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Review

The Impact of Chromatin Organization of Vitamin D Target Genes

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Abstract. *The vitamin D receptor (VDR), the nuclear receptor for 1α,25-dihydroxyvitamin D3 (1α,25(OH)$_2$D$_3$), controls gene expression by binding discrete DNA sequences in promoter regions of target genes, referred to as 1α,25(OH)$_2$D$_3$ response elements (VDREs). Although these elements are well characterized in vitro, the function of VDREs in living cells in the context of chromatin is still largely unknown. To resolve this issue, 7 to 8 kB of the promoter regions of the primary 1α,25(OH)$_2$D$_3$ target genes CYP24, cyclin C and p21$^{(Waf1/Cip1)}$ were studied by chromatin immunoprecipitation (ChIP) assays using antibodies against acetylated histone H4 (to assess the global chromatin status) and various other components of VDR-dependent gene activation, such as VDR, retinoid X receptor (RXR), coactivator (CoA) and corepressor proteins. This approach identified three to four functional VDREs per gene promoter. In parallel, the extended analysis of the gene areas, of all six members of the insulin-like growth factor binding protein (IGFBP) family (i.e., 10 kB of promoter, introns, exons and 10 kB of the downstream region) were screened in silico for putative VDR-binding sites. Gel shift, reporter gene and ChIP assays identified, in total, ten functional VDREs in the genes IGFBP1, 3 and 5 and real-time PCR confirmed that these genes are primary VDR targets. Taken together, these results suggest that a reasonable proportion of all VDR target genes, if not all, are under the control of multiple VDREs. These results will have an impact on the development of therapeutic regimes for diseases, such as cancer, that use 1α,25(OH)$_2$D$_3$ and its analogs.*

Abbreviations: 1α,25(OH)$_2$D$_3$, 1α,25-dihydroxyvitamin D$_3$; CDK, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; CoA, coactivator; CYP24, 25-hydroxyvitamin D$_3$-24-hydroxylase; DR, direct repeat; ER, everted repeat; IGFBP, insulin-like growth factor binding protein; LBD, ligand-binding domain; Pol II, RNA polymerase II; RE, response element; RXR, retinoid X receptor; TSS, transcription start site; VDR, vitamin D$_3$ receptor; VDRE, 1α,25(OH)$_2$D$_3$ response element.

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The most biologically-active vitamin D$_3$ metabolite, 1α,25(OH)$_2$D$_3$, is essential for mineral homeostasis and skeletal integrity (1). However, it also has important roles in the control of growth and differentiation in normal tissues and malignant cells derived from prostate, breast and bone (2). 1α,25(OH)$_2$D$_3$ is generated from sequential hydroxylations of vitamin D$_3$, which in turn is obtained from the diet or produced in the skin upon exposure to UV light (3). The first hydroxylation of vitamin D$_3$ occurs at the C25 position and is catalyzed by vitamin D-25-hydroxylase in the liver to produce 25-hydroxyvitamin D$_3$, the major circulating form of vitamin D$_3$ in mammals. 25-hydroxyvitamin D$_3$ is the substrate for a second hydroxylase, the renal enzyme 25-hydroxyvitamin D$_3$-1-hydroxylase resulting in the production of 1α,25(OH)$_2$D$_3$. Catabolism of vitamin D$_3$ metabolites is initiated by the widely expressed enzyme, 25-hydroxyvitamin D$_3$-24-hydroxylase, which is encoded by the gene *CYP24*. 1α,25(OH)$_2$D$_3$ mediates its genomic effects *via* the nuclear receptor VDR, the only nuclear protein that binds the nuclear hormone with high affinity (K_d=0.1 nM) (4). The *CYP24* gene is the most responsive primary VDR target, whose steady state mRNA expression level is very low in the absence of ligand, but is up to 1000-fold induced by stimulation with 1α,25(OH)$_2$D$_3$ (5). This tight regulation prevents excessive amounts of the hormone to build up.

