex that interacts with nVDRE$_{hPTHrP.}$ This anti-VDR antibody blocks the interaction of the VDR with its response element [117]. Here we show that nuclear proteins from C4-2 cells interact with the nVDRE$_{hPTHrP}$ sequence in a similar manner, and that this interaction is also blocked by an anti-VDR antibody, confirming VDR involvement (Fig. 3.1). To confirm the specificity of the anti-VDR antibody interactions, normal rat IgG was included in the binding reactions. Inhibition by this non-specific antibody was negligible or significantly less than that by the anti-VDR antibody (Fig. 3.1).

Regulation of gene expression via the VDR may involve other binding partners of the VDR [80,99]. Two candidate proteins shown in other systems to be involved with the VDR in the negative regulation of the PTHrP gene by 1,25$(OH)_2D_3$ are the RXR and Ku protein. The involvement of these two proteins in the interaction of the VDR with the nVDRE$_{hPTHrP}$ sequence was also assessed by EMSA. Inclusion in the binding reaction of an anti-RXRα antibody that blocks the RXRα-DNA interaction [137] inhibited complex formation between the nVDRE$_{hPTHrP}$ oligonucleotide and nuclear proteins from LNCaP

and C4-2 cells (Fig. 3.1). A faint upper band, formed only with the $nVDRE_{hPTHrP}$ and LNCaP nuclear proteins, was also blocked by inclusion of the anti-VDR and the anti-RXRα antibodies. The formation of this upper band was not always reproducible. The $1,25(OH)_2D_3$-mediated transcriptional regulation of mouse osteopontin gene expression involves an interaction of the VDR and RXRα on $VDRE_{mop}$, which is comprised of a DR3 motif (two directly-repeating hexanucleotide motifs separated by three nucleotides [128,138]). This sequence was used as a positive control in our studies. A similar binding profile was obtained with $VDRE_{mop}$, in that the presence of the anti-VDR or anti-RXRα antibody inhibited complex formation (Fig. 3.1).

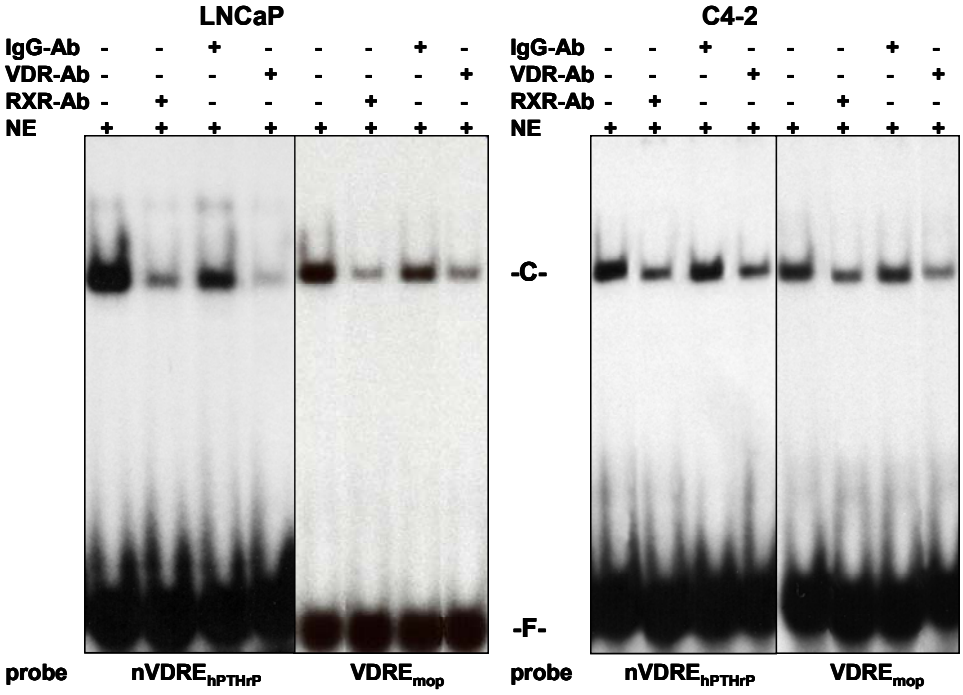


Figure 3.1. Effects of anti-VDR and anti-RXRα antibodies on the interaction of $nVDRE_{hPTHrP}$ with nuclear proteins from LNCaP and C4-2 cells. $VDRE_{mop}$ was used as a positive control. Nuclear extract (NE; 3 μg) from LNCaP or C4-2 cells was incubated with poly (dI-dC)·poly (dI-dC) (nonspecific competitor) and the indicated probe DNA for 40 min. After addition of anti-VDR antibody (VDR-Ab), anti-RXRα antibody (RXR-Ab) or the normal polyclonal IgG antibody (IgG-Ab), incubation was continued for a further 30 min. The reactions were then fractionated through a native 5% polyacrylamide gel. C, DNA/nuclear protein complex; F, free probe.

Conversely, inclusion of an anti-Ku-70 antibody, which supershifts the nuclear protein-DNA complex [139], had no effect on complex formation via the $nVDRE_{mop}$ or $nVDRE_{hPTHrP}$ (Fig. 3.2).

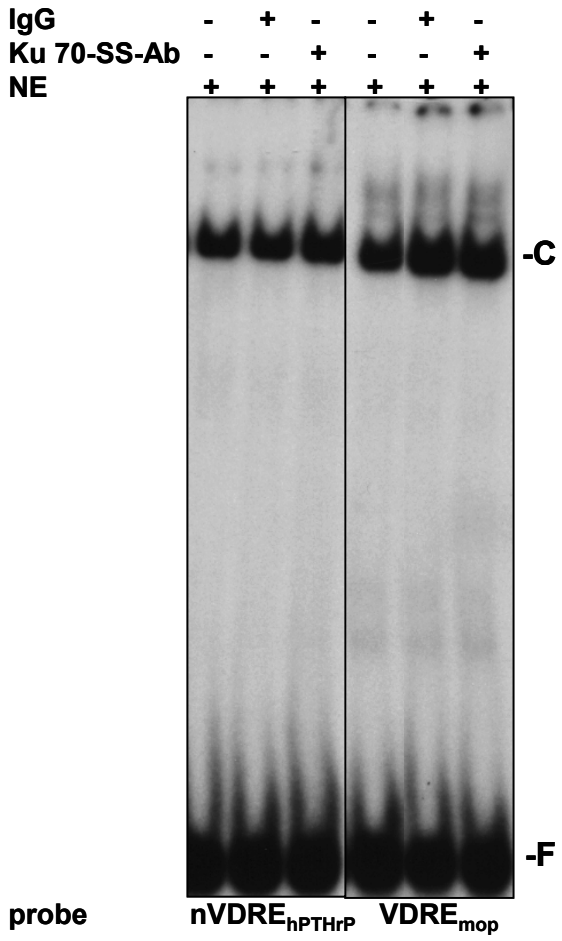


Figure 3.2. Effects of anti-Ku antibody on the interaction of $nVDRE_{hPTHrP}$ and $VDRE_{mop}$ with nuclear proteins from LNCaP cells. Nuclear extract (NE; 3 μg) from LNCaP cells was incubated with poly (dI-dC)·poly (dI-dC) (nonspecific competitor) and the indicated probe DNA for 40 min. After addition of the anti-Ku 70 supershift antibody (Ku 70-SS-Ab) or the normal polyclonal IgG antibody (IgG-Ab), incubation was continued for a further 30 min. The reactions were then fractionated through a native 5% polyacrylamide gel. C, DNA/nuclear protein complex; F, free probe.

Ku antigen is not involved in the $1,25(OH)_2D_3$-mediated transcriptional activation of mouse osteopontin gene expression. Taken together, these results indicate that the VDR along with the RXRα binds to the $nVDRE_{hPTHrP}$ sequence in LNCaP and C4-2 cells.

$1,25(OH)_2D_3$ and EB1089, alone or in combination with 9-cis-retinoic acid, decrease PTHrP mRNA and protein levels in LNCaP and C4-2 cells

We previously demonstrated that $1,25(OH)_2D_3$ and EB1089 decrease PTHrP mRNA levels in a concentration-dependent manner in LNCaP cells, with a maximal effect observed after treatment with 10^{-8} M $1,25(OH)_2D_3$ or EB1089 for 24 h or 48 h [82].

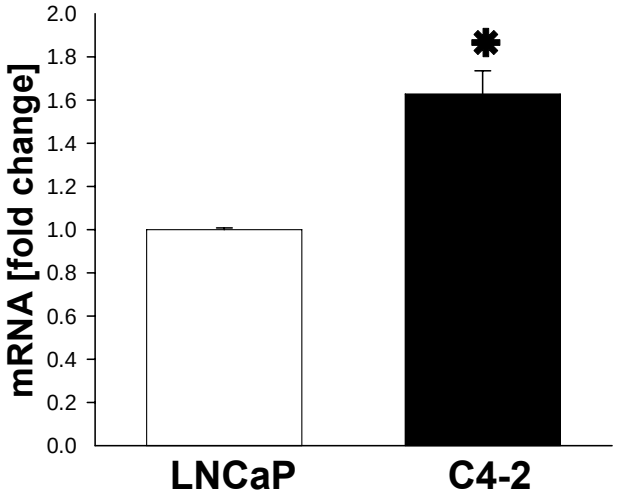


Figure 3.3. PTHrP mRNA levels in LNCaP and C4-2 cells. Cells were cultured in RPMI medium containing 10% FBS until they reached ~ 70% confluence. The PTHrP mRNA levels were assessed by RT-Real-time PCR. Each bar represents the mean ± S.E.M. of three independent experiments. Where no error bar is shown, the S.E.M. is smaller than the bar line. ✱, Significantly different from LNCaP at $P < 0.01$.

In the present study, we first compared PTHrP mRNA levels in LNCaP and C4-2 cells, and report that PTHrP expression was significantly higher (~ 2-fold) in C4-2 vs. LNCaP cells (Fig. 3.3).

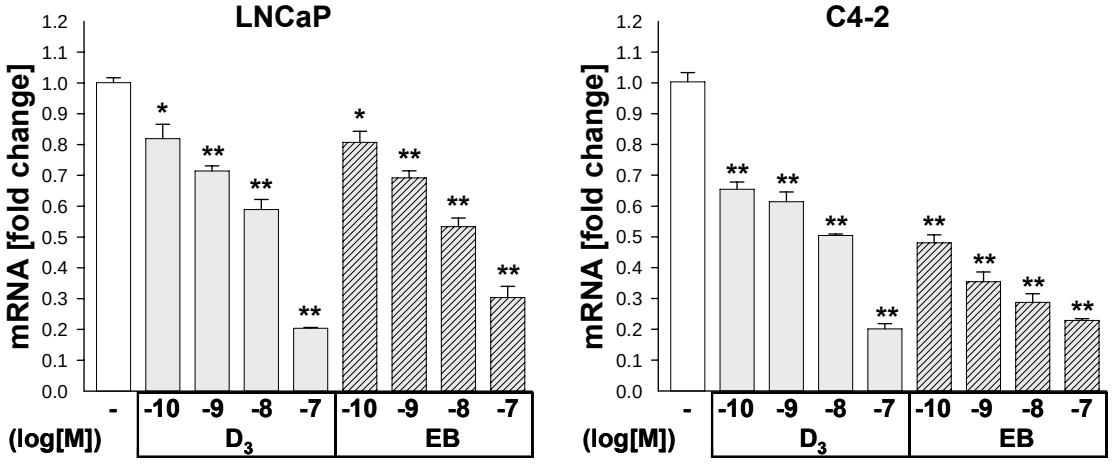


Figure 3.4. Effects of 1,25(OH)$_2$D$_3$ and EB1089 on PTHrP mRNA levels in LNCaP and C4-2 cells. Cells were cultured in the presence of the indicated concentrations (log of molarity) of 1,25(OH)$_2$D$_3$ (D$_3$) or EB1089 (EB) for 48h. Control cells (-) received ethanol (vehicle control). The PTHrP mRNA levels were assessed by RT-Real-time PCR. Each bar represents the mean ± S.E.M. of three independent experiments. Where no error bar is shown, the S.E.M. is smaller than the bar line. * = Significantly different from the control (-) at $P < 0.01$; ** = significantly different from the control at $P < 0.001$.

We also compared the effects of 1,25(OH)$_2$D$_3$ and EB1089 on PTHrP mRNA levels in these two cell lines. The two compounds produced a concentration-dependent decrease in PTHrP mRNA levels after 48 h of treatment in both cell lines (Fig. 3.4). This decrease was more pronounced in C4-2 than in LNCaP cells, especially at low concentrations of the compounds (Fig. 3.4).

We also measured the effects of 9-*cis*-RA on PTHrP mRNA levels in LNCaP and C4-2 cells. Treating LNCaP cells for 48 h with 9-*cis*-RA caused a concentration-dependent decrease in PTHrP mRNA levels (Fig. 3.5). Treating C4-2 cells for 48 h with

9-*cis*-RA also produced a decrease in the PTHrP mRNA levels. However, this decrease was significantly less pronounced and was not classically concentration-dependent (Fig. 3.5).

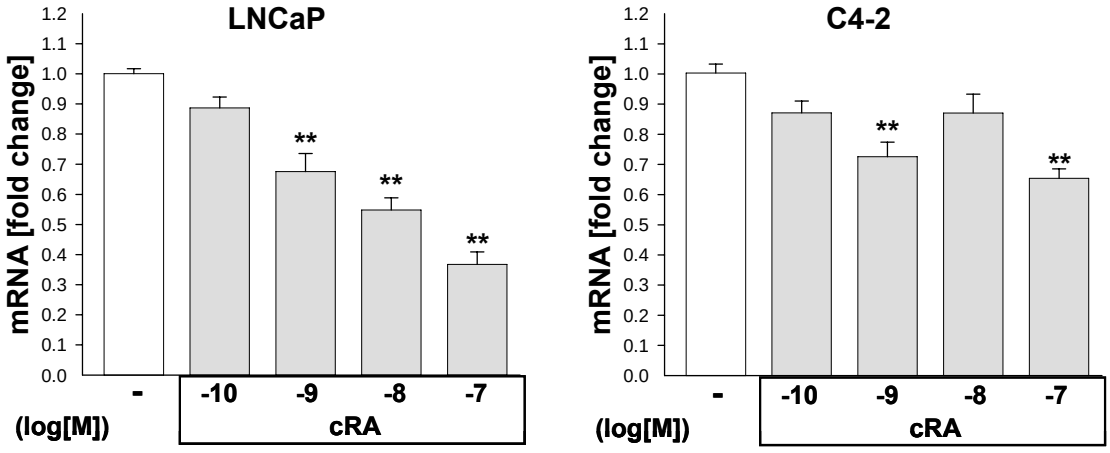


Figure 3.5. Effects of 9-*cis*-retinoic acid on PTHrP mRNA levels in LNCaP and C4-2 cells. Cells were cultured in the absence (-) or presence of the indicated concentrations (log of molarity) of 9-*cis*-retinoic acid (cRA) for 48h. Control cells (-) received ethanol (vehicle control). The PTHrP mRNA levels were assessed by RT-Real-time PCR. Each bar represents the mean ± S.E.M. of three independent experiments. ** = Significantly different from the control (-) at $P < 0.001$.

