2004

Studies on the molecular recognition of the nuclear vitamin D3 receptor

Ludmila T. Stoyanova
San Jose State University

Follow this and additional works at: https://scholarworks.sjsu.edu/etd_theses

Recommended Citation

Stoyanova, Ludmila T., "Studies on the molecular recognition of the nuclear vitamin D3 receptor" (2004).
Master's Theses. 2619.
DOI: <https://doi.org/10.31979/etd.6ds2-dp9t>
https://scholarworks.sjsu.edu/etd_theses/2619

This Thesis is brought to you for free and open access by the Master's Theses and Graduate Research at SJSU ScholarWorks. It has been accepted for inclusion in Master's Theses by an authorized administrator of SJSU ScholarWorks. For more information, please contact scholarworks@sjsu.edu.

**STUDIES ON THE MOLECULAR RECOGNITION OF THE NUCLEAR
VITAMIN D₃ RECEPTOR**

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

By

Ludmila T. Stoynova

August 2004

UMI Number: 1424490

Copyright 2004 by
Stoyanova, Ludmila T.

All rights reserved.

INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

UMI[®]

UMI Microform 1424490

Copyright 2005 by ProQuest Information and Learning Company.

All rights reserved. This microform edition is protected against
unauthorized copying under Title 17, United States Code.

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

© 2004

Ludmila T. Stoyanova

ALL RIGHTS RESERVED

APPROVED FOR THE DEPARTMENT OF CHEMISTRY

Elaine D. Collins

Dr. Elaine D. Collins

Pamela Stacks

Dr. Pamela Stacks

Roger Terrill

Dr. Roger Terrill

APPROVED FOR THE UNIVERSITY

Pamela C Stacks

ABSTRACT

STUDIES ON THE MOLECULAR RECOGNITION OF THE NUCLEAR VITAMIN D₃ RECEPTOR

by Ludmila T. Stoynova

Site-specific and random mutagenesis were used to study the structure-function relationship of the nuclear vitamin D₃ receptor (VDR), a transcription factor activated by binding to 1 α ,25-dihydroxyvitamin D₃. A screening assay for selecting VDR variants with altered molecular recognition was developed. The effect of amino acid region 165-215 on receptor function in conjunction with tyrosine 143 and serine 278 was studied. These amino acids participate in hydrogen bonding with the 3-hydroxyl group of the ligand. Several VDR deletion mutants were generated (VDR $_{\Delta 165-215}$, VDR $_{\Delta 165-215}$ Y143F, VDR $_{\Delta 165-215}$ S278A, VDR $_{\Delta 165-215}$ Y143F/S278A) by a novel method developed during this work. Functional studies showed that the deletion is not significant for ligand binding and transactivation. The binding affinities of the point mutants, both deleted and full-length, were essentially identical to that of native VDR, $K_d=0.61\pm0.33$ nM. Only VDR $_{\Delta 165-215}$ Y143F/S278A showed lower binding affinity than wild-type, $K_d=1.15\pm0.7$ nM. All point mutations, in both deleted and full-length VDR variants, proved important for transcriptional activation.

ACKNOWLEDGEMENTS

I would like to express my greatest appreciation and gratitude to my research advisor Dr. Elaine D. Collins for giving me the opportunity to work on such an exciting research project, and for the invaluable advice and guidance throughout this work. It was a rewarding experience.

I would also like to thank the members of my graduate committee, Dr. Pamela Stacks and Dr. Roger Terrill, for their help and suggestions in perfecting this thesis.

Special thanks to all my fellow students and friends for the good times we shared together and particularly to Alex, not only for her friendship but also for her invaluable help in the lab.

Finally, I would like to thank my family, and especially my husband, for all the patience, love, and support provided throughout the long years of my research.

TABLE OF CONTENTS

1. INTRODUCTION	1
1.1. Hormones	1
1.2. Vitamin D ₃	2
<i>Structural Features of the Vitamin D Family</i>	3
<i>Biological Functions of Vitamin D₃</i>	6
<i>Tissue Distribution of VDR</i>	7
<i>Genomic and Non-genomic Effects of 1,25(OH)₂D₃</i>	9
<i>Biosynthesis and Metabolism of Vitamin D₃</i>	10
1.3. Nuclear Vitamin D ₃ Receptor	15
<i>Domain Structure of Nuclear VDR</i>	16
<i>Mode of Action of 1,25(OH)₂D₃</i>	19
<i>Ligand Binding and Conformational Changes in VDR</i>	21
1.4. Putative Membrane Receptor for 1,25(OH) ₂ D ₃	27
2. RESEARCH OBJECTIVES	30
3. MATERIALS AND METHODS	33
3.1. Preparation of Buffers and Solutions	33
<i>Buffers</i>	33
<i>Bacterial Cell Culture</i>	33
<i>Eukaryotic Cell Solutions</i>	35
<i>Yeast Cell Stock Solutions and Growth Media</i>	35
<i>Plasmids</i>	38

<i>VDR Ligands</i>	38
3.2. PCR Amplification of VDR-LBD	39
<i>Primer Design</i>	39
<i>PCR Protocol</i>	39
<i>Purification of PCR Products</i>	40
3.3. Cloning of VDR-LBD PCR Product into pGEM®-T Easy Vector	41
<i>Ligation</i>	41
<i>Transformation of Competent Bacterial Cells</i>	42
<i>Plasmid Purification</i>	42
<i>Determination of DNA Concentration</i>	43
3.4. Cloning of VDR-LBD into pGilda Vector	45
<i>Endonuclease Digestion and Generation of Cohesive Ends</i>	45
<i>DNA Purification by Phenol/Chloroform Extraction</i>	46
<i>Column Purification of DNA from Agarose Slices</i>	47
<i>Cohesive End Ligation and Transformation</i>	48
3.5. Error-prone PCR	49
3.6. Yeast Screening Assay	50
<i>Construction of Yeast Reporter Strain EGY48[p8op-lacZ]</i>	51
<i>Transformation of Yeast Reporter Strain with pGilda/VDR-LBD</i>	52
<i>Screening for VDR-LBD Interaction with 1,25(OH)₂D₃</i>	53
<i>Control Transformations</i>	55
<i>Yeast Plasmid Isolation</i>	55
3.7. Generation of VDR _{Δ165-215} Deletion Mutants	56

3.8. DNA Sequencing	58
3.9. Cell Culture and Transient Transfection of COS-7 Cells	60
3.10. Saturation Binding Assay	61
3.11. Transcriptional Activation Assay	62
4. RESULTS	64
4.1. Random Mutagenesis of VDR-LBD	64
<i>PCR Amplification of VDR-LBD</i>	66
<i>Error-prone PCR and Gene Shuffling</i>	68
<i>Cloning of VDR-LBD</i>	70
<i>Yeast Screening Assay</i>	75
4.2. VDR Deletion Mutants	82
<i>Generation of VDR_{Δ165-215} Deletion Mutants</i>	82
<i>Saturation Binding Assays</i>	94
<i>Transcriptional Activation Assays</i>	95
5. DISCUSSION	98
5.1. Directed Evolution of VDR-LBD	99
5.2. VDR Deletion Mutants	110
6. CONCLUSIONS	119
REFERENCES	121
APPENDICES	133
A. List of Abbreviations	134
B. Single Letter Abbreviations for Amino Acids	136
C. Lambda DNA (<i>Hind</i> III/ <i>Eco</i> RI) Markers	137

D. Nucleotide and Amino Acid Sequence of Human VDR	138
E. Deletion and Point-Mutations within the VDR Sequence	139
F. Sequencing Data for Deletion Mutants	140
<i>Nucleotide and Amino Acid Sequence of VDR_{Δ165-215}</i>	140
<i>Nucleotide and Amino Acid Sequence of VDR_{Δ165-215}Y143F</i>	141
<i>Nucleotide and Amino Acid Sequence of VDR_{Δ165-215}S278A</i>	142
<i>Nucleotide and Amino Acid Sequence of VDR_{Δ165-215}Y143F/S278A</i>	143

LIST OF TABLES

Table	Page
1. Selection of pGilda/VDR-LBD transformed colonies	53
2. Control transformations for the yeast screening assay	54
3. Primers used for DNA sequencing	58
4. Summary of results	97

LIST OF FIGURES

Figure	Page
1. Structures of vitamin D ₂ , vitamin D ₃ , and 1,25-dihydroxyvitamin D ₃	3
2. Conformational equilibrium of ring A in the vitamin D ₃ molecule	5
3. Biosynthesis of vitamin D ₃	11
4. Enzymatic hydroxylations of vitamin D ₃	13
5. Domain structure of the nuclear vitamin D ₃ receptor	17
6. Modulation of gene transcription by the nuclear vitamin D ₃ receptor	20
7. Three-dimensional structure of the ligand-binding domain of the nuclear vitamin D ₃ receptor bound to 1,25(OH) ₂ D ₃	22
8. The 1,25(OH) ₂ D ₃ ligand docked in the hydrophobic pocket of VDR ligand-binding domain	24
9. Overview of the random mutagenesis experiment	64
10. Validation of the yeast screening assay for wild type VDR-LBD	65
11. PCR optimization experiments for amplification of hVDR-LBD	67
12. Error-prone PCR optimization	69
13. Map of pGEM®-T Easy cloning vector	70
14. Results from VDR-LBD cloning into pGEM®-T Easy vector	72
15. Map of pGilda yeast vector	73
16. Cloning of VDR-LBD into pGilda vector	73
17. Results from VDR-LBD cloning into pGilda vector	74
18. Principle of the yeast screening assay for functional VDR-LBD	76

19. One-hybrid yeast assay for VDR-LBD: experimental approach	77
20. Experimental validation of the yeast assay for VDR-LBD	81
21. Inverse PCR	83
22. Structure of phosphorothioate oligonucleotides	84
23. Complementarity of the 5' ends of the PCR Primers	84
24. Schematic representation of the phosphorothioate method for generating large deletion mutants from plasmid DNA templates	85
25. Inverse PCR optimization at various annealing temperatures	86
26. T7 gene 6 exonuclease treatment of non-modified and of phosphorothioate-modified PCR products	88
27. Optimization of T7 gene 6 exonuclease digestion	90
28. Generation of “sticky ends” in the amplicon with T7 gene 6 exonuclease treatment	91
29. Sequencing results for VDR _{Δ165-215} Y143F deletion mutant	93
30. Saturation binding assays	94
31. Transcriptional activation assays	96
32. Generation of library of random mutants by gene shuffling	104

1. INTRODUCTION

1.1. Hormones

Hormones are defined as chemical compounds that play the role of chemical messengers in living organisms. From a chemical point of view, they are very diverse and can be derivatives of amino acids, peptides or proteins, steroids, and fatty acids. Regardless of the complexity of their chemical structure, all hormones have two common features: first, the ability to travel various distances from the points of their biosynthesis to various other cells in the same organism, and second, their ability to bind with high affinity and specificity to other components of the signal transduction system, namely the hormone receptors. It is through this binding to their specific receptors and the ensuing biochemical processes that hormones deliver their messages to the target cells and organs (Norman and Litwack, 1997a). The origins of the high binding affinity between hormones and their receptors, as well as the precise nature of the biochemical consequences thereof, has been and continues to be an area of intense research.

This work has focused on a hormone whose main role is to maintain the concentration of calcium in serum within certain limits. This hormone is $1\alpha,25$ -dihydroxy vitamin D_3 , abbreviated as $1,25(OH)_2D_3$. More specifically, the binding of this hormone to its cognate receptor, the nuclear vitamin D_3 receptor (VDR) is of interest. VDR is a ligand-dependent transcription factor and is part of a superfamily of nuclear receptors (Collins and Norman, 2001). All nuclear receptors are DNA binding proteins, and their most important biological function

is to regulate the transcription of target genes. Many nuclear receptors modulate gene transcription as a result of ligand binding. The ligands of some nuclear receptors are still unknown, and therefore they are called “orphan” receptors. A large group of nuclear receptors, including VDR, bind hormones as their cognate ligands. More than 300 proteins are currently identified as members of the nuclear receptor superfamily including the thyroid receptor (Weinberger et al., 1986), receptors for various steroid hormones (Evans, 1988), hormonal forms of vitamin A (Giguere et al., 1987; Petkovich et al., 1987), peroxisomal activators (Schmidt et al., 1992), and various orphan receptors (Evans, 1988; Mangelsdorf et al., 1995).

1.2. Vitamin D₃

Vitamin D₃ is a member of the class of closely related sterol compounds called vitamin D. Other members of this class are vitamin D₂ and the various hydroxyl-derivatives of vitamin D₃ that will be described in more detail in the following pages. Thus the term “vitamin D” encompasses all of the members of this group and does not refer to any particular member.

A notable aspect of vitamin D₃ is the fact that this compound is not a vitamin in the classical definition of the word. Vitamins are defined as essential, trace nutrients that cannot be produced by the body and have to be supplied through the diet. This is not the case with vitamin D₃, which can be biosynthesized in the body by a photochemical reaction from 7-dehydrocholesterol, itself a derivative of the ubiquitous cholesterol. The

necessary catalyst of the photochemical reaction for the process is sunlight, and lack thereof can lead to vitamin D₃ deficiency. Under these circumstances, the compound becomes a true vitamin and has to be supplied by the diet. Indeed, vitamin D was initially discovered as a component in cod liver oil that could reverse the symptoms of the childhood disease rickets. Further studies then led to the identification and structure determination of vitamin D₃ in the 1930s (Collins and Norman, 2001; Norman et al., 2002).

Structural Features of the Vitamin D Family

Figure 1 shows the structures of three members of the vitamin D group, namely vitamin D₂, vitamin D₃, and its di-hydroxylated derivative, 1,25(OH)₂D₃.

Figure 1. Structures of vitamin D₂, vitamin D₃, and 1,25-dihydroxyvitamin D₃. The 1,25(OH)₂D₃ derivative is the hormonally active form of vitamin D₃.

All of these compounds are secosteroids. Structurally they are closely related to cholesterol and to the other steroid hormones with their cyclopentanoperhydrophenanthrene fused ring structure. However, the 9,10-carbon bond of ring B has undergone fission, and as a result the vitamin D molecule consists of only three intact rings and a conjugated system of three double bonds (Collins and Norman, 2001). Vitamin D₃, also known as cholecalciferol, is the naturally occurring form of vitamin D. Vitamin D₂, (or ergocalciferol), does not occur naturally, but is synthetically derived from the plant steroid ergosterol. Note the presence of an additional methyl group and a double bond in the side chain of this compound that are lacking in vitamin D₃. In the diet vitamin D₂ can substitute for vitamin D₃ and reverse the symptoms of vitamin D deficiency. For this reason, ergocalciferol was used as a food supplement for many years until the mid-1960s when vitamin D₃ (cholecalciferol) became readily available synthetically.

The lack of ring B and the presence instead of a conjugated double bond system in the vitamin D compounds introduce considerable flexibility in the ring structure, compared to the rigidity characteristic of other steroids (Okamura et al., 1974). X-ray crystallography shows that the conjugated diene system extending between C5 and C8 is nearly planar and has a trans configuration (Crowfoot and Dunitz, 1948; Hodgkin et al., 1963). However, the C6-C19 diene system is not planar and has a cis configuration, with the C10-C19 double bond twisted out of plane by 60°. This allows for two possible chair conformations of ring A. As a result the substituents on ring A are constantly interchanging

between the equatorial and axial positions. This conformational mobility is unique for vitamin D and is not observed for other steroid hormones (Collins and Norman, 2001; Norman and Litwack, 1997b). The conformational equilibrium of the vitamin D₃ molecule is shown in Figure 2. The two fused rings (the cyclohexane C and the cyclopentane D) are conformationally rigid and the alkyl side chain preferentially assumes an extended configuration.

Figure 2. Conformational equilibrium of ring A in the vitamin D₃ molecule

Although vitamin D₃ was recognized early on as a potent antirachitic agent, it was not until the early 1960s when it became clear that this effect is not due to cholecalciferol but to its hormonal metabolites. Vitamin D₃ has no inherent biological activity on its own and all biological responses to vitamin D₃ are the result of the hormonal action of its two metabolites: 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] and 24,25-dihydroxyvitamin D₃ [24,25(OH)₂D₃]. The existence of an endocrine system for the production and processing of vitamin D₃ into hormonally active metabolites is now a well-established fact (Norman et al., 2002).

Biological Functions of Vitamin D₃

Vitamin D₃ is involved in a variety of biological processes. The most important biological function of vitamin D₃ is to maintain calcium and phosphate concentrations in the blood within narrowly defined limits, which is essential for normal bone mineralization (DeLuca, 1979). Calcium deficiency leads to serious bone diseases such as rickets in children and osteomalacia in adults. However, excessive levels of serum calcium lead to hypercalcemia, a life-threatening condition. Calcium is essential not only for bone building, but also for blood clotting, nerve pulse transmission, muscle contraction, membrane structure, as a cofactor for lipases and ATPases, and in the formation of egg shells in birds (Collins and Norman, 2001). Calcium ions are involved in various signal transduction pathways as intracellular signals. Phosphorus is a structural component of DNA, RNA, and membrane lipids. It is part of ATP-energy-transferring systems. In addition, the phosphorylation of proteins is very important for the regulation of various metabolic pathways.

There are three separate processes that work together to keep calcium and phosphorus levels within the required narrow limits: absorption of dietary calcium through the intestines, accretion and resorption by bones, and retention and excretion by the kidney (Collins and Norman, 2001; Norman and Litwack, 1997b). Three hormones are involved in the maintenance of mineral homeostasis: 1,25(OH)₂D₃ (the active hormonal metabolite of vitamin D₃), calcitonin and parathyroid hormone (PTH). Therefore, the classical target tissues

for $1,25(\text{OH})_2\text{D}_3$ are those involved in the regulation of mineral homeostasis, namely the intestines, kidney, liver, and bone, where vitamin D_3 receptors are found in abundance.

Tissue Distribution of VDR

The classical function of $1,25(\text{OH})_2\text{D}_3$ is to regulate the concentration of calcium in the blood, and this process is intimately related to bone formation and to remodeling. This complex process is carefully regulated through several signal pathways. Although vitamin D_3 is mostly known as a potent antirachitic agent, its most important direct effect on bone is the stimulation of calcium resorption (release of calcium phosphate from bone tissue) in response to even minor decreases in blood calcium levels. This process leads to a rapid increase of calcium and phosphate concentrations in the bloodstream and is essential for maintaining mineral homeostasis (Underwood and DeLuca, 1984). The antirachitic properties and the stimulating effect of $1,25(\text{OH})_2\text{D}_3$ on bone growth and mineralization are indirect consequences of the increased absorption of dietary calcium and phosphate through the intestine. This process is also regulated at least partially by $1,25(\text{OH})_2\text{D}_3$.

The presence of nuclear receptors for $1,25(\text{OH})_2\text{D}_3$ in various tissues is indirect evidence of the regulatory role that the hormone plays there. For example, nuclear receptors for $1,25(\text{OH})_2\text{D}_3$ have been identified in normal osteoblasts and in osteoblast-like osteosarcoma cells in bone tissue, but none were found in osteoclasts (Collins, 2000). Osteoblasts are specialized cells from which

new bone tissues develops; osteoclasts are large, multinucleated cells in grown bone tissue that are involved in resorption of old bone as well as in bone modeling and remodeling (Marks and Hermey, 1996). One of the functions of $1,25(\text{OH})_2\text{D}_3$ is to increase the differentiation of precursor cells into osteoclasts.

In the last thirty years nuclear receptors for $1,25(\text{OH})_2\text{D}_3$ were discovered in many other tissues and cells not directly involved in mineral homeostasis. As a result, the overall understanding of the role of the vitamin D endocrine system was extended to include general effects on cell regulation and differentiation (Walters, 1992; Reichel et al., 1989). Nuclear VDRs were found in muscle, hematolymphopoietic, reproductive and nervous tissues as well as in other endocrine tissues (Collins and Norman, 2001). This implies that $1,25(\text{OH})_2\text{D}_3$ most likely plays an important role in the regulation of different processes in those tissues. The effect $1,25(\text{OH})_2\text{D}_3$ has on some of those various systems and the mechanism of its signal transduction are still under investigation. The $1,25(\text{OH})_2\text{D}_3$ hormone was found to trigger biological responses in skeletal and in cardiac muscle tissues, and to affect cellular differentiation of skin and hematopoietic cells, melanin synthesis, insulin secretion, prolactin secretion, immune and stress response, and muscle function (Lowe et al., 1992; Walters, 1992; Walters, 1995; Bouillon et al., 1995). Receptors for $1,25(\text{OH})_2\text{D}_3$ have also been found in human and mouse skin cells, and it appears that the presence of $1,25(\text{OH})_2\text{D}_3$ there affects cellular growth and differentiation. The same outcome is observed for some cancer cell lines, including certain types of leukemia and prostate cancer.

In general, the function of $1,25(\text{OH})_2\text{D}_3$ in those non-classical target cells is to promote differentiation and to inhibit cell proliferation. Inadequate levels of $1,25(\text{OH})_2\text{D}_3$ could lead to the onset of various illnesses and restoring the level of $1,25(\text{OH})_2\text{D}_3$ in the affected tissues could be a potential cure for these conditions. However, using $1,25(\text{OH})_2\text{D}_3$ directly for treatment is problematic because at high concentrations it can cause hypercalcemia, which can be fatal. This side effect could be overcome by designing analogs of $1,25(\text{OH})_2\text{D}_3$ that have the same anti-proliferation properties but lack its hypercalcemic effects. This is actively explored for the development of treatments for certain diseases. For example, a non-hypercalcemic analog of $1,25(\text{OH})_2\text{D}_3$, sold under the commercial name Dovonex® (Leo Laboratories Ltd.), is used successfully to treat the symptoms of some forms of psoriasis, a chronic, hyper-proliferative skin disorder.

Vitamin D_3 receptors were identified also in hematopoietic cells, monocytes, activated lymphocytes, and other cells of the immune system, indicating that $1,25(\text{OH})_2\text{D}_3$ is involved in the modulation of immune responses (Collins, 2000). VDRs were found also in certain areas of the brain but the exact function of $1,25(\text{OH})_2\text{D}_3$ there is not known yet.

Genomic and Non-genomic Effects of $1,25(\text{OH})_2\text{D}_3$

The transcription of over fifty genes, including some oncogenes, is now known to be regulated by $1,25(\text{OH})_2\text{D}_3$ (Hannah and Norman, 1994). For example, $1,25(\text{OH})_2\text{D}_3$ is known to induce the expression of a calcium binding protein, called calbindin-D in a variety of tissues. Different forms of the

calbindin-D protein might be produced depending on the species and the type of tissue where the protein is expressed but the expression of most calbindin proteins is regulated to at least some degree by $1,25(\text{OH})_2\text{D}_3$ (Cancela et al., 1992). Another role of $1,25(\text{OH})_2\text{D}_3$ is its involvement in the regulation of bone cell differentiation by affecting a variety of osteoblast-related events. The $1,25(\text{OH})_2\text{D}_3$ hormone is known to increase the production of osteocalcin, alkaline phosphatase, and the proliferation of cultured osteoblasts, and to decrease the production of type I collagen.

In addition to its genomic actions, $1,25(\text{OH})_2\text{D}_3$ is also involved in non-genomic signal-transduction pathways. One example is the rapid induction of changes in cytosolic calcium ion levels in osteoblast and osteosarcoma cells regulated by $1,25(\text{OH})_2\text{D}_3$ through the opening of voltage gated calcium ion channels (Caffray and Farach-Carson, 1989; Lieberherr, 1987). Some experiments suggest that $1,25(\text{OH})_2\text{D}_3$ is also involved in the regulation of the growth and differentiation of osteoclasts. However, osteoclasts do not have nuclear vitamin D receptors. Hence, the effect of $1,25(\text{OH})_2\text{D}_3$ on osteoclasts must be either indirect or non-genomic (Collins, 2000).

Biosynthesis and Metabolism of Vitamin D₃

Vitamin D₃ is obtained from the diet or is biosynthesized via a photochemical reaction when the skin is exposed to sunlight (Figure 3). The diet becomes an important source of vitamin D₃ largely when there is inadequate exposure of the skin to sunlight.

The precursor of vitamin D₃ is 7-dehydrocholesterol (provitamin D₃), a metabolite of cholesterol present in the skin. When the skin is exposed to sunlight, a photochemical reaction takes place leading to fission of the 9,10-carbon bond in ring B of the 7-dehydrocholesterol molecule. The immediate product of this photochemical transformation is previtamin D₃, which is slowly converted over a period of several days into vitamin D₃ via an intramolecular 1,7 sigmatropic hydrogen shift (Holick, 1981). Both steps of the vitamin D₃ biosynthesis take place in the skin, and only a short daily exposure to sunlight is sufficient for the production of the required amounts of vitamin D₃.

Figure 3. Biosynthesis of vitamin D₃

From the skin, vitamin D₃ is transferred into the bloodstream, and it circulates there bound to a carrier protein, called the vitamin D binding protein

(DBP) (Collins and Norman, 2001). The vitamin D binding protein is a multifunctional protein. It has a single ligand-binding site for all vitamin D secosterols, and one of its functions is to transport the parent vitamin D₃ molecule and its metabolites 25(OH)D₃, 1,25(OH)₂D₃, and 24,25(OH)₂D₃ through the bloodstream (Norman and Litwack, 1997b). Another function for DBP is to bind with high affinity to actin monomers, preventing the formation of actin polymers in the blood. DBP might also be involved in the cellular internalization of vitamin D and its metabolites.

Once in the bloodstream, the vitamin D₃ pro-hormone is transported by DBP to the liver, where it is enzymatically hydroxylated in the side chain at C-25 by vitamin D₃-25-hydroxylase (Henry and Norman, 1974; Tucker et al., 1973). The product, 25-hydroxyvitamin D₃ [25(OH)D₃], is the major vitamin D₃ metabolite circulating in the bloodstream. Vitamin D₃-25-hydroxylase is a poorly regulated enzyme (Tucker et al., 1973), therefore the level of circulating 25(OH)D₃ corresponds to the available vitamin D₃ level in the bloodstream (Mawer, 1980). This intermediate metabolite, like its parent vitamin D₃, is not biologically active. After it is released from the liver into the circulatory system, 25(OH)D₃ is transported to the kidney by DBP. In the kidney 25(OH)D₃ is hydroxylated either by 25-hydroxyvitamin D₃-1 α -hydroxylase (1-hydroxylase) or by 25-hydroxyvitamin D₃-24R-hydroxylase (24-hydroxylase) resulting in 1 α ,25(OH)₂D₃ or 24R,25(OH)₂D₃ respectively (Henry, 1992). The 1 α ,25(OH)₂D₃ metabolite is the biologically active form of vitamin D₃ and functions as a hormone. There are some indications that 24R,25(OH)₂D₃ might have some

unique biological activity (Norman and Litwack, 1997b) but this view is still controversial. Under normal physiological conditions both metabolites are secreted from the kidney and circulate in the plasma bound to DBP. The enzymatic hydroxylations of vitamin D₃ and the resulting metabolites are illustrated in Figure 4.

Figure 4. Enzymatic hydroxylations of vitamin D₃

The hydroxylations in the kidney are carefully regulated (Henry et al., 1992). Both 1,25(OH)₂D₃ and 24,25(OH)₂D₃ are involved in the control of this balancing process and operate in a feedback loop to modulate the activity of 1-hydroxylase, 24-hydroxylase, and parathyroid hormone (PTH), another regulator of renal 1,25(OH)₂D₃ production. When the circulating levels of 1,25(OH)₂D₃ are

high, the activity of 1-hydroxylase is repressed and 24-hydroxylase is induced. In a state of vitamin D deficiency the activity of 1-hydroxylase is high and the activity of 24-hydroxylase is low. Both enzymes are also regulated by the parathyroid hormone. PTH production is induced in a hypocalcemic state when the plasma calcium concentration is low, usually the result of vitamin D deficiency. Elevated levels of PTH increase the activity of 1-hydroxylase and suppress the activity of 24-hydroxylase. As a result, the secretion of $1,25(\text{OH})_2\text{D}_3$ by the kidney is elevated. The hormone is secreted and circulates in the bloodstream bound to the carrier DBP. At target tissues $1,25(\text{OH})_2\text{D}_3$ binds to specific intracellular receptors and is translocated inside the nucleus, where most of its biological effects are mediated through its interaction with the nuclear vitamin D_3 receptor (Jurutka et al., 2001).

