REVIEW ARTICLE

Genomic mechanisms involved in the pleiotropic actions of 1,25-dihydroxyvitamin D_3

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The biologically active metabolite of vitamin D (cholecalciferol), i.e. 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], is a secosteroid hormone whose mode of action involves stereospecific interaction with an intracellular receptor protein (vitamin D receptor; VDR). 1,25$(OH)_2D_3$ is known to be a principal regulator of calcium homeostasis, and it has numerous other physiological functions including inhibition of proliferation of cancer cells, effects on hormone secretion and suppression of T-cell proliferation and cytokine production. Although the exact mechanisms involved in mediating many of the different effects of 1,25$(OH)_2D_3$ are not completely defined, genomic actions involving the VDR are clearly of major importance. Similar to other steroid receptors, the VDR is phosphorylated; however, the exact functional role of the phosphorylation of the VDR remains to be determined. The VDR has been reported to be regulated by 1,25$(OH)_2D_3$ and also by activation of protein kinases A and C, suggesting co-operativity between signal transduction pathways and 1,25$(OH)_2D_3$ action. The VDR binds to vitamin D-responsive elements (VDREs) in the 5′ flanking region of target genes. It has been suggested that VDR homodimerization can occur upon binding to certain VDREs but that the VDR/retinoid X receptor (RXR) heterodimer is the functional transactivating species. Other factors reported to be involved in VDR-mediated transcription include chicken ovalbumin upstream promoter (COUP) transcription factor, which is involved in active silencing of transcription, and transcription factor IIB, which has been suggested to play a major role following VDR/RXR heterodimerization. Newly identified vitamin D-dependent target genes include those for Ca^{2+}/Mg^{2+}-ATPase in the intestine and p21 in the myelomonocytic U937 cell line. Elucidation of the mechanisms involved in the multiple actions of 1,25$(OH)_2D_3$ will be an active area of future research.

INTRODUCTION

It is known that the most active metabolite of vitamin D (cholecalciferol), i.e. 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], functions to regulate calcium homeostasis in intestine, bone and kidney [1,2]. 1,25$(OH)_2D_3$ has also been found to have numerous other physiological functions, including effects on cell growth and differentiation [2,3], secretion of hormones [4–6], T-cell proliferation and cytokine production [7,8], sterol metabolism [9] and cardiovascular function [10] (see [1,2,8,10–14] for reviews). 1,25$(OH)_2D_3$, similar to other steroid hormones, binds to a high-affinity, low-capacity receptor protein (the vitamin D receptor or VDR), resulting in concentration of the 1,25$(OH)_2D_3$–VDR complex in the target cell nucleus. Exactly how 1,25$(OH)_2D_3$ affects numerous different systems is a subject of continuing investigation; however, the VDR is clearly of major importance.

Although many gene products have been reported to be sensitive to 1,25$(OH)_2D_3$ (see [15] for a list of 51 genes reported to be regulated by the active form of vitamin D), little is known about the molecular mechanisms involved in the regulation by 1,25$(OH)_2D_3$ of most of these genes. Transcriptional regulation has been described for about 20% of the genes reported to be regulated by 1,25$(OH)_2D_3$, and only a small number of genes, including those encoding osteopontin, osteocalcin, 25-hydroxyvitamin D_3 24-hydroxylase (24-hydroxylase) and integrin $\alpha_v\beta_3$, have been reported to contain a vitamin D-responsive element (VDRE). It is likely that future research will result in the identification of a number of different mechanisms of regulation of these genes, including effects of 1,25$(OH)_2D_3$ on stability and the possibility that induction by 1,25$(OH)_2D_3$ may be secondary to a primary effect of 1,25$(OH)_2D_3$ on other factors.

In this review, recent findings related to genomic mechanisms of 1,25$(OH)_2D_3$ action will be discussed, including evidence for a role of transcription factor IIB (TFIIB) and other transcription factors in vitamin D-mediated transcription. An overview of newly identified vitamin D-responsive genes will also be presented. In addition, current controversies in the vitamin D field related to VDR regulation, the site of phosphorylation of the VDR and VDR homodimerization versus heterodimerization will be reviewed. It should be noted, however, that not all vitamin D-regulated biological responses may require nuclear-mediated mechanisms [16,17]; possible non-genomic mechanisms will not be discussed here (non-genomic effects of 1,25$(OH)_2D_3$ are discussed as part of a recent excellent review by Bouillon et al. [18]).