Combined treatment with 10^{-7} M $1,25(OH)_2D_3$ plus 10^{-7} M 9-*cis*-RA for 48 h produced a statistically significant enhancement of the effect of either compound alone in LNCaP, but not in C4-2 cells (Fig. 3.6,A). Similarly, treatment with 10^{-9} M $1,25(OH)_2D_3$ plus 10^{-9} M 9-*cis*-RA enhanced the $1,25(OH)_2D_3$-mediated downregulation of PTHrP expression. This effect was observed in both LNCaP and C4-2 cells, though it was more pronounced in LNCaP cells (Fig. 3.6,B).

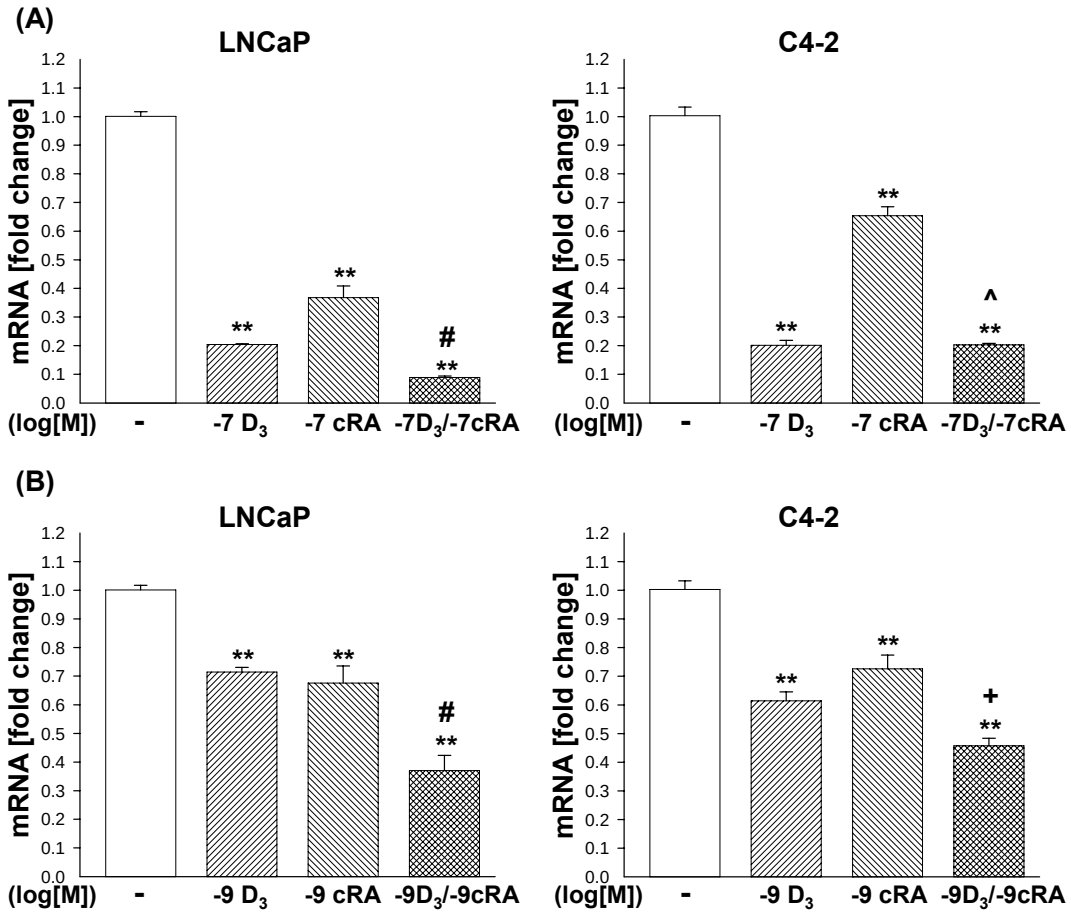


Figure 3.6. Effects of 1,25(OH)$_2$D$_3$ and/or 9-*cis*-retinoic acid on PTHrP mRNA levels in LNCaP and C4-2 cells. Cells were cultured in the presence of (A) 10^{-7} M and (B) 10^{-9} M of 1,25(OH)$_2$D$_3$ (D$_3$) and/or 9-*cis*-retinoic acid (cRA) for 48h. Control cells (-) received ethanol (vehicle control). The PTHrP mRNA levels were measured by RT-Real-time PCR. Each bar represents the mean ± S.E.M. of three independent experiments. Where no error bar is shown, the S.E.M is smaller than the bar line. ** = Significantly different from the control (-) at $P < 0.001$; # = significantly different from D$_3$ at $P < 0.001$ and from cRA at $P < 0.001$; ^ = significantly different from cRA at $P < 0.001$; and + = significantly different from D$_3$ at $P < 0.05$ and from cRA at $P < 0.001$.

In contrast, PTHrP mRNA levels were not significantly different in LNCaP cells treated with 10^{-8} M 1,25(OH)$_2$D$_3$ or 10^{-8} M 9-*cis*-RA alone, or with the combination of the two compounds (Fig. 3.7). At the same concentration, combined treatment of C4-2

cells produced a small, though significant increase in the effect obtained with $1,25(OH)_2D_3$ alone (Fig. 3.7). Similar effects were observed with combined treatment with EB1089 and 9-*cis*-RA.

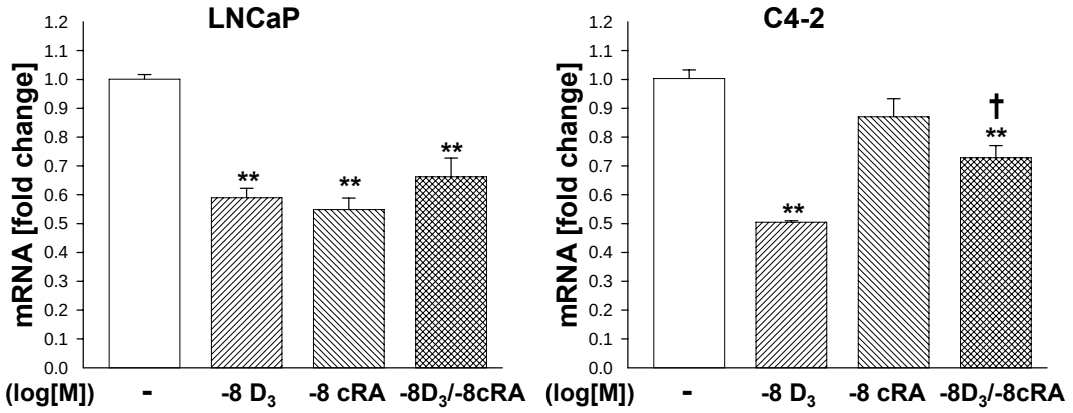


Figure 3.7. Effects of 10^{-8} M of $1,25(OH)_2D_3$ and/or 9-cis-retinoic acid on PTHrP mRNA levels in LNCaP and C4-2 cells. Cells were cultured in the presence of 10^{-8} M of $1,25(OH)_2D_3$ (D_3) and/or 9-*cis*-retinoic acid (cRA) for 48h. Control cells (-) received ethanol (vehicle control). The PTHrP mRNA levels were measured by RT-Real-time PCR. Each bar represents the mean ± S.E.M. of three independent experiments. Where no error bar is shown, the S.E.M is smaller than the bar line. ** = Significantly different from the control (-) at $P < 0.001$; † = significantly different from D_3 at $P < 0.05$

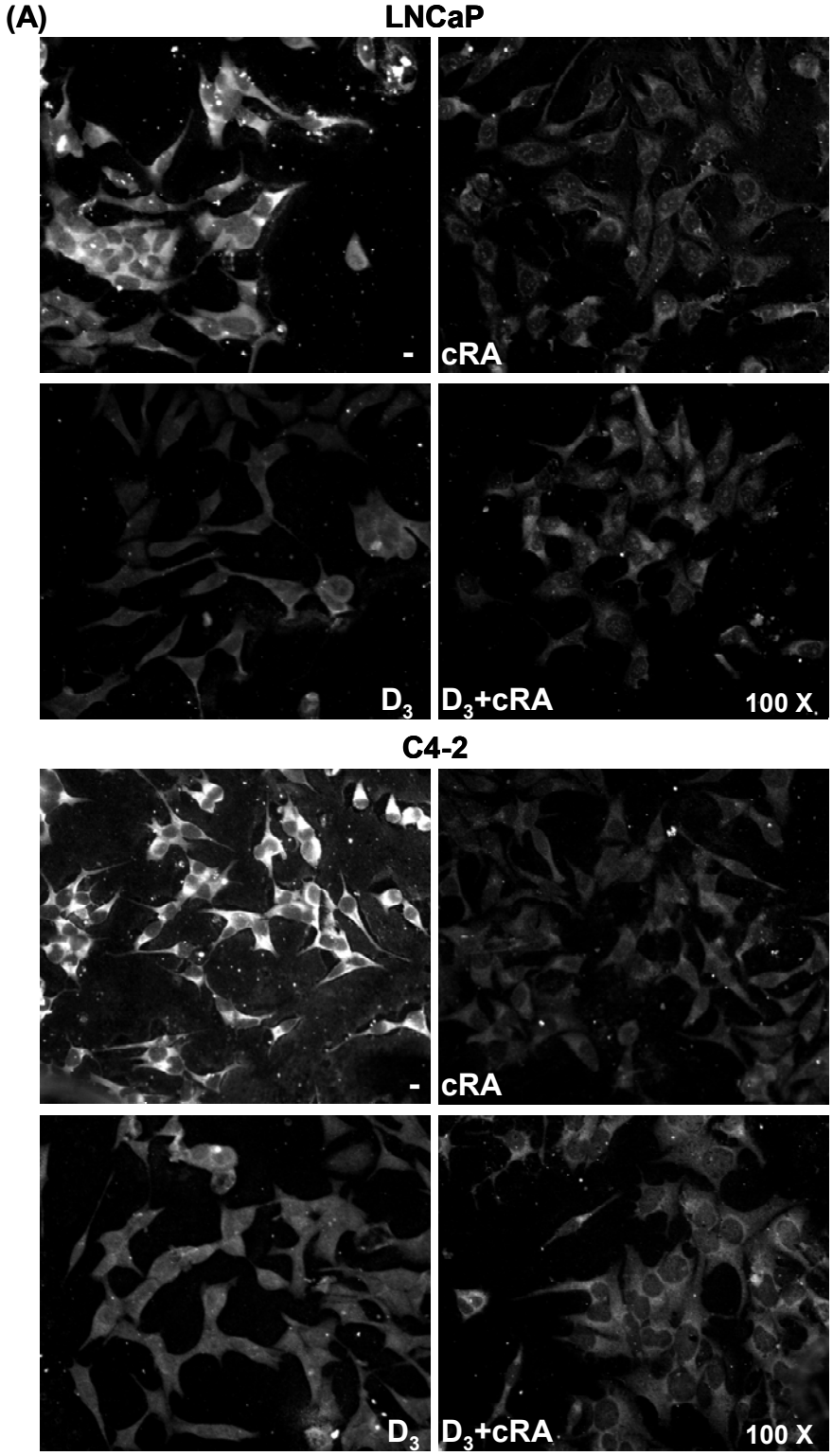
The effect of $1,25(OH)_2D_3$ and EB1089, either alone or in combination with 10^{-7} M 9-*cis*-RA acid, on total cellular PTHrP levels was assessed by fluorescence immunocytochemistry. In agreement with our previous study [82], treating LNCaP cells with 10^{-7} M $1,25(OH)_2D_3$ or EB1089 for 72 h decreased total cellular PTHrP levels (Fig. 3.8,A). The same effects on total cellular PTHrP levels were observed in C4-2 cells (Fig. 3.8,A). Treating LNCaP and C4-2 cells with 10^{-7} M 9-*cis*-RA also decreased cellular PTHrP levels (Fig. 3.8,A). Quantitative analysis of the fluorescence using a program written in the Matlab System by Dr. Gilbert R. Hillman showed that, in LNCaP cells, combination treatment with $1,25(OH)_2D_3$ or EB1089 plus 9-*cis*-RA enhanced the

effect of either compound alone on total cellular and nuclear PTHrP levels (Fig. 3.8, B and D). In C4-2 cells, the combination treatment did not augment the decrease in cellular PTHrP levels produced by 9-*cis*-RA alone (Fig. 3.8, C and E).

The nVDRE$_{hPTHrP}$ confers vitamin D responsiveness on C4-2 cells

We previously demonstrated that the nVDRE$_{hPTHrP}$ sequence, located between nucleotides -546 and -517 of the human PTHrP gene, confers responsiveness to 1,25(OH)$_2$D$_3$ and EB1089 in the weakly metastatic LNCaP, but not in the highly metastatic PC-3, cells [82]. To determine whether the nVDRE$_{hPTHrP}$-mediated response to these compounds correlates with the metastatic potential of different prostate cancer cell lines, we compared the responses of LNCaP and C4-2 cells to treatment with 1,25(OH)$_2$D$_3$ and EB1089. For this purpose, the nVDRE$_{hPTHrP}$ sequence was cloned upstream of the SV40 promoter in the vector pCAT promoter (Promega), and this construct (pCAT-nVDRE$_{hPTHrP}$) was transiently transfected into LNCaP and C4-2 cells, which were then treated with 1,25(OH)$_2$D$_3$ or EB1089.

To ascertain whether the response of C4-2 cells to these compounds is comparable to that of LNCaP cells, the two cell lines were transfected with a construct containing the VDRE from the mouse osteopontin gene (VDRE$_{mop}$ [111]) cloned in pCAT promoter (positive control).