The natural hormonal ligand of the nuclear VDR is $1,25(\text{OH})_2\text{D}_3$, and they bind together with very high affinity ($K_d = 0.1 - 5.0 \text{ nM}$, where K_d is the dissociation equilibrium constant) (Wecksler and Norman, 1980; Wecksler et al., 1980a; Wecksler et al., 1980b). The intermediate metabolite $25(\text{OH})\text{D}_3$ can also bind to VDR but with much lower affinity ($\text{RCI} = 0.15$, where RCI is the relative competitive index) (Bouillon et al., 1995). The second hormonal metabolite of vitamin D_3 , $24,25(\text{OH})_2\text{D}_3$, also binds to the nuclear VDR but with even lower affinity than $25(\text{OH})\text{D}_3$ ($\text{RCI} = 0.03$) (Bouillon et al., 1995). There are some indications that a specific receptor for $24,25(\text{OH})_2\text{D}_3$ might exist, but none have been isolated yet (Haussler, 1998; Norman and Litwack, 1997b). It is not quite clear if $24,25(\text{OH})_2\text{D}_3$ has unique biological functions, but there is some evidence

that it might play a role in bone mineralization (Evans et al., 1982; Yamato et al., 1993), fracture healing (Lidor et al., 1987a; Lidor et al., 1987b), and in suppressing the secretion of PTH (Canterbury et al., 1980; Henry et al., 1977). Therefore, not only $1,25(\text{OH})_2\text{D}_3$ but also $24,25(\text{OH})_2\text{D}_3$ are probably needed for some of the known biological responses to vitamin D_3 .

A number of other vitamin D_3 metabolites besides $25(\text{OH})\text{D}_3$, $1,25(\text{OH})_2\text{D}_3$, and $24,25(\text{OH})_2\text{D}_3$ have been isolated and characterized. Most of them seem to be intermediates from the degradation of $1,25(\text{OH})_2\text{D}_3$ and do not display any biological activity. The only exception is $1,25(\text{OH})_2\text{D}_3$ -26,23-lactone produced by the kidney when the circulating levels of $1,25(\text{OH})_2\text{D}_3$ in the bloodstream are very high (Norman and Litwack, 1997b). Although its precise function is not determined yet, it appears to act as a natural antagonist of $1,25(\text{OH})_2\text{D}_3$. This view is supported by experiments suggesting that the lactone blocks the $1,25(\text{OH})_2\text{D}_3$ mediated resorption of calcium from bones (Ishizuka et al., 1988). Other experiments demonstrated that the presence of the lactone leads to a decrease in serum calcium concentrations in rats. This apparent antagonistic effect of $1,25(\text{OH})_2\text{D}_3$ -26,23-lactone is probably a defense mechanism for prevention of the toxic hypercalcemic effects resulting from overproduction of $1,25(\text{OH})_2\text{D}_3$ (Collins, 2000).

1.3. Nuclear Vitamin D_3 Receptor

The nuclear vitamin D_3 receptor (VDR) is a ligand-dependent transcription modulator and is a member of a superfamily of homologous nuclear receptors.

VDR is located primarily in the nucleus. The translocation of the receptor from the cytosol into the nucleus occurs mainly by binding of VDR to importin α , a nuclear localization signal (NLS) receptor associated with the nuclear import machinery. This process is independent from $1,25(\text{OH})_2\text{D}_3$. VDR can also translocate into the nucleus in a hormone-dependent pathway (Racz and Barsony, 1999).

The nuclear vitamin D_3 receptor is a protein of 427 amino acid residues with molecular weight of approximately 50,000 Da. It was first isolated from the intestine of vitamin D-deficient chicks in 1969 (Haussler et al., 1969). The VDR cDNA was cloned and sequenced in 1988 (Baker et al., 1988). VDR has been thoroughly studied, and up to date only one gene for the receptor has been identified.

Domain Structure of Nuclear VDR

All nuclear hormone receptors have similar domain structures. VDR is a typical representative of the superfamily and has a modular, four-domain structure (Figure 5). The individual domains can operate as distinct units, retaining their unique functions and acting independently from each other (Beato, 1989; Green and Chambon, 1988; Ham and Parker, 1989).

The N-terminal domain A/B is poorly conserved within the family of nuclear receptors and varies not only in amino acid sequence but also in size. The A/B domain of the vitamin D_3 receptor is the smallest within the nuclear receptor family consisting of only 24 amino acids. The function of this domain is

not clearly defined although in many receptors it possesses immunogenic properties. Most receptors have an autonomous transactivation function (AF-1) within domain A/B that is independent of ligand binding. The presence of this function and its importance for transcriptional activation vary widely within the family. VDR does not have an independent AF-1 function.

Figure 5. Domain structure of the nuclear vitamin D₃ receptor

The DNA binding domain (DBD), designated as domain C, is the most conserved domain for all receptors in the superfamily. DBD is responsible for DNA recognition and binding to specific DNA sequences on the promoters of target genes, called DNA response elements. In the case of the nuclear vitamin D₃ receptor, the DNA-binding domain consists of 66 amino acids folded into two zinc-finger motifs. The first zinc finger contains several hydrophobic amino acid residues and four conserved cysteines, coordinated by zinc into a tetrahedral

arrangement. The first zinc finger binds to the DNA response element and determines its sequence specificity. The second zinc finger consists of five cysteine residues and several basic amino acids. It binds to the DNA of target genes as well but is also involved in the dimerization of the receptor protein in a hormone-independent fashion. The binding of the first zinc finger to the DNA response element aligns the second zinc finger in a proper position for binding to DNA as well as for protein-protein contacts in the formation of the receptor dimer.

The second most conserved domain within the family of nuclear receptors is the ligand-binding domain (LBD), designated as domain E. This is the largest domain for the nuclear VDR and consists of 302 amino acids. The ligand-binding domain is responsible for high-affinity binding of the nuclear receptors to their cognate hormonal ligands. The VDR ligand-binding domain is made up of predominantly hydrophobic amino acid residues that fold together to form a hydrophobic pocket for hormone binding. However, VDR-LBD has also other important functions besides ligand binding. At the N-terminus of LBD there is a region involved in ligand-dependent dimerization of VDR with other nuclear receptors, most often with the retinoic X receptor (RXR). Close to the C-terminus there is a highly conserved region, which encodes a transcriptional activation sub-domain (AF-2). The activity of this sub-domain is dependent on binding of $1,25(\text{OH})_2\text{D}_3$ to VDR. AF-2 is involved in coactivator binding and is essential for transcriptional activation (Barsony et al., 1990; Matsuyama et al., 1997). AF-2 is

also involved in hormone-dependent nuclear translocation of VDR from the cytosol (Racz and Barsony, 1999).

Domain D is a weakly conserved, flexible hinge region and its function is not very well defined. The hinge region of VDR consists of 35 amino acids and has antigenic properties. Amino acid residues at the C-terminus of the hinge region, adjacent to the DNA binding domain, are involved in dimerization and determine the spacing requirement for the formation of heterodimers. The hinge region plays the role of a connector between the DNA-binding domain and the ligand-binding domain. It is conformationally quite flexible permitting proper interactions for both DBD and LBD.

Mode of Action of 1,25(OH)₂D₃

All nuclear hormone receptors have a similar mode of action, and VDR is no exception. The 1,25(OH)₂D₃ hormone is carried through the bloodstream bound to DBP. At the target cell, the hormone is released and passively diffuses through the cell membrane into the cytosol. Next, the 1,25(OH)₂D₃ is translocated into the nucleus, where it binds to its cognate receptor. This leads to a conformational change in the receptor protein. The usual response of most nuclear receptors to ligand binding is dimerization, either as homodimers or as heterodimers with another nuclear receptor. In the case of VDR, ligand binding typically leads to heterodimer formation with the unliganded RXR (Haussler et al., 1998). The presence of the cognate ligand for RXR, 9-*cis* retinoic acid, leads to preferential formation of RXR homodimers instead of VDR-RXR heterodimers.

This results in the suppression of gene transcription normally stimulated by $1,25(\text{OH})_2\text{D}_3$ hormone (Haussler et al., 1997).

Figure 6. Modulation of gene transcription by the nuclear vitamin D_3 receptor

The formation of the VDR-RXR heterodimer is followed by its high affinity binding to specific sequences on the DNA of target genes called vitamin D_3 response elements (VDREs). Although the VDR dimers can bind to various types of response elements, the preferred VDRE sequence appears to be a direct repeat of the hexamer AGGTCA with three base pair spacing in between (Norman et al., 2002). This chain of events leads to modulation of gene transcription, and as a result, the expression of target genes is either up- or down-regulated. The changes in gene expression determine the level of protein production, leading to

different biological responses. VDR alone can bind to VDRE sequences but it does so with much lower affinity than the VDR-RXR heterodimer and cannot successfully regulate gene transcription (Norman et al. 2002). The mechanism of interaction between the nuclear vitamin D₃ receptor and its cognate ligand 1,25(OH)₂D₃ is illustrated in Figure 6.

Ligand Binding and Conformational Changes in VDR

Ever since the VDR cDNA sequence was determined (Baker et al., 1988) and cloned into eukaryotic expression plasmids, the molecular details of the binding between VDR and its cognate ligand 1,25(OH)₂D₃ have been a subject of intense study. One major goal was to identify the amino acid residues involved in ligand binding, the changes in the receptor protein caused by this interaction, and how these changes are linked to the receptor's function and biological responses. It is now known that the binding of 1,25(OH)₂D₃ to VDR induces a conformational change in the receptor protein that leads to the activation of the transcription of target genes (Carlberg et al., 1993; Peleg et al., 1995). Most of these studies involved site-directed mutagenesis of specific amino acid residues in the receptor protein or studies of various analogs of the hormonal vitamin D₃. Another approach has been to use computer modeling of VDR-LBD based on sequence alignment with other receptors from the same family with known crystal structures. In the 1990s the crystal structures of RXR, the retinoic acid receptor (RAR), the thyroid receptor (TR), the estrogen receptor (ER), the progesterone receptor (PR), the androgen receptor (AR), and the peroxisome

proliferator-activated receptor (PPAR γ) were determined (Collins, 2000). Surprisingly, the crystal structures of all these receptors turned out to be very similar. The receptors with sequences most closely aligned with the sequence of VDR-LBD were used to generate computer models of probable binding sites within the VDR-LBD that are in contact with 1,25(OH) $_2$ D $_3$. In one computer modeling study, the VDR-LBD was aligned to the sequence of RAR (Wurtz et al., 1997). In another study, the VDR-LBD was aligned with the sequence of TR (Norman et al., 1999).

Figure 7. Three-dimensional structure of the ligand-binding domain of the nuclear vitamin D $_3$ receptor bound to 1,25(OH) $_2$ D $_3$. All α -helices are represented as green barrels, β -sheets as bronze arrows and 1,25(OH) $_2$ D $_3$ is depicted in grey, with red dots representing the three hydroxyl groups. N(A) and C(A) denote the N- and C-termini respectively. The arrow points to the position of Δ 165-215 deletion, depicted in yellow (Rochel et al., <http://www.ncbi.nlm.nih.gov>).

The crystal structure of a truncated version of the ligand-binding domain of the human vitamin D₃ receptor with the 1,25(OH)₂D₃ anchored inside was resolved and published (Rochel et al., 2000). It finally established the three-dimensional composition of the ligand-binding pocket around the hormone, and determined the active conformation of 1,25(OH)₂D₃ when it is bound to the receptor. However, the protein was crystallized only after 51 amino acids (Δ165-215) were removed from a poorly defined region at the N-terminus of the ligand-binding domain. The crystal structures of the entire VDR protein or of the intact VDR ligand-binding domain are still unsolved. A three-dimensional representation of VDR-LBD based on its crystal structure is shown on Figure 7.

The ligand-binding domain of VDR consists of twelve alpha helices and two beta-sheets that fold together in three layers, forming a large hydrophobic pocket. The large cavity is needed to accommodate the conformationally flexible ligand. The interior of VDR-LBD consists of predominantly hydrophobic amino acid residues and several hydrophilic residues clustered at the ends of the ligand-binding domain. Direct hydrogen bonding of the three hydroxyl groups of 1,25(OH)₂D₃ with several specific hydrophilic amino acids in the binding pocket are responsible for docking of the ligand. The 25-hydroxyl group located on the 1,25(OH)₂D₃ side chain is involved in hydrogen bond formation with two histidine amino acid residues at positions 305 and 397 of the VDR ligand-binding domain. The 1-hydroxyl group of the hormone forms hydrogen bonds with serine-237 and arginine-274; the 3-hydroxyl group forms hydrogen bonds with tyrosine-143 and

serine-278 (Rochel et al., 2000). The contact points of 1,25(OH)₂D₃ within the ligand binding pocket of VDR-LBD are illustrated in Figure 8.

Figure 8. The 1,25(OH)₂D₃ ligand docked in the hydrophobic pocket of VDR ligand-binding domain. Only amino acid residues forming hydrogen bonds with the three hydroxyl groups of the ligand are shown. Oxygen atoms are denoted in red, nitrogen atoms are blue, and carbon-carbon bonds are grey.

Most of the nuclear receptors contain a variable loop region located between helix 1 and helix 3, and in VDR this region is larger than in the rest of the receptors. This variable loop with poorly defined secondary structure makes the crystallization of the protein very difficult. Therefore, 51 amino acid residues, from amino acid 165 to 215, were deleted from this region to facilitate the crystallization of the VDR-LBD (Rochel et al., 2000). This loop region is not

well conserved among the nuclear vitamin D₃ receptors from different species and therefore is presumed not to be implicated in ligand binding.

Comparative studies of the crystal structures of several nuclear receptors, either bound or unbound to their cognate ligands, revealed that there is a major conformational difference between the structures of unoccupied receptor and receptor bound to its cognate ligand (Norman et al., 2002). The crystal structures of all family members determined so far show a high degree of similarity. The most dramatic change that takes place upon binding of 1,25(OH)₂D₃ is the repositioning of helix 12 to pack tightly against helix 3 and to close the access to the hydrophobic cavity of VDR-LBD, thus completely burying the ligand inside. This conformational change exposes new surfaces on the outside of the receptor, including some involved in dimerization and transcriptional activation. Helix 12 contains the transactivation AF-2 sub-domain, and after its repositioning, a hydrophobic cleft for binding of coactivators is exposed on the outer side of the protein interface (Norman et al., 2002). Coactivators are adapter proteins involved in the regulation of gene transcription. They serve as a bridge between transcriptional factors such as VDR and the basal transcriptional machinery (BTM), and trigger the transcription of target genes (Moras and Gronemeyer, 1998).

The coactivators that interact with nuclear receptors belong to three families: SRC-1/NcoA-1 (Lavinski et al., 1998; Onate et al., 1998), TIF2/GRIP-1 (Hong et al., 1997; Voegel et al., 1996), and ACTR/pCIP (Torchia et al., 1997). They all contain a common, conserved amino acid motif consisting of several

leucine residues (LXXLL, where L is leucine, and X is any other amino acid). This region is involved in interaction with nuclear receptors (Mannervik et al, 1999). After binding to the nuclear receptor, coactivators recruit additional proteins to the complex, such as pCBP and CREB.

One function of the coactivator complex is to link upstream transcriptional activators to the RNA polymerase II transcription complex (RNA Pol II) through the interaction of proteins from both complexes, and therefore modulate gene transcription (Kingston, 1999; Narlikar et al., 2002). The coactivators can modify transcription also through other pathways, such as various enzymatic modifications of chromatin. The coactivator complexes often possess enzymatic activities needed to remodel the structure of chromatin in order to facilitate the binding of RNA Pol II to the promoter region on the DNA of target genes. Normally chromatin is densely packed and the promoters are not readily accessible. The histone acetyl transferase activity (Ogryzko et al., 1996) and methyl transferase activity (Chen, D. et al., 1999) of various coactivator complexes all play a role in decondensing chromatin to aid binding of RNA Pol II to DNA. Some coactivators, for example ACTC, can be acetylated as well (Chen, H. et al., 1999), and there is some evidence that nuclear receptors can also be modified by acetylation. For AR, this process is essential for transactivation (Fu et al., 2000), but for VDR and TR it leads to dissociation of the coactivator complex, which is then followed by the formation of a new complex on the receptor surface. The new complex involves fifteen proteins called DRIPs (Vitamin D Receptor Interacting Proteins) (Rachez et al., 1999). DRIPs bind only to ligand-

bound VDR at the same AF-2 region on helix 12 as the previous complex (Rachez et al., 2000). This interaction of VDR with DRIPs is a requisite for transcriptional activation to occur (Rachez and Freedman, 2000).

Overall, the regulation of gene transcription by VDR and other nuclear receptors is a very complex process. Ligand binding is an early step in this process, and the conformational changes that it triggers are of primary importance for mediating the biological effects that follow.

1.4. Putative Membrane Receptor for 1,25(OH)₂D₃

Most biological responses to 1,25(OH)₂D₃ are genomic in nature and are mediated through interactions with its nuclear receptor. These events are relatively slow and occur on a timescale of minutes, hours or even days (Norman et al., 2002). However, not all of the biological responses to 1,25(OH)₂D₃ can be explained by its interaction with the nuclear vitamin D₃ receptor (VDR_{nuc}). There is mounting evidence that a putative membrane receptor (VDR_{mem}) might exist (Nemere et al., 1993) that mediates the non-genomic responses to the hormone through a different pathway. The non-genomic actions of 1,25(OH)₂D₃ occur very rapidly, at a timescale of seconds to minutes. They develop at the plasma membrane of a variety of target cells and involve the activation of several signal transduction pathways such as changes in ion channel activities, activation of second messenger pathways and elevation of calcium concentration in the cytosol (Caffrey and Farach-Carson, 1989; Song et al., 1998; Zanello and Norman, 1997). Rapid responses to 1,25(OH)₂D₃ have been reported in various tissues, both on

the cellular and sub-cellular level such as calcium transport across tissues or across the cell membrane (Farach-Carson et al., 1991; Nemere and Norman, 1987; Zhou et al., 1992). Those effects appear to be mediated by a membrane receptor protein specific for $1,25(\text{OH})_2\text{D}_3$ that is probably identical or slightly different from VDR_{nuc} . This putative VDR_{mem} has different ligand-binding properties than the nuclear receptor and is most likely located on the external cell surface (Baran et al., 1994; Nemere et al., 1994).

The presence of a vitamin D_3 receptor protein in the cell membrane is still a controversial issue in the vitamin D_3 field. Some researchers suggest that the rapid, non-genomic responses to $1,25(\text{OH})_2\text{D}_3$ are the result of its interaction with a separate membrane receptor, not related to VDR. Annexin II has been suggested as a possible membrane receptor for $1,25(\text{OH})_2\text{D}_3$ in rat osteoblast-like cells (Baran et al, 2000). The detection and isolation of an unique membrane receptor for $1,25(\text{OH})_2\text{D}_3$ that is not related to VDR_{nuc} has been reported by Nemere and Farach-Carson at the 12th Vitamin D Workshop in Maastricht, The Netherlands (July 6-10, 2003). This receptor, designated $1,25\text{D}_3$ -MARRS (for membrane-associated, rapid response, steroid-binding protein) is associated with $1,25(\text{OH})_2\text{D}_3$ -dependent regulation of phosphate uptake in chick intestinal epithelial cells. The complete cDNA sequence of the $1,25\text{D}_3$ -MARRS was published in 2004 (Nemere et al., 2004). However, another study published in 2004 determined that in calvarial osteoblasts the presence of a fully functional nuclear VDR is crucial for rapid induction of membrane-associated electrical responses to

1,25(OH)₂D₃ stimulation and that this effect is linked to exocytosis (Zanello and Norman, 2004).

Membrane effects and rapid, non-genomic responses to hormonal stimulation are not unique to 1,25(OH)₂D₃. Similar effects have been reported for other steroid hormones as well, including estrogen, progesterone, testosterone, and thyroid hormone (Chambliss et al., 2002; Nemere et al., 1993). It is still not clear if rapid, non-genomic responses to 1,25(OH)₂D₃ and other steroid hormones are induced by binding of the hormone to a specific membrane receptor presumably present in the plasma membrane or if these effects are the result of the stimulation of signal transduction pathways directly by the hormone/nuclear receptor complex through cross-talk with various signal transduction factors (Cato et al., 2002; Chambliss et al., 2002; Norman et al., 2002). Determining the mechanism of non-genomic actions of 1,25(OH)₂D₃ and the exact physiological role of non-genomic actions at the single cell level is presently an area of intense research.

2. RESEARCH OBJECTIVES

The overall goal of this work was to generate and characterize mutated variants of the human vitamin D₃ receptor (hVDR) in order to investigate the structure-function relationship of the receptor protein. One set of experiments was aimed at the generation of a large library of random VDR-LBD mutants by applying methods such as error-prone PCR and gene shuffling (Cadwell and Joyce, 1992; Stemmer, 1993). Such a library is expected to contain numerous random mutants of the wild-type receptor, including some that show an increased binding affinity for the intermediate vitamin D₃ metabolite 25(OH)D₃ instead of the natural ligand 1,25(OH)₂D₃. The identification, isolation, and characterization of such mutants are of significant theoretical and practical interest. Most importantly, by identifying which amino acid residues are changed to accommodate the loss of the 1-hydroxyl group of the ligand, significant information can be gained about the interaction between the VDR-LBD and the 1,25(OH)₂D₃ hormone.

In the course of this work, it became clear that screening of a large library of mutant protein receptors is a major bottleneck in the whole process. Therefore, significant effort was spent on the development and validation of a rapid and simple method for screening the library for VDR-LBD mutants with the desired altered binding properties. The results of these studies, using the natural ligand 1,25(OH)₂D₃ as a model are presented herein.

A second part of the project was the generation of a series of single and double point mutations of hVDR, all containing an additional deletion of 51 amino acids ($\Delta 165-215$). The introduction of this deletion allowed for the crystallization and three-dimensional structure determination of the VDR ligand-binding domain (Rochel et al., 2000). Some of the results published by Rochel and co-workers, regarding the role of amino acid residues tyrosine 143 and serine 278 of the receptor protein in the ligand binding the $1,25(\text{OH})_2\text{D}_3$ ligand, were in disagreement with results from this laboratory (Acevedo et al., 2004; Davis, 2002) and also with results published by others (Choi et al., 2001). Therefore, several VDR deletion variants were generated and characterized: a deletion variant $\text{VDR}_{\Delta 165-215}$ identical to that described by Rochel and co-workers as well as three other VDR variants with the same deletion but carrying in addition some point mutations, namely $\text{VDR}_{\Delta 165-215}\text{Y143F}$, $\text{VDR}_{\Delta 165-215}\text{S278A}$, and the double mutant $\text{VDR}_{\Delta 165-215}\text{Y143F/S278A}$. By comparing the binding affinities of the deleted variants and their ability to modulate the transcription of target genes to these of their undeleted counterparts, including the wild-type VDR, the role that these 51 amino acids play in the function of the receptor might be elucidated.

The generation of this relatively large, pre-defined cDNA deletion mutant with concomitant preservation of the correct reading frame of the protein was a significant challenge. During this part of the work a new, universally applicable technique for the rapid and efficient generation of such deletions was successfully developed (Stoynova et al., 2004). This new technique as well as the characterization of the binding affinity and transcription activation abilities of the

deleted VDR mutants are also described in this thesis.

3. MATERIALS AND METHODS

3.1. Preparation of Buffers and Solutions

Buffers

- TE buffer, pH 8.0: 10 mM Tris-(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), 1 mM ethylenediaminetetraacetic acid (EDTA).
- TAE buffer: 89 mM Tris, 89 mM acetic acid, 2 mM EDTA.
- TBE buffer: 89 mM Tris, 89 mM boric acid, 2 mM EDTA.

Bacterial Cell Culture

- Ampicillin solution (100 mg/mL): Dissolve 1 g ampicillin, sodium salt (Mediatech, Inc.) in 10 mL Millipore water. Filter-sterilize using 0.2 μ m sterile Acrodisc® 13 syringe filter (Gelman Sciences).
- 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) stock solution (100 mg/mL): Dissolve 250 mg of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (Eppendorf) into 2.5 mL of N,N-dimethylformamide (Sigma). Store in a dark container at -20°C.
- Isopropyl- β -D-thiogalactopyranoside (IPTG) stock solution (100 mM): Dissolve 1.2 g of isopropyl- β -D-thiogalactopyranoside (Acros) in Millipore water to a total volume of 50 mL. Filter-sterilize using 0.22 μ m cellulose acetate (CA) polystyrene filter (Corning, Inc.). Store at 4°C.
- NZY⁺ Broth: 10 g Bacto Casein Digest (Difco), 1 g Bacto Casamino Acids (Difco), 5 g Yeast Extract (Difco), 5 g Sodium Chloride (Sigma), 1 g

Magnesium Sulfate (anhydrous) in 1 L Millipore water. Autoclave at 121°C and 105 kPa chamber pressure for 20 min. Store at 4°C.

- LB Broth: 10 g Bacto Tryptone (Difco), 5 g Yeast Extract, 5g Sodium Chloride in 1 L Millipore water. Autoclave at 121°C and 105 kPa for 20 min. Store at 4°C.
- LB Agar selection plates: 10 g Bacto Tryptone, 5 g Yeast Extract, 5g Sodium Chloride, 15 g Bacto Agar (Difco) in 1 L Millipore water. Autoclave at 121°C and 105 kPa for 20 min. Cool to 55 °C and add 1 mL of ampicillin (100 mg/mL) to a final concentration of 100 µg/mL. Mix well and pour approximately 50 mL of the agar solution per plate. Keep overnight at room temperature, then store the plates inverted at 4°C for up to a month.
- LB Agar selection plates containing X-Gal: 10 g Bacto Tryptone, 5 g Yeast Extract, 5g Sodium Chloride, 15 g Bacto Agar in 1 L Millipore water. Autoclave at 121°C and 105 kPa for 20 min. Cool to 55°C, then add 1 mL of ampicillin (100 mg/mL) to a final concentration of 100 µg/mL, 5 mL of IPTG (100 mM) to 0.5 mM final concentration and 0.8 mL of X-Gal (100 mg/mL) to a final concentration of 80 µg/mL. Mix well and pour plates (50 mL per plate). Keep overnight at room temperature, then store the plates inverted at 4°C, protected from light for up to a month.

Eukaryotic Cell Solutions

- Chloroquine solution (8 mM): 0.041 g chloroquine diphosphate (Fluka), 10 mL Millipore water. Filter-sterilize using 2 μm sterile Acrodisc®13 syringe filter.
- DEAE solution (10 mg/mL): 1.0 g DEAE-dextran hydrochloride (Fluka) per 100 mL phosphate buffered saline (10 mM). Filter-sterilize using 0.22 μm polyether sulfone (PES) filter (Corning, Inc.).
- Dulbecco's Modification of Eagle's Medium (DMEM): 13.38 g DMEM powder (Cellgro), 1.4 g sodium bicarbonate, 10 mL antibiotic solution (Cellgro 3C-004-C1 100x: 100,000 U/mL Penicillin, 25 $\mu\text{g/mL}$ Amphotericin, and 10,000 $\mu\text{g/mL}$ Streptomycin), 10 % newborn calf serum (NCS) or fetal bovine serum (FBS) (BioWhittaker) in 1 L Millipore water. Filter-sterilize using 0.22 μm PES filter (Corning, Inc.).
- Phosphate Buffered Saline (PBS), pH 7.4 (10 mM): 9.55 g PBS powder (Cellgro) dissolved in 1 L Millipore water. Adjust to pH 7.4 if needed. Autoclave at 121°C and 105 kPa for 20 min or filter-sterilize using 0.22 μm PES filter.
- TED Buffer, pH 7.5: 10 mM Tris, 1 mM EDTA, 0.5 mM dithiothreitol (DTT) in 1 L Millipore water. Adjust to pH 7.5.

Yeast Cell Stock Solutions and Growth Media

- 10x BU salts stock solution: Dissolve 7.0 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ and 3.0 g of NaH_2PO_4 (Sigma) in Millipore water to a total volume of 100 mL. Adjust to

pH 7.0 with H_3PO_4 , then autoclave the solution at 121°C and 105 kPa for 20 min and store at room temperature.

- 40 % (w/v) Dextrose (Glucose): Dissolve 40 g dextrose (Sigma) in Millipore water to a final volume of 100 mL. Filter-sterilize through a 0.22 μm filter and store at room temperature.
- 40 % (w/v) [D(+)] Galactose (Gal): Dissolve 40 g [D(+)] galactose (Acros) in Millipore water to a final volume of 100 mL. Filter-sterilize through a 0.22 μm filter and store at room temperature.
- 40 % (w/v) Raffinose (Raf): Dissolve 40 g raffinose (Difco) in Millipore water to a final volume of 100 mL. Filter-sterilize through a 0.22 μm filter and store at room temperature.
- X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) stock solution (50 mg/mL in DMF): Dissolve 250 mg of X-Gal (Eppendorf) into 5 mL of N,N-dimethylformamide (Sigma). Store in a dark container at -20°C.
- 10x Dropout (DO) supplements: Dissolve 600 mg of DO Supplement/-His,-Leu,-Trp,-Ura (BD Biosciences-Clontech) and the appropriate amount of additional amino acids (see below) in 100 mL Millipore water. Autoclave at 121°C and 105 kPa for 20 min and store at 4°C for up to a year. An amino acid not indicated as missing is present in the DO supplement.
 - 10x DO/-Ura: Add 20mg L-Histidine HCl monohydrate, 100 mg L-Leucine, and 20 mg L-Tryptophan (Sigma).
 - 10x DO/-His,-Ura: Add 100 mg L-Leucine and 20 mg L-Tryptophan.

