11 Diversity of Vitamin D Target Genes

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Abstract The vitamin D receptor (VDR) is a ligand-inducible transcription factor, whose target genes play key roles in cellular metabolism, bone formation, cellular growth, differentiation, and in controlling inflammation. Many of these VDR target genes are also involved in dysregulated pathways leading to common human diseases, such as cancer, osteoporosis, or the metabolic syndrome. The activation of VDR by natural and synthetic ligands may improve such pathological conditions. On a genomic level these pathways converge on regulatory modules, some of which contain VDR-binding sites, so-called vitamin D response elements (VDREs). Transcriptome analysis, chromatin immunoprecipitation scans and in silico screening approaches already identified many genomic targets of the VDR. Important regulatory modules with VDREs should have a major impact on understanding the role and potential therapeutic value of VDR and its ligands.

Key Words: Vitamin D; vitamin D receptor; vitamin D response elements; in silico screening; gene regulation; nuclear receptor

Abbreviations

$1,25(OH)_2D$	1α,25-dihydroxyvitamin D_3
3C	chromosome-conformation-capture
ALOX5	5-lipoxygenase
CDK	cyclin-dependent kinase
ChIP	chromatin immunoprecipitation
CCNC	cyclin C
CDKN1	p21
CYP24A1	24-hydroxylase
CYP27B1	25-dihydroxyvitamin D_3 1α-hydroxylase
DR	direct repeat
ER	everted repeat
HAT	histone acetyltransferase
IGFBP	insulin-like growth factor binding protein
MAR	matrix attachment region
PPAR	peroxisome proliferator-activated receptor
RXR	retinoid X receptor
TSS	transcription start site
VDR	vitamin D receptor
VDRE	vitamin D response element

From: *Nutrition and Health: Vitamin D*
Edited by: M.F. Holick, DOI 10.1007/978-1-60327-303-9_11,

1. INTRODUCTION

The biologically active form of vitamin D, 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], acts as a ligand to the transcription factor vitamin D receptor (VDR). In order to directly activate a gene by $1,25(OH)_2D$ at least one VDR molecule has to bind in sufficient vicinity to the gene's transcription start site (TSS) *(1)*. However, "vicinity" could in some cases be a distance of up to 100 kB, irrespective if upstream or downstream of the TSS. Moreover, there are a number of evidences that most primary VDR target genes use multiple VDR-binding sites, the so-called vitamin D response elements (VDREs), for their full functionality *(2)*. The complete sequence of the human genome and also that of other mammalian species, such as chimp, dog, mouse, and rat, is now available, so that we are able to screen for all putative VDREs. However, the constant packaging of genomic DNA into chromatin provides a repressive environment, which in most cases denies the access to putative VDREs *(3)*. Fortunately, new experimental techniques for genome-wide analyses of chromatin modifications and transcription factor binding, such as chromatin immunoprecipitation (ChIP)-chip and massively parallel sequencing, are now available *(4)*. This will revolutionize our understanding of the genome-wide effects of the VDR and of the diversity of $1,25(OH)_2D$ target genes as outlined in this chapter.

2. VDR IS A NUCLEAR RECEPTOR

2.1. The Nuclear Receptor Superfamily

Nuclear receptors are the best-characterized representatives of approximately 3,000 different mammalian proteins that are involved in transcriptional regulation in human tissues *(5)*. They form a superfamily with 48 human members, of which the most have the special property to be ligand-inducible *(6, 7)*. Nuclear receptors modulate genes that affect processes as diverse as reproduction, development, inflammation, and general metabolism. They can be classified based on ligand sensitivity *(6)*, evolution of the nuclear receptor genes *(8),* and their physiological role as interpreted from tissue-specific expression patterns *(9)*.

The ligand sensitivity approach suggests three classes of nuclear receptors. Class I contains the endocrine receptors with high-affinity hormonal lipids, such as the receptors for the steroid hormones estradiol (estrogen receptors α and β), progesterone (progesterone receptor), testosterone (androgen receptor), cortisol (glucocorticoid receptor), and aldosterol (mineralocorticoid receptor), for thyroid hormones (thyroid hormone receptors α and β), and for the biologically active forms of the fat-soluble vitamins A and D, all-*trans* retinoic acid (retinoid acid receptors α, β, and γ) and $1,25(OH)_2D$ (VDR). In class II are adopted orphan receptors that bind to dietary lipids and xenobiotics with low affinity, such as peroxisome proliferator-activated receptors (PPARs) α, δ, and γ, constitutive androstane receptor, and pregnane X receptor. Finally, in class III orphan receptors are placed, such as estrogen-related receptors, for which a physiological ligand has not yet been identified.

When the sequences of nuclear receptors are compared on DNA and protein level, the grouping significantly differs from the ligand-centered view. For example, VDR is

in the same group with PPARs and the highly ligand-sensitive estrogen receptors and the orphan estrogen-related receptors are together in another group.

On the basis of mRNA expression of all nuclear receptor genes in 39 different tissues in two different mouse strains, nuclear receptors are divided into clades with distinct physiological roles. In this classification, for example VDR is grouped to bile acid and xenobiotic metabolism based on its high expression in gastroentric tissues and PPARs are linked to lipid metabolism and energy homeostasis.

2.2. Modular Structure of Nuclear Receptors

Nuclear receptors have a modular structure, onto which certain functions can be ascribed. The amino-terminus is of variable length and sequence in the different family members. It contains a transactivation domain, termed AF-1, which is recognized by co-activator proteins and/or other transcription factors, often in a ligand-independent fashion. The central DNA-binding domain has two zinc-finger motifs that are common to the entire family. The carboxy-terminal ligand-binding domain, whose overall architecture is well conserved between the various family members, nonetheless diverges sufficiently to guarantee selective ligand recognition as well as accommodate the broad spectrum of nuclear receptor ligand structures. The ligand-binding domain consists of 250–300 amino acids in 11–13 α-helices *(10)*. Ligand binding causes a conformational change within the ligand-binding domain, whereby, at least in the case of endocrine nuclear receptors, helix 12, the most carboxy-terminal α-helix (also called AF-2 domain), closes the ligand-binding pocket via a "mouse-trap like" intramolecular folding event *(11)*. The ligand-binding domain is also involved in a variety of interactions with nuclear proteins, such as other members of the nuclear receptor superfamily and co-regulator proteins.

2.3. The VDR

VDR is an endocrine member of the nuclear receptor superfamily *(7)*, because it is the only nuclear protein that binds 1,25$(OH)_2$D with high affinity ($K_d = 0.1$ nM) *(12, 13)*. VDR has been shown to form homodimers *(14, 15)* and heterodimers with thyroid hormone receptors *(16, 17)* and retinoid acid receptors *(18)*, but by far the strongest binding partner of VDR is one of the three retinoid X receptors (RXRs) α, β, and γ *(19)*. In mammals, the highest VDR expression is found in metabolic tissues, such as intestine and kidney, as well as in skin and the thyroid gland, but moderate expression is found in nearly all tissues *(9)*. Moreover, the receptor is also expressed in many malignant tissues *(20)*. Mice lacking a functional *VDR* gene develop alopecia (likewise found in many patients with mutations in the VDR) *(21)*; these mice also exhibit a defect in epidermal differentiation. Moreover, VDR-null mice also show an increased susceptibility to tumor formation *(22)*. More details on the receptor have been provided in the previous chapter.

3. VDR-BINDING SITES

3.1. DR3-Type VDREs

The binding of VDR–RXR heterodimers is achieved through the specific binding of the DNA-binding domain of the VDR to the major grove of a hexameric DNA sequence,

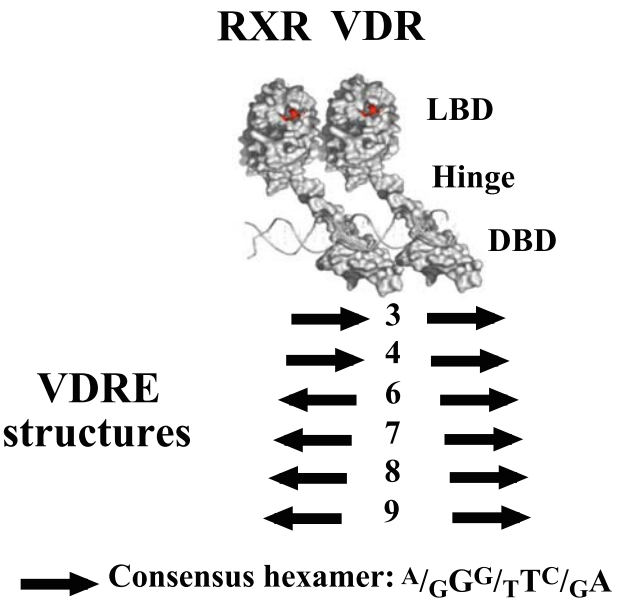

Fig. 1. VDR–RXR heterodimer on VDREs: VDR–RXR heterodimer binding to different types of VDREs is schematically depicted.

referred to as core-binding motif, with the consensus sequence RGKTSA (R = A or G, K = G or T, S = C or G) *(13)* (Fig. 1). Numerous studies (for example *(14, 23)*) have confirmed Umesomo's suggestion *(24)* that VDR–RXR heterodimers bind well to response elements (REs) that are formed by a direct repeat of the hexameric sequences with three spacing nucleotides. These DR3-type REs are therefore widely accepted as the classical structure of a VDRE.