(A)
LNCaP
-
cRA
D3
D3+cRA
100 X
C4-2
-
cRA
D3
D3+cRA
100 X

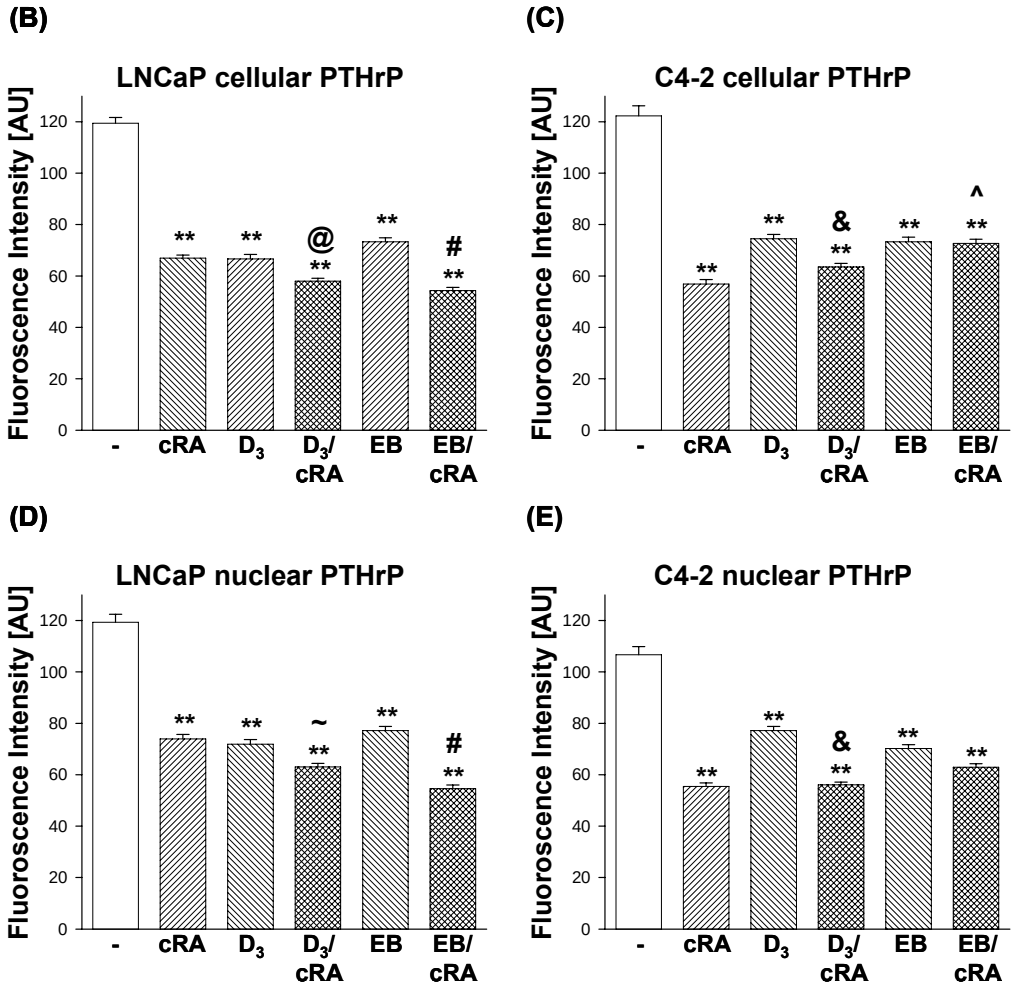


Figure 3.8. Effects of 1,25(OH)$_2$D$_3$ and/or 9-*cis*-retinoic acid on cellular PTHrP levels in LNCaP and C4-2 cells. Cells were cultured in the presence of 10^{-7} M 1,25(OH)$_2$D$_3$ and/or 9-*cis*-retinoic acid for 72 h. (A) Immunofluorescence staining for PTHrP was performed as described in the Materials and Methods. D$_3$, cells cultured in the presence of 1,25(OH)$_2$D$_3$; EB, EB1089; cRA, 9-*cis*-retinoic acid; -, vehicle (ethanol) control. (B-E) Quantitative analysis of total cellular (B, C) and nuclear (D, E) fluorescence intensity in LNCaP and C4-2 cells, calculated as described in the Materials and Methods. Each bar represents the mean ± SEM from 60 cells/treatment (10 cells from 6 individual slides). (B-E) **, Significantly different from vehicle control at $P < 0.001$. (B) @, Significantly different from D$_3$ and cRA at $P < 0.05$; #, significantly different from EB and cRA at $P < 0.001$. (C) &, Significantly different from D$_3$ at $P < 0.01$; ^, significantly different from cRA at $P < 0.001$. (D) ~, Significantly different from D$_3$ at $P < 0.05$ and from cRA at $P < 0.001$; #, significantly different from EB and cRA at $P < 0.001$. (E) &, Significantly different from D$_3$ at $P < 0.001$.

In LNCaP cells, the promoter activity driven by $nVDRE_{hPTHrP}$ is responsive to treatment with $1,25(OH)_2D_3$ or EB1089 in transient transfection assays [82]. Treating C4-2 cells with 10^{-7} M $1,25(OH)_2D_3$ or EB1089 for 48 h inhibited CAT activity driven by the $nVDRE_{hPTHrP}$ sequence by ~ 40 or 55% respectively, compared to ethanol (vehicle control)-treated cells (Fig. 3.9). This response was comparable to that observed in LNCaP cells (Fig. 3.9, [82]). Transfection with the pCAT-$VDRE_{mop}$ construct produced a significant increase in CAT activity in both LNCaP and C4-2 cells in response to treatment with $1,25(OH)_2D_3$ or EB1089; again this response was comparable in the two cell lines (Fig. 3.9). These data confirm the functionality of the VDR in LNCaP and C4-2 cells, and their ability to respond to these compounds. Treatment with $1,25(OH)_2D_3$ or EB1089 had no effect on promoter activity in pCAT-promoter (empty vector) transfectants (Fig. 3.9); ethanol treatment had no significant effect on CAT activity in either cell line (Fig. 3.9).

9-cis-retinoic acid enhances the $nVDRE_{hPTHrP}$-mediated effect of $1,25(OH)_2D_3$ and EB1089 in LNCaP, but not C4-2, cells

We have identified the RXR as the heterodimeric binding partner of the VDR on the $nVDRE_{hPTHrP}$ sequence in both LNCaP and C4-2 cells (Fig. 3.1). Here we investigated the effect of 9-*cis*-RA on the $1,25(OH)_2D_3$- and EB1089-mediated downregulation of PTHrP gene expression via the $nVDRE_{hPTHrP}$ in these cell lines. LNCaP and C4-2 cells were transfected with the CAT-$nVDRE_{hPTHrP}$ construct [82], and then treated with $1,25(OH)_2D_3$, EB1089 and/or 9-*cis*-RA. pCAT-$VDRE_{mop}$ was used as a positive control for the 9-*cis*-RA effect [99,140].

Treatment with 10^{-7} M 9-*cis*-RA alone caused a 48% decrease in CAT activity in LNCaP cells transfected with the pCAT-$nVDRE_{hPTHrP}$ construct (Fig. 3.9). In contrast, 9-*cis*-RA had no significant effect on CAT activity in C4-2 cells transfected with the same construct (Fig. 3.9). Co-treatment of pCAT-$nVDRE_{hPTHrP}$ transfectants with 10^{-7} M 9-*cis*-RA plus 10^{-7} M $1,25(OH)_2D_3$ or EB1089 enhanced the decrease in CAT activity obtained with $1,25(OH)_2D_3$ or EB1089 alone in LNCaP, but not in C4-2, cells (Fig. 3.9). Thus, the decrease in CAT activity obtained by co-treatment of LNCaP cells with either

1,25(OH)$_2$D$_3$ or EB1089 plus 9-cis-RA was significantly greater ($P < 0.01$) than the effect obtained with either compound alone (Fig. 3.9).

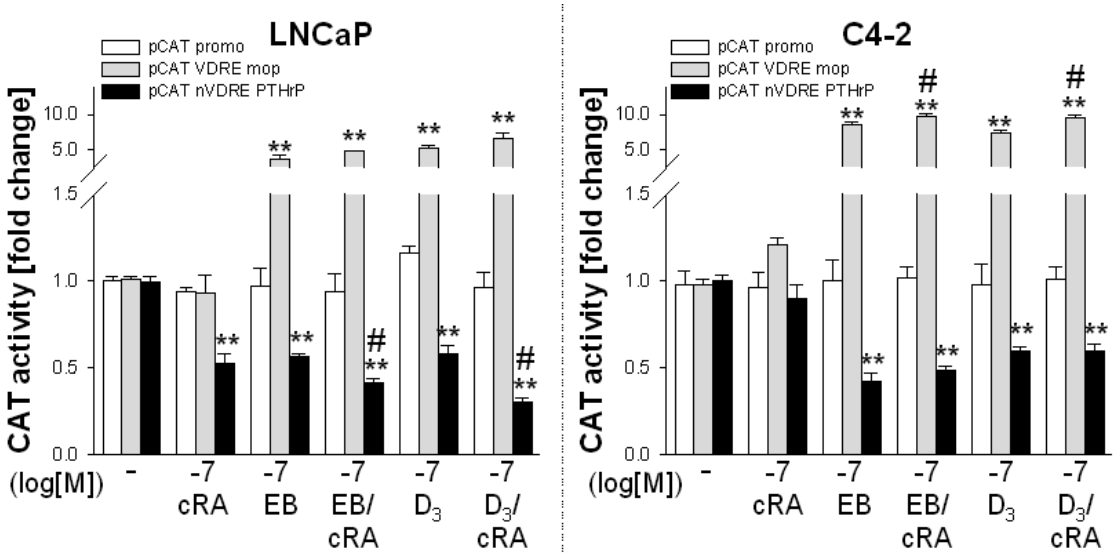


Figure 3.9. Effects of 1,25(OH)$_2$D$_3$, EB1089 and/or 9-*cis*-retinoic acid on promoter activity mediated via the nVDRE$_{hPTHrP}$ in LNCaP and C4-2 cells. The nVDRE$_{hPTHrP}$ sequence was cloned upstream of the SV40 promoter/CAT gene in the vector pCAT promoter (pCAT-nVDRE$_{hPTHrP}$), and transfected into LNCaP or C4-2 cells. pCAT-VDRE$_{mop}$ and pCAT-promo (empty vector) served as positive and negative controls, respectively. After transfection, the cells were treated with 10^{-7} M of 1,25(OH)$_2$D$_3$ (D$_3$), EB1089 (EB) and/or 9-*cis*-retinoic acid (cRA) for 48h. Control transfections (-) received ethanol (vehicle). Promoter activity was measured after 48 h by the CAT (Chloramphenicol Acetyl Transferase) assay. Each bar represents the mean ± S.E.M. of three independent experiments. ** = Significantly different from the control (-) at $P < 0.001$; # = significantly different from D$_3$ or EB at $P < 0.01$.

As described above, transfection with the pCAT-VDRE$_{mop}$ construct produced a significant increase in CAT activity in both LNCaP and C4-2 cells in response to 1,25(OH)$_2$D$_3$ or EB1089 (Fig. 3.9). In contrast, treatment with 9-*cis*-RA had no significant effect on promoter activity driven by pCAT-VDRE$_{mop}$ in either LNCaP or C4-2 cells (Fig. 3.9). Treatment of pCAT-nVDRE$_{mop}$-transfected C4-2 cells with 10^{-7} M 9-*cis*-RA in the presence of either 1,25(OH)$_2$D$_3$ or EB1089 enhanced the increase in CAT activity obtained with either 1,25(OH)$_2$D$_3$ or EB1089 alone by a small but significant

degree ($P < 0.01$) (Fig. 3.9). A similar trend was observed in LNCaP cells, although in this case the observed augmentation in CAT activity was not statistically significant (Fig. 3.9). Treatment with $1,25(OH)_2D_3$ or EB1089 and/or 9-*cis*-RA had no effect on promoter activity in pCAT promoter (empty vector) transfectants (Fig. 3.9). Ethanol (vehicle control) had no effect on CAT activity in either LNCaP or C4-2 cells (Fig. 3.9).

$1,25(OH)_2D_3$ and EB1089, alone or in combination with 9-cis-retinoic acid attenuate the binding between $nVDRE_{hPTHrP}$ and the VDR

The effects of $1,25(OH)_2D_3$ and EB1089, alone or in combination with 9-*cis*-RA on the $nVDRE_{hPTHrP}$-VDR interaction were investigated by incubating LNCaP and C4-2 nuclear extracts *in vitro* with either compound alone or in combination with 9-*cis*-RA for 30 min before addition of probe DNA ($nVDRE_{hPTHrP}$). Treating C4-2 nuclear extracts *in vitro* with $1,25(OH)_2D_3$ or EB1089 for 48 h weakened the binding between $nVDRE_{hPTHrP}$ and C4-2 nuclear proteins in a dose-dependent manner (Fig. 3.10). A decrease in the C4-2 nuclear proteins-$nVDRE_{hPTHrP}$-complex was also obtained with 10^{-7} M 9-*cis*-RA treatments of C4-2 nuclear extracts (Fig. 3.10). Treating LNCaP nuclear extracts under the same conditions also weakened the protein-DNA interactions (Chapter Two, Fig. 2.9,B).

Incubating C4-2 nuclear extracts *in vitro* with either $1,25(OH)_2D_3$ or EB1089 in combination with 10^{-7} M 9-*cis*-RA for 30 min prior to addition of probe DNA ($nVDRE_{hPTHrP}$) further decreased protein-DNA complex formation than either compound alone in a dose-dependent manner (Fig. 3.10).

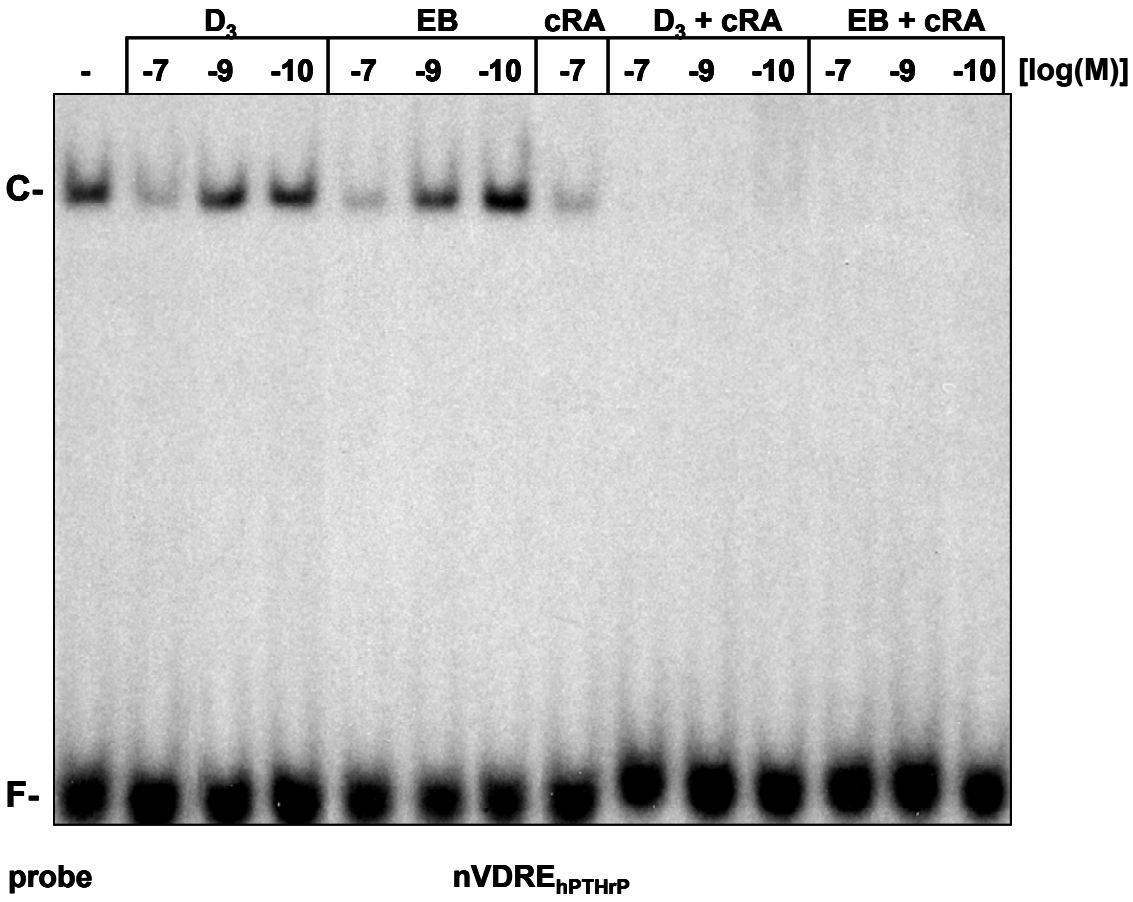


Figure 3.10. Effects of $1,25(OH)_2D_3$ and EB1089 and/or 9-*cis*-retinoic acid on the interaction between the $nVDRE_{hPTHrP}$ sequence and the VDR in nuclear extracts from C4-2 cells. Poly (dI-dC)·poly (dI-dC) (non-specific competitor) and 1.5 µl of nuclear extract from C4-2 cells were used in each binding reaction. D_3 = $1,25(OH)_2D_3$; EB = EB1089; cRA = 9-cis-retinoic acid;- = vehicle (ethanol) control. Nuclear extracts from C4-2 cells were treated with the indicated concentrations (log of molarity) of $1,25(OH)_2D_3$ or EB1089 and/or 9-cis-retinoic acid for 30 min before addition of probe DNA. C, DNA/nuclear protein complex; F, free probe.