THE VDR

General considerations

Although the VDR protein has been cloned from human [19], rat [20,21], mouse [22] and chicken [23] (for reviews concerning the VDR, see [24,25]), a complete analysis of the sequence and detailed structural and functional studies have not been published for a number of the VDRs. X-ray crystallographic data, which

Abbreviations used: 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; (h)VDR, (human) vitamin D receptor; 24-hydroxylase, 25-hydroxyvitamin D_3 24-hydroxylase; VDRE, vitamin D-responsive element; TFIIB, transcription factor IIB; RAR, retinoic acid receptor; PMA, phorbol 12-myristate 13-acetate; CAT, chloramphenicol acetyltransferase; calbindin D_{28k}, 28 kDa form of calbindin D; NFAT, nuclear factor of activated T-cells; RXR, retinoid X receptor; tk, thymidine kinase; COUP-TF, chicken ovalbumin upstream promoter transcription factor.

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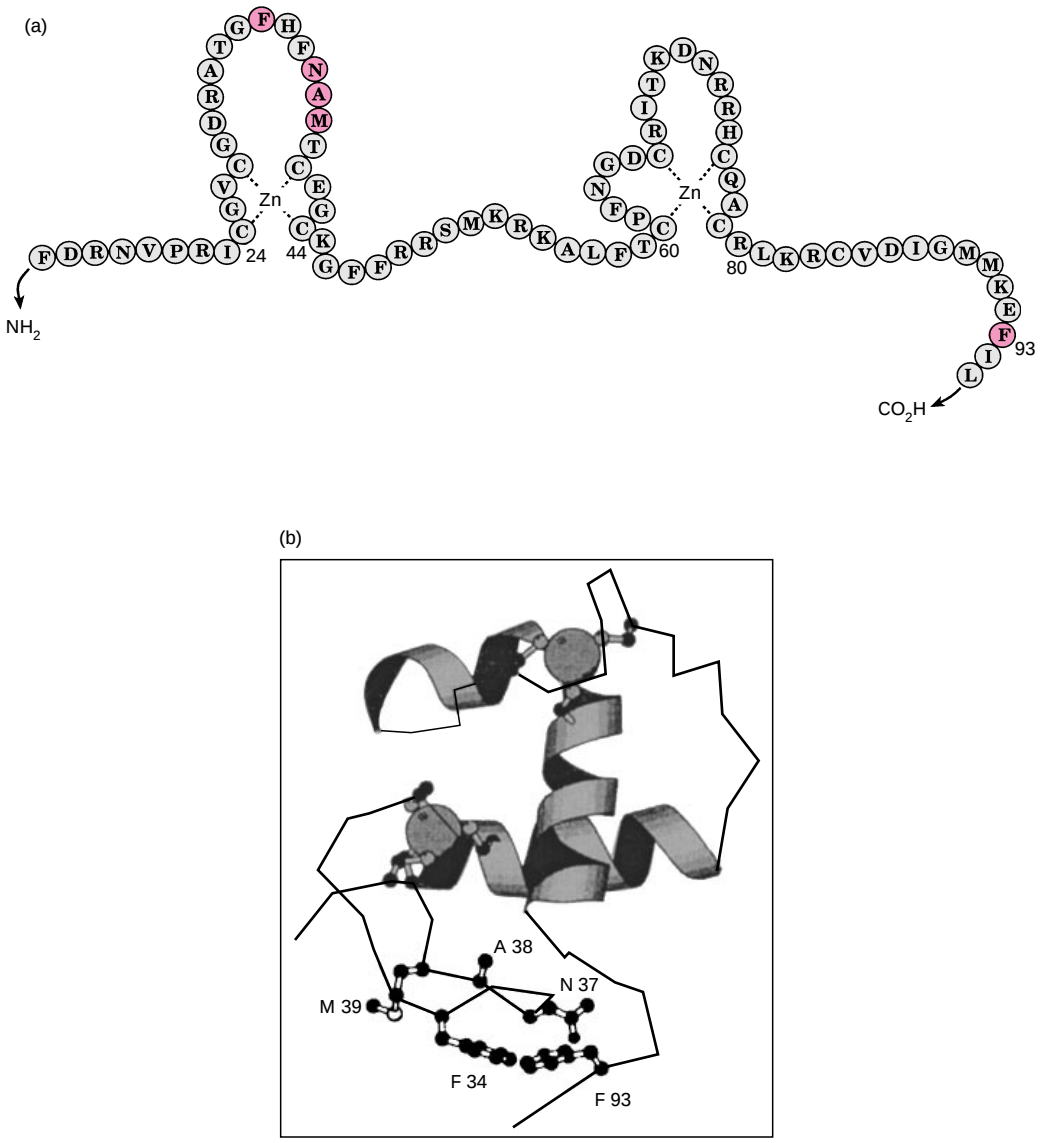