The VDR

The VDR is one of eleven members of the nuclear receptor superfamily that function as classic endocrine receptors. These include the receptors for the nuclear hormones retinoic acid, thyroid hormone, estradiol, progesterone, testosterone, cortisol and aldosterol, which bind their specific ligand with a

K_d of 1 nM or less (6). Like most members of the nuclear receptor superfamily, the VDR contains two zinc finger structures that form a characteristic DNA-binding domain of 66 amino acids (7). In addition, the carboxy-terminal of the protein contains a ligand-binding domain (LBD) of approximately 300 amino acids formed by twelve α-helices (8). Ligand binding causes a conformational change within the LBD, whereby helix 12, the most carboxy-terminal α-helix, closes the ligand-binding pocket *via* a "mouse-trap-like" intramolecular folding event (9). The LBD is also involved in a variety of interactions with nuclear proteins, such as other members of the nuclear receptor superfamily, CoA and corepressor proteins (10). Corepressor proteins, such as NCoR, SMRT and Alien, link non-liganded, DNA-bound VDR to enzymes with histone deacetylase activity that cause chromatin condensation (11). This provides VDR with intrinsic repressive properties comparable to retinoic acid and thyroid hormone receptors. The conformational change within VDR's LBD after binding of $1\alpha,25(OH)_2D_3$ results in the replacement of corepressor molecules by a CoA protein of the p160-family, such as SRC-1, TIF2 or RAC3 (12), in complex with more general CoAs, such as CREB binding protein (CBP) (13). These CoA complexes have histone acetyl-transferase activity, whose action on their major nuclear substrate, histone N-terminus tails have the net effect of causing chromatin relaxation (14). In a subsequent step, ligand-activated VDR changes rapidly from interacting with the CoAs of the p160-family to those of mediator complexes, such as thyroid hormone receptor-associated protein 220 (TRAP220) (15). The mediator complexes which consist of approximately 15-20 proteins build a bridge to the basal transcription machinery (16). In this way, ligand-activated VDR executes two tasks: the modification of chromatin and the regulation of transcription. These ligand-triggered protein-protein interactions are the central molecular events of nuclear receptor-dependent $1\alpha,25(OH)_2D_3$ signaling.

VDREs

An essential pre-requisite for the direct modulation of transcription by $1\alpha,25(OH)_2D_3$ is the location of at least one activated VDR protein close to the transcription start site (TSS) of the respective primary $1\alpha,25(OH)_2D_3$ target gene. In the vast majority of cases identified to date, this is achieved through the specific binding of VDR to a $1\alpha,25(OH)_2D_3$ response element (VDRE) (4). In detail, the DNA-binding domain of the VDR contacts the major grove of a hexameric sequence, referred to as core binding motif, with the consensus sequence RGKTSA (R=A or G, K=G or T, S=C or G). The affinity of monomeric VDR to a single core binding motif is not sufficient for the formation of a stable protein-DNA complex and thus VDR requires the formation of homo- and/or heterodimeric complexes with a partner nuclear receptor in order to allow efficient DNA binding (17). The nuclear retinoid X receptor (RXR) is the preferential heterodimeric partner of VDR (4).

VDR binds well to two hexameric core binding motifs in a direct repeat (DR)-type orientation with three intervening nucleotides (17-19). DR3-type response elements (REs) are therefore widely accepted as the classic VDRE structure. However, effective VDR binding was also observed on DR4-type REs (17, 20) and on everted repeat (ER)-type VDREs with seven to nine nucleotides (ER7, ER8, ER9) (21, 22). Additionally, most of the currently known natural VDREs also have a DR3-type structure and are located within the first 1,000 bp of a promoter sequence upstream of the TSS with a consensus VDR core binding motif of RGKTSA. However, it should be noted that most of these VDREs were identified before the genomes of *homo sapiens* and other species were sequenced and only limited promoter sequences were available, thus explaining the apparent proximity to their target gene TSS, as well as their uniformity in structure. Moreover, only a very few of these VDREs, such as that of the rat *osteocalcin* gene, are understood in their promoter context, where other factors, such as chromatin organization and flanking binding sites for other transcription factors were taken into consideration. Results from *in silico* promoter screening and a more detailed understanding of the chromatin organization of primary $1\alpha,25(OH)_2D_3$ target genes, as discussed below, are likely to revise the view of VDRE action *in vivo*.

Complex VDREs

All natural VDREs are formed by only two hexameric core binding motifs in a DR3-, DR4-, DR6- and ER7- to ER9-type arrangement (23). These may be considered as "simple" VDREs, but the question is whether the occurrence of one simple VDRE within a promoter is sufficient for gene responsiveness to $1\alpha,25(OH)_2D_3$. 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* (20, 24). However, the chromatin in the region of the *pit-1* gene promoter containing this RE seems to be closed in most adult rat cells, so that the responsiveness of the gene to $1\alpha,25(OH)_2D_3$ is lower than expected (14). This indicates that a high *in vitro* binding affinity of VDR-RXR heterodimers for a VDRE is not sufficient for *in vivo* responsiveness to $1\alpha,25(OH)_2D_3$.