9-cis-retinoic acid inhibits the growth of LNCaP, but not C4-2, cells

Previous studies have shown that $1,25(OH)_2D_3$ and EB1089 exert an anti-proliferative effect on LNCaP cells in culture [72,82,115] and on LNCaP tumor xenografts in nude mice [141]. The effect of $1,25(OH)_2D_3$ on C4-2 cell growth is similar to that on

LNCaP cells [142]. In agreement with the above studies, here we show that treating LNCaP and C4-2 cells with 10^{-7} M $1,25(OH)_2D_3$ or EB1089 caused a significant decrease in cell number, which became apparent after 3 days (Fig. 3.11). Treating LNCaP cells with 10^{-7} M 9-*cis*-RA for 3 or 7 days had no significant effect on cell number (Fig. 3.11). However, an 11-day treatment did cause a significant decrease in cell number (Fig. 3.11). In contrast, 9-*cis*-RA did not decrease C4-2 cell number at any of the time-points tested (Fig. 3.11).

We also measured the proliferation of LNCaP and C4-2 cells co-treated with $1,25(OH)_2D_3$ or EB1089 (10^{-7} M) plus 9-*cis*-RA (10^{-7} M). These concentrations were chosen since they produce maximal downregulation of PTHrP gene expression in both cell lines. Under our experimental conditions (cells cultured in the presence of 10% dialyzed FBS), combination treatment with $1,25(OH)_2D_3$ or EB1089 and 9-*cis*-RA did not augment the growth-inhibitory effects of either compound alone at any of the time points tested in C4-2 cells (Fig. 3.11).

However, in LNCaP cells, co-treatment with $1,25(OH)_2D_3$ or EB1089 plus 9-*cis*-RA augmented the effects of either compound alone after 7 days treatments, but not after 3 or 11 days treatments (Fig. 3.11). Blutt *et al*. [65] report a synergistic inhibition in the proliferation of LNCaP cells treated for 9 days with $1,25(OH)_2D_3$ (10^{-7} M or 10^{-8} M) plus 9-*cis*-RA (10^{-8} M) in 10% FBS containing medium. The 11 days combination treatment may have failed to augment the growth-inhibitory effects of either compound alone because cells growth was very low or almost negligible at this time point (Fig. 3.11).

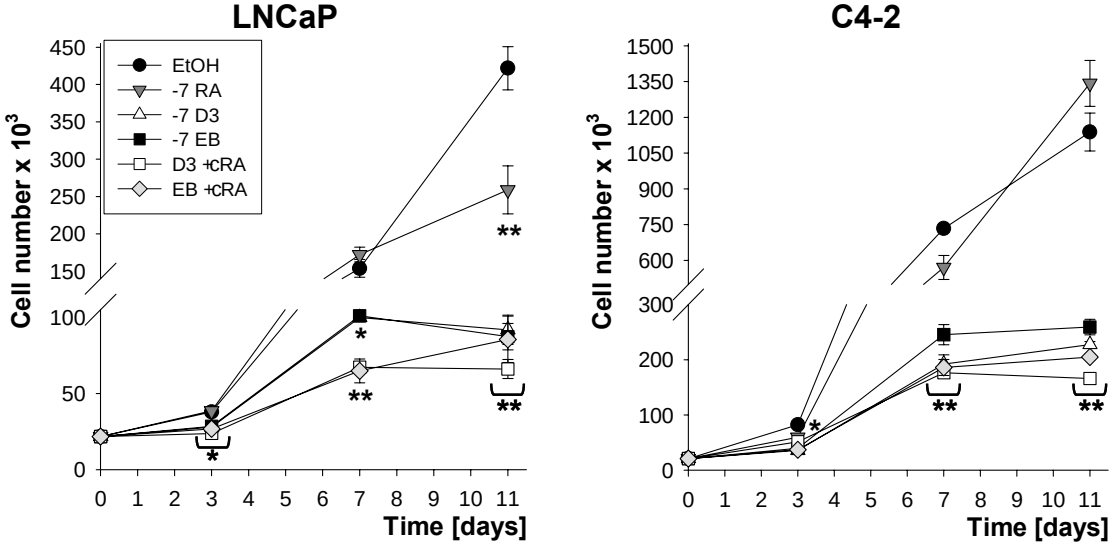


Figure 3.11. Proliferation of LNCaP and C4-2 cells treated with 1,25(OH)$_2$D$_3$, EB1089 and/or 9-*cis*-retinoic acid. Cells were plated in 24-well dishes in medium containing 10% FBS at a density of 10^4 cells/well. After 24 h, the medium was replaced with dialyzed FBS-containing medium (final FBS concentration, 10%). After a further 24 h, the cells were treated with 10^{-7} M 1,25(OH)$_2$D$_3$ (D$_3$), EB1089 (EB) and/or 9-*cis*-retinoic acid (cRA). Control cells were treated with ethanol (vehicle). At the indicated time intervals, the cells were trypsinized, and cell numbers determined using a Coulter Counter. Each point is the mean ± S.E.M. of three independent experiments (four wells per experiment). Where no error bar is shown, the S.E.M. is smaller than the symbol. * = Significantly different from control (vehicle only) at $P < 0.05$; and ** = significantly different from control at $P < 0.001$.

DISCUSSION

PTHrP plays an important role in the proliferation and survival of prostate cancer cells *in vitro* [37,38] and in the growth of prostate cancer *in vivo* [32,122]. The natural progression of human prostate cancer frequently proceeds to androgen independence and metastases, with a propensity for metastasis to the bone [103]. PTHrP also plays an important role in this process, increasing osteoclastic osteolysis in the presence of focal osseous metastases [32]. PTHrP enhances the cell surface expression of the pro-invasive integrins α1, α6, and β4, and increases adhesion to the extracellular matrix (ECM)

proteins collagen type I and laminin [39,40,143]; enhanced integrin expression and ECM adhesion may play a role in the proliferative, anti-apoptotic and invasive effects of PTHrP. Hence, downregulation of PTHrP gene expression may prove clinically important in the treatment of prostate cancer. In this study, we used the human prostate cancer cell lines LNCaP and C4-2 as models for prostate cancer of good and poor clinical outcomes, respectively, representing the natural progression of this cancer from primary to metastatic tumor.

The role of vitamin D in prostate cancer has been well studied. Vitamin D deficiency has been linked to an increase in the incidence of prostate cancer (reviewed in [54]). Several studies link the function of $1,25(OH)_2D_3$ to growth inhibition of both androgen-dependent and -independent human prostate cancer cell lines *in vivo* and *in vitro*, including LNCaP and C4-2 cells [72,82,115,141,142]. $1,25(OH)_2D_3$ also decreases integrin α6 and β4 expression, cell adhesion, migration, and invasion, and tumor invasion in many cell types, including human prostate cancer cell lines [123-126]. We have previously shown that $1,25(OH)_2D_3$ and its non-hypercalcemic analog EB1089 decrease steady-state PTHrP mRNA and secreted and cellular PTHrP levels in LNCaP and PC-3 cells [82,83]. We have now extended these studies to C4-2 cells, and show that $1,25(OH)_2D_3$ and EB1089 decrease PTHrP mRNA as well as total cellular PTHrP levels in these cells. Thus, downregulation of PTHrP expression by $1,25(OH)_2D_3$ and its non-hypercalcemic analogs may play a role in the beneficial effects of these compounds in prostate cancer. Since androgen ablation therapy for prostate cancer is initially effective but ultimately fails, compounds that have the potential to decrease prostate cancer cell growth and invasion have great therapeutic potential. Non-hypercalcemic $1,25(OH)_2D_3$ analogs fit into this class of compounds, since they may be therapeutically effective at multiple stages of prostatic carcinoma.

Three promoters have been identified within the human PTHrP gene [24,144]. A nVDRE ($nVDRE_{hPTHrP}$) is present within the promoter 1 region of the gene [80,82]; this nVDRE represents a *bona fide* VDRE, binding the VDR in a $1,25(OH)_2D_3$-responsive manner [80,82]. In this and a previous study [82], we show that downregulation of PTHrP gene

expression is mediated via this nVDRE in both LNCaP and C4-2 cells, which represent early- and advanced-stage prostate cancer, respectively. Thus, we can conclude that the $nVDRE_{hPTHrP}$ plays an important role in the regulation of PTHrP gene expression in different stages of prostate carcinoma. Understanding the transcriptional pathways involved in the downregulation of PTHrP gene expression via this nVDRE may therefore prove beneficial in the development of therapeutic regimens for the treatment of this cancer.

In MT-2 cells, negative regulation of PTHrP gene expression by $1,25(OH)_2D_3$ involves an interaction on $nVDRE_{hPTHrP}$ of the VDR and Ku antigen [80], a heterodimeric nuclear protein that associates with a 350 kDa catalytic subunit ($DNA\text{-}PK_{cs}$) to form a DNA-dependent protein kinase (DNA-PK). Ku antigen is thought to tether the serine-threonine kinase activity of DNA-PK to DNA, allowing phosphorylation of nearby substrates [132]. The model proposed by Nishishita *et al*. [80] involves binding of VDR and Ku in close proximity on the $nVDRE_{hPTHrP}$ sequence of the hPTHrP gene, thereby weakening the VDR's ability to bind the VDRE and decreasing promoter activity in response to $1,25(OH)_2D_3$. These investigators showed that anti-Ku antibody specifically supershifted the MT2 nuclear protein-$nVDRE_{hPTHrP}$ complex [80]. In contrast, here we report that, under the same conditions, anti-Ku antigen antibody had no effect on complex formation between nuclear extracts from LNCaP or C4-2 cells with the $nVDRE_{hPTHrP}$ sequence, as assessed by EMSA. Further, we also show by EMSA that both the VDR and RXRα interact with the $nVDRE_{hPTHrP}$. Thus, the molecular mechanisms of downregulation of PTHrP gene expression via the $nVDRE_{hPTHrP}$ may be cell type-specific. In fact, the $nVDRE_{hPTHrP}$-mediated response to the RXR ligand 9-*cis*-RA differs in LNCaP vs. C4-2 cells.

Multiple studies have shown that the VDR forms a heterodimer with the RXR to induce $1,25(OH)_2D_3$-mediated gene regulation [99,145]. The role of the RXR in $1,25(OH)_2D_3$-transcriptional repression is not as well defined. Here we show that treating LNCaP cells with 9-*cis*-RA decreased PTHrP mRNA levels in a concentration-dependent manner. In contrast, treating C4-2 cells with this RXR ligand produced a significantly

smaller, non-concentration-dependent decrease in PTHrP mRNA levels. However, the effect of 9-*cis*-RA on cellular PTHrP levels was comparable in the two cell lines. We also show that 10^{-7} M 9-*cis*-RA had no effect on the proliferation of C4-2 cells, but caused a significant decrease in LNCaP cell proliferation. 9-*cis*-RA, a metabolite of vitamin A, can bind with high affinity and activate both the RXRs (-α, -β, and -γ) and the retinoic acid receptors (RAR-α, -β, and -γ). Since RARs have been shown to regulate PTHrP gene expression differentially in a number of cell lines [146-148], the differing effects of 9-*cis*-RA on PTHrP mRNA levels and cell proliferation in the two cell lines may be due to differential regulation of both the RXRs and RARs by this ligand, or to limiting concentrations of RXR in C4-2 cells. In addition, 9-*cis*-RA may be exerting its effects independently of the VDR and nVDREs, and may be functioning through other response elements which may be present within the hPTHrP gene, such as retinoic acid response elements (RAREs [149]) or peroxisome proliferators response elements (PPREs [150]). Also the effects of 9-*cis*-RA on cellular PTHrP levels may be mediated not only through transcriptional, but also post-transcriptional and post-translational pathways.

Co-treating LNCaP cells with 10^{-7} M or 10^{-9} M $1,25(OH)_2D_3$ plus 9-*cis*-RA produced an enhanced decrease in PTHrP mRNA levels, in that PTHrP mRNA levels in co-treated cells were significantly lower than those in cells treated with either compound alone. In C4-2 cells, 9-*cis*-RA augmented the effect of $1,25(OH)_2D_3$ on PTHrP mRNA levels only at the 10^{-9} M concentration. Combination treatment with $1,25(OH)_2D_3$ or EB1089 plus 9-*cis*-RA also augmented the decrease by either compound alone on cellular PTHrP levels in LNCaP, but not in C4-2 cells. These results demonstrate that the 9-*cis*-RA response of the two cell lines is different, with C4-2 cells being less responsive than LNCaP cells to downregulation of PTHrP gene expression by either $1,25(OH)_2D_3$ or EB1089 plus 9-*cis*-RA. As discussed below, the effects of 9-*cis*-RA on $1,25(OH)_2D_3$-mediated transcriptional regulation are complex, and are not only cell type-specific, but also depend on the concentration of 9-*cis*-RA used.