Every $1,25(OH)_2D$ target gene has to contain at least one VDRE in its promoter region and the first VDREs have been identified rather close to the TSS of the genes. The strongest DR3-type VDREs has been identified within the *rat atrial natriuretic factor* promoter *(25)*, the mouse and pig *osteopontin* promoter *(26, 27)*, and the chicken *carbonic anhydrase II* promoter *(28)*. A number of other DR3-type VDREs have been published and were based on their in vitro binding affinity categorized into different classes *(29)*. The REs with the lowest affinity show a significant deviation from the RGKTSA consensus and may not be functional. However, it is possible that these VDREs may gain responsiveness to $1,25(OH)_2D$ in their natural promoter context through the help of flanking partner proteins (see also Section 3.4.). Moreover, the functionality of a $1,25(OH)_2D$ responding gene will also depend on a potential cooperative action of two or more VDREs, such as in the case of the *24-hydroxylase* (*CYP24A1*) gene *(30)*. Nevertheless, VDR–RXR heterodimers form identical complexes on all DR3-type VDREs and display no significant differences in their interaction with a given co-activator or co-repressor protein *(29)*.

3.2. Other Types of VDREs

There are also other VDRE structures, such as direct repeats with four intervening nucleotides (DR4). Effective VDR binding has also been observed on everted repeat (ER)-type REs with 6–9 spacing nucleotides (ER6, ER7, ER8, ER9) *(17, 31)* (Fig. 1). A VDRE classification according to the affinity for VDR–RXR heterodimers suggests that the degree of deviation from the core-binding motif consensus sequence RGKTSA *(32)* is proportional to the loss of in vitro functionality *(29)*. Interestingly, the DR4-type RE of the rat *pit1* gene *(33)*, which contains two perfect core-binding motifs, was found to be stronger than any known natural DR3-type VDRE *(29)*. However, one has to consider that a DR4-type REs is also recognized by the heterodimeric complexes of thyroid hormone receptor, constitutive active receptor and pregnane X receptor and other orphan nuclear receptors with RXR *(19, 34)*, whereas the same complexes bind to DR3-type REs less tightly than VDR–RXR heterodimers. The competitive situation on DR4-type REs may therefore be the reason why in vivo VDR–RXR heterodimers still prefer DR3-type REs. Moreover, VDR–RXR heterodimers bind to DR4-type REs in the same conformation as to DR3-type REs *(34)*, i.e., there seem to be no differential action of VDR on these elements due to a differential complex formation with RXR.

The VDRE of the human *osteocalcin* promoter was the first identified natural binding site for the VDR *(35, 36)*. It was initially described as a DR6-type structure but later a third cryptic hexamer was identified at a distance of 3 nucleotides, so that the whole VDRE is more likely a complex DR6/DR3-type RE (see Section 3.4.). The DR6 part of the VDRE has been shown to bind VDR homodimers *(14)* and VDR–RAR heterodimers *(18)*, whereas the DR3 part weakly binds VDR–RXR heterodimers. Other examples of a DR6-type VDREs that bind VDR homodimers and VDR–RAR heterodimers have been identified in the promoters of mouse *fibronectin (37)*, rat *CYP24A1 (38)*, and human *phospholipase C (39)*. Their functionality remain to be determined.

3.3. Chromatin and Co-factors

The major protein constituents of chromatin are the four different histones that form a nucleosome, around which DNA is wound. Covalent modifications of the lysines at the amino-terminal tails of these histone proteins neutralize their positive charge and thus their attraction for the negatively charged DNA backbone is diminished *(40)*. As a consequence, the association between the histone and the DNA becomes less stable. This influences the degree of chromatin packaging and regulates the access of transcription factors to their potential binding sites. When nuclear receptors are bound to REs in the regulatory regions of their target genes, they recruit positive and negative co-regulatory proteins, referred to as co-activators *(41)* and co-repressors *(42)*, respectively.

In a simplified view of nuclear receptor signaling, in the absence of ligand, the nuclear receptor interacts with co-repressor proteins, such as NCoR1, SMRT, hairless, and Alien, which in turn associate with histone deacetylases leading to a locally increased chromatin packaging *(43, 44)*. The binding of ligand induces the dissociation of the co-repressor and the association of a co-activator of the p160 family, such as SRC-1, TIF2, or RAC3 *(45)*. Some co-activators have histone acetytransferase (HAT) activity

or are complexed with proteins harboring such activity and this results in the net effect of local chromatin relaxation *(46)*. In a subsequent step, ligand-activated nuclear receptors change rapidly from interacting with the co-activators of the p160 family to those of the mediator complex, such as Med1 *(47)*. The mediator complex, which consists of approximately 15–20 proteins, builds a bridge to the basal transcriptional machinery *(48)*. In this way ligand-activated nuclear receptors execute two tasks, the modification of chromatin and the regulation of transcription.

Cell- and time-specific patterns of relative protein expression levels of some co-regulators can distinctly modulate nuclear receptor transcriptional activity. This aspect may have some diagnostic and therapeutic value in different types of cancer *(49)*. Concerning skin cancer it was postulated that the stoichiometric ratio between co-activators of the p160 family and Med1 might regulate a $1,25(OH)_2D$-dependent balance between proliferation and differentiation of keratinocytes *(50)*. However, the switch between gene repression and activation is more complex than a simple alternative recruitment of two different regulatory complexes *(51)*. Most co-regulators are co-expressed in the same cell type at relatively similar levels, which raises the possibility of their concomitant recruitment to a specific promoter. This has been resolved by the mutually exclusive binding of co-activators and co-repressors to ligand-bound and ligand-unbound nuclear receptors, respectively. Therefore, repression and activation are more likely achieved by a series of sequential multiple enzymatic reactions that are promoter and cell-type specific. Transcriptional regulation is a highly dynamic event of rapid association and dissociation of proteins and their modifications, including proteolytic degradation and de novo synthesis. A pattern of recruitment and release of cohorts of co-regulatory complexes was demonstrated on a single region of the *trefoil factor-1* promoter in breast cancer cells *(52)*. This study revealed detailed and coordinated patterns of co-regulator recruitment and preferential selectivity for factors that have similar enzymatic activities. Interestingly, similar cyclic behavior was also observed for the VDR *(53)*.

3.4. VDREs in the Chromatin Context

It is assumed that matrix attachment regions (MARs) subdivide genomic DNA into units of an average length of 100 kB containing the coding region of at least one gene *(54)* (Fig. 2). DNA looping should be able to bring any DNA site within the same chromatin unit close to the basal transcriptional machinery that is assembled on the TSS. This model suggests that also very distant sequences can serve as VDREs and that even sequences downstream of the TSS could serve as functional VDR-binding sites.