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 chromatin an essential pre-requisite for a functional VDRE. Chromatin decondensation is achieved by the activity of histone acetyltransferases, which are recruited to their local chromatin target by CoA proteins. In turn, these CoAs 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 promoter region contains and the more of these transcription factors are expressed in the respective cell, the higher is the chance that this area of the promoter becomes locally decondensed. The VDRE of the rat *osteocalcin* gene is flanked on both sides with a binding site for the transcription factor Runx2/Cbfa1 (25), which may act as a link to the nuclear scaffold. By contacting CoA proteins and histone acetyltransferases, 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 can become 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. This fact alone underlines the importance of experimentally verifying REs identified by both *in silico* and *in vitro* methods.

Whole Genome Screening for Putative VDREs

Considering both orientations of DNA, the nuclear receptor core binding motif RGKTSA should be found on average in every 256 bp of genomic DNA. Furthermore, dimeric assemblies of such hexamers should show up as DRs every 65,536 bp of the promoter sequence and as ERs every 32,768 bp. Since VDR-RXR heterodimers bind comparably well to DR3-, DR4- and ER9-type REs, an *in silico* screen would be expected to identify, on average every 16,384 bp, a putative VDR binding site. This would predict that nearly 200,000 putative VDREs exist within the human genome and, as a consequence, on average every gene would contain several VDREs in its promoter and should be responsive to $1\alpha,25(OH)_2D_3$. Similar calculations apply to other members of the nuclear receptor superfamily and for transcription factors with a shorter specific binding site even higher numbers would be predicted. A realistic number of $1\alpha,25(OH)_2D_3$-responding genes is far less than this, perhaps a few hundred per cell type. The number of VDR molecules varies from hundreds to several thousand molecules per cell.

These calculations make it obvious that not every putative VDR binding site is used in nature in any cell at any given time. The most obvious reason is that most of these sequences are effectively covered by nucleosomes, so that they are not accessible to the VDR. This applies, in particular, to those sequences that are isolated from other nuclear receptors or transcription factor binding sites or lie distant from the promoter. This perspective strongly discourages the idea that isolated, simple VDREs may be functional *in vivo*. Therefore, the currently identified simple VDREs may be parts of more complex VDREs, as already demonstrated for the *CYP24* and *osteocalcin* gene. An effective *in silico* prediction of novel VDREs has to focus on the identification of complex VDREs. Unfortunately, the *in silico* screening software, which is currently available, such as the NUBI-scan (26) and the NHR-scan (27), are unable to identify complex VDREs. Other available programs, such as TRANSFAC, have the capacity to identify larger numbers of REs for different transcription factors, making the identification of potential complex REs possible. However, even these programs are unable to predict chromatin condensation states and nucleosome positioning, which is essential information in determining the likelihood that a given RE lies in an accessible region of a promoter. General parameters, such as nucleosome positioning, interspecies homology screening of regulatory sequences, as well as the binding sites of all other transcription factors and their cell-specific expression patterns, have to be included in more effective versions of *in silico* screening software.

Chromatin

The major protein constituents of chromatin are the histones, which form nucleosomes. Covalent modifications of the lysines at the N-terminal tails of these histone proteins neutralize their positive charge and, thus, their attraction for the negatively-charged DNA is diminished (28). This influences the degree of chromatin packaging and regulates the access of transcription factors to their potential binding sites. More than ten specific modifications of histones are known, but the acetylation of the lysine at position 8 of histone 4 correlates most strongly with the activation of chromatin on a promoter preceeding the initiation of transcription (29). Therefore, in most cases, the histones associated with active regions of promoters have a higher degree of acetylation at certain positions than in repressed or silent regions. To date, most studies on transcriptional regulation have concentrated on isolated promoter regions or proximal promoters, where binding sites of nuclear receptors and other transcription factors have been localized (30).