The promoter activity of the cloned $nVDRE_{hPTHrP}$ in response to 9-*cis*-RA was also different in LNCaP and C4-2 cells, in that this RXR ligand decreased $nVDRE_{hPTHrP}$-

mediated promoter activity in LNCaP, but not in C4-2 cells. Furthermore, combination treatment with 1,25$(OH)_2D_3$ or EB1089 and 9-*cis*-RA produced an additive decrease in promoter activity, compared to treatment with 1,25$(OH)_2D_3$ or EB1089 alone, in LNCaP, but not in C4-2 cells. These data mirror the effects of these compounds on PTHrP mRNA levels in the two cell lines, and indicate that the effects of 9-*cis*-RA in LNCaP cells are partially mediated via the $nVDRE_{hPTHrP}$. Since 9-*cis*-RA had no effect on $nVDRE_{hPTHrP}$-mediated promoter activity, but downregulated PTHrP mRNA levels in C4-2 cells, we infer that additional nVDREs may be present within the PTHrP gene. In support of this idea, we previously reported that 1,25$(OH)_2D_3$ downregulated PTHrP expression in PC-3 cells via a transcriptional pathway, but had no effect on $nVDRE_{hPTHrP}$-mediated promoter activity [82]. Thus, downregulation of PTHrP gene expression by 1,25$(OH)_2D_3$ may depend on the presence of cell type-specific transcription factors and/or cell type-specific promoter utilization.

Multiple studies have shown that the effects of 9-*cis*-RA on the binding and transcriptional regulation of 1,25$(OH)_2D_3$-responsive genes by the VDR-RXR heterodimer are dependent on the concentration of 9-*cis*-RA used, and are also cell type-specific. For example, 9-*cis*-RA has been shown to repress the 1,25$(OH)_2D_3$-dependent accumulation of osteocalcin mRNA in osteoblast-like ROS 17/2.8 cells [114]. This effect, which is 9-*cis*-RA concentration-dependent, was attributed to diversion of RXRs away from VDR-mediated transcription and towards other RXR-dependent pathways [114]. In contrast, treating a number of transfected cell lines with both 1,25$(OH)_2D_3$ and 9-*cis*-RA enhanced the effect of 1,25$(OH)_2D_3$ alone [99,151]. In our system, co-treatment with 1,25$(OH)_2D_3$ or EB1089 plus 9-*cis*-RA augmented the decrease in promoter activity driven by $nVDRE_{hPTHrP}$ vs. that obtained with either compound alone in LNCaP, but not in C4-2, cells. A lack of effect of 9-*cis*-RA on 1,25$(OH)_2D_3$-mediated transcription has also been reported [140], analogous to our results with C4-2 cells. It therefore appears that in LNCaP cells, treatment with both the VDR and RXR ligands is required for optimal response in terms of both PTHrP mRNA levels and promoter activity driven by $nVDRE_{hPTHrP}$. Conversely, in C4-2 cells, 9-*cis*-RA does not appear to play a role in the

$1,25(OH)_2D_3$-mediated response via $nVDRE_{hPTHrP}$. These differences between the two cell lines may depend on the concentration of RXR ligand used, as well as on the levels of expression of the different RAR and RXR isoforms. For example, it has been shown that phosphorylation of the human RXRα in *ras*-transformed human keratinocytes through the activated Ras-Raf-mitogen-activated protein kinase (Ras-Raf-MAPK) pathway results in attenuated transactivation by the VDR and resistance to the growth-inhibitory effects of $1,25(OH)_2D_3$ and the RXR-specific agonist LG1069 [137]. C4-2 cells express nonmutationally active c-Ras, and inducible expression of a dominant negative form of Ras restores androgen sensitivity in these cells [152]. In contrast, LNCaP cells do not express active c-Ras [153]. Thus, the different profile of downregulation of PTHrP expression by 9-*cis*-RA plus $1,25(OH)_2D_3$ in C4-2 vs. LNCaP cells and the lack of effect of 9-*cis*-RA on $nVDRE_{hPTHrP}$-mediated downregulation of PTHrP gene expression in C4-2 cells may occur through the c-Ras-mediated inactivation of RXRα and the partial contribution of RXRβ and RXRγ in these cells.

In conclusion, the association between PTHrP expression and prostate cancer growth and metastasis clearly demonstrates the importance of controlling PTHrP production by prostate cancer cells. In this study, we show that $1,25(OH)_2D_3$ and its non-hypercalcemic analogue EB1089 downregulate PTHrP expression in prostate cancer cell lines exhibiting different metastatic potential, providing the rationale for the use of non-hypercalcemic $1,25(OH)_2D_3$ analogues to control PTHrP production in prostate cancer. Co-treatment with 9-*cis*-RA enhances the effect of $1,25(OH)_2D_3$ and EB1089 on PTHrP expression. The development of combination therapy with $1,25(OH)_2D_3$ plus 9-*cis*-RA analogues would therefore allow the use of lower concentrations of each compound to control PTHrP levels than would be required with either compound alone, thereby potentially decreasing the side-effects of the individual compounds.

CHAPTER FOUR: SUMMARY

PTHrP is expressed by prostate cancer cells. Since PTHrP increases the growth and enhances the osteolytic potential of prostate cancer cells, it is important to control PTHrP expression in these cells. The overall goal of this project was to study the effects of $1,25(OH)_2D_3$ and a non-hypercalcemic analog, EB1089, on PTHrP gene expression in prostate cancer cells and the underlying mechanisms involved. We were fortunate to have access to prostate cancer cells lines with different androgen responsiveness and metastatic potential. Thus, we were able to study the mechanisms underlying $1,25(OH)_2D_3$-mediated PTHrP gene regulation in cells with different androgen responsiveness and metastatic potential that represent the natural progression of lethal phenotypes of human prostate cancer to androgen independence and their propensity to form osseous metastases.

In this series of studies, we report that $1,25(OH)_2D_3$ and EB1089, downregulate PTHrP gene expression and production in three human prostate cancer cell lines, the AR-positive LNCaP cell line; the AR-positive, but androgen independent LNCaP-derived C4-2 cell line [82]; and the AR-negative PC-3 cell line. The results obtained with these cell lines are summarized in Table III and Table IV.

We also investigated whether the single heptameric motif located upstream of the P1 promoter transcription start site of the human PTHrP gene ($nVDRE_{hPTHrP}$ [80]) is responsible for the $1,25(OH)_2D_3$-mediated downregulation of PTHrP gene expression in these cell lines. Our results indicate that $nVDRE_{hPTHrP}$ mediates the PTHrP gene repression in response to $1,25(OH)_2D_3$ and EB1089 in LNCaP and C4-2, but not in the AR-negative and highly metastatic PC-3, cells (summarized in Tables V and VI). As $1,25(OH)_2D_3$ does downregulate PTHrP gene expression via a transcriptional pathway in PC-3 cells [83], these results suggest the presence of additional, as yet unidentified, more effective nVDRE(s) within the human PTHrP gene. Another possibility for these results

is that alternative collaborating sequence-context factors are required for negative regulator binding or stabilization in the downregulation of PTHrP gene expression in PC-3 and LNCaP cells via the $nVDRE_{hPTHrP}$, and that the 30 bp $nVDRE_{hPTHrP}$ sequence used here is not long enough to allow binding of these negative regulators in PC-3 cells. Additionally, these factors may not be present in the same relative levels in PC-3 cells. These results address the issue of whether the $nVDRE_{hPTHrP}$-mediated response to $1,25(OH)_2D_3$ correlates with the different stages of prostate cancer. We also report that, even though $1,25(OH)_2D_3$ and EB1089 downregulate $nVDRE_{hPTHrP}$-driven promoter activity in both LNCaP and C4-2 cells, the mechanisms underlying this response are cell type-specific and differ in prostate cancer cell lines that represent different stages of the disease.

Table III. Summary of the effects of $1,25(OH)_2D_3$ (D_3) and EB1089 (EB) on PC-3 and LNCaP cell lines

	PC-3		LNCaP	
	D_3	EB	D_3	EB
Cell proliferation	↓	↓	↓↓	↓↓
PTHrP mRNA	↓	↓	↓	↓
PTHrP secretion	↓	↓	↓	↓
Cellular PTHrP	↓	↓	↓	↓

Table IV. Summary of the effects of 1,25(OH)$_2$D$_3$ (D$_3$), EB1089 (EB), and/or 9-*cis*-retinoic acid (cRA) on LNCaP and C4-2 cell lines

	LNCaP				C4-2			
	D$_3$	EB	cRA	Comb.	D$_3$	EB	cRA	Comb.
Cell proliferation	↓	↓	↓	↓	↓	↓	-	↓
PTHrP mRNA	↓	↓	↓	↓	↓	↓	↓	↓
Cellular PTHrP	↓	↓	↓	↓	↓	↓	↓	↓

1,25(OH)$_2$D$_3$ upregulates gene transcription via binding to the VDR, a member of the superfamily of ligand-inducible nuclear transcription factors, and subsequent binding of the liganded VDR to VDREs. However, our results indicate that the interaction of the VDR with the nVDRE$_{hPTHrP}$ is decreased by 1,25(OH)$_2$D$_3$ in all three cell lines (summarized in Tables V and VI). The VDR binds to the VDRE in the target genes together with other proteins that sometimes function as co-activators or co-repressors. In this study, we also demonstrated that the RXR, together with the VDR, forms part of the nVDRE$_{hPTHrP}$-nuclear extract complex in PC-3 (Fig. 4.1), LNCaP, and C4-2 cells. In contrast, Nishishita *et al*. [80] reported that in the human T-cell line MT-2 (human adult T cell lymphoma/leukemia virus-infected T cells), the VDR along with the Ku-antigen bind to the nVDRE$_{hPTHrP}$, and that the RXR is not involved. Thus, our results suggest that the accessory proteins which interact with the VDR to promote PTHrP downregulation are cell-type specific, and might depend on the presence of cell type-specific transcription factors. In addition, since the human *PTHrP* gene has 9 exons and three distinctive promoters (P1, P2, and P3), the mechanism involved in the 1,25(OH)$_2$D$_3$ mediated downregulation of PTHrP may also depend on cell type-specific promoter utilization.

Table V. Summary of the effects of 1,25(OH)$_2$D$_3$ (D$_3$) and EB1089 (EB) mediated via the nVDRE$_{hPTHrP}$ on PC-3 and LNCaP cell lines

		PC-3		LNCaP	
EMSA	nVDRE	+		+	
	VDR-Ab	+		+	
	nVDRE-NE-complex	D$_3$	EB	D$_3$	EB
		↓		↓	
CAT→ nVDRE		D$_3$	EB	D$_3$	EB
		-		↓	

Table VI. Summary of the effects of 1,25(OH)$_2$D$_3$ (D$_3$), EB1089 (EB), and/or 9-*cis*-retinoic acid (cRA) mediated via the nVDRE$_{hPTHrP}$ on LNCaP and C4-2 cell lines

		LNCaP				C4-2			
EMSA with nVDRE	VDR-Ab	+				+			
	RXRα-Ab	+				+			
	nVDRE-NE-complex	D$_3$		EB	RA	D$_3$		EB	RA
		↓ (↓ comb.)				↓ (↓ comb.)			
CAT→ nVDRE		D$_3$	EB	RA	Comb.	D$_3$	EB	RA	Comb.
		↓	↓	↓	↓	↓	↓	-	↓

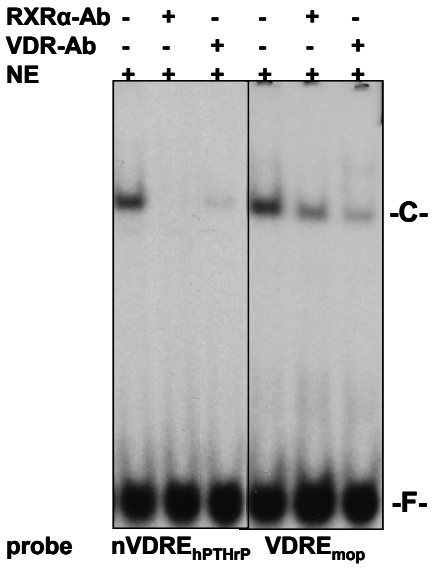


Figure 4.1. Effects of anti-VDR and anti-RXRα antibodies on the interaction of nVDRE$_{hPTHrP}$ with nuclear proteins from PC-3 cells. VDRE$_{mop}$ was used as a positive control. Nuclear extract (NE; 3 μg) from PC-3 cells was incubated with poly (dI-dC)·poly (dI-dC) (nonspecific competitor) and the indicated probe DNA for 40 min. After addition of anti-VDR antibody (VDR-Ab) or anti-RXRα antibody (RXR-Ab), incubation was continued for a further 30 min. The reactions were then fractionated through a native 5% polyacrylamide gel. C, DNA/nuclear protein complex; F, free probe.

We also report that the RXR ligand, 9-*cis*-RA, downregulates PTHrP mRNA levels in both LNCaP and C4-2 cells, though this effect is more pronounced in LNCaP cells (summarized in Table IV). Furthermore, our experiments using the nVDRE$_{hPTHrP}$ cloned upstream of the SV40 promoter show that treatment with 9-*cis*-RA decreases promoter activity driven by this construct in LNCaP, but not in C4-2 cells (summarized in Table VI). These results suggest that the downregulation of PTHrP mRNA levels by the combination of 1,25(OH)$_2$D$_3$ and 9-*cis*-RA in C4-2 cells might be mediated either via a VDRE other than the nVDRE$_{hPTHrP}$ or via binding of 9-*cis*-RA to either RAR or RXR promoting RAR-RXR heterodimer or RXR homodimer formation and subsequent binding to their respective retinoic acid response elements that might be present in the *PTHrP* gene. RAR homodimer and RAR-RXR heterodimer bind to RAREs [149]; and the

RXR homodimer transactivates through a response element with a single base-pair spacer between the two half-sites, DR1 [105,154]. Alternatively, 9-*cis*-RA could also be affecting PTHrP gene expression through either PPAR-RXR heterodimeric [155] or RXR-RXR homodimeric [156] binding to the Peroxisome Proliferators Response Elements (PPREs [150]). Furthermore, the effects of 9-*cis*-RA on cellular PTHrP levels may also be mediated through post-transcriptional and/or post-translational pathways.

We also show that the combination of $1,25(OH)_2D_3$ and 9-*cis*-RA allows the usage of lower concentration of each compound to reach significant downregulation of PTHrP gene expression in LNCaP and C4-2 cells (summarized in Table IV). Thus, understanding the mechanisms for downregulation of PTHrP expression by $1,25(OH)_2D_3$ may lead to the development of combination therapies for the treatment of prostate cancer that are more effective and result in lower side effect than the use of either compound alone.

In conclusion, downregulation of PTHrP expression via $1,25(OH)_2D_3$ and its non-hypercalcemic analogs may provide extra therapeutic benefits in neutralizing the contribution of PTHrP to the pathogenesis and progression of prostate carcinoma and the tendency of this tumor to metastasize to bone.

REFERENCES

1. **Parker, S. L., T. Tong, S. Bolden, and P. A. Wingo**. 1997. Cancer statistics, 1997. CA Cancer J Clin. **47**:5-27.

2. **Huggins, C. and C. V. Hodges**. 2002. Studies on prostatic cancer. I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. 1941. J. Urol. **167**:948-951.

3. **Huggins, C. B.** 1969. Commentary on the treatment of prostate cancer. J. Urol. **102**:119-120.

4. **Kirby, R. S., T. J. Christmas, and M. K. Brawer**. 2001. Prostate Cancer. Mosby.