Due to its optimized 5′-flanking dinucleotide and core-binding motif sequences the DR4-type RE of the rat *pit-1* gene is the most efficient known VDRE in vitro *(29, 34)*. However, the chromatin in the region of the *pit-1* gene promoter containing this RE seems to be closed in the adult rat, so that the responsiveness of the gene to $1,25(OH)_2D$ is lower than expected *(46)*. This indicates that a high in vitro binding affinity of VDR–RXR heterodimers for a VDRE is not sufficient for responsiveness to $1,25(OH)_2D$. When the promoter region that contains the VDRE is covered by condensed chromatin, VDR–RXR heterodimers are unable to bind there. This makes sufficiently decondensed

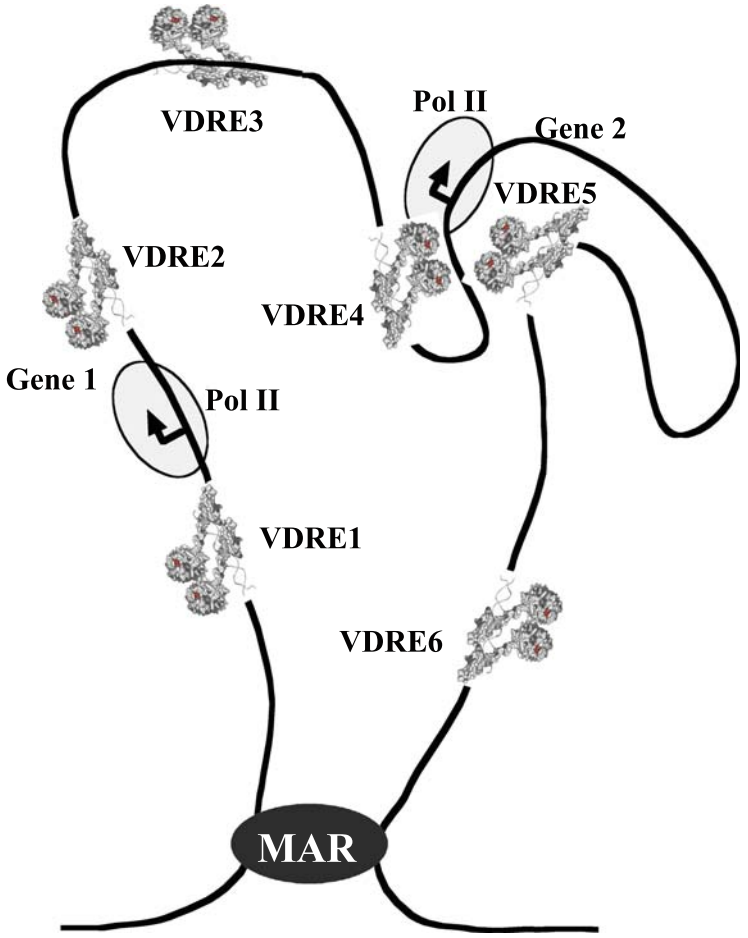

Fig. 2. 1,25(OH)$_2$D responsive chromatin units: A chromatin unit carrying two genes and six VDREs is schematically depicted. The loop may be formed by some 100 kB of genomic sequence, which is kept together by a matrix attachment region (MAR) complex. For clarity the some 500 nucleosome covering this genomic region are not shown. In this scenario VDREs 4 and 5 do sub-looping to the basal transcriptional machinery (Pol II) on the TSS (arrow) of gene 2 and induce transcription. Similarly all six VDREs have the potential to activate genes 1 and 2, i.e., both genes are supposed to be primary VDR target genes.

chromatin an essential prerequisite for a functional VDRE. Chromatin decondensation is achieved by the activity of HATs, which are recruited to their local chromatin target by co-activator proteins. In turn, these co-activators are transiently attracted to a promoter region by ligand-activated nuclear receptors and other active transcription factors. Therefore, the more transcription factor binding sites a given genomic region has and the more of these transcription factors are expressed in the respective cell, the higher is the chance that this area of the promoter gets locally decondensed. One example is the VDRE of the rat *osteocalcin* gene, which is flanked on both sides with a binding site for the transcription factor Runx2/Cbfa1 *(55)*. By contacting co-activator

proteins and HATs Runx2/Cbfa1 seems to mediate the opening of chromatin locally, which allows efficient binding of VDR–RXR heterodimers to this decondensed region to occur. This mechanism suggests that VDREs are better targets for VDR–RXR heterodimers, if other transcription factors are bound to the same chromatin region. In this respect, promoter context and cell-specific expression of other transcription factors may be of greater importance to VDRE functionality and specificity than its in vitro binding profile.

The DR6/DR3 and ER9/DR3 structures of the VDREs of the human and rat *osteocalcin* genes, respectively, are the first examples of complex VDREs. These two complex VDREs show only limited homology to each other, although they are derived from orthologous genes. This suggests that for an important primary $1,25(OH)_2D$ responding gene, such as *osteocalcin*, there may be limited evolutionary pressure for a specific VDRE structure. It seems to be more important to guarantee an efficient binding of the VDR to a promoter in competition with the tight packaging of nucleosomes. It is interesting to note that the complex VDRE of the human *osteocalcin* gene is overlaid by a binding site of the transcription factor AP-1 *(56)*, which provides the RE with an increased activity. These types of REs are also observed for other nuclear receptors and often referred to as composite REs *(57)*. Another interesting example of a complex/composite VDRE has been reported in the mouse c-*fos* promoter *(58)*. Within this VDRE three hexameric core-binding motifs are forming a DR7/DR7 structure, which contains an internal binding site for the transcription factor NF-1. Additional examples are the VDRE of the human and mouse *fibronectin* gene, which contains an internal binding site for the transcription factor CREB *(37)*, or the VDRE of the rat *bone sialo protein*, which also seems to bind the general transcription factor TBP *(59)*.

3.5. Negative VDREs

Expression profiling using microarray technology indicates that comparable numbers of genes are down-regulated by $1,25(OH)_2D$ as are up-regulated by the hormone *(60)*. In general, the mechanisms of the down-regulation of genes by $1,25(OH)_2D$ are much less understood, but they also seem to require the binding of an agonist to the VDR. It is obvious that only genes, which show basal activity, can be down-regulated, i.e., these genes exhibit basal activity due to other transcription factors binding to their promoter. There are several different models that attempt to explain, how $1,25(OH)_2D$ and the VDR can mediate down-regulation of genes, but the common theme is that VDR counteracts the activity of specific transcription factors. For the physiologically important down-regulation of the *25-dihydroxyvitamin D_3 1α-hydroxylase* (*CYP27B1*) gene by $1,25(OH)_2D$ a negative VDRE located at position –0.5 kB has been proposed, where VDR–RXR heterodimers do not bind directly, but via the transcription factor VDR-interacting repressor (VDIR, also called TCF3) *(61)*. In addition, two positive VDREs are located –2.6 and –3.2 kB upstream from the TSS and modulate the cell-specific activity of the negative VDRE *(62)*. Association of VDR–RXR heterodimers to TCF3-binding sites may also occur through ligand-dependent chromatin looping from more distal regions that directly bind the VDR *(62)*. In situations where these activating transcription factors are other nuclear receptors or transcription factors that bind

to composite nuclear receptor REs, VDR could simply compete for DNA-binding sites *(63, 64)*. In a similar way, VDR could also compete for binding to partner proteins, such as RXR, or for common co-activators, such as SRC-1 or CBP *(65)*. In all these situations the down-regulating effects of the VDR should be of general impact, i.e., the mechanism could apply to other genes in the same way. So far, however, no general down-regulating effects of $1,25(OH)_2D$ have been reported.

The concept that multiple VDREs (see also Fig. 2) together with other transcription factor binding sites regulate primary $1,25(OH)_2D$ responding genes (see Section 5.2.) suggests that a promoter may contain both negative and positive VDREs. The activities of the different VDREs are determined by the promoter context and may not be simultaneously active. One might imagine that prior to stimulation with $1,25(OH)_2D$ only the negative VDREs bind the VDR and recruit co-repressors. This would actively condense the chromatin on a particular promoter region. The addition of ligand induces the release of co-repressor proteins and reduces chromatin density. The VDR may then be transiently released from the negative VDRE and bind to a positive VDRE, which may be uncovered through $1,25(OH)_2D$-dependent local nucleosome acetylation. The VDR then interacts with the mediator protein complex on this positive VDRE leading to transient transcriptional activation. After a certain period of time, newly synthesized, unliganded VDR again binds to the negative VDRE, which initiates chromatin closing and inactivation of the positive VDRE *(62)*. In this or even more complex scenarios, the balance between negative and positive VDREs could explain the time course of the activation of primary $1,25(OH)_2D$ responding genes.