Primary VDR Target Genes with Impact in Cellular Growth

In recent years, $1\alpha,25(OH)_2D_3$ and its low-calcemic analogs have emerged as promising anticancer agents (31). However, the mechanisms of the antiproliferative, pro-differentiating and pro-apoptotic effects of VDR ligands vary and are cell-specific. They are mediated by the up-regulation of tumor suppressors, such as the cyclin-dependent kinase (CDK) inhibitors p21 and p27 (32) and the down-regulation of oncogene products, including Bcl-2 (33) and Myc (34). The *p21(waf1/cip1)* gene was first suggested by Jiang *et al.* (35) to be a key gene for understanding the antiproliferative action of $1\alpha,25(OH)_2D_3$. Moreover, the *CYP24* gene was proposed to

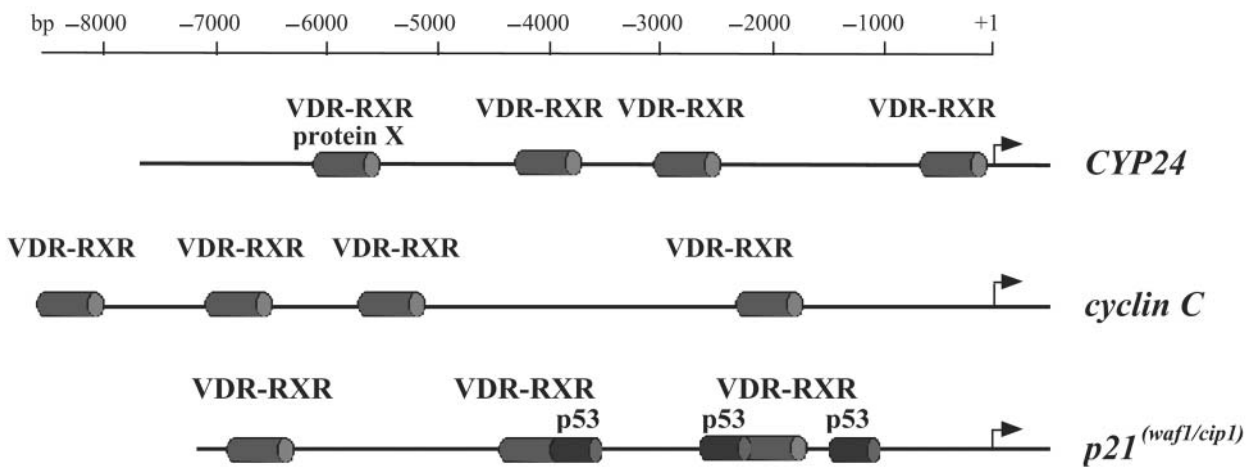

Figure 1. *1α,25(OH)$_2$D$_3$-responsive regions in the promoters of the human CYP24, cyclin C and p21$^{(waf1/cip1)}$ genes. Summary of the location of VDR-RXR heterodimer-associated regions as detected by the whole promoter ChIP approach. In the case of the p21$^{(waf1/cip1)}$ gene, p53 binding sites were also mapped and indicated.*

be an oncogene (36), since its overexpression significantly reduced beneficial 1α,25(OH)$_2$D$_3$ levels. The *CYP24* gene is the most responsive primary VDR target gene and showed at the mRNA level up to 1000-fold inducibility by 1α,25(OH)$_2$D$_3$ (37). Most other known primary 1α,25(OH)$_2$D$_3$ target genes, such as *cyclin C* and *p21$^{(waf1/cip1)}$*, are much less responsive and often show an inducibility of two-fold or less after short-term treatment with 1α,25(OH)$_2$D$_3$ (38, 39). All three genes were analyzed by studying 7.1 to 8.4 kB of their promoter in chromatin immunoprecipitation (ChIP) assays using each 20-25 overlapping promoter region. Promising promoter regions were then screened *in silico* for putative VDREs, whose functionality was analyzed in gel shift, reporter gene and re-ChIP assays. An alternative approach was performed with the six members of the *IGFBP* gene family by first *in silico* screening the whole gene area, which included 10 kB of both their upstream and downstream regions, followed by analysis of the candidate 1α,25(OH)$_2$D$_3$-responsive sequences by gel shift, reporter gene and re-ChIP assays.