5. **Amorino, G. P. and S. J. Parsons**. 2004. Neuroendocrine cells in prostate cancer. Crit Rev. Eukaryot. Gene Expr. **14**:287-300.

6. **Bonkhoff, H.** 1998. Neuroendocrine cells in benign and malignant prostate tissue: morphogenesis, proliferation, and androgen receptor status. Prostate Suppl **8**:18-22.

7. **Denis, L. J. and K. Griffiths**. 2000. Endocrine treatment in prostate cancer. Semin. Surg. Oncol. **18**:52-74.

8. **Broadus, A. E., M. Mangin, K. Ikeda, K. L. Insogna, E. C. Weir, W. J. Burtis, and A. F. Stewart**. 1988. Humoral hypercalcemia of cancer. Identification of a novel parathyroid hormone-like peptide. N. Engl. J. Med. **319**:556-563.

9. **Burtis, W. J., T. Wu, C. Bunch, J. J. Wysolmerski, K. L. Insogna, E. C. Weir, A. E. Broadus, and A. F. Stewart**. 1987. Identification of a novel 17,000-dalton parathyroid hormone-like adenylate cyclase-stimulating protein from a tumor associated with humoral hypercalcemia of malignancy. J. Biol. Chem. **262**:7151-7156.

10. **Moseley, J. M., M. Kubota, H. Diefenbach-Jagger, R. E. Wettenhall, B. E. Kemp, L. J. Suva, C. P. Rodda, P. R. Ebeling, P. J. Hudson, J. D. Zajac, and .** 1987. Parathyroid hormone-related protein purified from a human lung cancer cell line. Proc. Natl. Acad. Sci. U. S. A **84**:5048-5052.

11. **Suva, L. J., G. A. Winslow, R. E. Wettenhall, R. G. Hammonds, J. M. Moseley, H. Diefenbach-Jagger, C. P. Rodda, B. E. Kemp, H. Rodriguez, E. Y. Chen, and .** 1987. A parathyroid hormone-related protein implicated in malignant hypercalcemia: cloning and expression. Science **237**:893-896.

12. **Strewler, G. J.** 2000. The physiology of parathyroid hormone-related protein. N. Engl. J. Med. **342**:177-185.

13. **Strewler, G. J.** 2000. The parathyroid hormone-related protein. Endocrinol Metab Clin. North Am. **29**:629-645.

14. **Philbrick, W. M., J. J. Wysolmerski, S. Galbraith, E. Holt, J. J. Orloff, K. H. Yang, R. C. Vasavada, E. C. Weir, A. E. Broadus, and A. F. Stewart**. 1996. Defining the roles of parathyroid hormone-related protein in normal physiology. Physiol Rev. **76**:127-173.

15. **Fiaschi-Taesch, N. M. and A. F. Stewart**. 2003. Minireview: parathyroid hormone-related protein as an intracrine factor--trafficking mechanisms and functional consequences. Endocrinology **144**:407-411.

16. **Burtis, W. J.** 1992. Parathyroid hormone-related protein: structure, function, and measurement. Clin. Chem. **38**:2171-2183.

17. **Asadi, F. and S. Kukreja**. 2005. Parathyroid hormone-related protein in prostate cancer. Crit Rev. Eukaryot. Gene Expr. **15**:15-28.

18. **Kaiser, S. M., M. Sebag, J. S. Rhim, R. Kremer, and D. Goltzman**. 1994. Antisense-mediated inhibition of parathyroid hormone-related peptide production in a keratinocyte cell line impedes differentiation. Mol. Endocrinol. **8**:139-147.

19. **Henderson, J. E., N. Amizuka, H. Warshawsky, D. Biasotto, B. M. Lanske, D. Goltzman, and A. C. Karaplis**. 1995. Nucleolar localization of parathyroid hormone-related peptide enhances survival of chondrocytes under conditions that promote apoptotic cell death. Mol. Cell Biol. **15**:4064-4075.

20. **Lam, M. H., S. L. Olsen, W. A. Rankin, P. W. Ho, T. J. Martin, M. T. Gillespie, and J. M. Moseley**. 1997. PTHrP and cell division: expression and localization of PTHrP in a keratinocyte cell line (HaCaT) during the cell cycle. J. Cell Physiol **173**:433-446.

21. **Dingwall, C. and R. A. Laskey**. 1991. Nuclear targeting sequences--a consensus? Trends Biochem. Sci. **16**:478-481.

22. **Falzon, M. and P. Du**. 2000. Enhanced growth of MCF-7 breast cancer cells overexpressing parathyroid hormone-related peptide. Endocrinology **141**:1882-1892.

23. **Massfelder, T., P. Dann, T. L. Wu, R. Vasavada, J. J. Helwig, and A. F. Stewart**. 1997. Opposing mitogenic and anti-mitogenic actions of parathyroid hormone-related protein in vascular smooth muscle cells: a critical role for nuclear targeting. Proc. Natl. Acad. Sci. U. S. A **94**:13630-13635.

24. **Southby, J., L. M. O'Keeffe, T. J. Martin, and M. T. Gillespie**. 1995. Alternative promoter usage and mRNA splicing pathways for parathyroid hormone-related protein in normal tissues and tumours. Br. J Cancer **72**:702-707.

25. **Wu, T. L., R. C. Vasavada, K. Yang, T. Massfelder, M. Ganz, S. K. Abbas, A. D. Care, and A. F. Stewart**. 1996. Structural and physiologic characterization of the mid-region secretory species of parathyroid hormone-related protein. J. Biol. Chem. **271**:24371-24381.

26. **Jans, D. A., R. J. Thomas, and M. T. Gillespie**. 2003. Parathyroid hormone-related protein (PTHrP): a nucleocytoplasmic shuttling protein with distinct paracrine and intracrine roles. Vitam. Horm. **66**:345-384.

27. **Cornish, J., K. E. Callon, G. C. Nicholson, and I. R. Reid**. 1997. Parathyroid hormone-related protein-(107-139) inhibits bone resorption in vivo. Endocrinology **138**:1299-1304.

28. **Cornish, J., K. E. Callon, C. Lin, C. Xiao, J. M. Moseley, and I. R. Reid**. 1999. Stimulation of osteoblast proliferation by C-terminal fragments of parathyroid hormone-related protein. J. Bone Miner. Res. **14**:915-922.

29. **Kovacs, C. S., B. Lanske, J. L. Hunzelman, J. Guo, A. C. Karaplis, and H. M. Kronenberg**. 1996. Parathyroid hormone-related peptide (PTHrP) regulates fetal-placental calcium transport through a receptor distinct from the PTH/PTHrP receptor. Proc. Natl. Acad. Sci. U. S. A **93**:15233-15238.

30. **Juppner, H., A. B. Abou-Samra, M. Freeman, X. F. Kong, E. Schipani, J. Richards, L. F. Kolakowski, Jr., J. Hock, J. T. Potts, Jr., H. M. Kronenberg, and .** 1991. A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide. Science **254**:1024-1026.

31. **Abou-Samra, A. B., H. Juppner, T. Force, M. W. Freeman, X. F. Kong, E. Schipani, P. Urena, J. Richards, J. V. Bonventre, J. T. Potts, Jr., and .** 1992. Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: a single

receptor stimulates intracellular accumulation of both cAMP and inositol trisphosphates and increases intracellular free calcium. Proc. Natl. Acad. Sci. U. S. A **89**:2732-2736.

32. **Rabbani, S. A., J. Gladu, P. Harakidas, B. Jamison, and D. Goltzman**. 1999. Over-production of parathyroid hormone-related peptide results in increased osteolytic skeletal metastasis by prostate cancer cells in vivo. Int. J Cancer **80**:257-264.

33. **Deftos, L. J.** 2000. Prostate carcinoma: production of bioactive factors. Cancer **88**:3002-3008.

34. **Mahadevia, P. S., A. Ramaswamy, E. S. Greenwald, D. I. Wollner, and D. Markham**. 1983. Hypercalcemia in prostatic carcinoma. Report of eight cases. Arch. Intern. Med. **143**:1339-1342.

35. **Iwamura, M., G. Wu, P. A. Abrahamsson, P. A. di Sant'Agnese, A. T. Cockett, and L. J. Deftos**. 1994. Parathyroid hormone-related protein is expressed by prostatic neuroendocrine cells. Urology **43**:667-674.

36. **Wu, G., M. Iwamura, P. A. di Sant'Agnese, L. J. Deftos, A. T. Cockett, and S. Gershagen**. 1998. Characterization of the cell-specific expression of parathyroid hormone-related protein in normal and neoplastic prostate tissue. Urology **51**:110-120.

37. **Tovar Sepulveda, V. A. and M. Falzon**. 2002. Parathyroid hormone-related protein enhances PC-3 prostate cancer cell growth via both autocrine/paracrine and intracrine pathways. Regul. Pept. **105**:109-120.

38. **Dougherty, K. M., E. A. Blomme, A. J. Koh, J. E. Henderson, K. J. Pienta, T. J. Rosol, and L. K. McCauley**. 1999. Parathyroid hormone-related protein as a growth regulator of prostate carcinoma. Cancer Res. **59**:6015-6022.

39. **Shen, X. and M. Falzon**. 2003. PTH-related protein modulates PC-3 prostate cancer cell adhesion and integrin subunit profile. Mol Cell Endocrinol **199**:165-177.

40. **Shen, X. and M. Falzon**. 2003. Parathyroid hormone-related protein upregulates integrin expression via an intracrine pathway in PC-3 prostate cancer cells. Regul. Pept. **113**:17-29.

41. **Iwamura, M., P. A. Abrahamsson, K. A. Foss, G. Wu, A. T. Cockett, and L. J. Deftos**. 1994. Parathyroid hormone-related protein: a potential autocrine growth regulator in human prostate cancer cell lines. Urology **43**:675-679.

42. **Asadi, F., M. Faraj, S. Malakouti, and S. C. Kukreja**. 2001. Effect of parathyroid hormone related protein, and dihydrotestosterone on proliferation and ornithine decarboxylase mRNA in human prostate cancer cell lines. Int. Urol. Nephrol. **33**:417-422.

43. **Pizzi, H., J. Gladu, L. Carpio, D. Miao, D. Goltzman, and S. A. Rabbani**. 2003. Androgen regulation of parathyroid hormone-related peptide production in human prostate cancer cells. Endocrinology **144**:858-867.

44. **Cramer, S. D., D. M. Peehl, M. G. Edgar, S. T. Wong, L. J. Deftos, and D. Feldman**. 1996. Parathyroid hormone--related protein (PTHrP) is an epidermal growth factor-regulated secretory product of human prostatic epithelial cells. Prostate **29**:20-29.

45. **Yin, J. J., K. Selander, J. M. Chirgwin, M. Dallas, B. G. Grubbs, R. Wieser, J. Massague, G. R. Mundy, and T. A. Guise**. 1999. TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. J. Clin. Invest **103**:197-206.

46. **Sellers, R. S., B. E. LeRoy, E. A. Blomme, S. Tannehill-Gregg, S. Corn, and T. J. Rosol**. 2004. Effects of transforming growth factor-beta1 on parathyroid hormone-related protein mRNA expression and protein secretion in canine prostate epithelial, stromal, and carcinoma cells. Prostate **58**:366-373.

47. **Werkmeister, J. R., E. A. Blomme, M. T. Weckmann, A. Grone, L. K. McCauley, A. B. Wade, J. O'Rourke, C. C. Capen, and T. J. Rosol**. 1998. Effect of transforming growth factor-beta1 on parathyroid hormone-related protein secretion and mRNA expression by normal human keratinocytes in vitro. Endocrine. **8**:291-299.

48. **Lindemann, R. K., P. Ballschmieter, A. Nordheim, and J. Dittmer**. 2001. Transforming growth factor beta regulates parathyroid hormone-related protein expression in MDA-MB-231 breast cancer cells through a novel Smad/Ets synergism. J. Biol. Chem. **276**:46661-46670.

49. **Kakonen, S. M., K. S. Selander, J. M. Chirgwin, J. J. Yin, S. Burns, W. A. Rankin, B. G. Grubbs, M. Dallas, Y. Cui, and T. A. Guise**. 2002. Transforming growth factor-beta stimulates parathyroid hormone-related protein and osteolytic metastases via Smad and mitogen-activated protein kinase signaling pathways. J. Biol. Chem. **277**:24571-24578.

50. **Sinha, A. A., M. J. Wilson, and D. F. Gleason**. 1987. Immunoelectron microscopic localization of prostatic-specific antigen in human prostate by the protein A-gold complex. Cancer **60**:1288-1293.

51. **Cramer, S. D., Z. Chen, and D. M. Peehl**. 1996. Prostate specific antigen cleaves parathyroid hormone-related protein in the PTH-like domain: inactivation of PTHrP-stimulated cAMP accumulation in mouse osteoblasts. J. Urol. **156**:526-531.

52. **Iwamura, M., J. Hellman, A. T. Cockett, H. Lilja, and S. Gershagen**. 1996. Alteration of the hormonal bioactivity of parathyroid hormone-related protein (PTHrP) as a result of limited proteolysis by prostate-specific antigen. Urology **48**:317-325.

53. **Tovar Sepulveda, V. A., X. Shen, and M. Falzon**. 2002. Intracrine PTHrP protects against serum starvation-induced apoptosis and regulates the cell cycle in MCF-7 breast cancer cells. Endocrinology **143**:596-606.

54. **Feldman, D., R. J. Skowronski, and D. M. Peehl**. 1995. Vitamin D and prostate cancer. Adv. Exp. Med. Biol **375**:53-63.

55. **Christakos, S., P. Dhawan, Y. Liu, X. Peng, and A. Porta**. 2003. New insights into the mechanisms of vitamin D action. J Cell Biochem **88**:695-705.

56. **Giovannucci, E.** 2005. The epidemiology of vitamin D and cancer incidence and mortality: a review (United States). Cancer Causes Control **16**:83-95.

57. **Corder, E. H., H. A. Guess, B. S. Hulka, G. D. Friedman, M. Sadler, R. T. Vollmer, B. Lobaugh, M. K. Drezner, J. H. Vogelman, and N. Orentreich**. 1993. Vitamin D and prostate cancer: a prediagnostic study with stored sera. Cancer Epidemiol. Biomarkers Prev. **2**:467-472.

58. **Gann, P. H., J. Ma, C. H. Hennekens, B. W. Hollis, J. G. Haddad, and M. J. Stampfer**. 1996. Circulating vitamin D metabolites in relation to subsequent development of prostate cancer. Cancer Epidemiol. Biomarkers Prev. **5**:121-126.

59. **Nomura, A. M., G. N. Stemmermann, J. Lee, L. N. Kolonel, T. C. Chen, A. Turner, and M. F. Holick**. 1998. Serum vitamin D metabolite levels and the subsequent development of prostate cancer (Hawaii, United States). Cancer Causes Control **9**:425-432.