4. VDR TARGET GENES

4.1. Classical VDR Targets

The most striking effect of severe vitamin D deficiency is rickets. Rickets can also by inflicted by mutations in the *CYP27B1* or the *VDR* gene. $1,25(OH)_2D$ is essential for adequate Ca^{2+} and P_i absorption from the intestine and hence for bone formation *(66)*. Liganded VDR has been shown to induce expression of the gene encoding for the major Ca^{2+} channel in intestinal epithelial cell, *TRPV6*, by direct binding to a VDRE at -1.2 kB from the TSS *(67)*. The phosphate co-transporter *NaP(i)-IIb* gene was also found to be induced by $1,25(OH)_2D$ but no VDREs have yet been identified for this gene *(68)*. $1,25(OH)_2D$ also down-regulates the expression of the *PTH* gene that opposes $1,25(OH)_2D$ in regulation of serum Ca^{2+} and P_i levels, but up-regulates the *FGF23* gene, which, like PTH, lowers serum P_i levels *(69)*. The induction of the *RANKL* gene by liganded VDR via multiple distant VDREs (up to 70 kB from the TSS) leads to stimulation of osteoclast precursors to fuse and form new osteoclasts, resulting in enhanced resorption of bone *(70)*.

4.2. VDR Targets in Cell Cycle Regulation

The main anti-proliferative effect of $1,25(OH)_2D$ on cells is a cell cycle block at the G_1 phase. This can be explained by changed expression of multiple cell cycle regulator genes. Among the first targets described, expression of cyclin-dependent kinase

inhibitors (CDKIs) *p21* (*CDKN1A*) and *p27* were found to be up-regulated by ligand treatment *(71, 72)*. For the *CDKN1A* gene a VDRE in the proximal promoter was characterized, thus establishing *CDKN1A* as a direct $1,25(OH)_2D$ target gene *(73)*. Later on it has been questioned, whether *CDKN1A* truly is a primary target or a secondary target via up-regulation of TGFβ or of the insulin-like growth factor binding protein (IGFBP) 3, and whether the described VDRE is truly functional *(74, 75)*. However, by screening 7 kB of the *CDKN1A* promoter with overlapping ChIP regions three novel regions with $1,25(OH)_2D$-induced VDR enrichment, two of which harvested also p53 were identified *(76)*. The direct role of the characterized $1,25(OH)_2D$-responsive regions on regulation of *CDKN1A* expression is illustrated by the their association with VDR-RNA polymerase II complexes as well as by their ligand-dependent looping to the *CDKN1A* TSS *(51)*. Also other CDKIs, such as *p15*, *p16*, *p18,* and *p19*, show transcriptional response to $1,25(OH)_2D$, but for *p16* the response is secondary as it can be blocked by inhibition of protein synthesis *(73, 77)*. In addition, *cyclin E*, *cyclin D1,* and *CDK2* were found to be down-regulated by $1,25(OH)_2D$ *(74)*. It remains to be elucidated, whether these effects are primary and occur via functional VDREs on regulatory regions of these genes.

Another interesting $1,25(OH)_2D$-target gene is *cyclin C* (*CCNC*). The cyclin C-CDK8 complex was found to be associated with the RNA polymerase II basal transcriptional machinery *(78)* and is considered as a functional part of those mediator protein complexes that are involved in gene repression *(79)*. The fact that the *CCNC* gene, being located in chromosome 6q21, is deleted in a subset of acute lymphoblastic leukemias, suggests its involvement in tumorigenesis *(80)*. In addition, *GADD45A* and members of the *IGFBP* gene family respond to $1,25(OH)_2D$ *(81, 82)*. *GADD45A* plays an essential role in DNA repair and GADD45 proteins displace cyclin B1 from Cdc2 and thus inhibit the formation of M phase-promoting factor that is essential for G_2/M transition *(83)*. *GADD45A* has been shown to be a direct transcriptional target of $1,25(OH)_2D$ with a functional VDRE within the fourth exon of the gene *(84)*. IGFBPs modulate the activity of the circulating insulin-like growth factors (IGF) I and II. The *IGFBP-3* gene was first discovered to be up-regulated by $1,25(OH)_2D$ and contains a functional VDRE *(85)*. Later also *IGFBP-1* and *IGFBP-5* have been characterized as primary $1,25(OH)_2D$ target genes *(82)*. Another interesting primary $1,25(OH)_2D$ target is the *PPARD* gene, which carries a potent DR3-type VDRE in close proximity to its TSS *(86)*. PPARδ and VDR proteins are widely expressed and an apparent overlap in the physiological action of the two nuclear receptors is their involvement in the regulation of cellular growth, particularly in neoplasms. High *PPARD* expression in tumor seems to be positive for the prognosis of the respective cancer *(87)*.

Overall, $1,25(OH)_2D$ restricts cell cycle progression in several phases via multiple and partially redundant targets on parallel pathways, that when combined, provide robustness for its anti-proliferative effect.

4.3. Relative Expression of VDR Target Genes

The steady-state mRNA expression levels of some VDR target genes, such as that of the *CYP24A1* gene, are very low in the absence of ligand, but are up to 1,000-fold

induced by stimulation with 1,25$(OH)_2$D *(88)*. Most other known primary 1,25$(OH)_2$D target genes, such as *CCNC* and *CDKN1A*, often show an inducibility of twofold or less after short-term treatment with 1,25$(OH)_2$D *(89, 90)*. However, both genes have 10,000- to 100,000-fold higher basal expression levels compared to that of the *CYP24A1* gene. Therefore, when the relative levels are taken into account, 2- to 20-fold more *CCNC* and *CDKN1A* than *CYP24A1* mRNA molecules are produced after induction with 1,25$(OH)_2$D.

5. VDR TARGET GENE ANALYSIS

5.1. Transcriptome Analysis

There are a number of modern methods for the identification and characterization of VDR target genes. The effect of 1,25$(OH)_2$D on the mRNA expression, i.e., 1,25$(OH)_2$D-induced changes of the transcriptome, has been assayed by multiple microarray experiment in cellular models (either an established cell line or primary cells) or in in vivo models (mostly rodents). In case the focus is on the identification of primary VDR target genes, the stimulation times are short (2–6 h), but when the overall physiological effects are the center of the study, longer treatment times are used (24–72 h). For a limited number of putative VDR target genes quantitative PCR can be applied, but for a whole genome perspective on VDR signaling, microarrays have to be used.

A few years ago mostly cDNA arrays with an incomplete number of genes were used and rather short lists of VDR target genes from colon *(90)*, prostate *(49, 91–93)*, breast *(89)*, and osteoblasts *(94, 95)*. In squamous cell carcinoma more than 900 genes respond to 1,25$(OH)_2$D after 12 h treatment in the presence of the protein synthesis inhibitor cycloheximide *(96)*. However, the number of overlapping VDR target genes in these lists was low. Since the setups of these microarray analyses were different in treatment times and probe sets, this suggests that most VDR target genes reply to 1,25$(OH)_2$D in a very tissue-specific fashion and may have only a rather transient response. However, on the basis of these results the total number of convincing primary 1,25$(OH)_2$D target genes is in the order of 250. In addition, secondary 1,25$(OH)_2$D-responding genes contribute to the physiological effects of 1,25$(OH)_2$D, but their induction is delayed by a few hours or even days and are probably mediated by primary 1,25$(OH)_2$D-responding gene products, such as transcription factors or co-regulator proteins *(90)*. For a more detailed meta-analysis of VDR target genes, standardized microarray procedures performed on whole genome chips from Affymetrix, Illumina or other commercial suppliers are essential. Results from such approaches will be published soon.

5.2. ChIP Analysis

For a detailed analysis of the regulatory regions of primary VDR target genes since a couple of years the method of ChIP became the golden standard. For the genes *CYP24A1 (88)*, *CYP27B1 (62)*, *CCNC (97)*, and *CDKN1A (76)* 7.1–8.4 kB of their promoter regions were investigated by using in each case a set of 20–25 overlapping genomic region. The spatio-temporal, 1,25$(OH)_2$D-dependent chromatin changes in the

four gene promoters were studied by ChIP assays with antibodies against acetylated histone 4, VDR, RXR, and RNA polymerase II. Promising promoter regions were then screened *in silico* for putative VDREs, whose functionality was analyzed sequentially with gel shift, reporter gene, and re-ChIP assays. This approach identified four VDREs for both the *CYP24A1* and *CCNC* genes, three in the *CDKN1A* promoter and two in the *CYP27B1* gene. However, most of them are simultaneously under the control of other transcription factors, such as p53 in case of the *CDKN1A* gene *(76)*, and therefore possess significant basal levels of transcription.