a) The CYP24 gene: The spatio-temporal, 1α,25(OH)$_2$D$_3$-dependent chromatin acetylation status of the human *CYP24* promoter was analyzed by performing ChIP assays with antibodies against acetylated histone 4 (5). This was achieved by performing PCR on 25 contiguous genomic regions spanning the first 7.7 kB of the promoter. ChIP assays using antibodies against VDR revealed, in addition to the proximal promoter, three novel regions further upstream at approximate positions –2700, –4000 and –5800 relative to the TSS associated with VDR (Figure 1). Combined *in silico*/*in vitro* screening identified sequences in three out of the four promoter region sequences resembling known VDREs and reporter gene assays confirmed the inducibility of these regions by 1α,25(OH)$_2$D$_3$. In contrast, the fourth VDR-associated promoter region did not contain any recognizable classic VDRE that could account for the direct binding of VDR to DNA. However, re-ChIP assays monitored the simultaneous association of VDR with RXR, the CoAs RAC3 and TRAP220 and Pol II on all four promoter regions. These proteins showed a promoter region-specific association pattern, demonstrating the complex choreography of the *CYP24* gene promoter activation over a 300-min time-period.

Previously, the cluster of VDREs in the proximal *CYP24* promoter was found to be sufficient to explain the dominant role of the VDR in the regulation of the human, mouse and rat *CYP24* gene (40-42). Similar findings have been reported for other primary nuclear receptor responding genes, such as the *pS2* gene core promoter and its response to estrogen receptor signaling (30). However, these core promoter regions do not, on their own, reflect the *in vivo* activation of the genes that they serve. The two additional classic VDREs of the *CYP24* promoter, the DR4-type REs at positions –2700 and –4000, confirm the concept that each promoter may contain multiple functional REs to account for the *in vivo* gene activation profiles. The additional 1α,25(OH)$_2$D$_3$-responsive region at position –5800 also showed strong VDR association, but seemed not to contain any recognizable VDRE and did not mediate ligand inducibility in isolation. There are various explanations to account for the presence of VDR on this region. It is possible that other transcription factors that bind in the vicinity stabilize a VDR-RXR heterodimer to a VDRE, which is physically weak under the stringent *in vitro* binding conditions. Such a phenomenon has already been described to explain the binding of VDR-RXR heterodimers to the proximal VDRE of the rat *CYP24* gene promoter (43). An alternative indirect approach has been described for the *IL-2* gene

promoter. Here, VDR-RXR heterodimers bound on top of a complex consisting of the transcription factors NF-AT and AP-1 and did not directly bind to DNA (44). In a similar way, a currently unknown protein may allow contact of the VDR-RXR heterodimers to the distal region of the *CYP24* promoter.

The histone 4 proteins associated with the three novel distal, 1α,25$(OH)_2D_3$-responsive *CYP24* promoter regions were already acetylated to some degree at time-point 0 min and this endowed them with a quicker 1α,25$(OH)_2D_3$-dependent chromatin response than the proximal region (5). Having acetylated regions prior to activation would have advantages in developing a fast response to environmental conditions. Acetylated histone 4 would allow the remodeling complexes, such as SWI/SNF, quicker access to these regions and give them a "head start" in a particular promoter's activation. The distal promoter regions may, therefore, play a role in the implementation of gene activation, because they raise their 1α,25$(OH)_2D_3$-dependent histone 4 acetylation status earlier and either stay acetylated longer or decrease their levels of acetylated histone 4 earlier compared to the proximal promoter region.

b) The cyclin C gene: The human *cyclin C* gene is a primary 1α,25$(OH)_2D_3$ target gene that shows an inducibility of two-fold or less after short-term treatment with 1α,25$(OH)_2D_3$ (45). Functionally, cyclin C belongs to the cyclin protein superfamily, whose members control cell cycle transitions through the activation of CDKs. Interestingly, the cyclin C-CDK8 complex was found to be associated with the Pol II basal transcriptional machinery (46) and was considered to be a functional part of those mediator protein complexes that are involved in gene repression (47). The cyclin C-CDK8 complex phosphorylates the carboxy-terminal domain of Pol II (46) and the basal transcription factor TFIIH (48) and both of the phosphorylations terminate transcription. Another role of cyclin C, in complex with CDK3, seems to be the regulation of the G0 to G1 transition of the cell cycle through specific phosphorylation of the retinoblastoma protein pRb (49). Moreover, the fact that the *cyclin C* gene, being located in chromosome 6q21, was deleted in a subset of acute lymphoblastic leukemias, suggests its involvement in tumorigenesis (50).