- 10x DO/-Trp,-Ura: Add 20mg L-Histidine HCl monohydrate and 100 mg L-Leucine.
 - 10x DO/-His,-Leu,-Ura: Add 20 mg L-Tryptophan.
 - 10x DO/-His,-Trp,-Ura: Add 100 mg L-Leucine.
 - 10x DO/-His,-Leu,-Trp,-Ura: no addition of extra amino acids.
- Synthetic dropout (SD) media and SD agar selection plates (1 L): Dissolve 6.7 g of dehydrated yeast nitrogen base without amino acids (Difco) in 850 mL Millipore water, add 100 mL of the appropriate 10x DO solution and 50 mL of 40 % glucose stock solution to a final concentration of 2 %. Adjust to pH 5.8 if needed, add 20 g agar (for plates only) and autoclave at 121°C and 105 kPa for 20 min. Cool medium to approximately 55°C, then add X-Gal to a final concentration of 80 mg/L and pour plates.
- SD/Gal/Raf plates containing X-Gal and BU salts: Dissolve 6.7 g of dehydrated yeast nitrogen base without amino acids (Difco) in 725 mL Millipore water and add 100 mL of the appropriate 10x DO solution. Do not adjust pH. Autoclave at 121°C and 105 kPa for 20 min, cool medium to approximately 55°C and add 50 mL of sterile 40 % stock of galactose to a final concentration of 2 %, 25 mL of sterile 40 % stock of raffinose to a final concentration of 1 %, 100 mL of sterile 10x BU salts stock solution and 1.6 mL of X-Gal stock solution (50 mg/mL) to a final concentration of 80 mg/L. Pour plates, allow medium to harden at room temperature overnight, then store plates inverted in the dark at 4°C for up to two months.

- YPD Broth (1L): Dissolve 50 g YPD Broth powder (Difco) in 1 L Millipore water. Adjust to pH 5.8 if necessary and autoclave at 121°C and 105 kPa for 20 min.
- YPD agar plates: Dissolve 65 g YPD Agar powder (Difco) in 1 L Millipore water, autoclave at 121°C and 105 kPa for 20 min, cool to approximately 55°C and pour plates.

Plasmids

A construct plasmid, designated pcVDR, was used as template DNA for PCR amplification. It contains the wild-type cDNA sequence of the human vitamin D₃ receptor minus the first three codons, cloned into the *EcoRI* and *NsiI* restriction sites of pcDNA1.1/Amp (Invitrogen) and was kindly provided by Prof. Anthony W. Norman (University of California, Riverside). The pSEAP-VDRE construct plasmid used for gene transcription activation assay was also provided by Prof. Anthony W. Norman. The pGEM®-T Easy plasmid was purchased from Promega. The pGilda and p8op-lacZ plasmids were purchased from BD Biosciences-Clontech as part of the MATCHMAKER LexA Two-Hybrid System.

VDR Ligands

The two hormonal ligands, 1,25(OH)₂D₃ and its analog 25(OH)D₃, were supplied by Dr. M. Uskokovich from Roche Pharmaceuticals (Nutley, NJ, USA).

3.2. PCR Amplification of VDR-LBD

Primer Design

To amplify the ligand-binding domain of the vitamin D₃ receptor, two primers flanking the entire VDR-LBD, including the stop codon were designed. The forward primer, an oligonucleotide of 21 bases designated VDRLex-305, contained two mismatches (A → T, G → C) introducing a recognition site for *Bam*HI restriction endonuclease (G^{*}GATCC) at the 5' end of the PCR product. Care was taken to preserve the correct reading frame of the VDR-LBD. The sequence of the forward primer is 5'GTGCAGAGGAT**TC**CGGGAGATG^{3'}, with the two mismatched nucleotides shown in bold. The reverse primer, an oligomer of 17 bases designated VDRLex-1322, contained one mismatch (G → C) introducing a recognition site for *Xho*I restriction endonuclease (C^{*}TCGAG) at the 3' end of the PCR product. The stop codon was not part of the primer sequence. The sequence of the reverse primer is shown below with the mismatched residue highlighted in bold, 5'CGTGGCCCT**C**GAGGAGC^{3'}.

PCR Protocol

The VDR-LBD was amplified using pcVDR as template DNA. Reaction conditions for the amplifications of VDR-LBD were optimized to eliminate non-specific products and to increase the yield of the VDR-LBD amplicon. All PCR amplifications were performed in 50 µL reaction volumes, using the TaqMaster PCR kit (Eppendorf). For each amplification 10 ng of pcVDR were used as template DNA. The final concentrations of the remainder of the PCR reagents

were as follows: 0.5 μ M of each primer (Sigma-Genosys), 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 1x TaqMaster PCR enhancer and 1.5 U *Taq* DNA polymerase in 1x *Taq* DNA polymerase buffer. The PCR protocol consisted of initial denaturation of the double-stranded DNA at 95°C for 2 min, followed by 40 cycles of 95°C/30 s, 62°C/1 min, 72°C/2 min, a final extension step 72°C for 10 min and cooling to 4°C. All PCR amplifications were performed on the Mastercycler gradient instrument (Eppendorf). The PCR amplified fragments were identified by gel electrophoresis on 1 % agarose gels (BioRad) in 1x TAE buffer, containing 0.005 % ethidium bromide (Sigma). A mix of Lambda DNA fragments, digested with *HindIII* and *EcoRI* (Promega) were used as DNA size markers.

Purification of PCR Products

The PCR product was purified using the QIAquick® PCR Purification Kit (QIAGEN), following the instructions by the manufacturer. Five volumes of Buffer PB were added to one volume of crude PCR product, and the solution was transferred to a QIAquick® column, inserted in a collection tube. This assembly was centrifuged for 1 min at 13,000 x g, the flow-through was discarded, and the DNA attached to the column matrix was washed with 0.75 mL of Buffer PE, containing 70 % ethanol. The column/collection tube assembly was centrifuged again for 1 min at 13,000 x g, the flow-through was discarded, and the column was centrifuged once more at 13,000 x g for 1 min to remove residual ethanol. The column was transferred to a clean 1.5 mL microcentrifuge tube, 50 μ L of Buffer EB (10 mM Tris $\cdot$ Cl, pH 8.5) were added to the center of the column,

incubated for 1 min to allow the DNA to dissolve, and centrifuged at 13,000 x g for 1 min to elute the DNA. The concentration of the purified PCR product was determined by agarose gel electrophoresis.

3.3. Cloning of VDR-LBD PCR Product into pGEM®-T Easy Vector

Ligation

The VDR-LBD amplicon was cloned into pGEM®-T Easy vector (Promega) using the pGEM®-T Easy Cloning kit (Promega) and following the protocol recommended by the manufacturer. The ligation of PCR product to the pGEM®-T Easy vector was performed using 1:3 vector to insert molar ratio in 10 µL reaction volume by mixing 51 ng of the purified VDR-LBD PCR product (20 ng/µL), 1 µL pGEM®-T Easy (50 ng/µL, Promega), 5 µL of 2x Rapid Ligation Buffer containing ATP (Promega), 1 µL T4DNA ligase (3 Weiss U/µL, Promega), and nuclease-free water to a total volume of 10 µL. The reaction was incubated at 4 C° overnight (16-18 hr).

The amount of the insert DNA corresponding to a particular molar ratio needed for the ligation was calculated by the formula:

$$\text{ng insert} = (\text{ng vector/Kb insert}) \times \text{Kb insert} \times (\text{insert/vector})$$

*Kb is the size of the DNA in kilo base pairs.

Transformation of Competent Bacterial Cells

Ligation reactions (1 μ L and 4 μ L) were used to transform 50 μ L aliquots of JM109 competent *E. coli* cells (Promega). The defrosted competent cells were gently mixed with the DNA and incubated on ice for 30 min. Then the cells were heat-shocked at 42°C for 45 s, followed by 2 min incubation on ice and the addition of 450 μ L aliquots of NZY⁺ broth, pre-warmed to 37°C. The transformation reactions were incubated at 37°C and 180 rpm shaking for 60 min. Next, 100 μ L of the transformation mix was spread on agar plates, containing 100 μ g/mL ampicillin, 80 μ g/mL X-Gal, and 0.5 mM IPTG. The selection plates were incubated overnight at 37°C for a total of 18-20 hr.

Plasmid Purification

The recombinant plasmid was purified from selected transformants using the Perfectprep® Plasmid Mini kit (Eppendorf), following the protocol suggested by the manufacturer. Several single, white colonies were used to inoculate 5 mL aliquots of NZY⁺ media, supplemented with 5 μ L ampicillin (100 mg/mL). The bacterial cultures were incubated overnight at 37°C and 300 rpm shaking in Lab-Line® Orbit Environ-Shaker incubator (Lab-Line Instruments, Inc.) for a total of 18 hr. Next, 3 mL of each cell culture was transferred to a 1.5 mL minicentrifuge tube and pelleted by centrifugation at 13,000 x g for 30 s on a Sorval Biofuge pico microcentrifuge. The supernatant was aspirated, and the cells were completely resuspended in 100 μ L of resuspension solution. The resuspended cells were lysed with 100 μ L of lysing solution by repeated gentle inversion of the tube until

the solution became viscous. The bacterial lysate was treated immediately with 100 μ L of neutralization solution and completely mixed by inversion of the tube until a white, curd-like precipitate appeared. The neutralized lysate was centrifuged at 13,000 x g for 60 s to pellet bacterial cell wall debris, precipitated proteins, and chromosomal DNA. The supernatant, containing the plasmid DNA, was transferred into a spin column and then 450 μ L of DNA binding matrix was added. The spin column/collection tube assembly was centrifuged at 13,000 x g for 30 s, then washed with 400 μ L purification solution, containing 70 % ethanol and centrifuged again for 30 s at 13,000 x g. The filtrate was discarded and the spin column/collection tube assembly was centrifuged again to remove any remaining purification solution. Finally, the DNA was eluted from the DNA binding matrix by adding 60 μ L of nuclease-free water, pre-heated to 65°C, vortexing briefly and centrifuging at 13,000 x g for 60 s. The eluted DNA was stored at -20°C.

Determination of DNA Concentration

The concentrations of the purified plasmids were determined by agarose gel electrophoresis and by UV spectroscopy. Prior to agarose gel electrophoresis, the recombinant plasmids were linearized by *Eco*RI endonuclease digestion. Aliquots of 8 μ L of each plasmid preparation were digested with 1 μ L of *Eco*RI (20 U/ μ L, Promega) in 1x buffer H (Promega). All reactions were incubated at 37°C for 1 hr. Then 2 μ L of blue/orange 6x loading buffer (Promega) was added to each reaction, and the samples were resolved electrophoretically on 1 %

agarose gels containing 0.005 % ethidium bromide and 1x TAE buffer. The agarose gel electrophoresis was performed on a Fisher Scientific FB-SB-710 apparatus at 120 V for 75 min. The DNA bands on the agarose gel were visualized by UV irradiation on Stratagene Eagle Eye Junior gel documentation transilluminator 2020E with version 3.2 Eagle Eye software or on Alpha Innotech FluorChem® 8900 Imaging System with Alpha Ease®FC software.

The DNA concentration was determined by comparing the intensity of the DNA band to the intensities of the Lambda DNA (*Hind*III/*Eco*RI) markers. The Lambda DNA fragment with equivalent intensity to the DNA band of interest was used to calculate the DNA concentration by the following formula:

$$C_{DNA} (\mu\text{g}/\mu\text{L}) = (N_f/N_{total}) \times C_{Lambda}(\mu\text{g}/\mu\text{L}) \times V_{DNA} \times F_D$$

where C_{DNA} is the concentration of DNA in $\mu\text{g}/\mu\text{L}$,

N_f is the size of the matching fragment in base pairs,

N_{total} is the size of the total Lambda DNA (48,502 base pairs),

V_{DNA} is the volume of DNA sample loaded on the gel in microliters,

C_{Lambda} is the concentration of the Lambda DNA markers ($0.5 \mu\text{g}/\mu\text{L}$),

F_D is the dilution factor, if any.

The DNA concentration and purity were determined also by UV spectroscopy (Ausbel et al., 1997). Suitable dilutions, usually 1:500 of each plasmid DNA preparation were made, and their absorbance was measured at four

fixed wavelengths (230 nm, 260 nm, 280 nm, and 320 nm) on the Hewlet Packard 8452A Diode Array spectrophotometer. The concentration of the double-stranded DNA was calculated using the formula:

$$C_{\text{DNA}} (\mu\text{g}/\mu\text{L}) = [A_{260}/(0.02 \mu\text{L}/\mu\text{g})] \times F_D$$

where A_{260} is the absorbance at 260 nm and F_D is the dilution factor. The purity of the DNA was determined by the ratio of the absorbances measured at 260 nm and 280 nm (A_{260}/A_{280}). A ratio of 1.8 to 1.9 indicates a highly pure DNA preparation.

3.4. Cloning of VDR-LBD into pGilda Vector

Endonuclease Digestion and Generation of Cohesive Ends

The newly generated pGEM®-T/VDR-LBD construct plasmid was digested simultaneously at 37°C for 2 hr with two restriction endonucleases, *Bam*HI (10 U/ μ L, Promega) and *Xho*I (20 U/ μ L, New England Biolabs). The final concentration of each enzyme was 0.5 U/ μ L. The digestion reactions were carried out in 1x NBE buffer 2, provided by the vendor with the *Xho*I restriction endonuclease; *Bam*HI is 50-75 % efficient in this buffer. The reaction was incubated at 37°C for 1 hr, then heated to 70°C for 15 min and cooled to 4°C. Following the double digestion, the reaction mix was gel-purified on 0.7 % low melting agarose gel (Fisher Scientific). The band at approximately 1,000 bp,

corresponding to VDR-LBD, was excised from the gel, and the DNA was separated from the agarose by phenol/chloroform extraction described below.

The yeast vector pGilda (Invitrogen) was linearized by double digestion with *Bam*HI and *Xho*I. A total of 0.5 μ g pGilda was digested in 20 μ L reaction volume, using 1 μ L *Bam*HI (10 U/ μ L) and 0.5 μ L *Xho*I (20 U/ μ L) in 1x NBE buffer 2. The reaction was incubated at 37°C for 1 hr, then heated to 70°C for 15 min and cooled to 4°C. The linearized vector was purified on 0.7 % low melting agarose gel as described below, by excising the band at 6.6 Kb, corresponding to the pGilda and purifying the DNA by phenol/chloroform extraction and ethanol precipitation.

DNA Purification by Phenol/Chloroform Extraction

The agarose band, containing the desired DNA, was transferred to a 1.5 mL microcentrifuge tube, its volume was estimated, and the same volume of buffered phenol (pH 8.0) was added to the agarose. The mix was vortexed for 5 min, then subjected to three cycles of freezing at -70°C for 30 min, thawing at room temperature and vortexing for 2-3 min. The organic and aqueous phases were separated by centrifugation at 4°C on a Savant μ SpeedFuge SFA13K microcentrifuge at the maximum speed of 14,000 x g for 15 min. The upper, aqueous layer containing the DNA was carefully transferred to a clean tube, and 100 μ L of TE buffer (pH 8.0) was added to the phenol layer for extraction of residual DNA. The mix was vortexed for 2-3 min and centrifuged at 4°C for 15 min at maximum speed. The upper, aqueous phase was removed and combined

with the rest of the extracted DNA. One volume of chloroform/isoamyl alcohol (24:1) was added to the aqueous phase, the mix was vortexed for 30 s, and the organic and aqueous phases were separated by centrifugation at 4°C at the maximum speed of 14,000 x g for 15 min. The upper, aqueous layer containing the DNA was carefully transferred to a clean tube. The DNA was precipitated by adding 1/10 volume of 3 M sodium acetate (or 5.0 M sodium chloride), and two volumes of 100 % ice-cold ethanol to the aqueous layer, mixing well and cooling at -70°C for at least 30 min or at -20°C overnight. The mix was then centrifuged at 4°C for 30 min at 14,000 x g, the supernatant was carefully removed, and the pellet was washed with 100 µL of 70 % ethanol. The mix was centrifuged again at 4°C and maximum speed for 15 min, the supernatant was removed, and the DNA pellet was dried at room temperature for 15-20 min. The DNA was dissolved in 50 µL of nuclease-free water or TE buffer (pH 8.0) at 4°C overnight, then stored at -20°C (Sambrook et al., 1989). Aliquots (1 µL) of the purified DNA were run on 1 % agarose gel to determine their approximate concentration.

Column Purification of DNA from Agarose Slices

DNA was also purified from agarose gel slices using the QIAquick® Gel Extraction Kit (QIAGEN), following the protocol recommended by the manufacturer. The band, containing the desired DNA fragment was excised from the low melting agarose gel, transferred in a tared colorless 1.5 mL microcentrifuge tube and weighed. The volume of the excised agarose band was estimated by its weight, with 100 mg of agarose corresponding approximately to

100 μ L. Three volumes of Buffer QG were added to one volume of agarose, and the mix was incubated at 50°C for 10 min until the agarose was completely dissolved. The color of the mixture at that stage should be yellow, indicating a $\text{pH} \leq 7.5$. If the color is orange or violet, 10 μ L of 3 M sodium acetate (pH 5.0) should be added to the mix to adjust to the optimal pH. Then the same volume of isopropanol as the initial gel slice volume was added to the sample and mixed. The sample was transferred to the QIAquick® column, inserted in a collection tube, and centrifuged for 1 min at 13,000 x g. The flow-through was discarded, and extra 0.5 mL of Buffer QG was added to the column to remove residual traces of agarose. The assembly was centrifuged at 13,000 x g for 1 min, and the flow-through discarded. Then 0.75 mL of Buffer PE, containing 70 % ethanol was added to the column, incubated for 5 min at room temperature, and centrifuged at 13,000 x g for 1 min. The flow-through was discarded and the column centrifuged again for 1 min to remove residual ethanol. Finally, the QIAquick® column was transferred to a clean 1.5 mL microcentrifuge tube, 50 μ L of Buffer EB (10 mM Tris · Cl, pH 8.5) was pipetted into the center of the column, and kept for 2-3 min before centrifuging at 13,000 x g for 1 min to elute the DNA. The concentration of the purified DNA was determined by agarose gel electrophoresis.

Cohesive End Ligation and Transformation

The purified VDR-LBD and linear pGilda, both now containing compatible cohesive ends as a result of the endonuclease digestions described above, were ligated together by T4 DNA ligase to create a construct plasmid, pGilda/VDR-LBD.

The ligation was carried out in 20 μ L reaction volume, using 3:1 insert to vector molar ratio, corresponding to 50 ng of VDR-LBD insert and 110 ng of pGilda. A total of 15 μ L of purified VDR-LBD insert (3.3 ng/ μ L) and 0.5 μ L of linearized pGilda (224 ng/ μ L) were mixed with 2 μ L of 10x T4 DNA ligase buffer containing ATP (Promega), and 2.5 μ L T4 DNA ligase (20 U/ μ L, Promega). The reaction was incubated at 4°C overnight, then various aliquots of the ligation reaction (1 μ L, 3 μ L, and 6 μ L) were used to transform supercompetent XL-1 Blue *E-coli* cells (Stratagene). The transformation was carried out using the standard protocol described above. Transformants were grown on LB agar selection plates containing 100 μ g/mL ampicillin. Then clones were selected for plasmid isolation as described above, identified by agarose gel electrophoresis, and sent for sequencing at Davis Sequencing Inc.

3.5. Error-prone PCR

Error-prone PCR amplification of VDR-LBD was performed in 50 μ L reaction volume, using Diversify™ PCR random mutagenesis kit (BD Biosciences-Clontech), following the manufacture's instructions. Buffer condition 5, optimized for introduction of an average of 4.6 mutations per 1,000 bp, was selected. For each amplification 10 ng pcVDR was used as template DNA with 0.5 μ M final concentration of each primer (Sigma Genosys), 0.2 mM of each dNTP, 40 μ M additional dGTP, 3.5 mM MgCl₂, 640 μ M MnSO₄ and 1x AdvanTaq Plus DNA polymerase in 1x AdvanTaq Plus PCR buffer (BD Biosciences-Clontech). The PCR protocol consisted of initial denaturation of the double-stranded DNA at 95°C for

2 min, followed by 30 cycles of 95°C/30s, 62°C/1 min, 68°C/2 min, a final extension step 68°C for 10 min and cooling to 4°C. The error-prone PCR amplifications were performed on the Mastercycler gradient instrument (Eppendorf). The PCR products were resolved by gel electrophoresis on a 1 % agarose gel (BioRad), containing 0.005 % ethidium bromide (Sigma) in 1x TAE buffer and identified with Lambda DNA Markers, digested with *Hind*III and *Eco*RI (Promega).

Next, aliquots of the error-prone PCR product were digested to create a pool of about 50 bp long DNA fragments that were to be used for gene shuffling of VDR-LBD. The PCR product (2.0 µg) was combined with 10 µL of 10x in-house made digestion buffer (500 mM Tris · HCl, pH 7.4; 100 mM MgCl₂) and nuclease-free water up to 100 µL final volume. The mix was equilibrated at 25°C for 10 min on a MJ Research Minicycler before adding the enzyme. After equilibration, 0.15 U of DNase I (70 U/µL, TaKaRa Biomedicals) was added, and the reaction was incubated for 10 min at 25°C. The enzyme was deactivated at 80°C for 10 min, and the fragments resolved on 2 % agarose gel, containing 0.005 % ethidium bromide.

3.6. Yeast Screening Assay

The MATCHMAKER LexA Two-Hybrid System (BD Biosciences-Clontech) was adapted as a one-hybrid system for screening of VDR-LBD interactions with its hormonal ligand. All media and stock solutions were prepared according to the manufacturer instructions. The YEASTMAKER™ Yeast Transformation System (BD

Biosciences-Clontech) was used for transformation of yeast cells, and the YEASTMAKER™ Yeast Plasmid Isolation Kit (BD Biosciences-Clontech) was used for plasmid isolation.

Construction of Yeast Reporter Strain EGY48[p8op-lacZ]

The reporter strain EGY48[p8op-lacZ] was constructed by transformation of *Saccharomyces cerevisiae* EGY48 host strain with the reporter plasmid p8op-lacZ (BD Biosciences-Clontech). First, 1 mL aliquots of YPD media were inoculated with several large yeast colonies, at least 2-3 mm in diameter, and vortexed to fully disperse any cell clumps. The cells were then transferred into a culture flask, containing 50 mL of SD/-Ura media and incubated overnight at 30°C with 250 rpm shaking for 18 hr. The overnight culture was then transferred into 300 mL of YPD media and incubated for another 3 hr at 30°C with 230 rpm shaking. Then the cells were transferred into six sterile 50 mL tubes and pelleted by centrifugation on Beckman Model TJ-6 centrifuge at 1,000 x g for 5 min at room temperature. The supernatant was decanted, and the cells were resuspended by vortexing in 30 mL of sterile TE buffer (5 mL per tube). All cells were pooled together in one tube and centrifuged at 1,000 x g for 5 min at room temperature. The supernatant was discarded, and the cells were resuspended in 1.5 mL of freshly prepared, sterile 1x TE/CH₃COOLi solution.

Next, 1 µL of plasmid p8op-lacZ (100 ng/µL) and 1 µL of herring testes carrier DNA (100 ng/µL) were dispersed into a new 1.5 mL tube and mixed by vortexing with 100 µL of competent cells. Then 100 µL of freshly prepared sterile

PEG/CH₃COOLi solution was added to the tube and mixed with the cells by vortexing at high speed. The cells were incubated at 30°C with 200 rpm shaking for 30 min, then 70 µL of DMSO was added and mixed gently with the cells by inversion. The cells were heat-shocked for 15 min at 42°C, then cooled on ice for 1-2 min. Next, the cells were pelleted by centrifugation at 13,000 x g at room temperature for 5 s, the supernatant was decanted, and the cells were resuspended in 500 µL of 1x TE buffer. Finally, 100 µL of the cells were plated on SD/-Ura agar selection plates and incubated at 30°C for 3-5 days.

Transformation of Yeast Reporter Strain with pGilda/VDR-LBD

The preparation of competent EGY48[p8op-lacZ] yeast cells, and their transformation with pGilda/VDR-LBD construct plasmid followed the same protocol described above. However, SD/-Ura media was used instead of YPD Broth for preparation of the overnight and 3 hr cultures of the EGY48[p8op-lacZ] reporter yeast strain. Each 100 µL aliquot of competent cells was transformed with 100 ng of pGilda/VDR-LBD along with 100 ng of herring testes carrier DNA. Four identical transformations with pGilda/VDR-LBD were carried out simultaneously. Two of the transformation reactions were further treated with 1,25(OH)₂D₃ (see below), and the other two were left untreated as negative controls. Finally, 100 µL aliquots of the transformed yeast cells were plated on various nutrient deficient agar selection plates, all containing X-Gal and galactose and raffinose as the carbon source (SD/Gal/Raf). The plates were incubated at 30°C for 3-5 days. The expected results are listed in Table 1.

Screening for VDR-LBD Interaction with 1,25(OH)₂D₃

The transformed yeast cells were screened for VDR-LBD interaction with 1,25(OH)₂D₃ by one of two methods. First, the surface of nutrient deficient agar selection plates, containing galactose and raffinose as the carbon source (SD/Gal/Raf), were treated with 1,25(OH)₂D₃ (1 mM stock solution in ethanol) at a final concentration of 10 nM per plate, immediately followed by spreading of 100 μ L aliquots of transformed cells on the plates and incubation at 30°C for 3-5 days. The second approach was to initially incubate the transformed cells for 15 min at room temperature with 1,25(OH)₂D₃ at a final concentration of 10 nM, followed by spreading of 100 μ L aliquots of the treated cells on nutrient deficient agar selection plates, and incubation at 30°C for 3-5 days. The various conditions and the expected results from these transformations are summarized in Table 1.

Table 1. Selection of pGilda/VDR-LBD transformed colonies

Plasmid	SD/Gal/Raf with X-Gal	1,25(OH)₂D₃ [10 nM]	Expected colony color	Experimental goal
pGilda/VDR-LBD	-His,-Ura	no	White	Negative control
pGilda/VDR-LBD	-His,-Leu,-Ura	no	None	Negative control
pGilda/VDR-LBD	-His,-Ura	yes	Blue and/or white	VDR-LBD interacting with 1,25(OH) ₂ D ₃
pGilda/VDR-LBD	-His,-Leu,-Ura	yes	Blue only	VDR-LBD interacting with 1,25(OH) ₂ D ₃

Table 2. Control transformations for the yeast screening assay

Plasmid containing DBD	Plasmid containing activation domain AD	SD/Gal/Raf Selection Medium	Expected Colony Color	Type of control
pLexA-Pos	-	-His/-Ura	blue	β -galactosidase assay positive control
pGilda	-	-His/-Ura	white	Background control
pLexA-53	-	-His/-Ura	white	DBD fusion control plasmid
	pB42AD-T	-Trp/-Ura	white	AD fusion control plasmid
pLexA-53	pB42AD-T	-His/-Trp/-Ura	blue	Positive control for two-hybrid protein interaction
pLexA-Lam: DBD fusion with human lamin C protein	-	-His/-Ura	white	False-positive detection plasmid
pLexA-Lam	pB42AD-T	-His/-Trp/-Ura	white	Accidental interaction with unrelated protein

Control Transformations

Control transformations of EGY48[p8op-lacZ] yeast reporter strain, listed in Table 2, were performed using 1 μ L aliquots of various control plasmids (100 ng/ μ L, BD Biosciences-Clontech), and following the protocol described above. Aliquots (100 μ L) of each transformation mix were spread on the appropriate nutrient deficient agar selection plates, containing X-Gal and galactose and raffinose as the carbon source (SD/Gal/Raf). The plates were incubated at 30°C for 3-5 days. The various conditions and expected results are listed in Table 2.

Yeast Plasmid Isolation

The pGilda/VDR-LBD plasmid was isolated from blue yeast transformants to confirm the presence of the VDR-LBD. The colonies were picked after 3 days of incubation at 30°C from SD/Gal/Raf/X-Gal plates, treated with 1,25(OH)₂D₃ and each colony was used to inoculate 500 μ L aliquots of two types of liquid selection media: SD/Gal/Raf/-His,-Ura and SD/Gal/Raf/-His,-Leu,-Ura. The cultures were incubated overnight at 30°C and 250 rpm shaking. The cells were pelleted by centrifugation at 13,000 x g for 5 min, the supernatant was removed, and the cells were resuspended into 50 μ L of sterile 67 mM KH₂PO₄ (pH 7.5) by brief vortexing. Lysostatin (10 μ L, 5 U/ μ L, BD Biosciences-Clontech), mixed well, and incubated at 37°C for 1.5 hr and 240 rpm shaking to fully lyse the yeast cells. Then 10 μ L of SDS were added to each tube and vortexed vigorously for 1 min. The cell mix was frozen at -20°C overnight, then defrosted at room temperature and vortexed

briefly. The DNA was transferred in the center of a Chroma Spin-1000 DEPC-H₂O column (BD Biosciences-Clontech), pre-treated as recommended by the manufacturer, and eluted by centrifuging for 5 min at 700 x g. The purified plasmid DNA was used as template DNA for PCR amplification of VDR-LBD, using the protocol described above, then stored at -20°C. The PCR products were resolved on 1 % agarose gel, containing 0.005 % ethidium bromide in 1x TAE buffer.