An alternative approach to the identification of primary $1,25(OH)_2D$ target genes was performed with the six members of the *IGFBP* gene family. Here first an *in silico* screen was performed, which was then followed by the analysis of candidate $1,25(OH)_2D$-responsive sequences by gel shift, reporter gene, and re-ChIP assays *(82)*. Induction of gene expression was confirmed independently using quantitative PCR. By using this approach, the genes *IGFBP1*, *IGFBP3,* and *IGFBP5* were demonstrated to be primary $1,25(OH)_2D$ target genes. The *in silico* screening of the 174 kB of genomic sequence surrounding all six *IGFBP* genes identified 15 candidate VDREs, 10 of which were shown to be functional in ChIP assays. Importantly, the *in silico* screening approach was not restricted to regulatory regions that comprise only maximal 2 kB of sequence up- and downstream of the TSS, as in a recent whole genome screen for regulatory elements *(98)*, but involved up to 10 kB of flanking sequences as well as intronic and intergenic sequences. In a similar approach the *5-lipoxygenase* (*ALOX5*) gene has been analyzed and confirmed to be a primary $1,25(OH)_2D$ target gene. From the 22 putative VDREs identified in the whole *ALOX5* gene sequence (–10 kB to +74 kB) by *in silico* screening, at least two have been validated to be functional in vitro and in the living cells. One of these VDREs is located far downstream of the TSS (+42 kB) and is one of the strongest known VDREs of the human genome *(99)*. No functional VDRE had been reported for the *ALOX5* gene before, since previous studies had been restricted to the proximal promoter region *(100, 101)*. Therefore, this approach revealed candidate VDREs that are located more than 30 kB distant from their target gene's TSS. Based on the present understanding of enhancers, DNA looping and chromatin units being flanked by insulators or MARs, these distances are not limiting *(102)*.

5.3. ChIP-Chip Assays

The combination of ChIP assays with hybridization of the resulting chromatin fragments on microarrays, the so-called ChIP-chip assays, provides an additional step for a larger scale analysis of VDR target genes. The ChIP-chip technology has been applied for the analysis of the *VDR* gene itself *(103)*, the intestinal calcium ion channel gene *TRPV6 (67)*, the Wnt signaling co-regulator *LRP5 (104),* and the TNF-like factor *RANKL* that promotes the formation of calcium-resorbing osteoclasts *(105)*. For all those genes a number of VDR-associated chromatin regions were identified, some of which were far upstream of the gene's TSS. These studies confirmed that many, if not all, VDR target genes have multiple VDR-associated regions. However, not all of these VDREs may be functional, i.e., they may not contact the gene's TSS via DNA looping. Therefore, it is necessary to apply an additional method, the so-called

chromosome-conformation-capture (3C) assay. So far, 3C assays confirmed the functionality of the VDREs in the *CYP27B1 (62)* and the *CDKN1A (51)* genes.

The next step in genome-wide association studies will be massively parallel sequencing of genomic fragments obtained after ChIP assays, also referred to as ChIP-Seq, with antibodies against VDR and its partner proteins. Results will be published soon.

5.4. In Silico Screening of VDREs

The specificity of VDR for its DNA-binding sites allows constructing a model to describe the VDRE properties that can be used to predict potential binding sites in genomic sequences. For this the VDR-binding preference, often expressed as position weight matrix, has to be described on the basis of experimental data, such as series of gel shift assays with a large number of natural binding sites *(106–109)*. However, VDR–RXR heterodimers do not only recognize a pair of the nuclear receptor consensus binding motifs AGGTCA, but also a number of variations to it. Dependent on the individual position weight matrix description this leads to a prediction of VDREs every 1,000–10,000 bp of genomic sequence. This probably contains many false-positive predictions, which is mainly due to scoring methodology and the limitations that are imposed by the available experimental data. Wang et al. combined microarray analysis and *in silico* genome-wide screens for DR3- and ER6-type VDREs *(96)*. This approach identified several novel VDREs and VDR target genes, but most of the VDREs await a confirmation by ChIP and 3C assays.

In a position frequency matrix the quantitative characteristics of a transcription factor, i.e., its relative binding strength to a number of different binding sites, are neglected, since simply the total number of observations of each nucleotide is recorded for each position. Moreover, in the past there was a positional bias of transcription factor binding sites upstream in close vicinity to the TSS. This would be apparent from the collection of identified VDREs *(13)*, but is in contrast with a multi-genome comparison of nuclear receptor binding site distribution *(98)* and other reports on wide-range associations of distal regulatory sites *(110)*.

Internet-based software tools, such as TRANSFAC *(111)*, screen DNA sequences with databases of matrix models. The accuracy of such methods can be improved by taking the evolutionary conservation of the binding site and that of the flanking genomic region into account. Moreover, cooperative interactions between transcription factors, i.e., regulatory modules, can be taken into account by screening for binding site clusters. The combination of phylogenetic footprinting and position weight matrix searches applied to orthologous human and mouse gene sequences reduces the rate of false predictions by an order of magnitude, but leads to some reduction in sensitivity *(112)*. Recent studies suggest that a surprisingly large fraction of regulatory sites may not be conserved but yet are functional, which suggests that sequence conservation revealed by alignments may not capture some relevant regulatory regions *(113)*.

The recently published classifier method for the *in silico* screening of transcription factor binding sites *(114)* showed at the example of PPAR–RXR heterodimers, how a set of in vitro binding preferences of the three PPAR subtypes can be used as an experimental data set. Single nucleotide variants were sorted into three classes, where in

class I the PPAR subtypes are able to bind the sequence with a strength of 75 ± 15% of that of the consensus PPAR-binding site, in class II with 45 ± 15%, and in class III with 15 ± 15%. Additional 130 PPREs were sorted on the basis of counting increasing number of variations from the consensus and taking into account the single nucleotide variant binding strength. Those variants that alone decrease the binding only modestly (class I) could be combined with even three deviations from consensus still resulting in more than 20% binding relative to consensus. Other combinations resulted in faster loss of binding detailed in 11 categories, where such combinations still resulted in more than 1% relative binding. The main advantage, when comparing the classifier to position weight matrix methods, is a clear separation between weak PPREs and those of medium and strong strength *(114)*. With this method the gene-dense human chromosome 19 (63.8 MB, 1,445 known genes) and its syntenic mouse regions (956 genes have known orthologs) were screened. Twenty percent of genes of chromosome 19 were found to contain a strong RE and additional 4% have more than two medium REs or one proximal medium RE. These numbers suggest a total of 4,000–5,000 targets for PPARs in the human genome. Presently, the same approach is used for a genome-wide screening of VDREs and the results will be published soon. First results already indicated that the number of putative VDR target genes is in the same order as that of possible PPAR targets. Certainly not all sites will be accessible and the human genome also contains weak binding sites that could gain function via interaction with other transcription factors.

In effect, these approaches and tools are still insufficient and there has to be a focus on the creation of bioinformatics resources that include more directly the biochemical restraints to regulate gene transcription. One important aspect is that most putative transcription factor binding sites are covered by nucleosomes, so that they are not accessible to the transcription factor. This repressive environment is found in particular for those sequences that are either contained within interspersed sequences, are located isolated from transcription factor modules, or lie outside of insulator sequences marking the border of chromatin loops *(115)*. This perspective strongly discourages the idea that isolated, simple VDREs may be functional in vivo. In turn, this idea implies that the more transcription factor binding sites a given promoter region contains and the more of these transcription factors are expressed, the higher is the chance that this area of the promoter becomes locally decondensed.

6. CONCLUSIONS

The sequencing of the complete human genome and the genome of other species, i.e., the availability of all regulatory sequences, enable a more mature understanding of the diversity of $1,25(OH)_2D$ target genes. Perhaps the idea of simple isolated VDREs should shift to the concept of complex VDREs, of which a simple DR3-, DR4- or ER-type VDRE represents the core. Depending on the temporal presence of cell-specific transcription factors, these complex REs may act positively or negatively in respect to $1,25(OH)_2D$. The coordinated action of these different types of VDREs could explain the individual response of target genes to $1,25(OH)_2D$.

Methods incorporating both experimental- and informatics-derived evidence to arrive at a more reliable prediction of VDR targets and binding modules can bring all available

data together with the aim to predict the outcome in a specific context. We envision that in future the emphasis will shift from target genes to target regulatory modules to alter a physiological response and from individual genes to whole genome response. Therefore, a much larger challenge lies ahead when we would be confronted with the higher order of regulated networks of genes, where the sum effect of ligand treatment may reveal itself. In an effort to study this, we have started applying systems biology to the field of nuclear receptor biology, through an EU-funded Marie Curie Research Training Network, NUCSYS (www.uku.fi/nucsys).