The basal expression of the *cyclin C* gene was 10,000 to 100,000 times higher than that of the CYP24 gene (51), suggesting that other transcription factors also contribute to the gene's activity. Concerning 1α,25$(OH)_2D_3$-mediated activation, 8.4 kB of the human *cyclin C* promoter were analyzed using ChIP assays with antibodies against acetylated histone 4, VDR and RXR (51). Interestingly, in contrast to our experience with the *CYP24* promoter (5), 1α,25$(OH)_2D_3$ treatment did not change the acetylation status of histone 4 on any region of the *cyclin C* promoter. This confirms the dominating role of VDR-RXR heterodimers for the *CYP24* gene regulation but reduces their impact for the *cyclin C* gene. However, four promoter regions showed a consistent, 1α,25$(OH)_2D_3$-dependent association with VDR and RXR over time and re-ChIP assays confirmed the simultaneous association of VDR with RXR, RAC3, TRAP220 and Pol II. Furthermore, *in silico* screening, gelshift and reporter gene assays identified, in each of these four regions, either a DR3- or DR4-type VDRE at approximate positions –2100, –5600, –7000 and –8300 relative to the TSS (Figure 1). The strong DR4-type VDRE at position –2100 was nearly three-times more potent than the DR3-type VDRE of the human *CYP24* proximal promoter when *in vitro* assays were used (24). This makes this *cyclin C* VDRE, in isolation, the most potent known human VDRE.

c) The p21$^{(waf1/cip1)}$ gene: The *p21$^{(waf1/cip1)}$* gene is a transcriptional target of the tumor suppressor protein p53, which binds as a tetramer to REs at positions –1400 and –2300 of the promoter (52). The p53 protein is a master regulator of a prominent transcriptional network that can control the fate of cells in response to stress and can be induced by chemotherapeutic agents, such as 5-fluorouracil (53). The previously reported VDRE within the human *p21$^{(waf1/cip1)}$* promoter (32) was characterized to be very weak (24) and many studies questioned the *p21$^{(waf1/cip1)}$* gene as being a primary VDR target gene (54-56). In order to challenge these questions, 20 overlapping regions covering the first 7.1 kB of the *p21$^{(waf1/cip1)}$* promoter were analyzed in MCF-7 human breast cancer cells using ChIP assays with antibodies against p53 and VDR (57). The p53 binding promoter regions at positions –1400 and –2300 (58) were confirmed but, due to the fact that most analyses of the *p21$^{(waf1/cip1)}$* promoter were restricted to the first 3 kB (59, 60), the novel p53 binding site at position –4000 has been overlooked so far. In addition, three VDR-associated promoter regions at positions –2200, –4400 and –6900 were found, *i.e.,* two regions showed binding for both p53 and VDR. *In silico* screening and *in vitro* binding assays using recombinant and *in vitro* translated proteins identified five p53 binding sites within the three p53-positive promoter regions as well as five VDREs within the three VDR-positive regions (57). Reporter gene assays confirmed the expected responsiveness of the respective promoter regions to the p53 inducer 5-fluorouracil and 1α,25$(OH)_2D_3$. Moreover, re-ChIP assays confirmed the functionality of the three 1α,25$(OH)_2D_3$-reponsive promoter regions by monitoring the simultaneous occupancy of VDR with the CoA proteins CBP, SRC-1 and TRAP220.

Cells, such as the MCF-7 breast cancer cells that were used for the screening of the *p21$^{(waf1/cip1)}$* promoter, have high basal *p21$^{(waf1/cip1)}$* mRNA expression because they contain a functional wild-type *p53* gene and resulting in only a minor 1.6-fold induction of gene expression when they were treated with 1α,25$(OH)_2D_3$. This may indicate that cells with the wild-