3.7. Generation of VDR_{Δ165-215} Deletion Mutants

The Δ165-215 deletion was engineered by inverse PCR (Imai et al., 1991) using phosphorothioate modified primers with the following sequences: 5'GGAGGGTCTGTGA**C*C*C***TAGAGCTG3' for the forward primer and 5'CACAGACCCTCCA**A*C*C*A***TCATTAC3' for the reverse primer (bold residues followed by asterisks denote phosphorothioate modifications). All deletion mutants were generated from plasmid DNA templates, containing the entire VDR sequence, either wild-type VDR (pcVDR) or VDR with previously incorporated point mutations (pcVDR-Y143F, pcVDR-S278A, pcVDR-Y143F/S278A). The inverse PCR amplifications were performed in 50 μL reaction volume, using 10-20 ng of template DNA, 0.2 mM of each dNTP (Eppendorf), 0.5 μM final concentration of each primer (Sigma-Genosys), and 2.5 U native *Pfu* DNA polymerase (Stratagene) in 1x native *Pfu* DNA polymerase buffer containing 2.0 mM MgSO₄. The PCR protocol consisted of initial denaturation step at 95°C for 2 min, followed by 35 cycles of 95°C/30 s, 58°C/1 min, 72°C/12 min, and a final

extension step at 72°C for 15 min, then cooling to 4°C. All PCR amplifications were performed on the Mastercycler gradient instrument (Eppendorf). Following the amplifications, 2.5 μ L *DpnI* (10 U/ μ L, Promega) was added to each PCR reaction and incubated at 37°C for 1 hr. Digestion with T7 gene 6 exonuclease (50 U/ μ L, Amersham Biosciences) was carried out at a final concentration of 2.0 U/ μ L, and the reactions were incubated at 37°C for 10 min (or alternatively at 22°C for 1 hr). The enzyme was deactivated by heating to 80°C for 10 min, followed by cooling to 4°C. Next, 1 μ L of each reaction mix was used to transform 50 μ L aliquots of XL-1 Blue *E. coli* competent cells (Stratagene). Alternatively, 10 μ L aliquots of *DpnI*/T7 gene 6 exonuclease treated PCR products were covalently recircularized by self-ligation, using the Quick ligation kit (New England Biolabs), following the conditions recommended by the manufacturer. Next, 1 μ L of each ligation reaction was used to transform 50 μ L aliquots of XL-1 Blue *E. coli* competent cells (Stratagene). Clones were selected for DNA isolation and purification using the Perfectprep® Plasmid Mini kit (Eppendorf) according to the manufacturer instructions. The success of the deletion mutagenesis was determined by in-house sequencing, using the dideoxy method described below. Once the presence of the deletion was confirmed, the clones containing the deletion were propagated to isolate a larger amount of the desired DNA, using the EndoFree™ Plasmid Maxi purification kit (QIAGEN), and following the protocol recommended by the manufacturer. The concentration and purity of the plasmid preparations were determined by UV spectroscopy and agarose gel electrophoresis as described above. All deletion mutants were sent to Davis

Sequencing, Inc. for additional sequencing of the entire VDR cDNA to ensure that no random mutations were incorporated during the inverse PCR.

3.8. DNA Sequencing

All DNA sequencing was performed by the dideoxy method using the *fmol*[®] DNA Cycle Sequencing System (Promega), following the vendor's instructions. First, the selected pcVDR sequencing primer (Sigma Genosys) was labeled at the 5' end with ³³P, following Promega's end-labeled primer protocol. The reaction was performed using 10 pmol of primer, 10 μ Ci/ μ L of [γ -³³P]ATP (2,500 Ci/mmol specific activity, Amersham Biosciences), 1 μ L of T4 polynucleotide kinase 10x buffer, 1 μ L of T4 polynucleotide kinase (10 U/ μ L), and nuclease-free water to a final volume of 10 μ L. The reaction was incubated at 37°C for 30 min, then heated at 90°C for 2 min to inactivate the enzyme, and cooled to 4°C. The sequences of all primers used are listed in Table 3.

Table 3. Primers used for DNA sequencing

Primer	Sequence
T7 (forward)	5' TAATACGACTCACTATAGGG 3'
VDR- 305 (forward)	5' GCATCACCAAGGACA 3'
RVDR-1425 (reverse)	5' GGACAGGAGAGAGAATGG 3'

For each set of sequencing reaction, four 0.5 mL microcentrifuge tubes were labeled G, A, T, or C, and 2 μ L of the corresponding d/ddNTP mix was added into each tube and kept at 4°C until needed. Separately, a master mix was prepared for each DNA template by combining 10 ng of the template DNA, 5 μ L of *fmol*[®] sequencing 5x buffer, 1.5 pmol of end-labeled primer, and nuclease-free water to a final volume of 16 μ L. Finally, 1 μ L of sequencing grade *Taq* DNA polymerase (5 U/ μ L) was added. After brief mixing and centrifugation, 4 μ L of the master mix was distributed into each of the tubes, labeled G, A, T, and C. The reactions were briefly mixed, centrifuged, and immediately placed into the thermocycler, pre-heated to 95°C. The cycle sequencing protocol consisted of an initial denaturation step at 95°C for 2 min, and 30 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s, and extension at 70°C for 3 min (30 s per 1,000 bp). After the cycle sequencing was completed, 3 μ L of *fmol*[®] sequencing stop solution was added to each tube to terminate the reaction. The samples were stored at -20°C.

The sequencing reactions were resolved electrophoretically on a 6 % polyacrylamide denaturing gel containing 6.0 M urea. The gel was prepared by mixing 48 mL SequaGel-6 (National Diagnostics), 12 mL SequaGel-6 Buffer (National Diagnostics) and 480 μ L of 10 % ammonium persulfate (Sigma). The gel was pre-run before loading the samples at 70 Watts for 45 min. Just prior to loading, the sequencing reactions were heated at 90°C for 2 min to denature the DNA, then briefly centrifuged to collect any condensation, and cooled on ice for about 1 min. The gel was run at 70 Watts for 2 hr on a Sequencing Gel Apparatus

model S2 (Life Technologies, Inc.). Then it was dried under vacuum (Savant Gel Dryer) at 80°C for 1 hr. Next, the gel was placed in autoradiography cassette along with an X-ray BioMax film (Kodak). The film was exposed for 5 days, and then developed on the Kodak RP-X-Omat Model M-7B Processor. The DNA sequence was determined, and the presence of the deletion and the lack of additional random mutations were confirmed by BLAST 2 sequences (bl2seq) alignment with the wild-type VDR cDNA sequence, accession number J03258 (National Center for Biological Information, BLAST, Blast 2 Sequences. <http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html>).

3.9. Cell Culture and Transient Transfection of COS-7 Cells

COS-7 monkey kidney cells (American Type Culture Collection) were maintained in continuous culture in Dulbecco's modification of Eagle medium, DMEM, (Cellgro) with 10 % fetal bovine serum, FBS, (BioWhittaker) at 37°C in a 92 % humidified atmosphere with 5 % CO₂ in a Forma Scientific 3860 S/N incubator. Cells were seeded for transfection at 1.2x10⁵ cells/mL in 150-mm tissue culture dishes (Corning, Inc.) and grown to 80-90 % confluency. After 36 to 48 hr of incubation, the media was removed by aspiration, and the cells were washed with phosphate buffered saline (PBS, pH 8.0), pre-heated to 37°C, and allowed to sit for 15 min. The PBS was aspirated, and the cells were treated with 1.0 mg/mL diethylaminoethyl (DEAE)-dextran (Sigma) in PBS for 9 min to facilitate DNA transfection (McCutchan and Pagano, 1968). After exactly 9 min the DEAE-dextran was removed by aspiration, and the cells were washed twice

with PBS. The cells in each tissue culture dish were treated with 10.6 μ g DNA in PBS and incubated at 37°C for 30 min, with gentle rocking every 5 min. The construct expression plasmid pcVDR, containing the cDNA for either wild-type or mutant VDR was used for transfection. Following the short incubation, DMEM media supplemented with 10 % FBS and 80 μ M chloroquine (Sigma) was added to each dish, and the cells were incubated at 37°C for 4 hr. The media was then aspirated and fresh DMEM with 10 % FBS but no chloroquine was added to the transfected cells, followed by incubation at 37°C for 60 to 72 hr.

3.10. Saturation Binding Assay

The cells were harvested 72 hr post-transfection with a solution containing 0.25 % trypsin and 0.1 % EDTA (Cellgro). First, the media was removed from the tissue culture dishes by aspiration, then the cells were washed with 4 mL of PBS, and the buffer removed. Then the cells were incubated with 2 mL of trypsin/EDTA at 37°C for 10-30 min with gentle rocking every 5 min until all cells were detached from the bottom of the dishes. The trypsin digestion was stopped by the addition of 4 mL of DMEM media containing 10 % FBS to each dish. The combined cells were transferred into a 50 mL sterile conical tube (Fisher Scientific), and pelleted by centrifugation at 3/4 speed on an IEC HN-S II Centrifuge (International Equipment Company). The supernatant was removed, and the cells were immediately lysed in 3 mL of ice-cold TED buffer by aspirating five times with a 20 mL gauge needle syringe. Aliquots (100 μ L) of crude lysates from COS-7 cells transfected with cDNA for wild-type or mutant VDR were

incubated for 4 hr at 4°C with increasing concentrations (0.125 nM to 6.0 nM) of [26,27-methyl-³H]-1,25(OH)₂D₃ (specific activity 90-93 Ci/mmol, Amersham Biosciences) in the presence or absence of a 100-fold excess of non-radiolabeled 1,25(OH)₂D₃. A hydroxyapatite batch assay was used to separate 1,25(OH)₂D₃ bound to the receptor from free 1,25(OH)₂D₃ by treating each sample with 300 µL of 50 % hydroxyapatite (BioRad) in TED buffer (Wecksler and Norman, 1979). Then, the hydroxyapatite was washed three times with TED buffer, supplemented with 0.5 % Triton X-100 solution (Sigma) and centrifuged for 1 min at 2,245 x g on a swing-bucket centrifuge (Beckman, Model TJ-6). The bound ligand was extracted by washing the final pellet with 1 mL of ice-cold, 200-proof ethanol. The ethanol containing the ligand was transferred into labeled scintillation vials, 3 mL of Ecoscint scintillation liquid (National Diagnostics) were added to each vial, and the bound ligand was quantified on a Beckman LS 6500 scintillation counter. Specific binding was calculated by subtracting non-specific binding from total binding.

3.11. Transcriptional Activation Assay

COS-7 cells were seeded at 1.2x10⁵ cells/mL in twenty-four well plates (Corning, Inc.) and co-transfected as described above with 0.5 µg/well of pcVDR plasmid and 1.5 µg/well pSEAP-VDRE plasmid, containing the osteocalcin gene vitamin D₃ response element (VDRE) linked to secreted alkaline phosphatase (SEAP) reporter gene. The transfected cells were incubated at 37°C in a 92 % humidified atmosphere with 5 % CO₂. Next, 24 hr post-transfection, the cell

medium was supplemented with ten-fold increasing concentrations of $1,25(\text{OH})_2\text{D}_3$ (from 1×10^{-11} M to 1×10^{-6} M) in 0.1 % final concentration of ethanol in MEM media without phenol red, followed by incubation at 37°C. The cell media was harvested after 30 hr of incubation in the presence of $1,25(\text{OH})_2\text{D}_3$ and assayed for SEAP activity using the Phospha-Light™ System (Tropix). Aliquots of the culture media (30 μL) from each well were transferred into a 96-well plate, incubated at 65°C for 30 min, then cooled on ice.

Meanwhile, the CSPD® chemiluminescent substrate (1.25 nM) was prepared by diluting with Phospha-Light™ Reaction Buffer Diluent in 1:20 volume ratio and equilibrated at room temperature, along with Phospha-Light™ Assay Buffer. Then, 20 μL of 1x Dilution Buffer was added to the medium in each well, followed by the addition of 50 μL /well of Assay Buffer. The plate was incubated at room temperature for 5 min, then 50 μL of diluted CSPD® substrate was added in each well, and the plate was incubated for 20 min at room temperature. The light generated in each well was measured at 10 min intervals using a chemiluminometer (Luminoscan, Ascent Labsystems) at a default PMT voltage of 835 V. All experiments were done in triplicate.

4. RESULTS

4.1. Random Mutagenesis of VDR-LBD

One of the main objectives of this research project was the generation of mutants of the ligand-binding domain of the vitamin D receptor protein that have enhanced binding affinity for the intermediate vitamin D₃ metabolite 25(OH)D₃ over the natural VDR ligand 1,25(OH)₂D₃. To achieve this goal, the following experimental scheme was envisioned (Figure 9).

Figure 9. Overview of the random mutagenesis experiment

In the first step, random mutations were to be introduced into the VDR-LBD gene by two PCR-based mutagenesis methods, error-prone PCR and gene shuffling. The mutated PCR products were to be cloned into suitable expression vectors and screened for binding affinity using the desired vitamin D₃ metabolite.

Mutants displaying high binding affinity were to be isolated and characterized further.

As can be seen from Figure 9, a crucial step in the overall strategy is the screening of the mutated proteins for their binding affinity towards the desired vitamin D analog. Such a method, should be relatively simple and fast, and most importantly, should allow the simultaneous screening of the relatively large number of mutants expected to be generated by the PCR process. In the following paragraphs, the development and validation of such a method is described. This initial work was done with the wild-type VDR-LBD and its natural ligand 1,25(OH)₂D₃, but once validated should be applicable to screening of the mutated proteins with any ligand of interest. The experimental steps involved in the validation of the yeast assay are schematically represented in Figure 10.

Figure 10. Validation of yeast screening assay for wild-type VDR-LBD

The screening method developed in this work is based on the known yeast two-hybrid assay (Fields and Song, 1989; Chien et al., 1991; Fritz and Green, 1992; Fields and Sternglanz, 1994). The readout of the method is based on the discrimination between blue and white yeast colonies following transformation with the desired plasmid. To obtain a plasmid suitable for cloning into a yeast expression vector, the desired PCR product had to be obtained, propagated in a suitable bacterial plasmid and transferred into the final yeast expression plasmid. These steps are described in more detail below.

PCR Amplification of VDR-LBD

The VDR ligand-binding domain was amplified by PCR using the plasmid pcVDR as the starting template and the following PCR primers:

- Forward primer, 5'GTGCAGAGGAT**TCC**GGGAGATG3'
- Reverse primer, 5'CGTGGCCCT**TCG**AGGAGC3'

In order to facilitate the subsequent cloning steps of the PCR product, these PCR primers contained a few mutations (shown in bold above) that were designed to introduce recognition sequences for the restriction endonucleases *Bam*HI (GATCC) and *Xho*I (CTCGAG) at the 5' and 3' ends of the PCR product.

The amplification of the desired VDR-LBD coding sequence required optimization of the PCR conditions with respect to several factors: annealing temperature, Mg²⁺ concentration, *Taq* DNA polymerase concentration, and the number of cycles. The effect of these variables on the PCR outcome is shown in Figure 11.

Figure 11. PCR optimization experiments for amplification of hVDR-LBD (1,018 bp). All PCR amplifications were performed in 50 μ L reaction volume, using the TaqMaster PCR kit (Eppendorf) and 10 ng pcVDR as template DNA. Two parameters were optimized simultaneously in each PCR reaction; all other conditions were kept constant. Negative controls were performed for all PCR conditions. The products were resolved electrophoretically on 1 % agarose gels, containing 0.005 % ethidium bromide in 1x TAE buffer, using Lambda (HindIII/EcoRI) DNA Markers (Promega) for identification (see Appendix C).

A. Optimization of the annealing temperature (59°C, 62°C and 65°C) and Mg^{2+} concentration (1.5 mM to 3.5 mM). The exact combination of T_{ann} and Mg^{2+} concentration for each individual reaction is shown on top of each lane. The concentration of all other reagents was identical (0.5 mM of each primer, 0.2 mM of each dNTP, 1x TaqMaster PCR enhancer and 2.5 U Taq DNA polymerase in 1x Taq DNA polymerase buffer). The PCR protocol consisted of 30 cycles of 95°C/30s, 62°C/1 min, 72°C/2 min. PCR reactions were loaded on the top row of the agarose gel, and the corresponding negative controls were loaded in the middle row (double gel).

B. Optimization of the enzyme concentration (1.5 U - 2.5 U) and PCR cycles (30, 36, and 40). All other reaction conditions were identical (0.5 mM of each primer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, and 1x TaqMaster PCR enhancer in 1x Taq DNA polymerase buffer). Each PCR cycle consisted of 95°C/30s, 62°C/1 min, and 72°C/2 min. The T_{ann} (62°C) and Mg^{2+} concentration (2.5 mM) were selected based on the results from the optimization experiment, shown in **part A** of this figure. The PCR reactions and the corresponding negative controls were loaded in adjacent wells (single gel).

From these experiments, the following conditions were found to be optimal for the amplification of the 1,018 bp VDR-LBD product: 62°C for the annealing temperature, 2.5 mM Mg²⁺ concentration, 0.03 U/ μ L *Taq* DNA polymerase concentration (1.5 U for a 50 μ L reaction), and 40 PCR cycles.

Error-prone PCR and Gene Shuffling

The VDR-LBD was amplified also by error-prone PCR. In this case, random mutations are deliberately introduced throughout the amplified VDR-LBD sequence using the Diversify™ PCR Random Mutagenesis Kit (BD Biosciences-Clontech). The increased mutagenesis rate is achieved by PCR amplification at sub-optimal reaction conditions, namely increased Mg²⁺ concentration (3.5 mM), addition of Mn²⁺ (640 μ M), and extra dGTP (40 μ M). The reaction was optimized using various starting concentrations of template DNA as recommended by the kit's manufacturer. The VDR-LBD was obtained successfully by error-prone PCR, and the results are shown in Figure 12. However, at this stage of the experiment it is still not clear if any mutations were introduced in the amplified VDR-LBD sequence. Practically, this can be determined only with a high-throughput functional screening assay for VDR-LBD. The development of such an assay became a major goal of this research project.

Figure 12. Error-prone PCR optimization. All PCR reactions were performed in 50 μ L reaction volume, using Diversify[®] PCR Random Mutagenesis Kit (BD Biosciences-Clontech). Various starting amounts of template pcVDR (1 ng, 3 ng, 5 ng, and 10 ng) were used and all other conditions were kept constant: 0.5 μ M final concentration of each primer, 0.2 mM of each dNTP, 40 μ M extra dGTP, 3.5 mM MgCl₂, 640 μ M MnSO₄ and 1x AdvanTaq Plus DNA polymerase in 1x AdvanTaq Plus PCR buffer. A negative PCR control without DNA template was also performed. The PCR protocol consisted of initial denaturation of the double-stranded DNA at 95°C for 2 min, followed by 30 cycles of 95°C/30 s, 62°C/1 min, 68°C/2 min, a final extension step 68°C for 10 min and cooling to 4°C. The PCR products were resolved on 1 % agarose gel, containing 0.005 % ethidium bromide in 1x TAE buffer, using Lambda (HindIII/EcoRI) DNA Markers (Promega) for identification (see Appendix C). The starting amounts of template pcVDR for each reaction are denoted on top of each lane. A single band at approximately 1,000 bp corresponding to VDR-LBD is observed for all PCR amplifications.

Cloning of VDR-LBD

The newly generated PCR product was treated with the two restriction endonucleases, *Bam*HI and *Xho*I. Cloning of the product into the yeast plasmid pGilda was attempted, but did not result in any transformants. One possible explanation of this is the close proximity of the *Xho*I site to the 5' end of the PCR product, which has been shown before to be a suboptimal substrate for this endonuclease (Ausbel et al., 2001). Therefore, an intermediate cloning step into the bacterial plasmid pGEM®-T Easy (Promega) was added. The map of the pGEM®-T Easy vector is shown in Figure 13.

Figure 13. Map of pGEM®-T Easy cloning vector (3,015 bp).
Reprinted with permission from Promega Corporation.

Cloning of PCR products into the plasmid pGEM®-T Easy is based on the fact that PCR products generated with the enzyme *Taq* DNA polymerase typically are not blunt-ended, but have single A residues appending both 3' ends of the product. The pGEM®-T Easy plasmid DNA is a linearized plasmid containing single T residues at both 3' ends. Thus, annealing and ligation of the PCR product to the linearized vector DNA provides a simple but efficient means for the cloning of any PCR product without the need for any further enzymatic modification of either component. The success of the cloning step is easily evaluated by β -galactosidase complementation following transformation of the reaction mixture into competent bacterial cells. Successful cloning results in disruption of Lac Z and thus generates white colonies, whereas any blue colonies are indicative of re-circularization of the starting pGEM®-T Easy DNA.

In this experiment, following overnight incubation of the transformed colonies at 37°C, about 50-70 white colonies and 15-20 blue colonies per plate were obtained. Twelve white colonies were selected for plasmid isolation, followed by double digestion with the restriction enzymes *Bam*HI and *Xho*I. As the starting pGEM®-T Easy vector DNA does not contain the recognition sites for these two enzymes, only plasmid DNA containing the ligated PCR product was expected to be digested by these two enzymes. The results from these experiments are shown in Figure 14. Two bands with approximate sizes of 1,000 and 3,000 bp were seen, corresponding to the VDR-LBD and the pGEM®-T Easy DNA, respectively.

Figure 14. Results from VDR-LBD cloning into pGEM[®]-T Easy vector. 1 % Agarose gel electrophoresis of double digestions of pGEM[®]-T Easy/VDR-LBD recombinant plasmid with BamHI (12U) and XhoI (20U) at 37°C for 1 hr (lanes 5, 6, 8-16), undigested controls (lanes 4 and 7), pGEM[®]-T Easy control (lane 2), Lambda DNA (HindIII/EcoRI) markers (lanes 1 and 17). The bands at approximately 1,000 bp correspond to VDR-LBD; the bands at approximately 3,000 bp correspond to the pGEM[®]-T Easy linear vector (see Appendix C for fragment sizes of Lambda DNA Markers).

The desired VDR-LBD product was gel purified and ligated into the BamHI/XhoI treated yeast pGilda vector (BD Biosciences-Clontech). The map of the pGilda vector is shown in Figure 15. This vector contains sequences allowing its

autonomous replication in either bacteria or yeast cells. A schematic representation of the ligation of VDR-LBD into pGilda is depicted in Figure 16.

Figure 15. Map of the pGilda yeast vector.
Reprinted with permission from Becton, Dickinson and Company.
The two arrows indicate the cloning sites for VDR-LBD.

Figure 16. Cloning of VDR-LBD into the yeast pGilda vector
(MCS = multiple cloning site).

Aliquots of the ligation reaction were transformed into XL-1 Blue competent cells (Stratagene) and transformants were selected on ampicillin containing LB agar plates. A total of 28 colonies were obtained, 22 of which were selected for plasmid isolation. The purified plasmids were screened for the presence of the insert by *Bam*HI/*Xho*I double digestion. The results are shown in Figure 17. Only one colony was found to contain an insert of the correct size. This single product was subjected to DNA sequence analysis and found to contain the desired VDR ligand-binding domain, without any random mutations. This plasmid was used in the subsequent experiments.

Figure 17. Results from VDR-LBD cloning into pGilda vector. 1 % Agarose gel electrophoresis of double digestions of pGilda/VDR-LBD recombinant plasmid with *Bam*HI (12 U) and *Xho*I (20 U) at 37°C for 1 hr (lane 7), undigested control (lane 9), VDR-LBD control (lane 3), pGilda control (lane 5) and Lambda DNA (*Hind*III/*Eco*RI) markers (lane 1). The bands at approximately 1.0 Kb correspond to VDR-LBD; the bands at approximately 6.6 Kb correspond to the linear pGilda vector (see Appendix C for sizes of Lambda DNA Markers).

Yeast Screening Assay

The recombinant pGilda/VDR-LBD vector was used further for the development of an one-hybrid yeast screening assay to detect interactions between the nuclear vitamin D₃ receptor and its cognate ligand 1,25-dihydroxyvitamin D₃ (Figure 18). The assay takes advantage of the modular domain structure of eukaryotic transcriptional regulators. Each domain is like an independent, physically separable, but still functional module. Two common domains, the DNA binding domain (DBD) and the activation domain (AD), are crucial for the activation of gene transcription. The DNA binding domain interacts with a specific promoter sequence on the target gene and the activation domain guides the RNA Polymerase II complex to transcribe genes downstream of the DNA binding site. Both interactions are necessary for transcription to occur. In this yeast assay, the DNA binding domain is supplied by the prokaryotic LexA protein. It binds specifically to LexA operators on target genes. The activation function is provided by VDR. The vitamin D₃ receptor does not have a separate activation domain but it has activation function (AF-2) as part of its ligand-binding domain. When VDR-LBD and LexA-DBD are fused together, a hybrid protein with binding affinity for LexA operators is created. It can activate transcription of reporter genes under the control of upstream LexA operators. However, VDR becomes an active transcription factor only after binding to its ligand and undergoing a conformational change. Therefore, if there is no ligand binding, no expression of the reporter genes will be observed.

Figure 18. Principle of the yeast screening assay for functional VDR-LBD.

There are two reporter genes used in this assay, *LEU2* and *LacZ*, both under the control of multiple LexA operators. Their promoters vary in the sequences flanking the LexA operators. This sequence difference helps to eliminate false positives. *LEU2* is a nutritional reporter gene and is integrated into the genome of the EGY48 yeast strain used in this assay. The EGY48 host cells are normally *Leu*⁻ auxotrophic. If the transcription of the *LEU2* reporter gene is activated, it will allow the transformed cells to grow on leucine deficient induction medium. The *LacZ* gene encodes for the enzyme β -galactosidase. When this reporter gene is

activated in the modified EGY48[p8opLacZ] yeast strain, the cells produce β -galactosidase. The activity of β -galactosidase is monitored through the hydrolysis of its substrate X-Gal present in the growth medium. The resulting colonies are blue. If the transcription of the lacZ reporter gene is not activated and β -galactosidase is not produced, then the colonies grown on X-Gal containing selection plates are white.

Figure 19. One-hybrid yeast screening assay for VDR-LBD: experimental approach. Vector maps reprinted with permission from Becton, Dickinson and Company.

The experimental set-up of the one-hybrid assay is illustrated in Figure 19. The protocol is comprised of several steps, starting with two sequential transformations of *Saccharomyces cerevisiae* EGY48 host strain. The cells are transformed with plasmids containing the coding sequences for genes that allow the cells to grow in media deficient in uracil and in specific amino acids. In general, the EGY48 yeast cells require the presence of all amino acids for their normal growth in addition to minerals and various sugars as carbon sources.

First, competent EGY48 yeast cells are transformed with the p8op-lacZ vector, which contains the coding sequences for the lacZ reporter genes and for the nutritional selection gene URA3. The nutritional URA3 selection gene allows the transformed colonies to grow in uracil deficient media. Then the modified EGY48[p8op-lacZ] yeast cells are transformed with the recombinant pGilda/VDR-LBD vector. The pGilda vector contains the gene for another nutrition selection marker (HIS3), which allows the transformed cells to grow on media lacking the amino acid histidine. The transformants are therefore selected on media deficient in both uracil and histidine after 3-5 days incubation at 30°C. However, all colonies containing the hybrid pGilda/VDR-LBD or pGilda alone will grow on these plates. To discriminate between colonies expressing active VDR-LBD variants and inactive proteins, the transformed cells have to be treated in parallel with 25(OH)D₃ and with 1,25(OH)₂D₃ as a control, then selected on induction media lacking not only uracil and histidine but also leucine. Only colonies that express the reporter gene LEU2 can grow on this induction medium. The transcription of the second reporter gene lacZ can be detected if X-Gal, the

substrate for β -galactosidase, and IPTG are included in the induction media. For the validation of the assay the EGY48[p8op-lacZ] yeast competent cells were transformed with the recombinant pGilda/VDR-LBD vector, containing only the wild-type VDR-LBD. The transformed yeast cells were then treated with $1,25(\text{OH})_2\text{D}_3$ or with $25(\text{OH})\text{D}_3$. The selection and screening of transformants can be done either consecutively or simultaneously.