Figure 1 The VDR DNA-binding domain

(**a**) The zinc fingers of the DNA-binding domain of the hVDR (primary sequence and secondary structure [47]). Residues discussed in (**b**) are coloured. (**b**) A model for intramolecular interaction of the T-box of the VDR (approx. five residues beyond the C-terminus of the second zinc finger) with the tip of the first zinc finger. Helices (ribbons), the C^{α} backbone trace (line), side chains of selected residues of the T-box and the tip of the first zinc finger (ball and stick) and metal co-ordinating cysteines are shown. The two zinc ions are represented by spheres. The recognition α-helix of the first zinc finger, which lies in the major groove of the DNA, is oriented horizontally. The principal helix of the second zinc finger lies vertically. Phe-93 of the T-box may pack against Asn-37 and Phe-34, which is supported by Ala-38 and probably Met-39. Reproduced with permission from [114].

are not as yet available, should result in refined structural analysis, including tertiary structure and distance geometry, which should greatly facilitate structure/function studies. The molecular mass of the VDR has been calculated to be between 48 kDa {for the human VDR (hVDR) [26]} and 60 kDa (for the avian VDR [23]). Among species there is between 93 and 95% sequence identity in the DNA- and hormone-binding domains. However, in the hinge region there was found to be significant variation in the sequences between species. Although detailed analysis of the structural organization of the VDR is not available as yet, 5′ and 3′ deletion of the hVDR were used to localize functional domains [26]. Data from these studies indicated that hormone binding was localized to a peptide fragment whose synthesis begins at residue 114 and ends at residue 373. The DNA-binding domain of the hVDR is the cysteine-rich region located at the extreme N-terminus (residues 1–114), i.e. the conserved zinc finger region. There are two zinc fingers. The zinc atom is proposed to be in a tetrahedral arrangement with the cysteine residues, which are highly conserved (Figure 1). It has been proposed for the VDR, similar to the glucocorticoid receptor and the oestrogen receptor, that an α-helical region residing at the C-terminal base of the first zinc finger may be responsible for receptor specificity for binding a specific DNA sequence [27]. It has also been proposed that specificity can be conferred by spacing between two half-sites of a VDRE [28], although strict adherence to spacer regions of 3, 4 and 5 nucleotides for VDR, thyroid hormone receptor and retinoic acid receptor (RAR) recognition respectively has been questioned [25]. In addition, auxiliary factors may contribute to VDR specificity in specific target tissues.

A region in the second zinc finger (five amino acids between the first two co-ordinating systems), the D-box, which is essential for dimer binding to the glucocorticoid response element [29], has been described in the crystal structure of the glucocorticoid receptor [30]. Freedman and Towers [31] reported that when the glucocorticoid receptor D-box is replaced by residues in that region of the VDR DNA-binding domain, co-operative DNA binding usually observed with the glucocorticoid receptor is abolished, suggesting that the VDR and the glucocorticoid receptor use different strategies to bind half-sites (the VDR DNA-binding domain appears to bind half-sites non-cooperatively, without the free-energy contribution of dimerization that has been observed for the glucocorticoid receptor), and that the VDR DNA-binding domain binds DNA independently of a glucocorticoid receptor-type finger-mediated dimer.