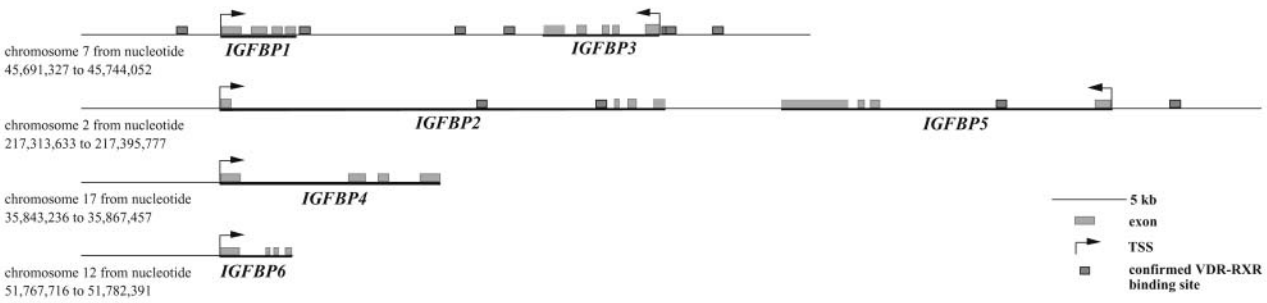

Figure 2. *Overview of the genomic organization and location of functional VDREs in the six human IGFBP family members. Human genomic DNA 174 kB (thin black line) from four different chromosomes comprising all six human IGFBP genes was screened in silico for putative VDREs. Ten VDR-RXR binding sites (gray boxes) were confirmed in genes IGFBP1, 2, 3 and 5, but none were in IGFBP4 or 6.*

type *p53* gene already have sufficiently high p21 protein levels, so that they may not need to take advantage of the $1\alpha,25(OH)_2D_3$ signaling pathway to further increase their $p21^{(waf1/cip1)}$ mRNA expression. This may also explain the varied observations concerning the response of the $p21^{(waf1/cip1)}$ gene to $1\alpha,25(OH)_2D_3$. However, even if not every type of cell may need to take full advantage of their $1\alpha,25(OH)_2D_3$ signaling system concerning a significant up-regulation of their $p21^{(waf1/cip1)}$ gene, the five VDREs in three regions of the $p21^{(waf1/cip1)}$ promoter are occupied with VDR and this system may be of greater importance in conditions where p53 protein is limiting. The demonstration of functional $1\alpha,25(OH)_2D_3$-responding promoter regions with the $p21^{(waf1/cip1)}$ promoter confirmed that the $p21^{(waf1/cip1)}$ gene is a primary $1\alpha,25(OH)_2D_3$-responding gene with at least three VDR-binding promoter regions, in two of which p53 also co-localizes.

d) The IGFBP gene family: Mitogens, such as insulin-like growth factors, have also been reported to be down-regulated by $1\alpha,25(OH)_2D_3$ (61). In addition, the up-regulation of factors, which control the actions of mitogens, such as the binding factors for insulin-like growth factors, the IGFBPs, have important anticancer effects (62). Recently, IGFBPs were found to be the primary mediators of the anti-proliferative actions of $1\alpha,25(OH)_2D_3$ in some cells, but depending on the cellular context, IGFBPs can also have a mitogenic effect. The *IGFBP3* gene was shown to be a primary VDR target (63) and is, therefore, of special interest in understanding the mechanisms of the cell-regulatory actions of $1\alpha,25(OH)_2D_3$. IGFBP3 belongs to the six-member IGFBP family that bind insulin-like growth factors with high affinity and specificity (64). IGFBPs also mediate insulin-like growth factor-independent actions, including the inhibition of cell growth and induction of apoptosis (65).

Comparative expression profiling of all six human *IGFBP* genes in prostate and bone cancer cells demonstrated that *IGFBP1, 3* and *5* are primary $1\alpha,25(OH)_2D_3$ target genes (66). *In silico* screening of the 174 kB of genomic sequence surrounding all six *IGFBP* genes identified 15 candidate VDREs close to or in *IGFBP1, 2, 3* and *5,* but not in the *IGFBP4* and *6* genes. The putative VDREs were evaluated *in vitro* by gelshift assays and in living cells by reporter gene and ChIP assays. Ten of these VDREs appeared to be functional (Figure 2). ChIP assays demonstrated an individual, stimulation time-dependent association profile for each of these, not only with the VDR, but also with RXR, other regulatory complex components and phosphorylated Pol II. Some of the VDREs are located distant from the TSS of *IGFBP1, 3* and *5*, but all ten VDREs seemed to contribute to the regulation of the genes by $1\alpha,25(OH)_2D_3$. This represents a 67% success rate of the *in silico* prediction of VDR binding sites, which was much higher than in comparable screenings (67). Moreover, non-ligand responsive genes, such as *IGFBP4* and *6*, did not contain any VDRE candidates, even though 24 and 15 kB, of their genomic sequence were screened, respectively. Finally, *in silico* analysis identified two VDREs within the first intron of the other non-ligand responsive gene, *IGFBP2*. However, ChIP analysis of the proximal promoter region of the *IGFBP2* gene indicated that these VDREs cannot be in contact with the TSS of this gene (66). It is, therefore, more likely that they are involved in the regulation of the neighboring *IGFBP5* gene. The high success rate of the combined *in silico* screen/ChIP analysis method suggests that its application on even larger screenings involving megabases of genomic sequence is possible.