One possibility is to initially select for pGilda/VDR-LBD transformants on induction media deficient only in uracil and histidine. The colonies are lifted and re-plated on X-Gal containing selection plates, deficient in nutrients: uracil, histidine and leucine. The surface of the selection plates is treated with 10 nM of $1,25(\text{OH})_2\text{D}_3$ or the analog $25(\text{OH})\text{D}_3$. Alternatively, the selected transformants are incubated with the hormone (10 nM) in uracil and amino acid deficient liquid media at room temperature for 15 min, then plated on uracil, histidine and leucine deficient selection plates, containing X-Gal, and incubated at 30°C for 3 to 5 days.

The second possibility is to simultaneously select and screen the transformed colonies based on their binding affinity for the hormonal ligand. Again, there are two options for delivery of the ligand: to treat the surface of the selection plates with the $1,25(\text{OH})_2\text{D}_3$ hormone or the analog $25(\text{OH})\text{D}_3$ at 10 nM concentration immediately prior to plating of the transformation reactions or to treat the transformed cells in hormone containing liquid media, followed by plating on X-Gal containing selection plates. The transformants containing a functional VDR-LBD, capable of binding to the hormone produced bright, blue

colonies on uracil, histidine, and leucine deficient induction media containing X-Gal. If the VDR-LBD does not bind to the hormone, no colonies should be produced on the selection plates lacking in uracil, histidine, and leucine. All options were tested, and the results were comparable. Therefore, the simplest option of simultaneous selection and screening for hormone binding to VDR-LBD was chosen.

A series of control transformations were performed along with the transformation with pGilda/VDR-LBD. Two positive transformation controls, one with the pGilda vector and one with the recombinant plasmid pGilda/VDR-LBD, were intended to verify the viability of the competent yeast cells. Both control transformations were plated on histidine and uracil deficient media, containing X-Gal and as expected, resulted in white colonies only. Another positive control with pLexA-Pos vector, encoding a fusion of Lex DBD and Gal4 AD, ensured that the reporter LacZ gene is activated and β -galactosidase is expressed. This control transformation resulted in blue colonies on growth medium lacking histidine and uracil. A positive control for interaction between two hybrid proteins made certain that the two-hybrid system is efficient. This control transformation involved the simultaneous transformation of the competent yeast cells with two control plasmids, pLexA53 encoding for DBD fusion protein and pB42AD-T, encoding an AD fusion protein. Both plasmids were provided with the commercial two-hybrid assay kit (BD Biosciences-Clontech). Aliquots of the transformation mix were plated on nutrient deficient growth media, lacking uracil

and the amino acids histidine and tryptophan but containing the substrate for β -galactosidase, X-Gal. All resulting colonies were blue.

A negative control for VDR interaction with $1,25(\text{OH})_2\text{D}_3$ was also routinely performed. In this case, the yeast cells were transformed with the pGilda/VDR-LBD recombinant plasmid, but were not treated with $1,25(\text{OH})_2\text{D}_3$ and no colonies were obtained on uracil, histidine, and leucine deficient selection plates containing X-Gal.

Figure 20. Experimental validation of the yeast assay for VDR-LBD. Transformation of the yeast strain EGY48[p8opLacZ] with pGilda/VDR-LBD with simultaneous screening for $1,25(\text{OH})_2\text{D}_3$. **A.** pGilda/VDR-LBD transformed yeast cells treated with 10 nM $1,25(\text{OH})_2\text{D}_3$ on His, Ura, Leu deficient agar plates with X-Gal. **B.** pGilda/VDR-LBD transformed yeast cells on His/Ura deficient agar plates with X-Gal without $1,25(\text{OH})_2\text{D}_3$ (negative control).

The one-hybrid yeast assay was successfully tested and validated for the wild-type VDR-LBD and its natural ligand $1,25(\text{OH})_2\text{D}_3$. These results are shown in Figure 20. The interaction of wild-type VDR-LBD with the analog $25(\text{OH})\text{D}_3$ at 10 nM concentration was also examined. As expected, not a single colony grew on nutritional selection plates lacking uracil, histidine, and leucine but containing X-Gal. This straightforward, blue-white screening protocol will be used in future studies for the evaluation of receptor-ligand binding specificity of random VDR variants generated by error prone PCR and gene shuffling.

4.2. VDR Deletion Mutants

Generation of VDR $_{\Delta 165-215}$ Deletion Mutants

One of the goals of this work was the generation and characterization of a series of mutant VDR proteins, all containing a relatively large deletion of the region between amino acid residues 165 and 215 ($\Delta 165-215$), in addition to several other point mutations. At the DNA level, this meant the deletion of 153 nucleotides. This had to be accomplished without altering the correct reading frame of the resulting sequence.

The known method of inverse PCR (Imai et al., 1991) was used to introduce this deletion into the VDR coding sequence. Inverse PCR is schematically represented in Figure 21. The pcVDR plasmid, containing the cDNA for the wild-type VDR, as well as several derivatives, containing desired point mutations, were used as starting DNA templates for the enzymatic amplification. These additional

mutations were Y143F, S278A, and Y143F/ S278A. These had been previously introduced into the pcVDR vector by site-directed mutagenesis.

Figure 21. Inverse PCR: Amplification of pcVDR

The PCR amplification of the desired 6,000 bp fragment was successful. However, initial attempts to recircularize the plasmid by blunt end self-ligation of the amplicon failed. Other known techniques to introduce the desired deletion were also unsuccessful. Finally, a new, universally applicable method was developed for the generation of such relatively large deletion mutants from cloned plasmid DNA and has been published (Stoynova et al., 2004). This method is based on the use of PCR primers that are partially modified by the introduction of phosphorothioate residues. Phosphorothioates are backbone-modified nucleotides where one of the non-bridging oxygen atoms is replaced by sulfur (Figure 22), and that makes them resistant to various endo- and exonucleases (Eckstein and Gish, 1989).

Figure 22. Structure of phosphorothioate oligonucleotides. One of the non-bridging oxygens of a phosphodiester bond is replaced by sulfur (shown in red), introducing a new chiral center in the molecule.

In addition to the introduction of phosphorothioates, the primers were designed to include 12 complementary bases at their 5' end. The sequences of the PCR primers used are shown in Figure 23. The complementarity of the 5' ends of the primers as well as the positions of the phosphorothioate residues are also depicted. These partially phosphorothioate modified primers were used to amplify, by inverse PCR, a 6,100 bp fragment from pcVDR and the additional plasmids containing various point mutations. Schematically this new method is shown in Figure 24.

Figure 23. Complementarity of the 5' ends of the PCR primers. Phosphorothioate residues are denoted with asterisks.

Figure 24. Schematic representation of the phosphorothioate method for generating large deletion mutants from a plasmid DNA template. The amino acid sequence shown at the top corresponds to part of the VDR protein, and the fragment that was to be deleted is indicated. The corresponding cDNA sequence as present in the pcVDR plasmid is shown below, together with the primers used in the inverse PCR. The positions of the phosphorothioates are indicated with asterisks. Note the six base extensions at the 5' ends of each primer, designed to generate complementary 3' overhangs after T7 gene 6 exonuclease treatment. The final deletion mutant retains the correct reading frame of the original protein.

The enzyme used for the amplification was *Pfu* DNA polymerase. It was essential to use this proofreading polymerase in order to prevent the addition of random mutations and the addition of extra nucleotides at the 3' ends of the amplification products as would be the case with other polymerases such as *Taq*. The PCR products were identified by 0.7 % agarose gel electrophoresis (Figure 25).

Figure 25. Inverse PCR optimization at various annealing temperatures. All PCR amplifications were performed in 50 μ L reaction volumes at different annealing temperatures, using 10-20 ng of template DNA, 0.2 mM of each dNTP (Eppendorf), 0.5 μ M final concentration of each primer and 2.5 U native *Pfu* DNA polymerase in 1x native *Pfu* DNA polymerase buffer containing 2 mM $MgSO_4$. The PCR protocol consisted of initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C/30 s, 58°C/1 min, 72°C/12 min, a final extension step at 72°C for 15 min and cooling to 4°C. The samples were resolved electrophoretically on 0.7 % agarose gel: *VDR* $_{\Delta 165-215}$ at 52°C, 54°C, 56°C, and 58°C (lanes 3, 4, 5, 6); *VDR* $_{\Delta 165-215}$ Y143F at 52°C, 54°C, 56°C, and 58°C (lanes 7, 8, 9, 10); *VDR* $_{\Delta 165-215}$ S278A at 52°C, 54°C, 56°C, and 58°C (lanes 12, 13, 14, 15); *VDR* $_{\Delta 165-215}$ Y143F/S278A at 52°C, 54°C, 56°C, and 58°C (lanes 16, 17, 18, 19); negative controls (lanes 1, 2 and 20), Lambda DNA (*Hind*III/*Eco*RI) markers (lane 11) were used for identification (see Appendix C).

A large band of approximately 6,000 bp, corresponding to the expected amplification product was identified. Some non-specific amplification products were also observed, with approximate sizes of 3,000 and 3,500 bp. Further optimization of the PCR conditions by varying the annealing temperature (Figure 25) and Mg^{2+} concentration (data not shown) enabled the reduction but not complete elimination of these non-specific products. Nevertheless, the yield of the desired specific product was high.

The inverse PCR was followed by *DpnI* restriction endonuclease digestion of the amplicon. The purpose of this treatment is to reduce the concentration of intact template plasmid molecules. This endonuclease recognizes the sequence $G^{me}ATC$, where ^{me}A denotes a methylated adenosine (Nelson and McClelland, 1992). The enzyme hydrolyzes the phosphodiester bond after this methylated residue, but no hydrolysis occurs if the residue is not methylated. Since none of the nucleotides used in a PCR reaction are methylated, the final amplification product is also not methylated. Therefore, no enzymatic digestion of the PCR product can occur. In contrast, the starting plasmid DNA template, which has been isolated from bacteria, typically contains numerous methylated nucleotides, including methylated adenosines within the *DpnI* recognition sequence. Thus, incubation of the amplified product with *DpnI* is expected to specifically reduce the amount of the intact starting plasmid DNA, which could compete in the final transformation step with the re-circularized PCR product. This step eliminates the need for a tedious purification of the PCR product from an agarose gel (Weiner et al., 1994).

The following step of the process was the treatment with T7 gene 6 exonuclease. This is a double-strand specific, 5'-3' exonuclease (Kerr and Sadowski, 1972). If a non-modified PCR product is treated with this enzyme, it is quickly converted into two half-length, single-stranded molecules. The enzyme is inhibited by phosphorothioate residues within the target sequence (Eckstein and Gish, 1989). Inclusion of phosphorothioate residues at the 5' end of only one PCR primer has been used before for the generation of single-stranded PCR products (Nikiforov et al., 1994). These results are illustrated in Figure 26.

Figure 26. T7 gene 6 exonuclease treatment of non-modified and a phosphorothioate-modified PCR products. The red squares denote the presence of phosphorothioate residues at the 5' end of one primer as described by Nikiforov et al. (1994).

In this experiment, both primers contained phosphorothioate primers located 12 bases away from the 5' end of the initial double-stranded PCR product. Treatment of the initially blunt-ended PCR product generated with these primers with T7 gene 6 exonuclease resulted in the formation of a PCR product with 12 base single-stranded 3' overhangs. By design, these overhangs are complementary to each other, so the product of this reaction can easily re-circularize by the hybridization of these single-stranded overhangs.

Next, the reaction conditions for the T7 gene 6 exonuclease digestion were selected in a series of optimization experiments. Aliquots of Lambda DNA (*HindIII*/*EcoRI*) markers (Promega), a mix of linear, double-stranded, unprotected DNA fragments, were digested with the T7 gene 6 exonuclease at 37°C and 22°C for various time intervals and in two different reaction buffers: the reaction buffer supplied with the exonuclease and the native *Pfu* DNA polymerase buffer used in the PCR amplification. The reaction mixtures were analyzed by agarose gel electrophoresis (Figure 27). Full digestion of all double-stranded fragments was achieved after 15 min at 37°C or after 60 min at 22°C. The digestion reaction appeared to be more efficient in the PCR buffer. Thus, it was established that the T7 gene 6 reactions can be carried out in the PCR reaction buffer, and no buffer exchange was needed.

Figure 27. Optimization of T7 gene 6 exonuclease digestion.

Lambda DNA Markers (*Hind*III/*Eco*RI) were used as a model to select optimal reaction condition for complete digestion of unprotected, double-stranded DNA by T7 gene 6 exonuclease. All reactions were performed in 25 μ L volumes with 0.5 μ g DNA, and 2 U/ μ L enzyme. Three parameters were varied: the reaction buffer (T7 reaction buffer or native Pfu DNA polymerase buffer with and without addition of extra Mg^{2+}), temperature (37°C or 22°C), and reaction time (15, 30 or 60 min). The products were resolved electrophoretically on 0.7 % agarose gel containing 0.005 % ethidium bromide in 1x TAE buffer: untreated *Lambda* DNA (*Hind*III/*Eco*RI) markers in TE buffer (lane 1); *Lambda* DNA (*Hind*III/*Eco*RI) treated with T7 gene 6 exonuclease at 37°C for 15 min in reaction buffer, PCR buffer, and in PCR buffer with extra $MgCl_2$ (lanes 2, 4 and 6 respectively); at 37°C for 30 min in the same buffers (lanes 8, 10, 12) and at 22°C for 1 hr (lanes 14, 16, 18). The corresponding undigested controls are shown in lanes 3, 5, 7, 9, 11, 13, 15, 17, and 19 (see Appendix C for fragment sizes of *Lambda* DNA Markers).

Once the optimal reaction conditions were established, all *DpnI* digested PCR products were treated with T7 gene 6 exonuclease to generate cohesive ends at the 3' ends of the amplicons (Figure 28). After the exonuclease was

Figure 28. Generation of “sticky ends” in the amplicon with T7 gene 6 exonuclease treatment. The crude PCR products (50 μ L) were first treated with 2.5 μ L *DpnI* (10 U/ μ L) at 37°C for 1 hr to eliminate the template pcVDR, followed by digestion with T7 gene 6 exonuclease (50 U/ μ L) at a final concentration of 2.0 U/ μ L. The reactions were incubated at 37°C for 10 min (or at 22°C for 1 hr). The enzyme was deactivated by heating to 80° for 10 min, followed by cooling to 4°C. The reactions were resolved electrophoretically on 0.7 % agarose gel, containing 0.005 % ethidium bromide in 1x TAE buffer: VDR $_{\Delta 165-215}$ (lane 4), VDR $_{\Delta 165-215}$ Y143F (lane 6), VDR $_{\Delta 165-215}$ S278A (lane 8), VDR $_{\Delta 165-215}$ Y143F/S278A (lane 10), undigested PCR controls (lanes 3, 5, 7, 9) and control reactions control for the efficiency of the enzyme: T7 gene 6 exonuclease treated unprotected PCR product (lane 11), undigested, unprotected PCR product (lane 12), T7 gene 6 exonuclease treated Lambda DNA (*HindIII*/*EcoRI*) markers (lane 1) and undigested Lambda DNA (*HindIII*/*EcoRI*) markers (lane 2), used also for identification (see Appendix C).

heat-inactivated, the amplicon was allowed to recircularize through hybridization of the single-stranded 3' ends. A ligase step was added to ensure the covalent closure of the molecule. It should be noted that the enzymatic hydrolysis with T7 gene 6 exonuclease proceeds in a way that leaves a phosphate group attached to the 5' end of the remaining DNA sequence (Kerr and Sadowski, 1972), thus allowing subsequent ligation without the need for enzymatic phosphorylation.

Finally, aliquots of the reaction mixtures were used to transform competent *E. coli* cells. Colonies were picked and sequenced to verify the presence of the desired 153 base pair deletion in the VDR sequence. On average, at least 50 % of the transformants contained the desired deletion. Subsequent experiments proved that the ligation step could be entirely omitted from the process, resulting in an even higher success rate of the overall procedure. The self-hybridization of the 3' overhangs generated by the T7 gene 6 exonuclease treatment of the PCR amplicon generated a nicked plasmid that was used successfully for transformation. The experiment was repeated several times with 35 to 60 colonies obtained per transformation. A total of 21 transformants were sequenced and 17 of them contained the desired deletion, a success rate of 81 %. When an enzymatic ligation step was included, transformation of competent *E. coli* cells resulted in 10 to 40 colonies per transformation. Of these, 26 were sequenced, and 13 contained the desired deletion, representing a 50 % success rate. The remaining 13 were undeleted, wild-type sequence. A representative sequencing gel of one of the deletion mutants is shown in Figure 29.

Figure 29. Sequencing results for $VDR_{\Delta 165-215}Y143F$ deletion mutant. The arrows indicate the position of the deleted region between amino acid residues 165 and 215.

From these results, it is clear that while the overall success rate of this method for introducing the desired deletion is quite high, a significant amount of the starting, unmodified plasmid DNA used as the template in the PCR step must also have survived the whole process and successfully transformed some of the competent cells. Apparently, the *DpnI* treatment had not successfully destroyed all of the starting DNA molecules. This step has not been optimized further, since at this stage of the work, all desired deletion mutants had been obtained and needed to evaluate their binding affinity toward $1,25(OH)_2D_3$.

Saturation Binding Assays

The effects of the $\Delta 165-215$ deletion mutation on ligand binding were assessed by saturation binding analysis. The results are summarized in Table 4 and representative graphs are shown in Figure 30. The full-length wild-type VDR,

Figure 30. Saturation binding assays. Wild-type VDR or mutant cDNA was transfected into COS-7 cells using DEAE-dextran. After 48-72 hr, the transfected cells were harvested and lysed. Increasing concentrations of 1,25-dihydro-[26,27-methyl- ^3H]-vitamin D_3 (180-186 Ci/mmol) were added in the presence or absence of 100-fold excess of non-radioactive $1,25(\text{OH})_2\text{D}_3$. The receptor-ligand complex was separated from the unbound ligand using hydroxyapatite batch assay. Specific binding data were determined by subtracting non-specific binding from total binding. The data for total binding are the mean of triplicate points and for non-specific binding are the mean of duplicate points.

the full-length single point mutants (Y143F, S278A), and the full-length double mutant (Y143/S278) displayed similar binding affinities to their deleted counterparts, VDR_{Δ165-215}, VDR_{Δ165-215}Y143F, VDR_{Δ165-215}S278A, and VDR_{Δ165-215}Y143F/S278A, respectively. Only the double mutant with the deletion (VDR_{Δ165-215}Y143F/S278A) showed decreased binding affinity for the hormone that was significantly different from both the full-length VDR and the deleted VDR_{Δ165-215}. These results suggest that amino acid residues 143 and 278 are not very important for binding but might be more important in the deletion VDR construct than in the full-length VDR (Acevedo et al., 2004).

Transcriptional Activation Assays

The functionality of the various VDR constructs was evaluated by gene transcription activation assays, and the data are summarized in Table 4. Representative transactivation data for each VDR variant as well as wild-type VDR are shown in Figure 31. The full-length wild-type VDR and the full-length point mutants Y143F, S278A, Y143F/S278A displayed similar transcriptional activation as their deleted counterparts: VDR_{Δ165-215}, VDR_{Δ165-215}Y143F, VDR_{Δ165-215}S278A, VDR_{Δ165-215}Y143F/S278A, respectively. All receptor variants containing the Y143F mutation required higher doses of hormone to induce gene transcription than both the full-length wild-type VDR and the deleted VDR_{Δ165-215}. This suggests that residue 143 is important for gene transcription in both the deleted VDR construct and the full-length VDR.

Figure 31. Transcriptional activation assays. Dose-response curves for full-length wild-type or mutant VDR and their respective VDR_{Δ165-215} pair. Plasmids containing hVDR cDNA and SEAP-VDRE were co-transfected into COS-7 cells using DEAE-dextran. After 24 hr, ethanol or increasing amounts of 1,25(OH)₂D₃ were added to the cells. Culture medium was collected 48 hr after hormone addition and secreted alkaline phosphatase levels were determined by chemiluminescence. Each point represents triplicate transfections. RLU refers to relative light units.

All experimental results from saturation binding analysis and transcriptional activation assays for all deleted VDR mutants (VDR_{Δ165-215},

VDR_{Δ165-215}Y143F, VDR_{Δ165-215}S278A, VDR_{Δ165-215}Y143F/S278A) and their full-length counterparts (VDR, Y143F, S278A, and Y143F/S278A) are summarized in Table 4.

Table 4. Summary of Results

Receptor	Saturation Binding K _D (nM)	Transactivation EC ₅₀ (nM)
w.t. VDR	0.61 ± 0.33 (n=7)	0.27 ± 0.24 (n=4)
VDR _{Δ165-215}	0.42 ± 0.08 (n=4)	0.13 ± 0.11 (n=4)
Y143F	0.46 ± 0.16 (n=4)	3.08 ± 2.75 (n=4) ^b
VDR _{Δ165-215} Y143F	0.64 ± 0.34 (n=6)	0.88 ± 0.79 (n=4) ^c
S278A	0.43 ± 0.17 (n=4)	0.66 ± 0.68 (n=3)
VDR _{Δ165-215} S278A	0.51 ± 0.20 (n=6)	0.27 ± 0.04 (n=3)
Y143F/S278A	0.56 ± 0.17 (n=7)	126 ± 5.7 (n=3) ^b
VDR _{Δ165-215} Y143F/S278A	1.15 ± 0.70 (n=5) ^{a, c}	30 ± 13 (n=4) ^{b, d}

Data are expressed as means and SD of “n” independent experiments. One-way ANOVA with Turkey’s Multiple Comparison post-test was performed using GraphPad Prism version 3.00 for Windows, GraphPad Software, CA.

^asignificantly different than w.t. VDR, p<0.05

^bsignificantly different than w.t. VDR, p<0.001

^csignificantly different than VDR_{Δ165-215}, p<0.05

^dsignificantly different than VDR_{Δ165-215}, p<0.001

5. DISCUSSION

The overall objective of this research project is to examine the structure-function relationships of the vitamin D₃ receptor. This is of fundamental importance not only for understanding how VDR modulates the transcription of targeted genes but also for the design of various hormone analogs with selective biological activity. There are several possible approaches to solving this problem. Site-directed mutagenesis targets specific amino acid residues in order to evaluate their contribution to ligand binding and transcriptional activation. Another approach is to modify the receptor's molecular recognition through random mutagenesis in a process known as directed evolution (Stemmer, 1994). However, both methods provide only an indirect assessment of the receptor structure. Real structural data obtained by X-ray crystallography and NMR is needed to determine the exact structure of the receptor protein and the amino acid residues involved in anchoring the ligand. The only structural data available for VDR is the crystal structure of its ligand-binding domain with the 1,25(OH)₂D₃ hormone bound (Rochel et al., 2000). The crystal structure allowed the identification of the contact amino acid residues involved in anchoring the hormone inside the hydrophobic pocket of the VDR-LBD. However, high quality protein crystals were only obtained after 51 amino acids (Δ 165-215) were deleted from a poorly defined region at the N-terminus of the VDR-LBD. The determination of the crystal structure of the entire native VDR protein is still elusive. NMR structural data, important for revealing the protein structure in

solution, are also not available for the full-length VDR or its ligand-binding domain. Therefore, the knowledge that can be gained from mutagenesis studies about the structure-function relationships of the vitamin D₃ receptor is still of great value. The overall aim of this work was centered around the generation and characterization of a number of mutated variants of the human vitamin D₃ receptor protein (hVDR) and the evaluation of the effects of these mutations on the receptor function.

5.1. Directed Evolution of VDR-LBD

When this research project was initiated, the crystal structure of VDR-LBD had not been published and no structural data were available for the vitamin D₃ receptor. Several computer-modeling studies were published in the late 1990s predicting the conformation of the protein and the contact points with the hormone. These models were based on sequence alignment of the VDR-LBD with the ligand-binding domains of other members of the nuclear receptor family with known crystal structures, namely TR and γ -RAR (Norman et al., 1999; Wurtz et al., 1997). In addition, site-directed mutagenesis studies of VDR-LBD had previously identified many amino acid residues important for ligand binding and activation of gene transcription (Bouillon et al., 1995). Even after solving the crystal structure of VDR-LBD, the question remained whether other amino acid residues in the VDR-LBD are involved in ligand binding or affecting the receptor functions *in vivo*. Despite all advances in understanding the VDR structure and function, there are many aspects of the protein function that are not easily predictable. To

shed light on some of the protein functions, a library of randomly mutated variants of the vitamin D₃ receptor ligand-binding domain was generated with the intent to select those members of the library with an increased binding affinity for the intermediate vitamin D₃ metabolite, 25(OH)D₃, instead of the natural ligand 1,25(OH)₂D₃. The overall process of constructing such a library is known as directed molecular evolution (Stemmer, 1994), and it leads to the creation of variant receptors with altered molecular recognition. The change in protein function can easily be traced back to the relevant amino acids by sequence alignments between the wild-type and the altered VDR-LBD variants.

Although the main reason for the generation of VDR-LBD with altered molecular recognition was to study the structure-function relationships of the receptor, the modified receptors could also be used to shed some light on another important and controversial problem in the VDR field, namely the possible existence of a membrane VDR involved in rapid responses to 1,25(OH)₂D₃ (Farach-Carson et al., 1991; Nemere and Norman, 1987; Nemere et al., 1993; Zhou et al., 1992). The generation of VDR variants with increased binding affinity for 25(OH)D₃ would allow the binding of 1,25(OH)₂D₃ to a putative membrane receptor (VDR_{mem}) independent of its binding to the nuclear VDR. This could potentially be achieved by *in vitro* studies of cell lines lacking endogenous VDR_{nuc} but still displaying rapid responses to 1,25(OH)₂D₃. These cells will be transfected with the modified receptor for binding of 25(OH)D₃. This intermediate metabolite is the major circulating form of vitamin D₃ in the bloodstream. Unlike 1,25(OH)₂D₃, it binds to wild-type VDR_{nuc} with relatively low affinity and is not

biologically active. However, the altered nuclear vitamin D₃ receptors would bind selectively and with high affinity to 25(OH)D₃ and at the high physiological concentration of this intermediate, the modified nuclear receptors would be fully saturated. This could permit independent investigation of the interaction of 1,25(OH)₂D₃ with its putative membrane receptor without its competing binding to nuclear VDR. In addition, the interaction between the two systems (VDR_{nuc} and VDR_{mem}) could be investigated by treating the cells independently with varying concentrations of either 1,25(OH)₂D₃ or 25(OH)D₃ and comparing the results with the response observed when the transfected cells are treated with both ligands simultaneously.

Another attractive application for a nuclear vitamin D₃ receptor with altered molecular recognition pattern is its potential usefulness in gene therapy. Gene therapy could provide an alternative treatment for various diseases caused by insufficient concentration of 1,25(OH)₂D₃, for example prostate cancer. Such an approach could circumvent the problem with the toxic, hypercalcemic effects of 1,25(OH)₂D₃ at high therapeutic concentrations, and also avoid the potential toxicity and side effects of synthetic, non-hypercalcemic analogs of 1,25(OH)₂D₃.

To achieve these goals and change the molecular recognition of VDR, the techniques of error-prone PCR (Cadwell and Joyce, 1992; Cadwell and Joyce, 1994) and gene shuffling (Stemmer, 1993; Stemmer, 1994) were applied. Both error-prone PCR and gene shuffling are molecular techniques used to generate libraries of partially randomized genes by deliberate introduction of numerous

random mutations in the encoding DNA sequence of a protein. These libraries are then screened and variant proteins with the desired functionality are selected.

Error-prone PCR relies on suboptimal, low-fidelity reaction conditions to substantially increase the rate of random mutations introduced by *Taq* DNA polymerase during the extension step of the amplification process. These suboptimal conditions usually include increased concentration of Mg^{2+} , the addition of small amounts of Mn^{2+} and biased concentrations of dNTPs (Cadwell and Joyce, 1992; Cadwell and Joyce, 1994; Cirino et al., 2003; Leung et al., 1989). Most often an excess of dGTP and/or dTTP is used as these deoxynucleotide triphosphates are preferentially incorporated by the DNA polymerase during amplification. The rate of mutagenesis, which is defined as the number of mutations introduced per 1,000 base pairs, is affected by the Mn^{2+} concentration and the number of PCR cycles (Cirino et al., 2003). The presence of Mn^{2+} along with excess dGTP and dTTP in the PCR reaction mix usually results in error rates of approximately one nucleotide per kilobase in the final library (Neylon, 2004). Higher rates of mutagenesis can be achieved, for example, by using dNTP analogs (Zaccolo et al., 1996) or by using a recombinant, highly error prone DNA polymerase.

In general a mutagenesis rate of two to six mutations per gene is considered optimal for the synthesis of libraries of mutant proteins with selectively enhanced activity (Shafikhani et al., 1997). Higher mutational rates often lead to a total loss of protein activity (Suzuki et al., 1996) although some proteins can tolerate much higher rates of mutagenesis (Vartanian, 1996). If a

higher mutational rate is desired, this can be achieved by consecutive rounds of error-prone PCR or by a combination of error-prone PCR with gene shuffling.