ACKNOWLEDGMENTS

The University of Luxembourg, the Academy of Finland, the Juselius Foundation, and the EU (Marie Curie RTN NucSys) supported the work.

REFERENCES

1. Carlberg C, Dunlop TW (2006) The impact of chromatin organization of vitamin D target genes. Anticancer Res 26:2637–2645
2. Carlberg C, Dunlop TW, Saramäki A, Sinkkonen L, Matilainen M, Väisänen S (2007) Controlling the chromatin organization of vitamin D target genes by multiple vitamin D receptor binding sites. J Steroid Biochem Mol Biol 103:338–343
3. Demeret C, Vassetzky Y, Mechali M (2001) Chromatin remodelling and DNA replication: from nucleosomes to loop domains. Oncogene 20:3086–3093
4. Valouev A, Johnson DS, Sundquist A et al (2008) Genome-wide analysis of transcription factor binding sites based on ChIP-Seq data. Nat Methods
5. Maglich JM, Sluder A, Guan X et al (2001) Comparison of complete nuclear receptor sets from the human, *Caenorhabditis elegans* and *Drosophila* genomes. Genome Biol 2:0029
6. Chawla A, Repa JJ, Evans RM, Mangelsdorf DJ (2001) Nuclear receptors and lipid physiology: opening the X-files. Science 294:1866–1870
7. Nuclear-Receptor-Committee (1999) A unified nomenclature system for the nuclear receptor superfamily. Cell 97:161–163
8. Bertrand S, Brunet FG, Escriva H, Parmentier G, Laudet V, Robinson-Rechavi M (2004) Evolutionary genomics of nuclear receptors: from twenty-five ancestral genes to derived endocrine systems. Mol Biol Evol 21:1923–1937
9. Bookout AL, Jeong Y, Downes M, Yu RT, Evans RM, Mangelsdorf DJ (2006) Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network. Cell 126:789–799
10. Rochel N, Wurtz JM, Mitschler A, Klaholz B, Moras D (2000) Crystal structure of the nuclear receptor for vitamin D bound to its natural ligand. Mol Cell 5:173–179
11. Moras D, Gronemeyer H (1998) The nuclear receptor ligand-binding domain: structure and function. Curr Opin Cell Biol 10:384–391
12. Bikle DD, Gee E, Pillai S (1993) Regulation of keratinocyte growth, differentiation, and vitamin D metabolism by analogs of 1,25-dihydroxyvitamin D. J Invest Dermatol 101:713–718
13. Carlberg C, Polly P (1998) Gene regulation by vitamin D_3. Crit Rev Eukaryot Gene Expr 8:19–42
14. Carlberg C, Bendik I, Wyss A et al (1993) Two nuclear signalling pathways for vitamin D. Nature 361:657–660
15. Cheskis B, Freedman LP (1994) Ligand modulates the conversion of DNA-bound vitamin D_3 receptor (VDR) homodimers into VDR-retinoid X receptor heterodimers. Mol Cell Biol 14:3329–3338
16. Schräder M, Carlberg C (1994) Thyroid hormone and retinoic acid receptors form heterodimers with retinoid X receptors on direct repeats, palindromes, and inverted palindromes. DNA Cell Biol 13: 333–341

17. Schräder M, Müller KM, Nayeri S, Kahlen JP, Carlberg C (1994) VDR-T_3R receptor heterodimer polarity directs ligand sensitivity of transactivation. Nature 370:382–386
18. Schräder M, Bendik I, Becker-Andre M, Carlberg C (1993) Interaction between retinoic acid and vitamin D signaling pathways. J Biol Chem 268:17830–17836
19. Mangelsdorf DJ, Evans RM (1995) The RXR heterodimers and orphan receptors. Cell 83:841–850
20. Norman AW (1998) Receptors for 1,25$(OH)_2$D: past, present, and future. J Bone Miner Res 13:1360–1369
21. Kato S, Takeyama K-I, Kitanaka S, Murayama A, Sekine K, Yoshizawa T (1999) In vivo function of VDR in gene expression-VDR knock-out mice. J Steroid Biochem Mol Biol 69:247–251
22. Welsh J (2004) Vitamin D and breast cancer: insights from animal models. Am J Clin Nutr 80: 1721S–1724S
23. MacDonald PN, Dowd DR, Nakajima S et al (1993) Retinoid X receptors stimulate and 9-*cis* retinoic acid inhibits 1,25-dihydroxyvitamin D_3-activated expression of the rat osteocalcin gene. Mol Cell Biol 13:5907–5917
24. Umesono K, Murakami KK, Thompson CC, Evans RM (1991) Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D_3 receptors. Cell 65:1255–1266
25. Kahlen JP, Carlberg C (1996) Functional characterization of a 1,25-dihydroxyvitamin D_3 receptor binding site found in the rat atrial natriuretic factor promoter. Biochem Biophys Res Commun 218:882–886
26. Zhang Q, Wrana JL, Sodek J (1992) Characterization of the promoter region of the porcine opn (osteopontin, secreted phosphoprotein 1) gene. Eur J Biochem 207:649–659
27. Noda M, Vogel RL, Craig AM, Prahl J, DeLuca HF, Denhardt DT (1990) Identification of a DNA sequence responsible for binding of the 1,25-dihydroxyvitamin D_3 receptor and 1,25-dihydroxyvitamin D_3 enhancement of mouse secreted phosphoprotein 1 (*Spp-1* or osteopontin) gene expression. Proc Nat Acad Sci USA 87:9995–9999
28. Quelo I, Machuca I, Jurdic P (1998) Identification of a vitamin D response element in the proximal promoter of the chicken carbonic anhydrase II gene. J Biol Chem 273:10638–10646
29. Toell A, Polly P, Carlberg C (2000) All natural DR3-type vitamin D response elements show a similar functionality in vitro. Biochem J 352:301–309
30. Kerry DM, Dwivedi PP, Hahn CN, Morris HA, Omdahl JL, May BK (1996) Transcriptional synergism between vitamin D-responsive elements in the rat 25-hydroxyvitamin D_3 24-hydroxylase (CYP) promoter. J Biol Chem 271:29715–29721
31. Schräder M, Nayeri S, Kahlen JP, Müller KM, Carlberg C (1995) Natural vitamin D_3 response elements formed by inverted palindromes: polarity-directed ligand sensitivity of vitamin D_3 receptor-retinoid X receptor heterodimer-mediated transactivation. Mol Cell Biol 15:1154–1161
32. Carlberg C (1995) Mechanisms of nuclear signalling by vitamin D_3. Interplay with retinoid and thyroid hormone signalling. Eur J Biochem 231:517–527
33. Rhodes SJ, Chen R, DiMattia GE et al (1993) A tissue-specific enhancer confers Pit-1-dependent morphogen inducibility and autoregulation on the *pit-1* gene. Genes Dev 7:913–932
34. Quack M, Carlberg C (2000) Ligand-triggered stabilization of vitamin D receptor/retinoid X receptor heterodimer conformations on DR4-type response elements. J Mol Biol 296:743–756
35. Morrison NA, Shine J, Fragonas J-C, Verkest V, McMenemey ML, Eisman JA (1989) 1,25-dihydroxyvitamin D-responsive element and glucocorticoid repression in the osteocalcin gene. Science 246:1158–1161
36. Ozono K, Liao J, Kerner SA, Scott RA, Pike JW (1990) The vitamin D-responsive element in the human osteocalcin gene. Association with a nuclear proto-oncogene enhancer. J Biol Chem 265:21881–21888
37. Polly P, Carlberg C, Eisman JA, Morrison NA (1996) Identification of a vitamin D_3 response element in the fibronectin gene that is bound by a vitamin D_3 receptor homodimer. J Cell Biochem 60: 322–333
38. Kahlen JP, Carlberg C (1994) Identification of a vitamin D receptor homodimer-type response element in the rat calcitriol 24-hydroxylase gene promoter. Biochem Biophys Res Commun 202: 1366–1372