Importantly, the *in silico* screening approach was not restricted to regulatory regions that comprise only a maximum 2 kB of the sequence up- and downstream of the TSS, as in a recent whole genome screen for regulatory elements (68), but involved up to 10 kB of flanking sequences, as well as intronic and even intergenic sequences. Therefore, this approach revealed candidate VDREs that are located more than 30 kB distant from their target gene TSS. Based on the present understanding of enhancers, DNA looping and chromatin

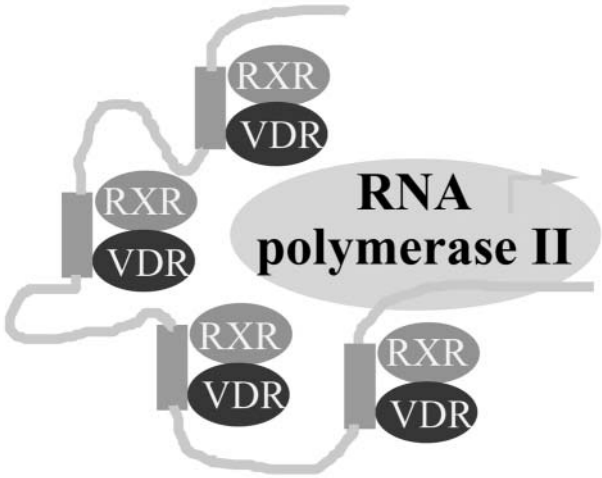

Figure 3. *A model of multiple VDRE action on a single promoter. Simultaneous communication of the individual promoter regions with the Pol II complex occurs through a discrete 3-dimensional organization of the promoter that is achieved via a large protein conglomeration such as the mediator complex. This arrangement would, therefore, allow the close contact of distant regions.*

units flanked by insulators or matrix attachment sites (69), these distances are no limitations.

Impact of Multiple VDREs

Although individual REs have been shown to be able to induce transactivation on their own, multiple VDREs, as now observed in the primary VDR target genes discussed above (5, 51, 57, 66), seem to favor the prominent response of the gene's transcription to VDR. Although the *in vitro* DNA binding affinity of VDR-RXR heterodimers to the VDREs described for these genes differ (compare (5, 51, 57, 66)), at the chromatin level all VDRE-containing promoter regions show a comparable association strength with VDR and RXR. Each of the multiple 1α,25$(OH)_2D_3$-responsive promoter regions is able to independently contact the basal transcriptional machinery (Figure 3). This suggests that the simultaneous communication of the individual promoter regions with the Pol II complex occurs through a discrete three-dimensional organization of the promoter within the nucleus of a cell and that this is achieved *via* a large protein conglomeration such as the mediator complex. This arrangement would, therefore, allow the close contact of distant regions. Such a model could resemble the traditional "DNA looping model" discussed to explain the activity of upstream enhancer elements (70).

Interestingly, the number of VDREs within a promoter does not correlate with the inducibility of a VDR target gene, since the average short-term transcriptional response of most primary VDR target genes was only two-fold or less (56). However, most of them are simultaneously under the control of other transcription factors, such p53, in case of the *p21(waf1/cip1)* gene.

Conclusion

The spatio-temporal pattern of the promoters of the human genes *CYP24, cyclin C* and *p21(waf1/cip1)* is complex and is far from being fully understood. However, these studies have indicated the importance of analyzing the chromatin organization of nuclear receptor responsing genes over time and to investigate larger promoter regions, including those which contain no obvious REs. The sequencing of the complete human genome and the genome of other species and, therefore, the availability of all regulatory sequences, enables a more mature understanding of the diversity of 1α,25$(OH)_2D_3$ target genes. Perhaps the idea of simple isolated VDREs should shift to the concept of multiple complex VDREs, of which the simple VDRE represents the core. The coordinated action of these different VDREs could explain the individual response of target genes to 1α,25$(OH)_2D_3$.

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