60. **Platz, E. A., M. F. Leitzmann, B. W. Hollis, W. C. Willett, and E. Giovannucci**. 2004. Plasma 1,25-dihydroxy- and 25-hydroxyvitamin D and subsequent risk of prostate cancer. Cancer Causes Control **15**:255-265.

61. **Jacobs, E. T., A. R. Giuliano, M. E. Martinez, B. W. Hollis, M. E. Reid, and J. R. Marshall**. 2004. Plasma levels of 25-hydroxyvitamin D, 1,25-

dihydroxyvitamin D and the risk of prostate cancer. J. Steroid Biochem. Mol Biol. **89-90**:533-537.

62. **Ahonen, M. H., L. Tenkanen, L. Teppo, M. Hakama, and P. Tuohimaa**. 2000. Prostate cancer risk and prediagnostic serum 25-hydroxyvitamin D levels (Finland). Cancer Causes Control **11**:847-852.

63. **Tuohimaa, P., L. Tenkanen, M. Ahonen, S. Lumme, E. Jellum, G. Hallmans, P. Stattin, S. Harvei, T. Hakulinen, T. Luostarinen, J. Dillner, M. Lehtinen, and M. Hakama**. 2004. Both high and low levels of blood vitamin D are associated with a higher prostate cancer risk: a longitudinal, nested case-control study in the Nordic countries. Int. J. Cancer **108**:104-108.

64. **Campbell, M. J., E. Elstner, S. Holden, M. Uskokovic, and H. P. Koeffler**. 1997. Inhibition of proliferation of prostate cancer cells by a 19-nor-hexafluoride vitamin D3 analogue involves the induction of p21waf1, p27kip1 and E-cadherin. J Mol Endocrinol **19**:15-27.

65. **Blutt, S. E., E. A. Allegretto, J. W. Pike, and N. L. Weigel**. 1997. 1,25-dihydroxyvitamin D3 and 9-cis-retinoic acid act synergistically to inhibit the growth of LNCaP prostate cells and cause accumulation of cells in G1. Endocrinology **138**:1491-1497.

66. **Blutt, S. E., T. C. Polek, L. V. Stewart, M. W. Kattan, and N. L. Weigel**. 2000. A calcitriol analogue, EB1089, inhibits the growth of LNCaP tumors in nude mice. Cancer Res. **60**:779-782.

67. **Blutt, S. E. and N. L. Weigel**. 1999. Vitamin D and prostate cancer. Proc. Soc. Exp. Biol Med. **221**:89-98.

68. **Zhuang, S. H. and K. L. Burnstein**. 1998. Antiproliferative effect of 1alpha,25-dihydroxyvitamin D3 in human prostate cancer cell line LNCaP involves reduction of cyclin-dependent kinase 2 activity and persistent G1 accumulation. Endocrinology **139**:1197-1207.

69. **Polek, T. C. and N. L. Weigel**. 2002. Vitamin D and prostate cancer. J Androl **23**:9-17.

70. **Peehl, D. M., R. J. Skowronski, G. K. Leung, S. T. Wong, T. A. Stamey, and D. Feldman**. 1994. Antiproliferative effects of 1,25-dihydroxyvitamin D3 on primary cultures of human prostatic cells. Cancer Res. **54**:805-810.

71. **Zhao, X. Y., D. M. Peehl, N. M. Navone, and D. Feldman**. 2000. 1alpha,25-dihydroxyvitamin D3 inhibits prostate cancer cell growth by androgen-dependent and androgen-independent mechanisms. Endocrinology **141**:2548-2556.

72. **Zhao, X. Y. and D. Feldman**. 2001. The role of vitamin D in prostate cancer. Steroids **66**:293-300.

73. **Carlberg, C., I. S. Mathiasen, J. H. Saurat, and L. Binderup**. 1994. The 1,25-dihydroxyvitamin D3 (VD) analogues MC903, EB1089 and KH1060 activate the VD receptor: homodimers show higher ligand sensitivity than heterodimers with retinoid X receptors. J Steroid Biochem Mol Biol **51**:137-142.

74. **Ikeda, K., C. Lu, E. C. Weir, M. Mangin, and A. E. Broadus**. 1989. Transcriptional regulation of the parathyroid hormone-related peptide gene by glucocorticoids and vitamin D in a human C-cell line. J Biol Chem. **264**:15743-15746.

75. **Kremer, R., A. C. Karaplis, J. Henderson, W. Gulliver, D. Banville, G. N. Hendy, and D. Goltzman**. 1991. Regulation of parathyroid hormone-like peptide in cultured normal human keratinocytes. Effect of growth factors and 1,25 dihydroxyvitamin D3 on gene expression and secretion. J Clin. Invest **87**:884-893.

76. **Sebag, M., J. Henderson, D. Goltzman, and R. Kremer**. 1994. Regulation of parathyroid hormone-related peptide production in normal human mammary epithelial cells in vitro. Am. J Physiol **267**:C723-C730.

77. **Falzon, M.** 1997. The noncalcemic vitamin D analogues EB1089 and 22-oxacalcitriol interact with the vitamin D receptor and suppress parathyroid hormone-related peptide gene expression. Mol Cell Endocrinol **127**:99-108.

78. **Falzon, M. and J. Zong**. 1998. The noncalcemic vitamin D analogs EB1089 and 22-oxacalcitriol suppress serum-induced parathyroid hormone-related peptide gene expression in a lung cancer cell line. Endocrinology **139**:1046-1053.

79. **Abe, M., N. Akeno, S. Ohida, and N. Horiuchi**. 1998. Inhibitory effects of 1,25-dihydroxyvitamin D3 and 9-cis-retinoic acid on parathyroid hormone-related protein expression by oral cancer cells (HSC-3). J Endocrinol **156**:349-357.

80. **Nishishita, T., T. Okazaki, T. Ishikawa, T. Igarashi, K. Hata, E. Ogata, and T. Fujita**. 1998. A negative vitamin D response DNA element in the human parathyroid hormone-related peptide gene binds to vitamin D receptor along with Ku antigen to mediate negative gene regulation by vitamin D. J Biol Chem. **273**:10901-10907.

81. **El Abdaimi, K., V. Papavasiliou, D. Goltzman, and R. Kremer**. 2000. Expression and regulation of parathyroid hormone-related peptide in normal and malignant melanocytes. Am. J Physiol Cell Physiol **279**:C1230-C1238.

82. **Tovar Sepulveda, V. A. and M. Falzon**. 2003. Prostate cancer cell type-specific regulation of the human PTHrP gene via a negative VDRE. Mol Cell Endocrinol **204**:51-64.

83. **Tovar Sepulveda, V. A. and M. Falzon**. 2002. Regulation of PTH-related protein gene expression by vitamin D in PC-3 prostate cancer cells. Mol Cell Endocrinol **190**:115-124.

84. **Haussler, M. R., G. K. Whitfield, C. A. Haussler, J. C. Hsieh, P. D. Thompson, S. H. Selznick, C. E. Dominguez, and P. W. Jurutka**. 1998. The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. J Bone Miner. Res. **13**:325-349.

85. **Cheskis, B. and L. P. Freedman**. 1994. Ligand modulates the conversion of DNA-bound vitamin D3 receptor (VDR) homodimers into VDR-retinoid X receptor heterodimers. Mol. Cell Biol. **14**:3329-3338.

86. **Koszewski, N. J., T. A. Reinhardt, and R. L. Horst**. 1996. Vitamin D receptor interactions with the murine osteopontin response element. J. Steroid Biochem. Mol. Biol. **59**:377-388.

87. **Koszewski, N. J., S. Kiessling, and H. H. Malluche**. 2001. Isolation of genomic DNA sequences that bind vitamin D receptor complexes. Biochem. Biophys. Res. Commun. **283**:188-194.

88. **Darwish, H. M. and H. F. DeLuca**. 1999. Identification of a transcription factor that binds to the promoter region of the human parathyroid hormone gene. Arch. Biochem Biophys. **365**:123-130.

89. **Mackey, S. L., J. L. Heymont, H. M. Kronenberg, and M. B. Demay**. 1996. Vitamin D receptor binding to the negative human parathyroid hormone vitamin D response element does not require the retinoid x receptor. Mol Endocrinol **10**:298-305.

90. **Demay, M. B., M. S. Kiernan, H. F. DeLuca, and H. M. Kronenberg**. 1992. Sequences in the human parathyroid hormone gene that bind the 1,25-dihydroxyvitamin D3 receptor and mediate transcriptional repression in response to 1,25-dihydroxyvitamin D3. Proc. Natl. Acad. Sci. U. S. A **89**:8097-8101.

91. **Wu, H. C., J. T. Hsieh, M. E. Gleave, N. M. Brown, S. Pathak, and L. W. Chung**. 1994. Derivation of androgen-independent human LNCaP prostatic cancer cell sublines: role of bone stromal cells. Int. J. Cancer **57**:406-412.

92. **Thalmann, G. N., P. E. Anezinis, S. M. Chang, H. E. Zhau, E. E. Kim, V. L. Hopwood, S. Pathak, A. C. von Eschenbach, and L. W. Chung**. 1994. Androgen-independent cancer progression and bone metastasis in the LNCaP model of human prostate cancer. Cancer Res. **54**:2577-2581.

93. **Mader, S., J. Y. Chen, Z. Chen, J. White, P. Chambon, and H. Gronemeyer**. 1993. The patterns of binding of RAR, RXR and TR homo- and heterodimers to direct repeats are dictated by the binding specificites of the DNA binding domains. EMBO J. **12**:5029-5041.

94. **Kersten, S., M. I. Dawson, B. A. Lewis, and N. Noy**. 1996. Individual subunits of heterodimers comprised of retinoic acid and retinoid X receptors interact with their ligands independently. Biochemistry **35**:3816-3824.

95. **Zhang, X. K., J. Lehmann, B. Hoffmann, M. I. Dawson, J. Cameron, G. Graupner, T. Hermann, P. Tran, and M. Pfahl**. 1992. Homodimer formation of retinoid X receptor induced by 9-cis retinoic acid. Nature **358**:587-591.

96. **Germain, P., J. Iyer, C. Zechel, and H. Gronemeyer**. 2002. Co-regulator recruitment and the mechanism of retinoic acid receptor synergy. Nature **415**:187-192.

97. **Kersten, S., L. Pan, and N. Noy**. 1995. On the role of ligand in retinoid signaling: positive cooperativity in the interactions of 9-cis retinoic acid with tetramers of the retinoid X receptor. Biochemistry **34**:14263-14269.

98. **Gampe, R. T., Jr., V. G. Montana, M. H. Lambert, G. B. Wisely, M. V. Milburn, and H. E. Xu**. 2000. Structural basis for autorepression of retinoid X receptor by tetramer formation and the AF-2 helix. Genes Dev. **14**:2229-2241.

99. **Carlberg, C., I. Bendik, A. Wyss, E. Meier, L. J. Sturzenbecker, J. F. Grippo, and W. Hunziker**. 1993. Two nuclear signalling pathways for vitamin D. Nature **361**:657-660.

100. **de Vos, S., M. I. Dawson, S. Holden, T. Le, A. Wang, S. K. Cho, D. L. Chen, and H. P. Koeffler**. 1997. Effects of retinoid X receptor-selective ligands on proliferation of prostate cancer cells. Prostate **32**:115-121.

101. **Mangelsdorf, D. J., S. A. Kliewer, A. Kakizuka, K. Umesono, and R. M. Evans**. 1993. Retinoid receptors. Recent Prog. Horm. Res. **48**:99-121.

102. **Giguere, V.** 1994. Retinoic acid receptors and cellular retinoid binding proteins: complex interplay in retinoid signaling. Endocr. Rev. **15**:61-79.

103. **Thalmann, G. N., R. A. Sikes, T. T. Wu, A. Degeorges, S. M. Chang, M. Ozen, S. Pathak, and L. W. Chung**. 2000. LNCaP progression model of human prostate cancer: androgen-independence and osseous metastasis. Prostate **44**:91-103.

104. **Feldman, D., P. J. Malloy, and C. Gross**. 1996. Vitamin D: metabolism and action, p. 205-235. *In* R. Marcus, D. Feldman, and J. Kelsey (eds.), Osteoporosis. Academic Press, New York.

105. **Mangelsdorf, D. J. and R. M. Evans**. 1995. The RXR heterodimers and orphan receptors. Cell **83**:841-850.

106. **Evans, R. M.** 1988. The steroid and thyroid hormone receptor superfamily. Science **240**:889-895.

107. **Liu, S. M., N. Koszewski, M. Lupez, H. H. Malluche, A. Olivera, and J. Russell**. 1996. Characterization of a response element in the 5'-flanking region of the avian (chicken) PTH gene that mediates negative regulation of gene transcription by 1,25-dihydroxyvitamin D3 and binds the vitamin D3 receptor. Mol. Endocrinol. **10**:206-215.

108. **Inoue, D., T. Matsumoto, E. Ogata, and K. Ikeda**. 1993. 22-Oxacalcitriol, a noncalcemic analogue of calcitriol, suppresses both cell proliferation and parathyroid hormone-related peptide gene expression in human T cell lymphotrophic virus, type I-infected T cells. J. Biol. Chem. **268**:16730-16736.

109. **Yang, K. H. and A. F. Stewart**. 1996. The PTH-related protein gene and protein products, p. 347-376. *In* J. P. Bilezikian, L. Riasz, and G. Rodan (eds.), Principles of Bone Biology. Academic.

110. **Danielson, P. E., S. Forss-Petter, M. A. Brow, L. Calavetta, J. Douglass, R. J. Milner, and J. G. Sutcliffe**. 1988. p1B15: a cDNA clone of the rat mRNA encoding cyclophilin. DNA **7**:261-267.

111. **Noda, M., R. L. Vogel, A. M. Craig, J. Prahl, H. F. DeLuca, and D. T. Denhardt**. 1990. Identification of a DNA sequence responsible for binding of the 1,25-dihydroxyvitamin D3 receptor and 1,25-dihydroxyvitamin D3 enhancement of mouse secreted phosphoprotein 1 (SPP-1 or osteopontin) gene expression. Proc. Natl. Acad. Sci. U. S. A **87**:9995-9999.

112. **Fraser, W. D., J. Robinson, R. Lawton, B. Durham, S. J. Gallacher, I. T. Boyle, G. H. Beastall, and F. C. Logue**. 1993. Clinical and laboratory studies of a new immunoradiometric assay of parathyroid hormone-related protein. Clin. Chem. **39**:414-419.

113. **Liu, Y. Y., C. Nguyen, and S. Peleg**. 2000. Regulation of ligand-induced heterodimerization and coactivator interaction by the activation function-2 domain of the vitamin D receptor. Mol Endocrinol **14**:1776-1787.

114. **MacDonald, P. N., D. R. Dowd, S. Nakajima, M. A. Galligan, M. C. Reeder, C. A. Haussler, K. Ozato, and M. R. Haussler**. 1993. Retinoid X receptors stimulate and 9-cis retinoic acid inhibits 1,25-dihydroxyvitamin D3-activated expression of the rat osteocalcin gene. Mol Cell Biol **13**:5907-5917.