39. Xie Z, Bikle DD (1997) Cloning of the human phospholipase C-γ1 promoter and identification of a DR6-type vitamin D-responsive element. J Biol Chem 272:6573–6577
40. Jenuwein T, Allis CD (2001) Translating the histone code. Science 293:1074–1080
41. Aranda A, Pascual A (2001) Nuclear hormone receptors and gene expression. Physiol Rev 81: 1269–1304
42. Burke LJ, Baniahmad A (2000) Co-repressors 2000. FASEB J 14:1876–1888
43. Polly P, Herdick M, Moehren U, Baniahmad A, Heinzel T, Carlberg C (2000) VDR-Alien: a novel, DNA-selective vitamin D_3 receptor-corepressor partnership. FASEB J 14:1455–1463
44. Privalsky ML (2004) The role of corepressors in transcriptional regulation by nuclear hormone receptors. Annu Rev Physiol 66:315–360
45. Leo C, Chen JD (2000) The SRC family of nuclear receptor coactivators. Gene 245:1–11
46. Castillo AI, Jimenez-Lara AM, Tolon RM, Aranda A (1999) Synergistic activation of the prolactin promoter by vitamin D receptor and GHF-1: role of coactivators, CREB-binding protein and steroid hormone receptor coactivator-1 (SRC-1). Mol Endocrinol 13:1141–1154
47. Rachez C, Suldan Z, Ward J et al (1998) A novel protein complex that interacts with the vitamin D_3 receptor in a ligand-dependent manner and enhances transactivation in a cell-free system. Genes Dev 12:1787–1800
48. Rachez C, Lemon BD, Suldan Z et al (1999) Ligand-dependent transcription activation by nuclear receptors requires the DRIP complex. Nature 398:824–828
49. Khanim FL, Gommersall LM, Wood VH et al (2004) Altered SMRT levels disrupt vitamin D_3 receptor signalling in prostate cancer cells. Oncogene 23:6712–6725
50. Oda Y, Sihlbom C, Chalkley RJ et al (2003) Two distinct coactivators, DRIP/mediator and SRC/p160, are differentially involved in vitamin D receptor transactivation during keratinocyte differentiation. Mol Endocrinol 17:2329–2339
51. Malinen M, Saramäki A, Ropponen A, Degenhardt T, Väisänen S, Carlberg C (2008) Distinct HDACs regulate the transcriptional response of human cyclin-dependent kinase inhibitor genes to Trichostatin A and 1α,25-dihydroxyvitamin D_3. Nucl Acids Res 36:121–132
52. Metivier R, Penot G, Hubner MR et al (2003) Estrogen receptor a directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. Cell 115:751–763
53. Kim S, Shevde NK, Pike JW (2005) 1,25-Dihydroxyvitamin D_3 stimulates cyclic vitamin D receptor/retinoid X receptor DNA-binding, co-activator recruitment, and histone acetylation in intact osteoblasts. J Bone Miner Res 20:305–317
54. Hancock R (2000) A new look at the nuclear matrix. Chromosoma 109:219–225
55. Sierra J, Villagra A, Paredes R et al (2003) Regulation of the bone-specific osteocalcin gene by p300 requires Runx2/Cbfa1 and the vitamin D_3 receptor but not p300 intrinsic histone acetyltransferase activity. Mol Cell Biol 23:3339–3351
56. Schüle R, Umesono K, Mangelsdorf DJ, Bolado J, Pike JW, Evans RM (1990) Jun-Fos and receptors for vitamins A and D recognize a common response element in the human osteocalcin gene. Cell 61:497–504
57. Miner JN, Yamamoto KR (1992) The basic region of AP-1 specifies glucocorticoid receptor activity at a composite response element. Genes Dev 6:2491–2501
58. Candeliere GA, Jurutka PW, Haussler MR, St-Arnaud R (1996) A composite element binding the vitamin D receptor, retinoid X receptor a, and a member of the CTF/NF-1 family of transcription factors mediates the vitamin responsiveness of the c-*fos* promoter. Mol Cell Biol 16: 584–592
59. Kim RH, Li JJ, Ogata Y, Yamauchi M, Freedman LP, Sodek J (1996) Identification of a vitamin D_3-response element that overlaps a unique inverted TATA box in the rat bone sialoprotein gene. Biochem J 318:219–226
60. White JH (2004) Profiling 1,25-dihydroxyvitamin D_3-regulated gene expression by microarray analysis. J Steroid Biochem Mol Biol 89–90:239–244
61. Murayama A, Kim MS, Yanagisawa J, Takeyama K, Kato S (2004) Transrepression by a liganded nuclear receptor via a bHLH activator through co-regulator switching. EMBO J 23: 1598–1608

62. Turunen MM, Dunlop TW, Carlberg C, Väisänen S (2007) Selective use of multiple vitamin D response elements underlies the 1α,25-dihydroxyvitamin D_3-mediated negative regulation of the human CYP27B1 gene. Nucleic Acids Res 35:2734–2747
63. Towers TL, Freedman LP (1998) Granulocyte-macrophage colony-stimulating factor gene transcription is directly repressed by the vitamin D_3 receptor: implications for allosteric influences on nuclear receptor structure and function by a DNA element. J Biol Chem 273:10338–10348
64. Towers TL, Staeva TP, Freedman LP (1999) A two-hit mechanism for vitamin D_3-dediates transcriptional repression of the granulocyte-macrophage colony-stimulating factor gene: vitamin D receptor completes for DNA binding with NFAT1 and stabilizes c-Jun. Mol Cell Biol 19:4191–4199
65. Polly P, Carlberg C, Eisman JA, Morrison NA (1997) 1α,25-dihydroxyvitamin D_3 receptor as a mediator of transrepression of retinoid signaling. J Cell Biochem 67:287–296
66. Renkema KY, Alexander RT, Bindels RJ, Hoenderop JG (2008) Calcium and phosphate homeostasis: concerted interplay of new regulators. Ann Med 40:82–91
67. Meyer MB, Watanuki M, Kim S, Shevde NK, Pike JW (2006) The human transient receptor potential vanilloid type 6 distal promoter contains multiple vitamin D receptor binding sites that mediate activation by 1,25-dihydroxyvitamin D_3 in intestinal cells. Mol Endocrinol 20:1447–1461
68. Xu H, Bai L, Collins JF, Ghishan FK (2002) Age-dependent regulation of rat intestinal type IIb sodium-phosphate cotransporter by 1,25-$(OH)_2$ vitamin D_3. Am J Physiol Cell Physiol 282: C487–C493
69. Saito H, Maeda A, Ohtomo S et al (2005) Circulating FGF-23 is regulated by 1α,25-dihydroxyvitamin D_3 and phosphorus in vivo. J Biol Chem 280:2543–2549
70. Kim S, Yamazaki M, Zella LA et al (2007) Multiple enhancer regions located at significant distances upstream of the transcriptional start site mediate RANKL gene expression in response to 1,25-dihydroxyvitamin D_3. J Steroid Biochem Mol Biol 103:430–434
71. Jiang H, Lin J, Su Z-z, Collart FR, Huberman E, Fisher PB (1994) Induction of differentiation in human promyelotic HL-60 leukemia cells activates p21, WAF1/CIP1, expression in the absence of p53. Oncogene 9:3397–3406
72. Wang QM, Jones JB, Studzinski GP (1996) Cyclin-dependent kinase inhibitor p27 as a mediator of the G1-S phase block induced by 1,25-dihydroxyvitamin D_3 in HL60 cells. Cancer Res 56:264–267
73. Liu M, Lee M-H, Cohen M, Bommakanti M, Freedman LP (1996) Transcriptional activation of the Cdk inhibitor p21 by vitamin D_3 leads to the induced differentiation of the myelomonocytic cell line U937. Genes Dev 10:142–153
74. Verlinden L, Verstuyf A, Convents R, Marcelis S, Van Camp M, Bouillon R (1998) Action of $1,25(OH)_2D_3$ on the cell cycle genes, cyclin D1, p21 and p27 in MCF-7 cells. Mol Cell Endocrinol 142:57–65
75. Danielsson C, Mathiasen IS, James SY et al (1997) Sensitive induction of apoptosis in breast cancer cells by a novel 1,25-dihydroxyvitamin D_3 analogue shows relation to promoter selectivity. J Cell Biochem 66:552–562
76. Saramäki A, Banwell CM, Campbell MJ, Carlberg C (2006) Regulation of the human $p21^{(waf1/cip1)}$ gene promoter via multiple binding sites for p53 and the vitamin D_3 receptor. Nucleic Acids Res 34:543–554
77. Tavera-Mendoza L, Wang TT, Lallemant B et al (2006) Convergence of vitamin D and retinoic acid signalling at a common hormone response element. EMBO Rep 7:180–185
78. Rickert P, Seghezzi W, Shanahan F, Cho H, Lees E (1996) Cyclin C/CDK8 is a novel CTD kinase associated with RNA polymerase II. Oncogene 12:2631–2640
79. Bourbon HM, Aguilera A, Ansari AZ et al (2004) A unified nomenclature for protein subunits of mediator complexes linking transcriptional regulators to RNA polymerase II. Mol Cell 14:553–557
80. Li H, Lahti JM, Valentine M et al (1996) Molecular cloning and chromosomal localization of the human cyclin C (CCNC) and cyclin E (CCNE) genes: deletion of the CCNC gene in human tumors. Genomics 32:253–259
81. Akutsu N, Lin R, Bastien Y et al (2001) Regulation of gene Expression by 1α,25-dihydroxyvitamin D_3 and Its analog EB1089 under growth-inhibitory conditions in squamous carcinoma Cells. Mol Endocrinol 15:1127–1139