The commercial Diversify PCR Random Mutagenesis Kit from BD Biosciences-Clontech, which is based on *Taq* DNA polymerase, was used to generate a library of VDR-LBD random mutants. The rate of mutagenesis here is controlled by varying the concentrations of Mn^{2+} and dGTP. The VDR-LBD was amplified using reaction conditions favoring a rate of 4.6 mutations per 1,000 bp, a Mg^{2+} concentration of 3.5 mM, Mn^{2+} at 640 μM , and extra dGTP at 40 μM .

The attractiveness of error-prone PCR is that the mutations are introduced along the entire DNA sequence and ideally should lead to a library where all mutations are equally represented. However, there is an inherent bias in error-prone PCR due to the characteristics of the DNA polymerase favoring the incorporation of some mismatched nucleotides more often than others (Cirino et al., 2003; Zaccolo et al., 1996). Another source of bias is the degenerate nature of the genetic code. Error-prone PCR results in single nucleotide substitutions in the DNA sequence thus leading to a bias in the mutated amino acids encoded by the DNA. Amino acid mutations resulting from a single nucleotide substitution in their codon will be represented more often than amino acids that require two or three nucleotide substitutions. Therefore, some amino acid mutations will be much more common in the final library constructed by error-prone PCR. Finally, there is also an amplification bias, inherent to the PCR technique that contributes to the uneven distribution of mutations. The amplification process in PCR is exponential by nature and any mutation introduced in the early cycles will be

copied in all of the remaining cycles and therefore will be over-represented in the final library (Neylon, 2004). All these problems can be alleviated and a truly random library can be created by combining error-prone PCR with gene shuffling.

Gene shuffling is a novel technique for directed evolution based on the random recombination of DNA sequences (Stemmer, 1993; Stemmer, 1994). The method is schematically represented in Figure 32.

Figure 32. Generation of a library of random mutants by gene shuffling

Initially, the gene of interest is amplified by PCR and is then subjected to random fragmentation by treatment with DNase I endonuclease. The size of the resulting fragments is important for the overall number of mutations introduced

in the gene in the following step of the gene shuffling protocol. It appears that the optimal size of the random fragments is between 50-200 base pairs (Zhang et al., 1997). These random fragments are then reassembled by self-priming PCR, generating a family of closely related DNA sequences. Numerous mutations are introduced at this step into the starting DNA sequence. Most of these mutations will be silent but some may lead to the desired change in activity. The random fragmentation and reassembly PCR steps are repeated for this new family of related DNA sequences creating a large library of recombinant genes.

Next, the library is screened to detect and isolate desired mutations and eliminate negative mutations for the next round of gene shuffling. In general, most of the newly introduced random mutations have no effect on the protein function. However, accumulation of some mutations might lead to enhancement of the selected function over the normal function of the protein. For example, an enzyme could be modified by random mutagenesis to have a preference for a different substrate (Cramer et al., 1996; Cramer et al., 1998; Giver et al., 1998; Kong et al., 2001; Moore et al., 1997; Zhang et al., 1997; Zhao and Arnold, 1999). The clones with the enhanced functionality are selected using a functional assay, then subjected to another round of random mutagenesis and screening until the new function becomes the predominant feature of the protein. This whole process mimics the pathway of evolutionary changes in nature but at a much more accelerated rate and is therefore known as directed evolution.

Both error-prone PCR and gene shuffling require fast and efficient screening to select mutants with enhanced activity. Therefore, the development

of a method for rapid screening of the binding properties of the library of mutant VDR proteins became a primary goal of this project. The simplest eukaryotic system, yeast, was chosen to develop a screening assay to select for VDR-LBD mutants that bind to $25(\text{OH})\text{D}_3$ instead of $1,25(\text{OH})_2\text{D}_3$. The assay is based on the fact that most eukaryotic transcriptional regulators have a modular domain structure with physically separable and independently functioning domains. In general, the DNA-binding domain (DBD) binds to a specific promoter sequence on the target gene and the activation domain (AD) directs the RNA Polymerase II complex to transcribe the gene downstream of the DNA binding site. A fusion protein, containing the VDR-LBD and the DBD of the prokaryotic LexA protein that binds to LexA operators on target genes, was constructed. The activation function of the hybrid protein is provided by the ligand-binding domain of VDR. The vitamin D_3 receptor does not have a separate activation domain but it has activation regions that are part of its ligand-binding domain. Therefore, when the VDR-LBD and the LexA-DBD are fused together, the result is a hybrid protein with binding affinity for LexA operators that can activate the transcription of reporter genes under the control of upstream LexA operators. However, the VDR-LBD becomes an active transcription factor only after binding to its cognate ligand and undergoing a conformational change. Hence, in the absence of ligand binding no expression of the reporter gene will be observed. If the hormonal ligand binds to the VDR-LBD, the transcription of the reporter gene will be activated and the gene expressed. This will be the indication that the modified VDR-LBD binds indeed to $25(\text{OH})\text{D}_3$.

Two reporter genes, both under the control of multiple LexA operators, were used in this assay: LEU2 and LacZ. Their promoters vary in the sequences flanking the LexA operators, and this sequence difference helps to eliminate false positives. LEU2 is a nutritional reporter gene and is integrated into the genome of the EGY48 yeast strain. The EGY48 host cells are normally Leu⁻ auxotrophic and can not grow in the absence of the amino acid leucine. If the transcription of the LEU2 reporter gene is activated, the transformed cells can then grow on leucine deficient induction medium. The second reporter gene, LacZ, encodes for the enzyme β -galactosidase. When this reporter gene is activated in the modified EGY48[p8opLacZ] yeast strain, the cells produce β -galactosidase. The activity of β -galactosidase is monitored through the hydrolysis of its substrate, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal), present in the growth medium, resulting in blue colonies. The intensity of the colony's blue color is an indication of how well the variant VDR-LBD binds the alternative ligand 25(OH)D₃ instead of 1,25(OH)₂D₃. This provides an easy visual screening for selection of mutants with the most altered molecular recognition.

However, before the assay can be applied for screening a randomly mutated VDR-LBD library, it had to be validated for the wild-type VDR-LBD. Competent yeast cells EGY48[p8opLacZ] were transformed with the recombinant plasmid pGilda/VDR-LBD. The pGilda/VDR-LBD plasmid contains a nutritional selection gene that allows transformed colonies to grow on histidine deficient induction media. After transformation, equal aliquots of cells were spread on uracil, leucine and histidine deficient selection plates that were either treated with

1,25(OH)₂D₃ or not. As expected, blue colonies were observed only if the transformed cells were incubated with the hormone, and no colonies grew on the plates if the transformed cells were not exposed to 1,25(OH)₂D₃. As a control, the same experiment was repeated but this time the transformed cells were treated with 25(OH)D₃ instead of 1,25(OH)₂D₃. As mentioned above, 25(OH)D₃ binds to wild-type VDR with relatively low affinity and does not induce transcription. Hence, in the control experiment with 25(OH)D₃ no colonies were expected to grow on any amino acid deficient selection plate, and this was indeed observed.

Overall, these results are very encouraging. The assay clearly works and can be used as a screening tool to select VDR-LBD variants with altered molecular recognition. There are still some issues with the reproducibility of the assay that need to be solved. The detection of the interaction of the wild-type VDR-LBD with 1,25(OH)₂D₃ was not always consistent. The assay was repeated six times and only in one experiment no colonies were obtained after incubating the VDR-LBD transformed cells with 1,25(OH)₂D₃. In about half of the successful experiments, the colonies that grew on the selection plates were only faintly bluish or displayed a range of blue color, from weak to intense. These somewhat inconsistent results require further optimization until the assay is completely reliable in detecting the interaction between 1,25(OH)₂D₃ and the wild-type VDR-LBD. Only then can it be applied for screening and selection of VDR-LBD variants with altered molecular recognition.

One way to improve the assay reliability is to optimize the delivery of the hormone. Two different approaches for treating the yeast cells transformed with

the pGilda/VDR-LBD plasmid were tested. Either the transformed cells were incubated prior to plating with $1,25(\text{OH})_2\text{D}_3$ at 10 nM concentration of the hormone or the surface of the selection plate was treated with the same concentration of the hormone immediately prior to spreading the cells. Both methods resulted in similar outcomes but the application of the hormone directly on the surface of the selection plate appeared to be slightly better.

Another way to improve the reliability of the assay is to convert it into a two-hybrid yeast assay by introducing the retinoic X receptor (RXR) along with VDR-LBD. In the current assay, transcriptional activation of the reporter genes depends solely on the conformational changes in VDR-LBD resulting from ligand binding. This is probably not always sufficient for efficient interaction with the basal transcription machinery. As mentioned before, the mechanism of transcription modulation by VDR involves not only hormone binding, but also the formation of a heterodimer with another nuclear receptor, RXR (Collins, 2000). However, RXR is not produced by yeast, and consequently the activation of the transcription in this screening assay depends only on the activation function, AF-2, of VDR-LBD. It is likely that the addition of RXR in another recombinant yeast plasmid, co-transformed with pGilda/VDR-LBD, will improve substantially the reproducibility of the assay.

In conclusion, the development of a screening assay for detecting the interaction between VDR-LBD and its cognate ligand was clearly successful. This assay will enable the high-throughput screening of a library of random VDR-LBD mutants to select variants that bind preferentially to the intermediate metabolite

25(OH)D₃ instead of the natural ligand for VDR 1,25(OH)₂D₃. Although the assay still needs some optimization, when this is accomplished, it will be applied for initial screening of the VDR-LBD library, generated earlier by error-prone PCR. The VDR-LBD library will be further randomized by gene shuffling methodology to increase the probability of generating mutants with altered molecular recognition. This modern approach has never been tried before to study the structure-function relationships of the vitamin D₃ receptor. If successful, it has also an immense potential for the development of novel approaches to the treatment of various cancers.

5.2. VDR Deletion Mutants

As mentioned earlier, during the course of this research a paper was published by Rochel and co-workers, describing the crystallization and three-dimensional structure determination of a truncated version of the human vitamin D₃ receptor ligand-binding domain (Rochel et al., 2000). In this work, the authors successfully crystallized this mutated VDR-LBD protein as a complex with 1,25(OH)₂D₃. The VDR-LBD protein was crystallized only after the removal of 51 amino acids (Δ 165-215) from a poorly conserved region of the wild-type protein, and the crystal structure described in this work is, therefore, not that of the wild-type protein but of this deletion mutant. Crystallization of the full-length wild-type vitamin D₃ receptor has remained an elusive goal. Binding studies and functional analysis indicated that the deletion has very little effect on the activity of the receptor (Rochel et al., 2001). The authors therefore argued that this

deletion is unlikely to cause any significant perturbations in the overall three dimensional structure of the protein, and that the conclusions drawn about the complex formation between $1,25(\text{OH})_2\text{D}_3$ and the ligand-binding domain of the $\text{VDR}_{\Delta 165-215}$ mutant are fully applicable to the wild-type VDR-LBD.

One of the important conclusions of the study by Rochel and co-workers is that amino acids tyrosine 143 and serine 278 are in direct contact with the 3-hydroxyl group of the bound ligand. These conclusions were surprising as no previous functional studies of VDR mutants have identified residues Y143 and S278 as important for ligand binding (Choi et al., 2001). To clarify this issue two single-point mutations (Y143F and S278A) and a double-point mutation (Y143/S278) were introduced in the full-length receptor protein but no significant differences were found in the binding affinities of any of these constructs and the wild-type VDR (Davis, 2002). There are several possible explanations for these results. It is known that the 3-hydroxyl group of $1,25(\text{OH})_2\text{D}_3$ is not as critical for high affinity binding to the receptor as the other two hydroxyl groups. This has been demonstrated by steroid competition binding assays with various ligand analogs that were missing one of the hydroxyl groups (Bouillon et al., 1995). Therefore, amino acid residues that interact with the 3-hydroxyl group may not contribute significantly to the overall binding and changing these residues may have little or no effect on the receptor binding affinity. Another possibility is that the 165-215 amino acid deletion has altered the binding pocket region that interacts with the 3-hydroxyl group of $1,25(\text{OH})_2\text{D}_3$, thus positioning different amino acid residues in proximity to the

3-hydroxyl group than in the full-length protein. Therefore, if the residues Y143 and S278 are substituted with non-polar amino acid residues in the deleted VDR, it might be expected that the binding affinity of the deletion mutants will decrease compared to the full-length constructs and the native protein. To investigate this possibility the exact same truncated version of VDR was constructed. In addition the single and double point mutations at the 143 and 278 sites were introduced in the deleted VDR.

Although this project appeared straightforward, it became soon clear that there were going to be significant challenges. To begin with, as no experimental details for the generation of the deletion mutant were available, a new method had to be developed for this project. The available starting material was a plasmid, designated pcVDR, containing a total of 6,200 base pairs. Of these, approximately 1,300 base pairs represent the entire coding sequence of VDR. At the DNA level, the deletion of 51 amino acids from this protein is equivalent to deleting 153 nucleotides from the total of 1,300 base pairs. Most importantly, these 153 amino acids had to be removed in such a way as to preserve the correct reading frame of the resulting deletion mutant.

Several methods for the introduction of such relatively large deletion mutation have been described. One of the simplest methods uses strategically located restriction enzyme recognition sites within the starting gene. If such sites are present, the desired fragment can be removed by treatment of the plasmid DNA with these restriction enzymes, followed by closure of the resulting gap with suitable linkers or adaptors to regenerate the correct reading frame. Such

conveniently located, unique restriction sites can be introduced into a gene of interest if it is generated by chemical synthesis, but such a situation occurs rarely in the case of native sequences, including the starting VDR cDNA. A version of the polymerase chain reaction known as inverse PCR, has been widely used to introduce such large deletions in plasmid DNA (Imai et al., 1991). Essentially, in this method the two PCR primers are designed to amplify the larger fragment of a circular plasmid DNA instead of the smaller. The smaller fragment is the fragment that is to be deleted. The primers hybridize in a tail-to-tail fashion at locations immediately adjacent to the sequence to be deleted. The resulting amplification product can then be re-circularized by enzymatic ligation, resulting in a product that has the desired sequence deleted. There are two problems with this approach. First, the relatively large size of the product makes the PCR amplification more difficult. A more critical problem is the low efficiency of the self-ligation of the resulting product, which is especially low in the case of a blunt-ended PCR product. This problem can be alleviated to some degree by the judicious introduction of strategic restriction enzyme sites via the PCR primers, followed by enzymatic digestion to generate sticky ends, and their ligation using suitable adapter molecules. Throughout the whole process, care has to be taken to ensure regeneration of the correct reading frame of the sequence.

A significant effort was invested in the inverse PCR method. Initially, an attempt was made to re-circularize the 6,050 base pair PCR product by blunt-end ligation. Various ligation conditions were tested at different amplicon concentrations and in the presence of compounds reported to enhance the

efficiency of blunt end ligations, such as polyethyleneglycol (PEG) (Pfeiffer and Zimmerman, 1983). All attempts to isolate recombinant colonies containing the desired ligation product after transformation of competent cells failed.

Next, the generation of complementary sticky ends at the PCR termini was attempted by introducing linker molecules having a *Bam*HI recognition site. However, the presence of an internal *Bam*HI site in the VDR sequence complicated this task and necessitated its protection from endonuclease digestion by methylation of the PCR product. The methylation of the internal *Bam*HI restriction site was followed by blunt-end ligation of *Bam*HI linkers and digestion of the resulting product with *Bam*HI restriction endonuclease. The desired DNA fragment was then gel-purified and recircularized by enzymatic ligation. Disappointingly, again no transformants containing the desired deletion were obtained.

The use of partially phosphorothioate-modified PCR primers and T7 gene 6 exonuclease hydrolysis was considered next as an alternative method for introducing complementary sticky ends at the termini of the PCR product. Phosphorothioate-modified residues are known to be resistant to various endo- and exonucleases (Eckstein and Gish, 1989). This property has been exploited in a method for the preparation of single-stranded PCR products by introducing phosphorothioate modifications at the 5' end of one of the PCR primers. These phosphorothioate residues become an integral part of the final PCR product. Treatment of this product with the enzyme T7 gene 6 exonuclease, a 5'-3' double-strand specific exonuclease, results in fast and quantitative hydrolysis of the

unmodified strand, leaving the strand carrying the phosphorothioate residues intact (Nikiforov et al., 1994).

For this project the method had to be modified further, as the goal was not to completely eliminate one of the strands of the PCR product but rather only to introduce short complementary overhangs at the 5' ends to facilitate the subsequent ligation step. As described in the Materials and Methods section and illustrated in Figure 23, both PCR primers were designed to incorporate four consecutive phosphorothioate residues located 12 bases away from the 5' ends of the primers. In addition, and this was extremely important for the regeneration of the correct reading frame after the subsequent ligation step, the outermost six bases of each PCR primer were designed to be complementary to the six bases at the 5' end of the opposite strand primer located immediately to the 5' end of its phosphorothioate residues. Thus, each primer initially carried an extension of six bases that would not hybridize to the target DNA. These extensions, however, assured that the 12 nucleotide long, single-stranded 3' tails obtained after the T7 gene 6 exonuclease treatment of the PCR product would be fully complementary and allow the self-circularization and ligation of the molecule (Figure 24).

Initially it was considered of significant importance that the enzymatic hydrolysis with T7 gene 6 exonuclease leaves a phosphate group attached to the 5' end of the remaining DNA sequence (Kerr and Sadowski, 1972), thus allowing subsequent ligation without the need for enzymatic phosphorylation. A ligation step was at first considered inevitable for regeneration of the circular DNA molecule. As it turned out, the ligation could be successfully omitted from the

procedure because the 12-base long, complementary overhangs generated by T7 gene 6 exonuclease digestion hybridize and form a nicked plasmid that can be used directly for transformation. Treatment of the PCR product with *DpnI* endonuclease, prior to the T7 gene 6 exonuclease digestion, was included to selectively hydrolyze the starting DNA template and reduce the transformation background. The overall method is very fast and efficient, requiring no buffer exchange or purification steps after the PCR amplification. This is a new and universally applicable technique for the generation of large deletion mutants from any plasmid DNA (Stoynova et al., 2004).

After generation of the four deletion mutants, their binding affinities were determined and compared to those of the wild-type VDR and to the full-length mutants (Acevedo et al., 2004). The K_D values for VDR $_{\Delta 165-215}$, VDR $_{\Delta 165-215}$ Y143F, and VDR $_{\Delta 165-215}$ S278A, determined by saturation binding assays, were not significantly different from the K_D values of the full-length mutants and the wild-type VDR. Only the double mutant containing the deletion, VDR $_{\Delta 165-215}$ Y143F/S278A, showed a slight decrease in binding affinity compared to both the deleted VDR $_{\Delta 165-215}$ and the wild-type VDR (Table 4). These results suggest that residues Y143 and S278 are not contributing significantly to the overall binding affinity of the receptor towards its cognate ligand 1,25(OH) $_2$ D $_3$. The decreased affinity of the double deleted mutant VDR $_{\Delta 165-215}$ Y143F/S278A for 1,25(OH) $_2$ D $_3$ compared to the full-length double mutant Y143F/S278A suggests the existence of subtle conformational differences between the full-length and the deleted VDR $_{\Delta 165-215}$. In addition, the crystal structure of VDR ligand-binding

domain indicated that several water molecules are present in the vicinity of the 1,25(OH)₂D₃ hormone A ring. These water molecules could substitute for the missing hydroxyl groups of tyrosine 143 and serine 278 in the mutant receptors by forming hydrogen bonds with the 3-hydroxyl group of the hormone. More detailed structural analysis studies are needed in order to distinguish between these two possibilities. For example, future work could use protease sensitivity assays to reveal conformational differences between the wild-type VDR and the mutant receptors. These assays are based on limited trypsin digestion of the receptor protein in the presence or absence of various concentrations of the hormone (Peleg et al., 1995). Changes in the conformation of the receptor protein might expose additional trypsin recognition sites on the protein surface, and the limited trypsin digestion then will result in protein fragments of different sizes.

The goal of the gene transactivation studies was to assess the ability of the VDR variants to activate the transcription of a receptor gene and to investigate if the deletion of 51 amino acids from the VDR-LBD had any impact on this most important function of the receptor. The results from these studies show that the $\Delta 165-215$ amino acid deletion does not significantly affect the ability of the receptor to induce gene transcription (Table 4). All deleted receptor variants, VDR $_{\Delta 165-215}$, VDR $_{\Delta 165-215}$ Y143F, VDR $_{\Delta 165-215}$ S278A, and VDR $_{\Delta 165-215}$ Y143F/S278A, showed similar transcription activation as their full-length counterparts, wild-type VDR, Y143F, S278A, and Y143F/S278A. However, the results of these transcriptional activation assays also demonstrate that both amino acid residues

tyrosine 143 and serine 278 are important for the proper function of the receptor. As seen from Table 4, both the deleted and full-length variants of VDR containing the single point mutation Y143F required higher doses of $1,25(\text{OH})_2\text{D}_3$ to reach maximum response compared to the wild-type VDR and VDR $_{\Delta 165-215}$. These results suggest that tyrosine 143 is important for gene activation in both the deleted and the wild-type VDR. On the other hand, the VDR variants containing only the S278A single point mutation did not show a significant difference in their ability to activate gene transcription than the wild-type protein. However, both the full-length and the deleted double mutants required even higher doses of the hormone to induce activation of gene transcription than the tyrosine 143 single mutants. These results indicate that serine 278 also likely plays some role in transactivation, albeit smaller than tyrosine 143.

In conclusion, this investigation on the possible effects of the 165-215 amino acid deletion on the binding affinity and the ability of the vitamin D_3 receptor to induce gene transcription showed that the removal of these 51 amino acids did not significantly affect the receptor functions. These results also hint that amino acid residues tyrosine 143 and serine 278, which according to the crystal structure are involved in direct hydrogen bond formation with the 3-hydroxyl group of $1,25(\text{OH})_2\text{D}_3$, are more important for activation of gene transcription than for ligand binding. More structural studies will be needed to clarify the role of these two amino acid residues in anchoring the ligand inside the hydrophobic pocket of the vitamin D_3 receptor ligand-binding domain.

6. CONCLUSIONS

The goal of this work was to investigate the structure–function relationship of the nuclear vitamin D₃ receptor. This is an extremely complex task and the present study succeeded in making some important contributions.

An easy, reliable, and straightforward screening assay for detecting the functional binding of the VDR-LBD with its cognate ligand was developed. This assay will be an important tool in future studies of the receptor protein by the novel method of directed evolution. This method typically generates large libraries of randomly mutated proteins, and the identification of variants with the desired activity becomes a critical step. The development and validation of the VDR screening assay is an important first step in the attempt to generate functional vitamin D₃ receptors with modified molecular recognition and binding selectivity for 25(OH)D₃ instead of 1,25(OH)₂D₃. Such studies are expected to provide new information for the function of the receptor protein at the molecular level, and in addition could prove useful in the treatment of various diseases related to 1,25(OH)₂D₃.

The role of a 51 amino acid fragment, between amino acid residues 165 and 215, on the function of the vitamin D₃ receptor was also examined. During this part of the work a new method for generating such a large deletion within the coding sequence of the protein was successfully developed. Functional studies of the deleted VDR_{Δ165-215} variants, with or without the additional point mutations Y143F and S278A, led to the conclusion that this region does not play a

substantial role in receptor function. Removal of these 51 amino acids ($\Delta 165-215$) did not significantly change the binding affinity of the deletion mutants compared to wild-type VDR. The amino acid residues tyrosine 143 and serine 278, based on the findings presented herein, seem to be more important for activation of gene transcription than for ligand binding. Surprisingly, substitution of these hydrophilic residues with non-polar amino acids (phenylalanine and alanine respectively) did not diminish the binding affinity of the receptor protein regardless of the presence or absence of the $\Delta 165-215$ amino acid deletion. However, water molecules present in the ligand-binding pocket might substitute for the missing polar amino acids and form hydrogen bonds with the 3-hydroxyl group of $1,25(\text{OH})_2\text{D}_3$. More structural data are needed to explain the exact role that tyrosine 143 and serine 278 play in ligand binding and transcriptional activation.

REFERENCES

- Acevedo, A., Stoyanova, L., Davis, K., Solórzano, R., Collins, E.D. (2004) Role of residues 143 and 278 of the human nuclear Vitamin D receptor in the full-length and Δ 165-215 deletion mutant. *J. Steroid Biochem. Mol. Biol.* 89-90, 83-87.
- Ausbel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., Eds. (1997) Detection of nucleic acids using absorption spectroscopy. In: *Short Protocols in Molecular Biology* (3rd edition), Wiley & Sons, Inc.: New York, pp A3/11-A3/12.
- Ausbel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., Eds. (2001) Introduction of restriction endonuclease sites by PCR. In: *Current Protocols in Molecular Biology*, Wiley & Sons, Inc.: New York, Vol. 1, pp 8.5.1-8.5.5.
- Baker, A.R., McDonnell, D.P., Hughes, M., Crisp, T.M., Mangelsdorf, D.J. Haussler, M.R., Pike, J.W., Shine, J., O'Malley, B.W. (1988) Cloning and expression of full-length cDNA encoding human vitamin D receptor. *Proc. Natl. Acad. Sci. USA* 85, 3294-3298.
- Baran, D.T., Quail, J.M., Rey, R., Leszyk, J., Honeyman, T. (2000) Annexin II is the membrane receptor that mediates the rapid actions of $1\alpha,25$ -dihydroxyvitamin D_3 . *J. Cell. Biochem.* 78, 34-46.
- Baran, D.T., Rey, R., Sorensen, A.M., Honeyman, T., Holick, M.F. (1994) Binding characteristics of a membrane receptor that recognizes $1\alpha,25$ -dihydroxyvitamin D_3 and its epimer, $1\alpha,25$ -dihydroxyvitamin D_3 . *J. Cell. Biochem.* 56, 510-517.
- Barsony, J., Pike, J.W., DeLuca, H.F., Marx, S.J. (1990) Immunocytology with microwave-fixed fibroblasts shows $1\alpha,25$ -dihydroxyvitamin D_3 -dependent rapid and estrogen-dependent slow reorganization of vitamin D receptors. *J. Cell Biol.* 111, 2385-2395.
- Beato, M. (1989) Gene regulation by steroid hormones. *Cell* 56, 335.
- Bouillon, R., Okamura W.H., Norman, A.W. (1995) Structure-function relationships in the vitamin D endocrine system. *Endocr. Rev.* 16, 200-257.
- Cadwell, R.C. and Joice, G.F. (1992) Randomization of genes by PCR mutagenesis. *PCR Methods Appl.* 2, 28-33.

- Cadwell, R.C. and Joice, G.F. (1994) Mutagenic PCR. *PCR Methods Appl.* 3, S136-S140.
- Caffrey, J.M., and Farach-Carson, M.C. (1989) Vitamin D₃ metabolites modulate dihydropyridine-sensitive calcium currents in clonal rat osteosarcoma cells. *J. Biol. Chem.* 264, 20265-20274.
- Cancela, L., Ishida, H., Bishop, J.E., Norman, A.W. (1992) Local chromatin changes accompany the expression of calbindin-D_{28k} gene: tissue specificity and effect of vitamin D activation. *Mol. Endocrinol.* 6, 468.
- Canterbury, J.M., Gavellas, G., Bourgoignie, J.J., Reise, E. (1980) Metabolic consequences of oral administration of 24R,25-dihydroxycholecalciferol to uremic dogs. *J. Clin. Invest.* 65, 571.
- Carlberg, C., Bendik, I., Wyss, A., Meier, E., Sturzenbecker, L.J., Grippo, J.F., Hunziker, W. (1993) Two nuclear signaling pathways for vitamin D. *Nature* 361 657-660.
- Cato, A.C., Nestl, A., Mink, S. (2002) Rapid actions of steroid receptors in cellular signaling pathways. *Science STKE* 138, RE9.
- Chambliss, K.L., Yuhanna, I.S., Anderson, R.G., Mendelsohn, M.E., Shaul, P.W. (2002) ER-beta has nongenomic action in caveolae. *Mol. Endocrinol.* 16, 938-946.
- Chen, D., Ma, H., Hong, H., Koh, S.S., Huang, S.-M., Schurter, B.T., Aswad, D.W., Stallcup, M.R. (1999) Regulation of transcription by a protein methyltransferase. *Science* 284, 2174-2177.
- Chen, H., Lin, R.J., Xie, W., Wilpitz, D., Evans, R.M. (1999) Regulation of hormone-induced histone hyperacetylation and gene activation via acetylation of an acetylase. *Cell*, 98, 675-686.
- Chien, C.T., Bartel, P.L., Sternglanz, R., Fields, S. (1991) The two-hybrid system: A method to identify and clone genes for proteins that interact with a protein of interest. *Proc Nat. Acad. Sci. USA* 88, 9578-9582.
- Choi, M., Yamamoto, K., Masuno, H., Nakashima, K., Taga, T., Yamada, S. (2001) Ligand recognition by the vitamin D receptor. *Bioorg. Med. Chem.* 9, 1721-1730.
- Cirino, P.C., Mayer, K.M., Umeno, D. (2003) Generating mutant libraries using error-prone PCR. *Methods Mol. Biol.* 231, 3-9.