Phosphorylation of the VDR

The VDR [32–34], similar to other steroid hormone receptors including those for progesterone [35], androgens [36], oestrogen [37,38] and glucocorticoids [39], is phosphorylated. Although it has been suggested that steroid receptor phosphorylation is involved in nuclear localization, hormone binding and transcriptional activation or repression, the exact functional role(s) of phosphorylation remains to be elucidated. Brown and DeLuca showed an increase in phosphorylation of the VDR within 15 min of $1,25(OH)_2D_3$ treatment, which continued for 1 h [33]. However, a low level of phosphorylated receptors was detectable even in the absence of $1,25(OH)_2D_3$. Thus ligand binding does not seem to be essential for phosphorylation, but does result in a substantial increase in phosphorylation. Several studies have shown that okadaic acid (an inhibitor of phosphatases 1 and 2A) can activate VDR-mediated transcription in the absence of ligand [40–42]. It is possible that activation of VDR-mediated transcription by okadaic acid is a result of direct phosphorylation of the VDR. However, it is also possible that okadaic acid may enhance the phosphorylation of an auxiliary transcription factor or of a member of the basal RNA polymerase II transcription machinery, which could further influence VDR-mediated transactivation. Although the exact nature of the effect of okadaic acid on VDR transactivation remains to be determined, these studies do suggest that ligand occupancy is not required for VDR-mediated transcription.

The subject of the site(s) of phosphorylation on the VDR has been somewhat controversial. The identification of the phosphoamino acid(s) of the hVDR was first reported by Hsieh et al. [43] and Jurutka et al. [44]. Hsieh et al. [43] reported that the hVDR is phosphorylated by protein kinase C-β on Ser^{51} in the zinc finger region, and Jurutka et al. [44] reported that the VDR is phosphorylated in the ligand-binding domain by casein kinase II at Ser^{208} (Figure 2). The magnitude of the phosphorylation at Ser^{208} was not affected by the addition of $1,25(OH)_2D_3$. These results were obtained using site-directed mutagenesis. When Ser^{51} or Ser^{208} was mutated to glycine, phosphorylation of the hVDR was reduced. However, studies by Brown and DeLuca, using the rat VDR, were not in agreement with Ser^{51} as a phosphorylation site, since they found that phosphorylation was localized only in the hormone-binding domain [45]. In a later study, Hilliard et al. [46], using selective manual Edman degradation of phosphorylated peptides coupled with direct amino acid sequence analysis of the isolated peptides, reported that a phosphate was released only at Ser^{205} (note that residue 1 of the hVDR reported in that paper was equivalent to residue 4 of the hVDR sequence reported by Baker et al. [47] which was used in the papers of Hsieh et al. [43] and Jurutka et al. [44]). Unlike the results reported by Jurutka et al. [44], the phosphorylation of Ser^{205} was strongly dependent on $1,25(OH)_2D_3$ treatment. Hilliard et al. [46] also showed that when Ser^{205} was modified by site-directed mutagenesis to alanine, the mutated protein continued to be phosphorylated in a $1,25(OH)_2D_3$-dependent manner. Jurutka et al. [44] reported that, when Ser^{208} was replaced with glycine, at least one additional phosphoacceptor site existed and that this site (not Ser^{208}) was increased in the presence of $1,25(OH)_2D_3$. Hilliard et al. [46] suggested that after site-directed mutagenesis an alternative site may become available due to structural alteration as a result of mutagenesis of the VDR. The authors therefore suggested that cautious interpretation should be made of data derived from mutagenesis experiments, particularly since the crystal structure of the VDR is unavailable at this time. Knowledge of this crystal structure will facilitate the resolution of the differences observed between the peptide mapping approach and mutagenesis experiments.

Regulation of the VDR

Up-regulation of VDRs by $1,25(OH)_2D_3$ has been demonstrated in several different systems, including rat intestine [48], pig kidney LLCPK-1 cells [49], 3T6 mouse fibroblasts [50], human HL60 leukaemia cells [51] and rat insulinoma cells [52]. However, whether the homologous up-regulation of the VDR involves induction of VDR mRNA is not clear. Mangelsdorf et al. [50] found an increase in VDR mRNA as well as an increase in VDR protein levels in mouse 3T6 cells in response to $1,25(OH)_2D_3$ treatment for 24–72 h. However, studies by Huang et al. [53] and Wiese et al. [54] indicated that administration of $1,25(OH)_2D_3$ to vitamin D-deficient rats did not alter VDR mRNA levels in intestine and kidney. Meyer et al. [55] reported that, when compared with control animals, chicks fed a low-calcium diet [which results in a marked increase in serum $1,25(OH)_2D_3$]