82. Matilainen M, Malinen M, Saavalainen K, Carlberg C (2005) Regulation of multiple insulin-like growth factor binding protein genes by 1α,25-dihydroxyvitamin D_3. Nucleic Acids Res 33: 5521–5532
83. Zhan Q, Antinore MJ, Wang XW et al (1999) Association with Cdc2 and inhibition of Cdc2/Cyclin B1 kinase activity by the p53-regulated protein Gadd45. Oncogene 18:2892–2900
84. Jiang F, Li P, Fornace AJ Jr., Nicosia SV, Bai W (2003) G2/M arrest by 1,25-dihydroxyvitamin D3 in ovarian cancer cells mediated through the induction of GADD45 via an exonic enhancer. J Biol Chem 278:48030–48040
85. Peng L, Malloy PJ, Feldman D (2004) Identification of a functional vitamin D response element in the human insulin-like growth factor binding protein-3 promoter. Mol Endocrinol 18:1109–1119
86. Dunlop TW, Väisänen S, Frank C, Molnar F, Sinkkonen L, Carlberg C (2005) The human peroxisome proliferator-activated receptor d gene is a primary target of 1α,25-dihydroxyvitamin D_3 and its nuclear receptor. J Mol Biol 349:248–260
87. Reed KR, Sansom OJ, Hayes AJ et al (2004) PPARδ status and Apc-mediated tumourigenesis in the mouse intestine. Oncogene 23:8992–8996
88. Väisänen S, Dunlop TW, Sinkkonen L, Frank C, Carlberg C (2005) Spatio-temporal activation of chromatin on the human CYP24 gene promoter in the presence of 1α,25-dihydroxyvitamin D_3. J Mol Biol 350:65–77
89. Swami S, Raghavachari N, Muller UR, Bao YP, Feldman D (2003) Vitamin D growth inhibition of breast cancer cells: gene expression patterns assessed by cDNA microarray. Breast Cancer Res Treat 80:49–62
90. Palmer HG, Sanchez-Carbayo M, Ordonez-Moran P, Larriba MJ, Cordon-Cardo C, Munoz A (2003) Genetic signatures of differentiation induced by 1α,25-dihydroxyvitamin D_3 in human colon cancer cells. Cancer Res 63:7799–7806
91. Peehl DM, Shinghal R, Nonn L et al (2004) Molecular activity of 1,25-dihydroxyvitamin D_3 in primary cultures of human prostatic epithelial cells revealed by cDNA microarray analysis. J Steroid Biochem Mol Biol 92:131–141
92. Krishnan AV, Shinghal R, Raghavachari N, Brooks JD, Peehl DM, Feldman D (2004) Analysis of vitamin D-regulated gene expression in LNCaP human prostate cancer cells using cDNA microarrays. Prostate 59:243–251
93. Ikezoe T, Gery S, Yin D et al (2005) CCAAT/enhancer-binding protein delta: a molecular target of 1,25-dihydroxyvitamin D3 in androgen-responsive prostate cancer LNCaP cells. Cancer Res 65: 4762–4768
94. Eelen G, Verlinden L, van Camp M et al (2004) The effects of 1α,25-dihydroxyvitamin D_3 on the expression of DNA replication genes. J Bone Miner Res 19:133–146
95. Eelen G, Verlinden L, Van Camp M et al (2004) Microarray analysis of 1α,25-dihydroxyvitamin D_3-treated MC3T3-E1 cells. J Steroid Biochem Mol Biol 89–90:405–407
96. Wang TT, Tavera-Mendoza LE, Laperriere D et al (2005) Large-scale *in silico* and microarray-based identification of direct 1,25-dihydroxyvitamin D_3 target genes. Mol Endocrinol 19:2685–2695
97. Sinkkonen L, Malinen M, Saavalainen K, Väisänen S, Carlberg C (2005) Regulation of the human cyclin C gene via multiple vitamin D_3-responsive regions in its promoter. Nucleic Acids Res 33: 2440–2451
98. Xie X, Lu J, Kulbokas EJ et al (2005) Systematic discovery of regulatory motifs in human promoters and 3′ UTRs by comparison of several mammals. Nature 434:338–345
99. Seuter S, Väisänen S, Radmark O, Carlberg C, Steinhilber D (2007) Functional characterization of vitamin D responding regions in the human 5-lipoxygenase gene. Biochim Biophys Acta 1771: 864–872
100. Klan N, Seuter S, Schnur N, Jung M, Steinhilber D (2003) Trichostatin A and structurally related histone deacetylase inhibitors induce 5-lipoxygenase promoter activity. Biol Chem 384:777–785
101. Sorg BL, Klan N, Seuter S et al (2006) Analysis of the 5-lipoxygenase promoter and characterization of a vitamin D receptor binding site. Biochim Biophys Acta 1761:686–697
102. Ogata K, Sato K, Tahirov TH (2003) Eukaryotic transcriptional regulatory complexes: cooperativity from near and afar. Curr Opin Struct Biol 13:40–48

103. Zella LA, Kim S, Shevde NK, Pike JW (2006) Enhancers located within two introns of the vitamin D receptor gene mediate transcriptional autoregulation by 1,25-dihydroxyvitamin D_3. Mol Endocrinol 20:1231–1247
104. Fretz JA, Zella LA, Kim S, Shevde NK, Pike JW (2007) 1,25-Dihydroxyvitamin D3 induces expression of the Wnt signaling co-regulator LRP5 via regulatory elements located significantly downstream of the gene's transcriptional start site. J Steroid Biochem Mol Biol 103:440–445
105. Kim S, Yamazaki M, Zella LA, Shevde NK, Pike JW (2006) Activation of receptor activator of NF-kappaB ligand gene expression by 1,25-dihydroxyvitamin D_3 is mediated through multiple long-range enhancers. Mol Cell Biol 26:6469–6486
106. Mader S, Leroy P, Chen J-Y, Chambon P (1993) Multiple parameters control the selectivity of nuclear receptors for their response elements. J Biol Chem 268:591–600
107. Schräder M, Müller KM, Becker-André M, Carlberg C (1994) Response element selectivity for heterodimerization of vitamin D receptors with retinoic acid and retinoid X receptors. J Mol Endocrinol 12:327–339
108. Schräder M, Müller KM, Carlberg C (1994) Specificity and flexibility of vitamin D signalling. Modulation of the activation of natural vitamin D response elements by thyroid hormone. J Biol Chem 269:5501–5504
109. Schräder M, Becker-Andre M, Carlberg C (1994) Thyroid hormone receptor functions as monomeric ligand-induced transcription factor on octameric half-sites. Consequences also for dimerization. J Biol Chem 269:6444–6449
110. Barski A, Cuddapah S, Cui K et al (2007) High-resolution profiling of histone methylations in the human genome. Cell 129:823–837
111. Matys V, Fricke E, Geffers R et al (2003) TRANSFAC: transcriptional regulation, from patterns to profiles. Nucleic Acids Res 31:374–378
112. Wasserman WW, Sandelin A (2004) Applied bioinformatics for the identification of regulatory elements. Nat Rev Genet 5:276–287
113. Odom DT, Dowell RD, Jacobsen ES et al (2007) Tissue-specific transcriptional regulation has diverged significantly between human and mouse. Nat Genet 39:730–732
114. Heinäniemi M, Uski JO, Degenhardt T, Carlberg C (2007) Meta-analysis of primary target genes of peroxisome proliferator-activated receptors. Genome Biol 8:R147
115. Burns JL, Jackson DA, Hassan AB (2001) A view through the clouds of imprinting. FASEB J 15:1694–1703