Collins, E.D. and Norman, A.W. (2001) Vitamin D. In: *Handbook of Vitamins* (3rd edition), Rucker, R., Suttie, J., McCormick, D., Machlin, L, Eds., Marcel Dekker, Inc.: New York, pp 51-113.

Collins, E.D. (2000) Molecular biology of the vitamin D endocrine system. *Recent Res. Devel. Steroid Biochem. Mol. Biol.* 1, 81-106.

Cramer, A., Whitehorn, E.A., Tate, E., Stemmer, W.P.C. (1996) Improved Green Fluorescent Protein by molecular evolution using DNA shuffling. *Nature Biotech.* 14, 315-319.

Cramer, A., Raillard, S.-A., Bermudez, E., Stemmer, W.P.C. (1998) DNA shuffling of a family of genes from diverse species accelerates directed evolution. *Nature* 39, 288-291.

Crowfoot, D. and Dunitz, J.D. (1948) Structure of calciferol. *Nature* 162, 608.

Davis, K. (2002) A study of amino acids 143 and 278 in the vitamin D nuclear receptor. *Master of Science Thesis*, San Jose State University, San Jose, CA, 43-61.

DeLuca, H.F. (1979) The vitamin D system in the regulation of calcium and phosphorus metabolism. *Nutr. Reviews* 37, 161-193.

Eckstein, F. and Gish, G. (1989) Phosphorothioates in molecular biology. *Trends Biochem. Sci.* 14, 97-100.

Evans, R.A., Hills, E., Wong, S.Y.P., Dunstan, C.R., Norman, A.W. (1982) The use of 24,25-dihydroxycholecalciferol alone and in combination with 1,25-dihydroxycholecalciferol in chronic renal failure. In: *Vitamin D: Chemical, Biochemical and Clinical Endocrinology of Calcium Metabolism*, Norman, A.W., Schaefer, K., Grigoleit, H.-G., and Herrath, D.V., Eds., Walter de Gruyter and Company: Berlin, pp 835-840.

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

Farach-Carson, M.C., Sergeev, I.N., Norman, A.W. (1991) Non-genomic actions of 1,25-dihydroxyvitamin D₃ in rat osteosarcoma cells: structure-function studies using ligand analogs. *Endocrinology* 129, 1876-1884.

Fields, S. and Song, O. (1989) A novel genetic system to detect protein-protein interactions. *Nature* 340, 245-247.

Fields, S. and Sternglanz, R. (1994) The two-hybrid system: an assay for protein-protein interactions. *Trends Genet.* 10, 286-292.

- Fritz, C.C. and Green, M.R. (1992) Fishing for proteins. *Current Biol.* 2, 403-405.
- Fu, M., Wang, C., Reutens, A.T., Wang, J., Angeletti, R.H., Siconolfi-Baez, L., Ogryzko, V., Avantaggiati, M.L., Pestell, R.G. (2000) *J. Biol. Chem.* 275, 20853-20860.
- Giguere, V., Ong E.S., Segui, P., Evans, R.M. (1987) Identification of a receptor for the morphogen retinoic acid. *Nature* 330, 624-629.
- Giver, L., Gershenson, A., Freskgard, P.-O., Arnold, F.H. (1998) Directed evolution of a thermostable esterase. *Proc. Natl. Acad. Sci. USA* 95, 12809-12813.
- Green, S. and Chambon, P. (1988) Nuclear receptors enhance our understanding of transcription regulation. *Trends Genet.* 4, 309.
- Grippo, J.F., Hunziker, W. (1993) Two nuclear signaling pathways for vitamin D. *Nature* 361, 657-660.
- Ham, J. and Parker, M.G. (1989) Regulation of gene expression by nuclear hormone receptors. *Curr. Opin. Cell. Biol.* 1, 503-511.
- Hannah, S.S. and Norman, A.W. (1994) 1 α ,25(OH) $_2$ -vitamin D $_3$ regulated expression of the eukaryotic genome. *Nutr. Reviews* 52, 376-382.
- Haussler, M.R., Haussler, C.A., Jurutka, P.W., Hsieh, J.-C., Remus, L.S., Selznick, S.H., Whitfield, G.K. (1997) The vitamin D hormone and its nuclear receptor: molecular actions and disease states. *J. Endocrinol.* 154, S57-S73.
- Haussler, M.R., Thomson, W.W., Norman, A.W. (1969) The isolation of purified nuclear and chromatin fractions from chick intestinal mucosa. *Exp. Cell Res.* 58, 234-242.
- Haussler, M.R., Whitfield, G.K., Haussler, C.A., Hsieh, J.-C., Thompson, P.D., Selznick, S.H., Dominguez, C.E., Jurutka, P.W. (1998) The nuclear vitamin D receptor: biological and molecular properties revealed. *J. Bone Min. Res.* 13, 325-349.
- Henry, H.L. (1992) Vitamin D hydroxylases. *J. Cell Biochem.* 49, 4-9.
- Henry, H.L., Dutta, C., Cunningham N., Blanchard, R., Penny, R., Tang, C., Marchetto, G., Chou S.-Y. (1992) The cellular and molecular regulation of 1,25(OH) $_2$ D $_3$ production. *J. Steroid Biochem. Mol. Biol.* 41, 401-407.

Henry, H.L. and Norman, A.W. (1974) Studies of calciferol metabolism. IX. Renal 25-hydroxyvitamin D₃-1-hydroxylase. Involvement of cytochrome P-450 and other properties. *J. Cell Chem.* 249, 7529-7535.

Henry, H.L., Taylor, A.N., Norman, A.W. (1977) Response of chick parathyroid glands to the vitamin D metabolites, 1,25-dihydroxycholecalciferol and 24,25-dihydroxycholecalciferol. *J. Nutr.* 107, 1918.

Hodgkin, D.C., Rimmer, B.M., Dunitz, J.D., Trueblood, K.N. (1963) The crystal structure of a calciferol derivative. *J. Chem. Soc.* 947, 4945.

Holick, M.F. (1981) The cutaneous photosynthesis of previtamin D₃: a unique photoendocrine system. *J. Invest. Dermatol.* 76, 51.

Hong, H., Kohli, K., Garabedian, M.J., Stallcup, M.R. (1997) GRIP1, a transcriptional coactivator for the AF-2 transactivation domain of steroid, thyroid, retinoid, and vitamin D receptors. *Mol. Cell Biol.* 17, 2735-2744.

Imai, Y., Matsushima, Y., Sugimura, T., Terada, M. (1991) A simple and rapid method for generating a deletion by PCR. *Nucleic Acids Res.* 19, 2785.

Ishizuka, S., Ohba, T., Norman, A.W. (1988) 1 α ,25(OH)₂D₃-26,23-Lactone is a major metabolite of 1 α ,25(OH)₂D₃ under physiological conditions. In: *Vitamin D: Molecular, Cellular and Clinical Endocrinology*, Norman, A.W., Schaefer, K., Griggolet, H.-G., and Herrath, D.V., Eds., Walter de Gruyter & Co.: Berlin, pp 143-144.

Jurutka, P.W., Whitfield, G.K., Hsieh, J.C., Thompson, P.D., Haussler, C.A., Haussler, M.R. (2001) Molecular nature of the vitamin D receptor and its role in regulation of gene expression. *Rev. Endocr. Metab. Disord.* 2, 203-216.

Kerr, C., and Sadowski, P.D. (1972) Gene 6 exonuclease of bacteriophage T7. II. Mechanism of the reaction. *J. Biol. Chem.* 247, 311-318.

Kingston, R.E. (1999) A shared but complex bridge. *Nature* 399, 199-200.

Kong, X., Liu, Y., Gou, X., Zhu, S. Zhang, X.W., Zhang, J. (2001) Directed evolution of α -aspartyl dipeptidase from *Salmonella typhimurium*. *Biochem. Biophys. Res. Comm.* 289, 137-142.

Lavinski, R.M., Jespen, K., Heinzl, T., Torchia, J., Mullen, T.M., Schiff, R., Del-Rio, A.L., Ricote, M., Ngo, S., Gemsch, S.G., Hilsenbeck, C.K., Osborne, C.K., Glass, C.K., Rosenfeld, M.G., Rose, D.W. (1998) Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. *Proc. Nat. Acad. Sci. USA* 95, 2920-2925.

Leung, D.W., Chen, E., Goeddel, D.V. (1989) A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. *Technique 1*, 11-15.

Lieberherr, M. (1987) Effects of vitamin D₃ metabolites on cytosolic free calcium in confluent mouse osteoblasts. *J. Biol. Chem.* 262, 13168-13173.

Lidor, C., Atkin, I., Ornoy, A., Dekel, S., Edelstein, S. (1987a) Healing of rachitic lesions in chicks by 24R,25-dihydroxycholecalciferol administered locally into bone. *J. Bone Miner. Res.* 2, 91.

Lidor, C., Dekel, S., Edelstein, S. (1987b) The metabolism of vitamin D₃ during fracture-healing in chicks. *Endocrinol.* 120, 389.

Lowe K.E., Maiyar, A.C., Norman, A.W. (1992) Vitamin D-mediated gene expression. *Crit. Rev. Eukar. Gene Exp.* 2, 65-109.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P. Schütz, G. Umesono, K. Blumberg, B. Kastner, P., Mark, M., Chambon, P., Evans, R.M. (1995) The nuclear receptor superfamily: The second decade. *Cell* 83, 835-839.

Mannervik, M., Nibu, Y., Zhang, H., Levine, M. (1999) Transcriptional coregulators in development. *Science* 284, 606-609.

Marks, S.C., Jr. and Hermey, D.C. (1996) The structure and development of bone. In: *Principles of Bone Biology*, Bilezikian, J.P., Raisz, L.G., Rodan, G.A., Eds., Academic Press: San Diego, CA, pp 3-15.

Matsuyama, H, Jefcoat, S.C. Jr., Macdonald, P.N. (1997) The N-terminal domain of transcription factor IIB is required for direct interaction with the vitamin D receptor and participates in vitamin D-mediated transcription. *Mol. Endocrinol.* 11, 218-228.

Mawer, E.B. (1980) Clinical implication of measurements of circulating vitamin D metabolites. *J. Clin. Endocrinol. Metab.* 9, 63.

McCutchan, J.H. and Pagano, J.S. (1968) Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. *J. Natl. Cancer Inst.* 41, 351-357.

Moore, J.C., Jin, H.-M., Kuchner, O., Arnold, F.H. (1997) Strategies for the *in vitro* evolution of protein function: enzyme evolution by random recombination of improved sequences. *J. Mol. Biol.* 272, 336-347.

Moras, D. and Gronemeyer, H. (1998) The nuclear receptor ligand-binding domain: structure and function. *Curr. Opin. Cell. Biol.* 10, 384-391.

Narlikar, G.J., Fan, H.Y., Kingston, R.E. (2002) Cooperation between complexes that regulate chromatin structure and transcription. *Cell* 108, 475-487.

National Center for Biological Information, BLAST, Blast 2 Sequences.
<http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html> (accessed in 2003)

Nelson, M. and McClelland, M. (1992) Use of DNA methyltransferase/endonuclease enzyme combinations for megabase mapping of chromosomes. *Methods Enzymol.* 216, 279-303.

Nemere, I., Dormanen, M.C., Hammond, Okamura, W.H., Norman, A.W. (1994) Identification of specific binding protein for $1\alpha,25$ -dihydroxyvitamin D_3 in basal-lateral membranes of chick intestinal epithelium and relationship to transcalcitachia. *J. Biol. Chem.* 269, 23750-23756.

Nemere, I., Farach-Carson, M.C., Rohe, B., Sterling, T.M., Norman, A.W., Boyan, B.D., Safford, S.E. (2004) Ribozyme knockdown functionally links a $1,25(OH)_2D_3$ membrane binding protein ($1,25D_3$ -MARS) and phosphate uptake in intestinal cells. *Proc. Natl. Acad. Sci. USA* 101, 7392-7397.

Nemere, I. and Norman, A.W. (1987) Studies on the mode of action of calciferol. LII. Rapid action of $1,25$ -dihydroxyvitamin D_3 on calcium transport in perfused chick duodenum: effect of inhibitors. *J. Bone Miner. Res.* 2, 99-107.

Nemere, I., Zhou L.-X., Norman, A.W. (1993) Non-transcriptional effects of steroid hormones. *Receptor* 32, 277-291.

Neylon, C. (2004) Chemical and biochemical strategies for the randomization of protein encoding DNA sequences: library construction methods for directed evolution. *Nucleic Acids Res.* 32, 1448-1459.

Nikiforov, T.T., Rendle, R.B., Kotewicz, M.L, Rogers, Y.-H. (1994) The use of phosphorothioate primers and exonuclease hydrolysis for the preparation of single-stranded PCR products and their detection by solid-phase hybridization. *PCR Methods Appl.* 3, 285-291.

Norman, A.W., Adams, D., Collins E.D., Okamura, W.H., Fletterick, R.J. (1999) Three-dimensional model of the ligand-binding domain of the nuclear receptor for $1\alpha,25$ -dihydroxyvitamin D_3 . *J. Cell. Biochem.* 74, 323-333.

Norman, A.W., and Litwack, G. (1997a) Steroid Hormones: Chemistry, biosynthesis, and metabolism. In: *Hormones* (2nd edition), Academic Press: San Diego, CA, pp 49-85.

Norman, A.W., and Litwack, G. (1997b) Calcium-regulating hormones: vitamin D, parathyroid hormone, and calcitonin. In: *Hormones* (2nd edition), Academic Press: San Diego, CA, pp 251-279.

Norman, A.W., Xu J., Collins E.D. (2002) The vitamin D endocrine system: metabolism, mode of action, and genetic evaluation. In: *Genetics in Endocrinology*, Baxter, J.D., Ed., Lippincott Williams & Wilkins, pp 445-472.

Ogryzko, V.V., Schiltz, R.L., Russanova, V., Howard, B.H., Nakatani, Y. (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* 87, 953-959.

Okamura, W.H., Norman, A.W., Wing, R.M. (1974) Vitamin D: concerning the relationship between molecular topology and biological function. *Proc. Natl. Acad. Sci. USA* 71, 4194-4197.

Onate, S.A., Boonyaratanakornkit, V., Spencer, T.E., Tsai, S.Y., Tsai, M.J., Edwards, D.P., O'Malley, B.W. (1998) The steroid receptor coactivator-1 contains multiple receptor interacting and activation domains that cooperatively enhance the activation function 1 (AF-1) and AF2 domains of steroid receptors. *J. Biol. Chem.* 273, 12101-12108.

Peleg, S., Sastry, M., Collins, E.D., Bishop, J.E., Norman, A.W. (1995) Distinct conformational changes induced by 20-epi analogs of 1 α ,25-dihydroxyvitamin D₃ are associated with enhanced activation of the vitamin D receptor. *J. Biol. Chem.* 270, 10551-10558.

Petkovich, M., Brand, N.J., Krust, A., Chambon, P. (1987) A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature* 330, 444-450.

Pfeiffer, B.H., and Zimmerman, S.R. (1983) Polymer stimulated ligation: enhanced blunt- or cohesive-end ligation of DNA or deoxyribonucleotides by T4DNA ligase in polymer solutions. *Nucleic Acids Res.* 11, 7853-7871.

Racz, A. and Barsony, J. (1999) Hormone-dependent translocation of vitamin D receptors is linked to transactivation. *J. Biol. Chem.* 274, 19352-19360.

Rachez, C., Lemon, B.D., Suldan, Z., Bromleigh, V., Gamble, M., Naar, A.M., Erdjument-Bromage, H., Tempst, P., Freedman, L.P. (1999) Ligand-dependent transcription activation by nuclear receptors requires the DRIP complex. *Nature* 398, 824-828.

- Rachez, C. and Freedman, L.P. (2000) Mechanisms of gene regulation by vitamin D₃ receptor: a network of coactivator interactions. *Gene* 246, 9-21.
- Rachez, C., Gamble, M., Chang, C.-P.B., Atkins, G.B., Lazar, M.A., Freedman, L.P. (2000) The DRIP complex and SCR-1/p160 coactivators share similar nuclear receptor binding determinants but constitute functionally distinct complexes. *Mol. Cell. Biol.* 20, 2718-2726.
- Reichel, H., Koeffler, H.P., Norman, A.W. (1989) The role of the vitamin D endocrine system in health and disease. *New Engl. J. Med.* 320, 980.
- Rochel, N., Tocchini-Valentini, G., Egea, P.F., Juntunen, K., Garnier, J.-M., Vihko, P., Moras, D. (2001) Functional and structural characterization of the insertion region in the ligand binding domain of the vitamin D nuclear receptor. *Eur. J. Biochem.* 268, 971-979.
- Rochel, N., Wurtz, J.M., Mitschler, A., Klaholz, B., Moras, D. (2000) The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand. *Moll. Cell* 5, 173-179.
- Rochel, N., Wurtz, J.M., Mitschler, A., Klaholz, B., Moras, D. NCBI MMDB Structure Summary: Crystal structure of the vitamin D receptor complexed to vitamin D. <http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?form=6&db=t&Dopt=s&uid=12831> (accessed in June, 2004).
- Sambrook, J., Fritsch, E.F., Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (2nd edition), Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, Vol. 3, pp E3-E5.
- Schmidt, A., Endo, N., Rutledge, S.J., Vogel, R., Shinar, D., Rodan, G.A. (1992) Identification of a new member of the steroid receptor superfamily that is activated by a peroxisome proliferator and fatty acids. *Mol. Endocrinol.* 6, 1634-1641.
- Shafikhani, S., Siegel, R.A., Ferrari, E. Schellenberger, V. (1997) Generation of large libraries of random mutants in *Bacillus subtilis* by PCR-based plasmid multimerization. *BioTechniques* 23, 304-310.
- Song, X., Bishop, J.E, Okamura, W.H., Norman, A.W. (1998) Stimulation of phosphorylation of mitogen-activated protein kinase by 1 α ,25-dihydroxyvitamin D₃ in promyelocytic NB4 leukemia cells: a structure-function study. *Endocrinology* 139, 457-465.

- Stemmer, W.P.C. (1993) DNA shuffling by random fragmentation and reassembly: *In vitro* recombination for molecular evolution. *Proc. Natl. Acad. Sci. USA* 91, 10747-10751.
- Stemmer, W.P.C. (1994) Rapid evolution in a protein *in vitro* by DNA shuffling. *Nature* 370, 389-391.
- Stoynova, L., Solórzano, R., Collins, E.D. (2004) Generation of large deletion mutants from plasmid DNA. *BioTechniques* 36, 402-408.
- Suzuki, M., Christians, F.C., Kim, B., Skandalis, A., Black, M.E., Loeb, L.A. (1996) Tolerance of different proteins for amino acid diversity. *Mol. Divers.* 2, 111-118.
- Torchia, J., Rose, D.W., Inostroza, J., Kamei, Y., Westin, S., Glass, C.K., Rosenfeld, M.G. (1997) The transcriptional co-activator p/CIP binds CBP and mediates nuclear receptor function. *Nature* 387, 677-684.
- Tucker, G., Gagnon, R.E., Haussler, M.R. (1973) Vitamin D₃-25-hydroxylase: tissue occurrence and apparent lack of regulation. *Arch. Biochem. Biophys.* 155, 47.
- Underwood, J.L., and DeLuca, H.F. (1984) Vitamin D is not directly necessary for bone growth and mineralization. *Am. J. Physiol.* 246, E493-E498.
- Vartanian, J.P., Henry, M., Wain-Hobson, S. (1996) Hypermutagenic PCR involving all four transitions and a sizeable proportion of transversions. *Nucleic Acids Res.* 24, 2627-2631.
- Voegel, J.J., Heine, M.J.S., Zechel, C., Chambon, P., Gronemeyer, H. (1996) TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. *EMBO J.* 15, 3667-3675.
- Walters, M.R. (1992) Newly identified actions of the vitamin D endocrine system. *Endocr. Rev.* 13, 719-764.
- Walters, M.R. (1995) Newly identified effects of the vitamin D endocrine system: update 1995. *Endocr. Rev.* 4, 47-56.
- Wecksler, W.R and Norman, A.W. (1979) Studies on the mode of action of calciferol XV: An hydroxylapatite batch assay for the quantitation of 1 α ,25-dihydroxyvitamin D₃-receptor complexes. *Anal. Biochem.* 92, 314-323.
- Wecksler, W.R and Norman, A.W. (1980) A kinetic and equilibrium binding study of 1 α ,25-dihydroxyvitamin D₃ with its cytosol receptor from chick intestinal mucosa. *J. Biol. Chem.* 255, 3571.

Wecksler, W.R., Ross, F.P., Mason, R.S., Posen, S., Norman, A.W. (1980a) Studies of the mode of action of calciferol. XXIV. Biochemical properties of the $1\alpha,25$ -dihydroxyvitamin D_3 cytoplasmic receptors from human and chick parathyroid glands. *Arch. Biochem. Biophys.* 201, 95.

Wecksler, W.R., Ross, F.P., Mason, R.S., Norman, A.W. (1980b) Studies of the mode of action of calciferol. XXI. Biochemical properties of the $1\alpha,25$ -dihydroxyvitamin D_3 cytosol receptors from human and chicken intestinal mucosa. *J. Clin. Endocr. Metab.* 50, 152.

Weinberger, C., Thompson, C.C., Ong, E.S., Lebo, R., Gruol, D.J., Evans, R.M. (1986) The c-erb-A gene encodes a thyroid hormone receptor. *Nature* 324, 641-646.

Weiner, M.P., Costa, G.L., Schoettlin, W., Cline, J., Mathur, E., Bauer, J.C. (1994) Site-directed mutagenesis of double-stranded DNA by the polymerase chain reaction. *Gene* 151, 119-123.

Wurtz, J-M., Guillot, B., Moras, D. (1997) 3D model of the ligand binding domain of the vitamin D nuclear receptor based on the crystal structure of holo RAR gamma. In: *Vitamin D: Chemistry, Biology and Clinical Applications of the Steroid Hormone*, Norman, A.W., Bouillon, R., Thomasset, M., Eds.; University of California - Riverside Printing and Reprographics: Riverside, CA, pp 165-172.

Yamato, H., Okazaki, R., Ishii, T., Ogata, E., Sato, T. Kumegawa, M., Akaogi, K., Taniguchi, N., Matsumoto, T. (1993) Effect of $24R,25$ -dihydroxyvitamin D_3 on the formation and function of osteoclastic cells. *Calcif. Tissue Int.* 52, 255.

Zaccolo, M., Williams, D.M., Brown, D.M., Gheradi, E. (1996) An approach to random mutagenesis of DNA using mixtures of triphosphate derivatives of nucleoside analogs. *J. Mol. Biol.* 255, 589-603.

Zanello, L. and Norman, A.W. (1997) Stimulation by $1\alpha,25(OH)_2$ -vitamin D_3 of whole cell chloride currents in osteoblastic ROS 17/2.8 cells. A structure-function study. *J. Biol. Chem.* 272, 22617-22622.

Zanello, L. and Norman, A.W. (2004) Rapid modulation of osteoblast ion channel responses by $1\alpha,25(OH)_2$ -vitamin D_3 requires the presence of a functional vitamin D nuclear receptor. *Proc. Nat. Acad. Sci. USA* 101, 1589-1594.

Zhang, J.-H., Dawest, G., Stemmer, W.P.C. (1997) Directed evolution of a fucosidase from a galactosidase by DNA shuffling and screening. *Proc. Natl. Acad. Sci. USA* 94, 4504-4509.

Zhao, H. and Arnold, F.H. (1999) Directed evolution converts subtilisin E into a functional equivalent of thermitase. *Protein Eng.* 12, 47-53.

Zhou L.-X., Nemere, I., Norman, A.W. (1992) 1,25(OH)₂-vitamin D₃ analog structure-function assessment of the rapid stimulation of intestinal calcium absorption (transcaltachia). *J. Bone Miner. Res.* 7, 457-463.

APPENDICES

Appendix A. List of Abbreviations

A	Adenine
AD	Activation Domain
AF	Activation Function
ATP	Adenosine Triphosphate
bp	base pair
C	Cytosine
C_i	Curies
cDNA	Complementary Deoxyribonucleic Acid
CTP	Cytidine Triphosphate
DBD	DNA Binding Domain
DBP	DNA Binding Protein
DMEM	Dulbecco's Modification of Eagle's Medium
DNA	Deoxyribonucleic Acid
DPM	Disintegrations per Minute
DTT	Dithiothreitol
EC₅₀	Effective Concentration at Half Maximal Response
EDTA	Ethylene Diamine Tetraacetic Acid
G	Guanine
GTP	Guanosine Triphosphate
FBS	Fetal Bovine Serum
LBD	Ligand Binding Domain

K_d	Equilibrium Dissociation Constant
Kb	Kilobase
MCS	Multiple Cloning Site
NCS	Newborn Calf Serum
dNTP	Deoxy-Nucleoside Triphosphate
ddNTP	Dideoxy-Nucleoside Triphosphate
PBS	Phosphate Buffered saline
PTH	Parathyroid Hormone
PMT	Photomultiplier Tube
RCI	Relative Competitive Index
RLU	Relative Light Units
RAR	Retinoic Acid receptor
RXR	Retinoic X Receptor
T	Thymine
TED	Tris-hydroxymethyl aminomethane; EDTA; DTT.
dTTP	Deoxythymidine Triphosphate
TR	Thyroid Receptor
U	Uracil
VDR	Vitamin D Receptor
VDRE	Vitamin D Response Element
w.t.	Wild-type

Appendix B. Single Letter Abbreviations for Amino Acids

A	Alanine	M	Methionine
C	Cysteine	N	Asparagine
D	Aspartic Acid	P	Proline
E	Glutamic Acid	Q	Glutamine
F	Phenylalanine	R	Arginine
G	Glycine	S	Serine
H	Histidine	T	Threonine
I	Isoleucine	V	Valine
K	Lysine	W	Tryptophan
L	Leucine	Y	Tyrosine

Appendix C. Lambda DNA (*Hind*III/*Eco*RI) Markers (Promega)

*Separation of Lambda DNA (*Hind*III/*Eco*RI) fragments on 1.2 % agarose gel in 1x TAE buffer and 0.005 % Ethidium Bromide.*

Appendix D. Nucleotide and amino acid sequence of human VDR

(National Center for Biological Information, <http://www.ncbi.nlm.nih.gov>, accession number J03258)

* VDR nucleotide sequence is in black, corresponding amino acid sequence is in blue; start and stop codons are designated in pink and underlined.