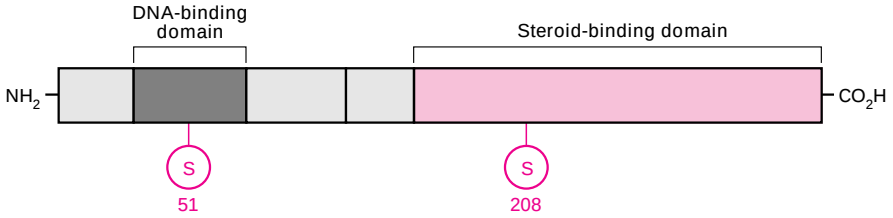

Figure 2 Schematic representation of the hVDR and proposed phosphorylation sites

Phosphorylation sites on the VDR are as reported by Hsieh et al. [43] and Jurutka et al. [44]. See the text for details.

exhibited a significant decrease in VDR mRNA. Reinhardt and Horst [56] reported that parathyroid hormone is a potent down-regulator of VDR mRNA. Thus it is possible that, in the low-calcium, vitamin D-deficient animals, up-regulation of VDR mRNA by $1,25(OH)_2D_3$ is not observed due, in part, to parathyroid hormone inhibition of VDR mRNA production. *In vitro* studies by Wiese et al. [54] indicated that VDR protein levels in 3T6 cells were induced 3-fold at 8 h after $1,25(OH)_2D_3$ treatment; however, VDR mRNA was not altered by $1,25(OH)_2D_3$ during this time. Similar findings indicating induction of VDR protein but not mRNA were noted in studies in rat epithelial cells [54], HL60 cells [51], rat insulinoma cells [52] and ROS 17/2.8 osteosarcoma cells [57], and for the VDR in transformed yeast cells [58]. It has been suggested that induction of $1,25(OH)_2D_3$ receptor protein following $1,25(OH)_2D_3$ administration is most likely due to altered stability of the occupied receptor [51,52,54,57,58].

Besides homologous up-regulation, the VDR has been reported to be regulated by a number of other factors, including glucocorticoids [53,59–63] and activation of protein kinase A [64–66] and protein kinase C [67]. Glucocorticoids have been reported to up-regulate as well as to down-regulate the VDR in a number of cells and tissues. However, it has been shown that up-regulation of rat intestinal VDR mRNA by glucocorticoids is not specific for VDR mRNA, but may reflect general effects of glucocorticoids on the intestine [53,63]. Concerning the effect of protein kinase C and protein kinase A, it has been suggested that the regulation of the VDR by signal transduction pathways may play an important role in modulating target cell responsiveness to $1,25(OH)_2D_3$. For example, Krishnan and Feldman [64] reported that in NIH3T3 mouse fibroblasts the elevation of intracellular cAMP by forskolin or dibutyryl-cAMP resulted in an 8–10-fold increase in VDR abundance. On the other hand, treatment of NIH3T3 cells with the phorbol ester phorbol 12-myristate 13-acetate (PMA), whose actions are mediated through the activation of protein kinase C, resulted in a time- and dose-dependent down-regulation of the VDR [67]. Up- and down-regulation of the VDR by cAMP and PMA respectively in NIH3T3 cells transfected with a plasmid containing the human osteocalcin VDRE fused to the reporter gene chloramphenicol acetyltransferase (CAT) resulted in a corresponding induction or attenuation in CAT activity. Thus the functional response corresponded to the changes in the VDR.