115. **Skowronski, R. J., D. M. Peehl, and D. Feldman**. 1993. Vitamin D and prostate cancer: 1,25 dihydroxyvitamin D3 receptors and actions in human prostate cancer cell lines. Endocrinology **132**:1952-1960.

116. **Zhuang, S. H., G. G. Schwartz, D. Cameron, and K. L. Burnstein**. 1997. Vitamin D receptor content and transcriptional activity do not fully predict antiproliferative effects of vitamin D in human prostate cancer cell lines. Mol Cell Endocrinol **126**:83-90.

117. **MacDonald, P. N., C. A. Haussler, C. M. Terpening, M. A. Galligan, M. C. Reeder, G. K. Whitfield, and M. R. Haussler**. 1991. Baculovirus-mediated expression of the human vitamin D receptor. Functional characterization, vitamin D response element interactions, and evidence for a receptor auxiliary factor. J Biol Chem. **266**:18808-18813.

118. **Colston, K. W., A. G. Mackay, S. Y. James, L. Binderup, S. Chander, and R. C. Coombes**. 1992. EB1089: a new vitamin D analogue that inhibits the growth of breast cancer cells in vivo and in vitro. Biochem. Pharmacol. **44**:2273-2280.

119. **Haq, M., R. Kremer, D. Goltzman, and S. A. Rabbani**. 1993. A vitamin D analogue (EB1089) inhibits parathyroid hormone-related peptide production and prevents the development of malignancy-associated hypercalcemia in vivo. J Clin. Invest **91**:2416-2422.

120. **Mathiasen, I. S., K. W. Colston, and L. Binderup**. 1993. EB 1089, a novel vitamin D analogue, has strong antiproliferative and differentiation inducing effects on cancer cells. J. Steroid Biochem. Mol. Biol. **46**:365-371.

121. **Kissmeyer, A. M., E. Binderup, L. Binderup, H. C. Mork, N. R. Andersen, H. L. Makin, N. J. Schroeder, V. N. Shankar, and G. Jones**. 1997. Metabolism of

the vitamin D analog EB 1089: identification of in vivo and in vitro liver metabolites and their biological activities. Biochem. Pharmacol. **53**:1087-1097.

122. **Goltzman, D.** 1997. Mechanisms of the development of osteoblastic metastases. Cancer **80**:1581-1587.

123. **Sung, V. and D. Feldman**. 2000. 1,25-Dihydroxyvitamin D3 decreases human prostate cancer cell adhesion and migration. Mol. Cell Endocrinol. **164**:133-143.

124. **Hansen, C. M., M. W. Madsen, B. Arensbak, T. Skak-Nielsen, S. Latini, and L. Binderup**. 1998. Down-regulation of laminin-binding integrins by 1 alpha,25-dihydroxyvitamin D3 in human melanoma cells in vitro. Cell Adhes. Commun. **5**:109-120.

125. **Metz, R. J., K. Vellody, S. Patel, R. Bergstrom, J. Meisinger, J. Jackson, M. A. Wright, and M. R. Young**. 1996. Vitamin D3 and ceramide reduce the invasion of tumor cells through extracellular matrix components by elevating protein phosphatase-2A. Invasion Metastasis **16**:280-290.

126. **Young, M. R. and Y. Lozano**. 1997. Inhibition of tumor invasiveness by 1alpha,25-dihydroxyvitamin D3 coupled to a decline in protein kinase A activity and an increase in cytoskeletal organization. Clin. Exp. Metastasis **15**:102-110.

127. **Rio-Tsonis, K. and P. A. Tsonis**. 1994. Expression of transforming growth factor-beta 1-4 in the chick limb bud mesenchymal cells: regulation by 1,25-dihydroxyvitamin D3. Biochem. Biophys. Res. Commun. **204**:975-982.

128. **Umesono, K., K. K. Murakami, C. C. Thompson, and R. M. Evans**. 1991. Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. Cell **65**:1255-1266.

129. **Falzon, M.** 1996. DNA sequences in the rat parathyroid hormone-related peptide gene responsible for 1,25-dihydroxyvitamin D3-mediated transcriptional repression. Mol Endocrinol **10**:672-681.

130. **Kremer, R., M. Sebag, C. Champigny, K. Meerovitch, G. N. Hendy, J. White, and D. Goltzman**. 1996. Identification and characterization of 1,25-dihydroxyvitamin D3-responsive repressor sequences in the rat parathyroid hormone-related peptide gene. J. Biol. Chem. **271**:16310-16316.

131. **Lees-Miller, S. P., K. Sakaguchi, S. J. Ullrich, E. Appella, and C. W. Anderson**. 1992. Human DNA-activated protein kinase phosphorylates serines 15 and 37 in the amino-terminal transactivation domain of human p53. Mol. Cell Biol. **12**:5041-5049.

132. **Gottlieb, T. M. and S. P. Jackson**. 1993. The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen. Cell **72**:131-142.

133. **Chung, U., T. Igarashi, T. Nishishita, H. Iwanari, A. Iwamatsu, A. Suwa, T. Mimori, K. Hata, S. Ebisu, E. Ogata, T. Fujita, and T. Okazaki**. 1996. The interaction between Ku antigen and REF1 protein mediates negative gene regulation by extracellular calcium. J. Biol. Chem. **271**:8593-8598.

134. **Giffin, W., H. Torrance, D. J. Rodda, G. G. Prefontaine, L. Pope, and R. J. Hache**. 1996. Sequence-specific DNA binding by Ku autoantigen and its effects on transcription. Nature **380**:265-268.

135. **Malloy, P. J., R. Xu, L. Peng, P. A. Clark, and D. Feldman**. 2002. A novel mutation in helix 12 of the vitamin D receptor impairs coactivator interaction and causes hereditary 1,25-dihydroxyvitamin D-resistant rickets without alopecia. Mol Endocrinol **16**:2538-2546.

136. **MacDonald, P. N., T. A. Baudino, H. Tokumaru, D. R. Dowd, and C. Zhang**. 2001. Vitamin D receptor and nuclear receptor coactivators: crucial interactions in vitamin D-mediated transcription. Steroids **66**:171-176.

137. **Solomon, C., J. H. White, and R. Kremer**. 1999. Mitogen-activated protein kinase inhibits 1,25-dihydroxyvitamin D3-dependent signal transduction by phosphorylating human retinoid X receptor alpha. J Clin Invest **103**:1729-1735.

138. **Towers, T. L., B. F. Luisi, A. Asianov, and L. P. Freedman**. 1993. DNA target selectivity by the vitamin D3 receptor: mechanism of dimer binding to an asymmetric repeat element. Proc. Natl. Acad. Sci. U. S. A **90**:6310-6314.

139. **Giampuzzi, M., G. Botti, M. Di Duca, L. Arata, G. Ghiggeri, R. Gusmano, R. Ravazzolo, and A. Di Donato**. 2000. Lysyl oxidase activates the transcription activity of human collagene III promoter. Possible involvement of Ku antigen. J Biol Chem. **275**:36341-36349.

140. **Ferrara, J., K. McCuaig, G. N. Hendy, M. Uskokovic, and J. H. White**. 1994. Highly potent transcriptional activation by 16-ene derivatives of 1,25-dihydroxyvitamin D3. Lack of modulation by 9-cis-retinoic acid of response to 1,25-dihydroxyvitamin D3 or its derivatives. J Biol Chem. **269**:2971-2981.

141. **Blutt, S. E., T. J. McDonnell, T. C. Polek, and N. L. Weigel**. 2000. Calcitriol-induced apoptosis in LNCaP cells is blocked by overexpression of Bcl-2. Endocrinology **141**:10-17.

142. **Stewart, L. V. and N. L. Weigel**. 2005. Role of insulin-like growth factor binding proteins in 1alpha,25-dihydroxyvitamin D(3)-induced growth inhibition of human prostate cancer cells. Prostate.

143. **Ye, Y., P. K. Seitz, and C. W. Cooper**. 2001. Parathyroid hormone-related protein overexpression in the human colon cancer cell line HT-29 enhances adhesion of the cells to collagen type I. Regul. Pept. **101**:19-23.

144. **Southby, J., L. M. Murphy, T. J. Martin, and M. T. Gillespie**. 1996. Cell-specific and regulator-induced promoter usage and messenger ribonucleic acid splicing for parathyroid hormone-related protein. Endocrinology **137**:1349-1357.

145. **Guo, B., F. Aslam, A. J. van Wijnen, S. G. Roberts, B. Frenkel, M. R. Green, H. DeLuca, J. B. Lian, G. S. Stein, and J. L. Stein**. 1997. YY1 regulates vitamin D receptor/retinoid X receptor mediated transactivation of the vitamin D responsive osteocalcin gene. Proc. Natl. Acad. Sci. U. S. A **94**:121-126.

146. **Chattopadhyay, N. and E. M. Brown**. 2001. Retinoic acid receptors are expressed in human primary astrocytes and their agonists inhibit parathyroid hormone-related peptide expression and secretion. Brain Res. Mol. Brain Res. **92**:172-176.

147. **Kremer, R., C. D. Woodworth, and D. Goltzman**. 1996. Expression and action of parathyroid hormone-related peptide in human cervical epithelial cells. Am. J. Physiol **271**:C164-C171.

148. **Karperien, M., H. Farih-Sips, C. W. Lowik, S. W. de Laat, J. Boonstra, and L. H. Defize**. 1997. Expression of the parathyroid hormone-related peptide gene in retinoic acid-induced differentiation: involvement of ETS and Sp1. Mol. Endocrinol. **11**:1435-1448.

149. **Wei, L. N.** 2003. Retinoid receptors and their coregulators. Annu. Rev. Pharmacol. Toxicol. **43**:47-72.

150. **Michalik, L., B. Desvergne, and W. Wahli**. 2004. Peroxisome-proliferator-activated receptors and cancers: complex stories. Nat. Rev. Cancer **4**:61-70.

151. **Schrader, M., S. Nayeri, J. P. Kahlen, K. M. Muller, and C. Carlberg**. 1995. Natural vitamin D3 response elements formed by inverted palindromes: polarity-directed ligand sensitivity of vitamin D3 receptor-retinoid X receptor heterodimer-mediated transactivation. Mol. Cell Biol. **15**:1154-1161.

152. **Bakin, R. E., D. Gioeli, E. A. Bissonette, and M. J. Weber**. 2003. Attenuation of Ras signaling restores androgen sensitivity to hormone-refractory C4-2 prostate cancer cells. Cancer Res. **63**:1975-1980.

153. **Bakin, R. E., D. Gioeli, R. A. Sikes, E. A. Bissonette, and M. J. Weber**. 2003. Constitutive activation of the Ras/mitogen-activated protein kinase signaling pathway promotes androgen hypersensitivity in LNCaP prostate cancer cells. Cancer Res. **63**:1981-1989.

154. **Holmbeck, S. M., H. J. Dyson, and P. E. Wright**. 1998. DNA-induced conformational changes are the basis for cooperative dimerization by the DNA binding domain of the retinoid X receptor. J. Mol. Biol. **284**:533-539.

155. **Tan, N. S., L. Michalik, B. Desvergne, and W. Wahli**. 2005. Multiple expression control mechanisms of peroxisome proliferator-activated receptors and their target genes. J. Steroid Biochem. Mol Biol. **93**:99-105.

156. **IJpenberg, A., N. S. Tan, L. Gelman, S. Kersten, J. Seydoux, J. Xu, D. Metzger, L. Canaple, P. Chambon, W. Wahli, and B. Desvergne**. 2004. In vivo activation of PPAR target genes by RXR homodimers. EMBO J. **23**:2083-2091.

VITA

Verónica Alejandra Tovar Sepúlveda was born on August 27, 1973 to Pablo and Gloria Tovar Sepúlveda. Verónica entered the Ruprecht-Karls-University at Heidelberg, Germany in 1992, as part of the "Programa Galileo" scholarship for the academic training of outstanding Venezuelan high school graduates at superior foreign Universities. There she received the degrees of Bachelor of Sciences and Master of Sciences in 1995 and 1998, respectively. After working for one and half years as a Research Associate at The University of Texas Medical Branch (UTMB), she enrolled in the Pharmacology and Toxicology (PHTO) Graduate Program also at UTMB as an advanced student in 2002.

While at graduate school, Verónica received several honors. In 2003, Verónica was awarded a Travel Award by the Sealy Center for Molecular Sciences & Department of Microbiology and Immunology Science Forum. In 2004 she received the Shirley Patricia Parker Scholarship for her research in the field of oncology, and in 2005 she received the George Palmer Saunders II Memorial Scholarship for her research in the field of Pharmacology. Verónica gained significant teaching experience as a tutor for the "Que quiere decir?" (QQD) Café, helping medical and graduate students to improve their Spanish, and published five peer-reviewed papers while at UTMB. She also served as a co-chair for the International Student Organization (ISO) at UTMB from 2003 to the present.

Verónica can be contacted through the graduate school at UTMB or at P.O. Box 15027, Galveston, TX 77555.

Education

B.S. (Vordiplom) in Biology, February 1995, Ruprecht-Karls-University, Heidelberg, B-W, Germany

M.S. (Diplom) in Biology, August 1998, Ruprecht-Karls-University, Heidelberg, B-W, Germany

Publications

Tovar Sepulveda, V.A., Berdel, B., Coffin, J.M., Reuss, F.U. 2000. Mouse Mammary Tumor Virus Superantigen Expression is reduced by Glucocorticoid Treatment. Virology **275**: 98-106.

Tovar Sepulveda, V.A., Shen, X., and Falzon, M. 2002. Intracrine parathyroid hormone-related protein protects against serum starvation-induced apoptosis and regulates the cell cycle in MCF-7 breast cancer cells. Endocrinology **143**: 596-606.

Tovar Sepulveda, V.A. and Falzon, M. 2002. Parathyroid hormone-related protein enhances PC-3 prostate cancer cell growth via both autocrine/paracrine and intracrine pathways. Regulatory Peptides **105**: 109-120.

Tovar Sepulveda, V.A. and Falzon, M. 2002. Regulation of parathyroid hormone-related protein gene expression by vitamin D in PC-3 prostate cancer cells. Mol. Cell. Endo. **190**: 115-124.

Tovar Sepulveda, V.A. and Falzon, M. 2003. Prostate cancer cell type-specific regulation of the human PTHrP gene via a negative VDRE. Mol. Cell. Endo. **204**: 54-64.

Narayanan, R., Tovar Sepulveda, V.A., Falzon, M., Weigel, N.L. 2004. The Functional Consequences of Cross Talk between the Vitamin D Receptor and ERK Signaling Pathways are Retinoid X Receptor Isoform Specific. J. Biol. Chem. **279**(45): 47298-310.

Tovar Sepulveda, V.A. and Falzon, M. 2002. The noncalcemic vitamin D analogue, EB1089, downregulates Parathyroid Hormone-related Protein (PTHrP) gene expression in the PC-3 prostate cancer cell line. Poster Presentation. *ASBMR 24th Annual Meeting* 2002. San Antonio, TX.