* Highlighted regions correspond to the sequence of PCR primers used for amplification of VDR-LBD. Nucleotides denoted in red are substituted in the primers to introduce suitable restriction sites.

```

1 - GAACAGCTTGTCCACCCGCCGCCGCCGACCAGAAGCCTTTGGGTCTGAAGTGTCTGTGAGA - 60
-
61 - CCTCACAGAAGAGCACCCCTGGGCTCCACTTACCTGCCCCCTGCTCCTTCAGGGAGGAG - 120
- M E 2
121 - GCAATGGCGGCCAGCACTTCCCTGCCTGACCTGGAGACTTTGACCGGAACGTGCCCCGG - 180
- A M A A S T S L P D P G D F D R N V P R 22
181 - ATCTGTGGGGTGTGTGGAGACCGAGCCACTGGCTTTTCACTTCAATGCTATGACCTGTGAA - 240
- I C G V C G D R A T G F H F N A M T C E 42
241 - GGCTGCAAAGGCTTCTTCAGGCGAAGCATGAAGCGGAAGGCACTATTCACCTGCCCCCTTC - 300
- G C K G F F R R S M K R K A L F T C P F 62
301 - AACGGGGACTGCCGCATCACCAAGGACAACCGACGCCACTGCCAGGCCTGCCGGGCTCAAA - 360
- N G D C R I T K D N R R H C Q A C R L K 82
361 - CGCTGTGTGGACATCGGCATGATGAAGGAGTTCATTCTGACAGATGAGGAAGTGCAGAGG - 420
- R C V D I G M M K E F I L T D E E V Q R 102
421 - AAGCGGGAGATGATCCTGAAGCGGAAGGAGGAGGAGGCCTGAAGGACAGTCTGCCGCCC - 480
- K R E M I L K R K E E A L K D S L R P 122
481 - AAGCTGTCTGAGGAGCAGCAGCGCATCATTGCCATACTGCTGGACGCCACCATAAGACC - 540
- K L S E E Q Q R I I A I L L D A H H K T 142
541 - TACGACCCACCTACTCCGACTTCTGCCAGTTCGGGCCTCCAGTTCGTGTGAATGATGGT - 600
- Y D P T Y S D F C Q F P P V R V N D G 162
601 - GGAGGGAGCCATCCTTCCAGGCCCACTCCAGACACACTCCCAGCTTCTCTGGGGACTCC - 660
- G G S H P S R P N S R H T P S F S G D S 182
661 - TCCTCCTCCTGCTCAGATCACTGTATCACCTCTTCAGACATGATGGACTCGTCCAGCTTC - 720
- S S C S D H C I T S S D M M D S S S F 202
721 - TCCAATCTGGATCTGAGTGAAGAAGATTGAGATGACCCTTCTGTGACCCTAGAGCTGTCC - 780
- S N L D L S E E D S D D P S V T L E L S 222
781 - CAGTCTCCATGCTGCCCCACCTGGCTGACCTGGTCAGTTACAGCATCCAAAAGGTCATT - 840
- Q L S M L P H L A D L V S Y S I Q K V I 242
841 - GGCTTTGCTAAGATGATACCAAGATTGAGAGACCTCACCTCTGAGGACCAGATCGTACTG - 900
- G F A K M I P G F R D L T S E D Q I V L 262
901 - CTGAAGTCAAGTGCCATTGAGGTTCATCATGTTGCGCTCCAATGAGTCCTTACCATGGAC - 960
- L K S S A I E V I M L R S N E S F T M D 282
961 - GACATGTCCTGGACCTGTGGCAACCAAGACTACAAGTACCGCGTCAGTGACGTGACCAAA - 1020
- D M S W T C G N Q D Y K Y R V S D V T K 302
1021 - GCCGGACACAGCCTGGAGCTGATTGAGCCCCCTCATCAAGTTCCAGGTGGGACTGAAGAAG - 1080
- A G H S L E L I E P L I K F Q V G L K K 322
1081 - CTGAACCTGCATGAGGAGGAGCATGTCCTGCTCATGGCCATCTGCATCGTCTCCCCAGAT - 1140
- L N L H E E E H V L L M A I C I V S P D 342
1141 - CGTCCTGGGGTGCAGGACGCCGCCGCTGATTGAGGCCATCCAGGACCGCCTGTCCAACACA - 1200
- R P G V Q D A A L I E A I Q D R L S N T 362
1201 - CTGCAGACGTACATCCGCTGCCGCCACCGCCCCCGGGCAGCCACCTGCTCTATGCCAAG - 1260
- L Q T Y I R C R H P P P G S H L L Y A K 382
1261 - ATGATCCAGAAGCTAGCCGACCTGCGCAGCCTCAATGAGGAGCACTCCAAGCAGTACCGC - 1320
- M I Q K L A D L R L N E E H S K Q Y R 402
1321 - TGCCTCTCCTTCCAGCCTGAGTGCAGCATGAAGCTAACGCCCTTGTGCTCGAAGTGTTT - 1380
- C L S F Q P E C S M K L T P L V L E V F 422
1381 - GGCAATGAGATCTCCCTAGGACAGCCTGTGCGGTGCCTGGGTGGGGCTGCTCCTCCA - 1440
- G N E I S 427
1441 - GGGCCACG .....

```


Appendix E. Deletion and point mutations within the VDR sequence

Sequence of wild-type VDR, accession number J03258 (National Center for Biological Information, <http://www.ncbi.nlm.nih.gov>)

- * The start and stop codons are shown in pink and underlined.
- * The nucleotide sequence of region to be deleted is in green.
- * The codons for Y143 and S278 amino acid residues to be mutated are in red.

```

1 - GAACAGCTTGTCCACCCGCCGGCCGGACCAGAAGCCTTTGGGTCTGAAGTGTCTGTGAGA - 60
-
61 - CCTCACAGAAGAGCACCCCTGGGCTCCACTTACCTGCCCCCTGCTCCTTCAGGGAGGAG - 120
- M E 2
121 - GCAATGGCGGCCAGCACTTCCCTGCCTGACCTGGAGACTTTGACCGGAACGTGCCCGG - 180
- A M A A S T S L P D P G D F D R N V P R 22
181 - ATCTGTGGGGTGTGTGGAGACCGAGCCACTGGCTTTTCACTTCAATGCTATGACCTGTGAA - 240
- I C G V C G D R A T G F H F N A M T C E 42
241 - GGCTGCAAAGGCTTCTTCAGGCGAAGCATGAAGCGGAAGGCACTATTACCTGCCCTTC - 300
- G C K G F F R R S M K R K A L F T C P F 62
301 - AACGGGGACTGCCGCATCACCAAGGACAACCGACGCCACTGCCAGGCCTGCCGGCTCAAA - 360
- N G D C R I T K D N R R H C Q A C R L K 82
361 - CGCTGTGTGGACATCGGCATGATGAAGGAGTTCACTCTGACAGATGAGGAAGTGCAGAGG - 420
- R C V D I G M M K E F I L T D E E V Q R 102
421 - AAGCGGGAGATGATCCTGAAGCGGAAGGAGGAGGAGGCCTGAAGGACAGTCTGCGGCCC - 480
- K R E M I L K R K E E E A L K D S L R P 122
481 - AAGCTGTCTGAGGAGCAGCAGCGCATCATTGCCATACTGCTGGACGCCACCATAAGACC - 540
- K L S E E Q Q R I I A I L L D A H H K T 142
541 - TACGACCCACCTACTCCGACTTCTGCCAGTTCCGGCCTCCAGTTCGTGTGAATGATGGT - 600
- Y D P T Y S D F C Q F R P P V R V N D G 162
601 - GGAGGGAGCCATCTTCCAGGCCCCAAGTCCAGACACACTCCAGCTTCTGTGGGAGCTCC - 660
- G G S H P S R P N S R H T P S F S G D S 182
661 - TCTCCTCCTGCTCAGATCACTGTATCACTTTCAGACATGATGGACTGGTCCAGCTTC - 720
- S S S C S D H C I T S S D M M D S S S F 202
721 - TCCAATCTGGATCTGAGTGAAGAAGATTGAGATGACCTTCTGTGACCCTAGAGCTGTCC - 780
- S N L D L S E E D S D D P S V T L E L S 222
781 - CAGCTCTCCATGCTGCCCCACCTGGCTGACCTGGTCAGTTACAGCATCCAAAGGTCATT - 840
- Q L S M L P H L A D L V S Y S I Q K V I 242
841 - GGCTTTGCTAAGATGATACCAGGATTGAGAGACCTCACCTCTGAGGACCAGATCGTACTG - 900
- G F A K M I P G F R D L T S E D Q I V L 262
901 - CTGAAGTCAAGTGCCATTGAGGTCATCATGTTGCGCTCCAATGAGTCCTTACCATGGAC - 960
- L K S S A I E V I M L R S N E S F T M D 282
961 - GACATGTCCTGGACCTGTGGCAACCAAGACTACAAGTACCGCGTCAGTGACGTGACCAAA - 1020
- D M S W T C G N Q D Y K Y R V S D V T K 302
1021 - GCCGGACACAGCCTGGAGCTGATTGAGCCCCTCATCAAGTTCCAGGTGGGACTGAAGAAG - 1080
- A G H S L E L I E P L I K F Q V G L K K 322
1081 - CTGAACCTTGCATGAGGAGGAGCATGTCCTGATCATGGCCATCTGCATCGTCTCCCCAGAT - 1140
- L N L H E E E H V L L M A I C I V S P D 342
1141 - CGTCCTGGGGTGCAGGACGCCGCGCTGATTGAGGCCATCCAGGACCGCCTGTCCACACA - 1200
- R P G V Q D A A L I E A I Q D R L S N T 362
1201 - TGCAGACGTACATCCGCTGCCGCCACCCGCCCGGGCAGCCACCTGCTCTATGCCAAG - 1260
- L Q T Y I R C R H P P P G S H L L Y A K 382
1261 - ATGATCCAGAAGCTAGCCGACCTGCGCAGCCTCAATGAGGAGCACTCCAAGCAGTACCGC - 1320
- M I Q K L A D L R S L N E E H S K Q Y R 402
1321 - TGCTCTCCTTCCAGCCTGAGTGACGATGAAGCTAACGCCCTTGTGCTCGAAGTGTTT - 1380
- C L S F Q P E C S M K L T P L V L E V F 422
1381 - GGCAATGAGATCTCCCTACTAGGACAGCCTGTGCGGTGCCTGGGTGGGGCTGCTCTCCA - 1440
- G N E I S 427

```

Appendix F. Sequencing data for deletion mutants

Nucleotide and amino acid sequence of VDR_{Δ165-215}

* The start and stop codons are shown in pink and underlined.

* The gap in the sequence represents Δ165-215 amino acid deletion.

```

1 - GAACAGCTTGTCCACCCGCCGGCCGGACCAGAAGCCTTTGGGTCTGAAGTGTCTGTGAGA - 60
-
61 - CCTCACAGAAGAGCACCCCTGGGCTCCACTTACCTGCCCCCTGCTCCTTCAGGGATGAG - 120
-                                     M E
121 - GCAATGGCGGCCAGCACTTCCCTGCCTGACCCCTGGAGACTTTGACCGGAACGTGCCCCGG - 180
- A M A A S T S L P D P G D F D R N V P R
181 - ATCTGTGGGGTGTGTGGAGACCGAGCCACTGGCTTTTCACTTCAATGCTATGACCTGTGAA - 240
- I C G V C G D R A T G F H F N A M T C E
241 - GGCTGCAAAGGCTTCTTCAGGCGAAGCATGAAGCGGAAGGCACTATTCACCTGCCCCCTC - 300
- G C K G F F R R S M K R K A L F T C P F
301 - AACGGGGACTGCCGCATCACCAAGGACAACCGACGCCACTGCCAGGCCTGCCGGCTCAAA - 360
- N G D C R I T K D N R R H C Q A C R L K
361 - CGCTGTGTGGACATCGGCATGATGAAGGAGTTCATTCTGACAGATGAGGAAGTGCAGAGG - 420
- R C V D I G M M K E F I L T D E E V Q R
421 - AAGCGGGAGATGATCCTGAAGCGGAAGGAGGAGGAGGCCTTGAAGGACAGTCTGCGGCCC - 480
- K R E M I L K R K E E E A L K D S L R P
481 - AAGCTGTCTGAGGAGCAGCAGCGCATCATTGCCATACTGCTGGACGCCACCATAAGACC - 540
- K L S E E Q Q R I I A I L L D A H H K T
541 - TACGACCCACCTACTCCGACTTCTGCCAGTTCGGGCCTCCAGTTCGTGTGAATGATGGT - 600
- Y D P T Y S D F C Q F R P P V R V N D G
601 - GGAGGG----- - 660
- G G -----
661 - ----- - 720
- ----- - 202
721 - -----TCTGTGACCCTAGAGCTGTCC - 780
- -----S V T L E L S
781 - CAGCTCTCCATGCTGCCCCACCTGGCTGACCTGGTCAGTTACAGCATCCAAAAGGTCATT - 840
- Q L S M L P H L A D L V S Y S I Q K V I
841 - GGCTTTGCTAAGATGATACCAGGATTACAGACCTCACCTCTGAGGACCAGATCGTACTG - 900
- G F A K M I P G F R D L T S E D Q I V L
901 - CTGAAGTCAAGTGCCATTGAGGTCATCATGTTGCGCTCCAATGAGTCCTTCACCATGGAC - 960
- L K S S A I E V I M L R S N E S F T M D
961 - GACATGTCCTGGACCTGTGGCAACCAAGACTACAAGTACCGCGTCAGTGACGTGACCAAA - 1020
- D M S W T C G N Q D Y K Y R V S D V T K
1021 - GCCGGACACAGCCTGGAGCTGATTGAGCCCCTCATCAAGTTCCAGGTGGGACTGAAGAAG - 1080
- A G H S L E L I E P L I K F Q V G L K K
1081 - CTGAACTTGCATGAGGAGGAGCATGTCCTGCTCATGGCCATCTGCATCGTCTCCCCAGAT - 1140
- L N L H E E E H V L L M A I C I V S P D
1141 - CGTCCTGGGGTGCAGGACGCCGCGCTGATTGAGGCCATCCAGGACCGCCTGTCCAACACA - 1200
- R P G V Q D A A L I E A I Q D R L S N T
1201 - CTGCAGACGTACATCCGCTGCCGCCACCCGCCCGGGCAGCCACCTGCTCTATGCCAAG - 1260
- L Q T Y I R C R H P P P G S H L L Y A K
1261 - ATGATCCAGAAGCTAGCCGACCTGCGCAGCCTCAATGAGGAGCACTCCAAGCAGTACCGC - 1320
- M I Q K L A D L R S L N E E H S K Q Y R
1321 - TGCCTCTCCTTCCAGCCTGAGTGACATGAAGCTAACGCCCTTGTGCTCGAAGTGTTT - 1380
- C L S F Q P E C S M K L T P L V L E V F
1381 - GGCAATGAGATCTCTTACTAGGACAGCCTGTGCGGTGCCTGGGTGGGGCTGCTCCTCCA - 1440
- G N E I S

```

Nucleotide and amino acid sequence of VDR_{Δ165-215}Y143F

- * The start and stop codons are shown in pink and underlined.
- * The gap in the sequence represents Δ165-215 amino acid deletion.
- * Codon in red translates to the desired Y143F point mutation.

```

1  - GAACAGCTTGTCCACCCGCCGGCCGGACCAGAAGCCTTTGGGTCTGAAGTGTCTGTGAGA - 60
-
61 - CCTCACAGAAGAGCACCCCTGGGCTCCACTTACCTGCCCCCTGCTCCTTCAGGGAGAGAG - 120
-                                     M E 2
121 - GCAATGGCGGCCAGCACTTCCCTGCCTGACCCTGGAGACTTTGACCGGAACGTGCCCCGG - 180
- A M A A S T S L P D P G D F D R N V P R 22
181 - ATCTGTGGGGTGTGTGGAGACCGAGCCACTGGCTTTCACTTCAATGCTATGACCTGTGAA - 240
- I C G V C G D R A T G F H F N A M T C E 42
241 - GGCTGCAAAGGCTTCTTCAGGCGAAGCATGAAGCGGAAGGCACTATTACCTGCCCTTTC - 300
- G C K G F F R R S M K R K A L F T C P F 62
301 - AACGGGGACTGCCGCATCACCAAGGACAACCGACGCCACTGCCAGGCCTGCCGGCTCAAA - 360
- N G D C R I T K D N R R H C Q A C R L K 82
361 - CGCTGTGTGGACATCGGCATGATGAAGGAGTTTATTCTGACAGATGAGGAAGTGCAGAGG - 420
- R C V D I G M M K E F I L T D E E V Q R 102
421 - AAGCGGGAGATGATCCTGAAGCGGAAGGAGGAGGAGGCCTTGAAGGACAGTCTCGGGCCC - 480
- K R E M I L K R K E E E A L K D S L R P 122
481 - AAGCTGTCTGAGGAGCAGCAGCGCATCATTGCCATACTGCTGGACGCCACCATAAGACC - 540
- K L S E E Q Q R I I A I L L D A H H K T 142
541 - TTCGACCCACCTACTCCGACTTCTGCCAGTTCCGGCTCCAGTTTCGTGTGAATGATGGT - 600
- F D P T Y S D F C Q F R P P V R V N D G 162
601 - GGAGGG----- - 660
- G G ----- 182
661 - ----- 720
- ----- 202
721 - -----TCTGTGACCCTAGAGCTGTCC - 780
- -----S V T L E L S 222
781 - CAGCTCTCCATGCTGCCCCACCTGGCTGACCTGGTCAGTTACAGCATCCAAAAGGTCATT - 840
- Q L S M L P H L A D L V S Y S I Q K V I 242
841 - GGCTTTTGCTAAGATGATACCAGGATTCAGAGACCTCACCTCTGAGGACCAGATCGTACTG - 900
- G F A K M I P G F R D L T S E D Q I V L 262
901 - CTGAAGTCAAGTGCCATTGAGGTCATCATGTTGCGCTCCAATGAGTCCTTACCATTGGAC - 960
- L K S S A I E V I M L R S N E S F T M D 282
961 - GACATGTCTGACCTGTGGCAACCAAGACTACAAGTACCGCGTCAGTGACGTGACCAAA - 1020
- D M S W T C G N Q D Y K Y R V S D V T K 302
1021 - GCCGGACACAGCCTGGAGCTGATTGAGCCCCATCAAGTTCCAGGTGGGACTGAAGAAG - 1080
- A G H S L E L I E P L I K F Q V G L K K 322
1081 - CTGAACCTTGCATGAGGAGGAGCATGTCCTGCTCATGGCCATCTGCATCGTCTCCCCAGAT - 1140
- L N L H E E E H V L L M A I C I V S P D 342
1141 - CGTCCTGGGGTGCAGGACGCCGCGCTGATTGAGGCCATCCAGGACCGCCTGTCCAACACA - 1200
- R P G V Q D A A L I E A I Q D R L S N T 362
1201 - CTGCAGACGTACATCCGCTGCCGCCACCCGCCCCGGGCAGCCACCTGCTCTATGCCAAG - 1260
- L Q T Y I R C R H P P P G S H L L Y A K 382
1261 - ATGATCCAGAAGCTAGCCGACCTGCGCAGCTCAATGAGGAGCACTCCAAGCAGTACCGC - 1320
- M I Q K L A D L R S L N E E H S K Q Y R 402
1321 - TGCCTCTCCTTCCAGCCTGAGTGCAGCATGAAGCTAACGCCCTTGTGCTCGAAGTGTTT - 1380
- C L S F Q P E C S M K L T P L V L E V F 422
1381 - GGCAATGAGATCTCCACTAGGACAGCCTGTGCGGTGCTGGGTGGGGCTGCTCCTCCA - 1440
- G N E I S 427

```

Nucleotide and amino acid sequence of VDR_{Δ165-215}S278A

- * The start and stop codons are shown in pink and underlined.
- * The gap in the sequence represents Δ165-215 amino acid deletion.
- * Codon in red translates to the desired S278A point mutation.

```

1 - GAACAGCTTGTCCACCCGCCGCCGACCAGAAGCCTTTGGGTCTGAAGTGTCTGTGAGA - 60
-
61 - CCTCACAGAAGAGCACCCCTGGGCTCCACTTACCTGCCCCCTGCTCCTTCAGGGAGAG - 120
-                                     M E 2
121 - GCAATGGCGGCCAGCACTTCCCTGCCTGACCCTGGAGACTTTGACCGGAACGTGCCCCGG - 180
- A M A A S T S L P D P G D F D R N V P R 22
181 - ATCTGTGGGGTGTGTGGAGACCGAGCCACTGGCTTTCACTTCAATGCTATGACCTGTGAA - 240
- I C G V C G D R A T G F H F N A M T C E 42
241 - GGCTGCAAAGGCTTCTTCAGGCGAAGCATGAAGCGGAAGGCACTATTCACCTGCCCCCTTC - 300
- G C K G F F R R S M K R K A L F T C P F 62
301 - AACGGGGACTGCCGCATACCAAGGACAACCGACGCCACTGCCAGGCCTGCCGGCTCAAA - 360
- N G D C R I T K D N R R H C Q A C R L K 82
361 - CGCTGTGTGGACATCGGCATGATGAAGGAGTTTCATTCTGACAGATGAGGAAGTGCAGAGG - 420
- R C V D I G M M K E F I L T D E E V Q R 102
421 - AAGCGGGAGATGATCCTGAAGCGGAAGGAGGAGGAGGCCTTGAAGGACAGTCTGCGGCCC - 480
- K R E M I L K R K E E E A L K D S L R P 122
481 - AAGCTGTCTGAGGAGCAGCAGCGCATCATTGCCATACTGCTGGACGCCCACCATAAGACC - 540
- K L S E E Q Q R I I A I L L D A H H K T 142
541 - TACGACCCACCTACTCCGACTTCTGCCAGTTCCGGCCTCCAGTTCGTGTGAATGATGGT - 600
- Y D P T Y S D F C Q F R P P V R V N D G 162
601 - GGAGGG----- - 660
- G G ----- 182
661 - ----- - 720
- ----- 202
721 - -----TCTGTGACCCTAGAGCTGTCC - 780
- -----S V T L E L S 222
781 - CAGCTCTCCATGCTGCCCCACCTGGGCTGACCTGGTCAGTTACAGCATCCAAAAGGTCATT - 840
- Q L S M L P H L A D L V S Y S I Q K V I 242
841 - GGCTTTGCTAAGATGATAACCAGGATTACAGACCTCACCTCTGAGGACCAGATCGTACTG - 900
- G F A K M I P G F R D L T S E D Q I V L 262
901 - CTGAAGTCAAGTGCCATTGAGGTCATCATGTTGCGCTCCAATGAGGGCTTCACCATGGAC - 960
- L K S S A I E V I M L R S N E A F T M D 282
961 - GACATGTCTTGACCTGTGGCAACCAAGACTACAAGTACCGCGTCAGTGACGTGACCAAA - 1020
- D M S W T C G N Q D Y K Y R V S D V T K 302
1021 - GCCGGACACAGCCTGGAGCTGATTGAGCCCCATCAAGTTCCAGGTGGGACTGAAGAAG - 1080
- A G H S L E L I E P L I K F Q V G L K K 322
1081 - CTGAACCTTGCATGAGGAGGAGCATGTCTGCTCATGGCCATCTGCATCGTCTCCCGAGAT - 1140
- L N L H E E E H V L L M A I C I V S P D 342
1141 - CGTCCTGGGGTGCAGGACGCCGCGCTGATTGAGGCCATCCAGGACCGCCTGTCCAACACA - 1200
- R P G V Q D A A L I E A I Q D R L S N T 362
1201 - CTGCAGACGTACATCCGCTGCCGCCACCCGCCGCGGACCCACCTGCTCTATGCCAAG - 1260
- L Q T Y I R C R H P P P G S H L L Y A K 382
1261 - ATGATCCAGAAGCTAGCCGACCTGCGCAGCCTCAATGAGGAGCACTCCAAGCAGTACCGC - 1320
- M I Q K L A D L R S L N E E H S K Q Y R 402
1321 - TGCCTCTCCTTCCAGCCTGAGTGCAGCATGAAGCTAACGCCCTTGTGCTCGAAGTGTTT - 1380
- C L S F Q P E C S M K L T P L V L E V F 422
1381 - GGCAATGAGATCTCCACTAGGACAGCCTGTGCGGTGCCTGGGTGGGGCTGCTCCTCCA - 1440
- G N E I S 427

```

Nucleotide and amino acid sequence of VDR_{Δ165-215}Y143F/S278A

- * The start and stop codons are shown in pink and underlined
- * The gap in the sequence represents Δ165-215 amino acid deletion.
- * Codons in red translate to the desired mutations Y143F and S278A.

```

1 - GAACAGCTTGTCCACCCGCCGGCCGACCAGAAGCCTTTGGGTCTGAAGTGTCTGTGAGA - 60
-
61 - CCTCACAGAAGAGCACCCCTGGGCTCCACTTACCTGCCCCCTGCTCCTTCAGGGATGGAG - 120
-                                     M E 2
121 - GCAATGGCGGCCAGCACTTCCCTGCCTGACCCTGGAGACTTTGACCGGAACGTGCCCCGG - 180
- A M A A S T S L P D P G D F D R N V P R 22
181 - ATCTGTGGGGTGTGTGGAGACCGAGCCACTGGCTTTCACTTCAATGCTATGACCTGTGAA - 240
- I C G V C G D R A T G F H F N A M T C E 42
241 - GGCTGCAAAGGCTTCTTCAGGCGAAGCATGAAGCGGAAGGCACTATTACCTGCCCCCTTC - 300
- G C K G F F R R S M K R K A L F T C P F 62
301 - AACGGGGACTGCCGCATCACCAAGGACAACCGACGCCACTGCCAGGCCTGCCGGCTCAAA - 360
- N G D C R I T K D N R R H C Q A C R L K 82
361 - CGCTGTGTGGACATCGGCATGATGAAGGAGTTTCTTCTGACAGATGAGGAAGTGCAGAGG - 420
- R C V D I G M M K E F I L T D E E V Q R 102
421 - AAGCGGGAGATGATCCTGAAGCGGAAGGAGGAGGAGGCTTGAAGGACAGTCTGCCGGCCC - 480
- K R E M I L K R K E E E A L K D S L R P 122
481 - AAGCTGTCTGAGGAGCAGCAGCGCATCATTGCCATACTGCTGGACGCCACCATAAGACC - 540
- K L S E E Q Q R I I A I L L D A H H K T 142
541 - TTCGACCCACCTACTCCGACTTCTGCCAGTTCCGGCCTCCAGTTCGTGTGAATGATGGT - 600
- F D P T Y S D F C Q F R P P V R V N D G 162
601 - GGAGGG----- - 660
- G G ----- 182
661 - ----- 720
- ----- 202
721 - -----TCTGTGACCCTAGAGCTGTCC - 780
- -----S V T L E L S 222
781 - CAGCTCTCCATGCTGCCCCACCTGGCTGACCTGGTCAGTTACAGCATCCAAAAGGTCATT - 840
- Q L S M L P H L A D L V S Y S I Q K V I 242
841 - GGCTTTGCTAAGATGATACCAAGGATTCAGAGACCTCACCTCTGAGGACCAGATCGTACTG - 900
- G F A K M I P G F R D L T S E D Q I V L 262
901 - CTGAAGTCAAGTGCCATTGAGGTCATCATGTTGCGCTCCAATGAGGGCTTCACCATGGAC - 960
- L K S S A I E V I M L R S N E A F T M D 282
961 - GACATGTCCTGGACCTGTGGCAACCAAGACTACAAGTACCGGTCAGTGACGTGACCAAA - 1020
- D M S W T C G N Q D Y K Y R V S D V T K 302
1021 - GCCGGACACAGCCTGGAGCTGATTGAGCCCCCTCATCAAGTCCAGGTGGGACTGAAGAAG - 1080
- A G H S L E L I E P L I K F Q V G L K K 322
1081 - CTGAACCTTGCATGAGGAGGAGCATGTCCTGCTCATGGCCATCTGCATCGTCTCCCCAGAT - 1140
- L N L H E E E H V L L M A I C I V S P D 342
1141 - CGTCCTGGGGTGCAGGACGCCGCGCTGATTGAGGCCATCCAGGACCGCCTGTCCAACACA - 1200
- R P G V Q D A A L I E A I Q D R L S N T 362
1201 - CTGCAGACGTACATCCGCTGCCGCCACCCGCCCCGGGCAGCCACCTGCTCTATGCCAAG - 1260
- L Q T Y I R C R H P P P G S H L L Y A K 382
1261 - ATGATCCAGAAGCTAGCCGACCTGCGCAGCCTCAATGAGGAGCACTCCAAGCAGTACCGC - 1320
- M I Q K L A D L R S L N E E H S K Q Y R 402
1321 - TGCTCTCTTCCAGCCTGAGTGCAGCATGAAGCTAACGCCCTTGTGCTCGAAGTGT - 1380
- C L S F Q P E C S M K L T P L V L E V F 422
1381 - GGCAATGAGATCTCTTACTAGGACAGCCTGTGCGGTGCCTGGGTGGGGCTGCTCCTCCA - 1440
- G N E I S 427

```

TO: San Jose State University Graduate School and Master's Student Ludmila Stoyanova

PERMISSION TO REPRODUCE COPYRIGHTED MATERIAL

Promega Corporation grants permission for your use of our pGEM®-T Easy Vector System map (Cat.# A1360) as an illustration in your Master's Thesis, provided that you give proper credit to Promega Corporation for use of the materials.

I/We grant permission for the use specified above.

Promega Corporation
(name of company)

Date: June 30, 2004

By:

(authorized signature)

Name (printed): Linda K. Schramer

Title: Intellectual Property Paralegal

G:\private\legal\template\copytem2.doc

PERMISSION AGREEMENT

Becton, Dickinson and Company ("BD") hereby grants to Ludmila Stoyanova (Licensee) the following permission to use certain images, copies of which are annexed hereto as Attachment A (hereinafter the "Material").

1. Licensee is permitted to use the Material for non-commercial use as illustrations in a Master's Thesis for San Jose State University.
2. The Material Licensee proposes to use shall be submitted for BD's prior approval in all cases.
3. Printing and reproduction of the Material shall be at the Licensee's expense and bear a notice in substantially the following form: Courtesy of Becton, Dickinson and Company.
4. If at any time BD, in its sole discretion, determines that the Material must be revised, then Licensee shall discontinue use of the Material and replace the same with a copy of the revised Material.
5. Licensee hereby acknowledges that BD is the owner of all right, title and interest in and to the Material and all revisions thereto as provided for herein.
6. Licensee is further hereby granted permission to use the BD name and logo and the other trademarks shown in Attachment B hereto, solely in connection with its exercise of rights licensed herein to publish and distribute the Material and to indicate BD as the source thereof. All rights in and to use of such name and logo and other trademarks shall inure to the benefit of BD.
7. All rights not expressly granted herein to Licensee, are reserved to BD which may fully exercise and exploit the same at all times.

BECTON, DICKINSON AND COMPANY

By: *[Signature]*

Date: 7/23/04

LICENSEE

By: *Ludmila Stoyanova*

Date: 7/19/2004

EXHIBIT "A"

EXHIBIT “B”

BD

BECTON DICKINSON