Treatment of mouse osteoblasts (MC3T3-E1 cells) and rat osteosarcoma (UMR-106-01) cells with forskolin or parathyroid hormone was also found to result in VDR up-regulation, and treatment with PMA was found to result in VDR down-regulation [66], similar to the results observed in NIH3T3 cells. Parathyroid hormone was found to enhance the $1,25(OH)_2D_3$-mediated induction of 24-hydroxylase mRNA in UMR cells. These findings have clinical implications, since in hyperparathyroid patients an augmented response to $1,25(OH)_2D_3$ may contribute to hypercalcaemia. It should be noted, however, that opposite findings have been reported by others. Reinhardt and Horst have shown that parathyroid hormone down-regulates, and activation of protein kinase C up-regulates, the VDR in rat osteosarcoma cells (ROS 17/2.8) [56,68]. They also reported that PMA enhanced the $1,25(OH)_2D_3$ induction of the VDR in these cells [68]. Van Leeuwen et al. [69] reported that in UMR-106-01 osteosarcoma cells, treatment with PMA down-regulated the VDR at 2–6 h but resulted in up-regulation after 24 h. These findings suggest that proliferation state, cell type and stage of differentiation may affect the interaction between $1,25(OH)_2D_3$ and signal transduction pathways. Potentiation rather than inhibition of the effect of $1,25(OH)_2D_3$ by activation of protein kinase C has also been observed using primary cultures of rat kidney cells [70] and intestinal epithelial cells [71]. In these cells, pharmacological doses of $1,25(OH)_2D_3$ resulted in induction of 24-hydroxylase mRNA. In the presence of PMA the dose–response curve shifted to the left, so that physiological concentrations of $1,25(OH)_2D_3$ were effective. The time course of the response was also shifted to the left. Thus PMA-induced second messenger pathways potentiated and accelerated the effect of $1,25(OH)_2D_3$ on 24-hydroxylase gene expression [70,71]. Potentiation of a response in the presence of both $1,25(OH)_2D_3$ and PMA was also observed by Chang and Prince [72], who reported that $1,25(OH)_2D_3$ is unable by itself to induce phosphorylation of osteopontin in JB6C141.5a epidermal cells or to induce tumorigenic transformation of these cells. However, $1,25(OH)_2D_3$ was found to enhance the synthesis and secretion of phosphorylated osteopontin induced by PMA and to enhance PMA-induced tumorigenic transformation of JB6C141.5a cells.

Collectively, these findings suggest co-operativity between signal transduction pathways and $1,25(OH)_2D_3$ action. The mechanism may involve an effect on the VDR, an effect on the promoter of the target gene and/or an effect on other transcription factors that may be interacting with the VDR to mediate $1,25(OH)_2D_3$-induced transcriptional activation. An interesting area of future research will be the study of the mechanisms and transcription factors involved in the interaction of these pathways.

VITAMIN D-MEDIATED TRANSCRIPTIONAL REGULATION

The genomic mechanism of $1,25(OH)_2D_3$ action involves the direct interaction of the VDR with DNA sequences. Although over 50 genes have been reported to be regulated by $1,25(OH)_2D_3$, only a small number have been reported to contain VDREs (Table 1). Although based on a very limited number of natural VDREs, in general the VDRE has been reported to consist of two direct imperfect repeats of the nucleotide sequence GGGTGA separated by three nucleotide pairs. The genes for osteocalcin, osteopontin and most recently 24-hydroxylase have provided the most information concerning transcriptional activation by $1,25(OH)_2D_3$. Although the exact function of osteocalcin is not known, its induction is positively correlated with new bone formation [73], whereas osteopontin promotes attachment of osteoclasts to the bone surface [74]. 24-Hydroxylase is thought to be the enzyme involved in the first step in the catabolism of $1,25(OH)_2D_3$ [75].

Both the human and rat osteocalcin VDREs have been well characterized [76–86]. Besides a VDRE, an AP-1 consensus sequence (TGACTCA) closely juxtaposed to the VDRE has been identified in the human osteocalcin promoter [78]. Ozono

Table 1 VDREs present in vitamin D-regulated genes

See the text for further details.

Gene product	VDRE sequence
Rat 24-hydroxylase	AGGTGA gtg AGGGCG (−151 to −137)
	CGCACC cgc TGAACC (−259 to −245)
Mouse osteopontin	GGTTCA cga GGTTCA (−757 to −743)
Human osteocalcin	GGGTCA acg GGGGCA (−499 to −485)
Rat osteocalcin	GGGTGA atg AGGACA (−455 to −441)
Mouse calbindin D_{28k}	GGGGGA tgtg AGGAGA (−198 to −183)
Rat calbindin D_{9k}	GGGTGT cgg AAGCCC (−489 to −475)
Avian integrin β_3	GAGGCA gaa GGGAGA (−770 to −756)

et al. [78] reported that the AP-1 site synergistically enhanced activation by $1,25(OH)_2D_3$. However, expression of c-*jun* and c-*fos* in ROS 17/2.8 cells has been reported to reduce both the basal activity of the osteocalcin gene and the response to $1,25(OH)_2D_3$ [86]. It has been proposed that the rat osteocalcin VDRE may also contain an AP-1 element within the VDRE [80] and that AP-1 proteins may function to induce or inhibit osteocalcin gene transcription, perhaps depending on the state of differentiation of the cell. Another well characterized VDRE, the mouse osteopontin VDRE, does not contain an AP-1 sequence [87]. Whether other vitamin D-responsive genes show similar cross-communication between the actions of jun and fos heterodimers remains an interesting area of investigation which may provide further insight concerning the inter-relationship between second messenger systems and steroid-receptor-mediated transcription.

Besides an AP-1 element, a glucocorticoid-responsive element has also been identified overlapping the TATA box in both the human and rat osteocalcin genes. It has been suggested that this element represses the inductive effect of $1,25(OH)_2D_3$ [77,88].

Most recently, VDREs have been identified in the rat 24-hydroxylase gene. Initial studies seemed to give conflicting results. The studies of Zierold et al. [89] indicated a VDRE located between nucleotides −262 and −238. However, Ohyama et al. [90] and Hahn et al. [91] reported that a VDRE at positions −150 to −136 in the promoter of the rat 24-hydroxylase gene was important in mediating $1,25(OH)_2D_3$-dependent transcription. This apparent conflict was resolved by recent findings by Jurutka et al. [92] and Ozono et al. [93], who reported that the 24-hydroxylase gene is actually the first vitamin D-stimulated gene to be controlled by two independent VDREs (at −264 to −238 and −150 to −136). Studies by Ozono et al. [93] suggested that the proximal VDRE has more of an effect on $1,25(OH)_2D_3$ inducibility than the distal VDRE. Recently Chen and DeLuca [94] reported that two VDREs are also present in the promoter of the human 24-hydroxylase gene. In intestine and kidney, the most pronounced effect of $1,25(OH)_2D_3$ is increased synthesis of 24-hydroxylase and of the vitamin D-dependent calcium-binding protein calbindin [95]. Unlike calbindin, which is only modestly transcriptionally responsive to $1,25(OH)_2D_3$ [96,97] [suggesting that the large induction in calbindin mRNA by $1,25(OH)_2D_3$ may be due primarily to post-transcriptional mechanisms], 24-hydroxylase is strongly responsive to $1,25(OH)_2D_3$ at the level of transcription. Further studies using the 24-hydroxylase gene will be important in order to understand other factors that may be involved in regulating vitamin D metabolism.

The integrin $\alpha_v\beta_3$ has been reported to be expressed in the osteoclast plasma membrane. Since integrin $\alpha_v\beta_3$ binds to osteopontin and bone sialoprotein through the amino acid sequence RGD (arginine, glycine, aspartic acid), and since antibodies against $\alpha_v\beta_3$ block bone resorption, it has been suggested that this integrin has an important role in the bone resorptive process [98,99]. $1,25(OH)_2D_3$ transcriptionally activates α_v and β_3 integrin genes, and recently a VDRE has been reported in the avian β_3 integrin gene (at positions −770 to −756). As for the genes encoding calbindin D_{28k} (the 28 kDa form of calbindin D) [96] and calbindin D_{9k} [97], the magnitude of the $1,25(OH)_2D_3$-enhanced transcription is modest.

The first demonstration of a negative VDRE was the report by Demay et al. [100] of sequences in the human parathyroid hormone gene (positions −125 to −101) that mediate transcriptional repression in response to $1,25(OH)_2D_3$. Unlike the other VDREs which are involved in induction of transcription by $1,25(OH)_2D_3$, only a single-copy motif (AGGTTCA) was noted within this region. The sequences contained in the 25 bp oligonucleotide mediated transcriptional repression in GH4C1 cells but not in ROS 17/2.8 cells, suggesting the requirement of tissue-specific cellular factors in addition to the VDR for $1,25(OH)_2D_3$-mediated transcription. In addition to the human parathyroid hormone gene, VDREs were also reported to be localized in the bovine parathyroid hormone gene (AGGTTA at −461 to −456 and AGTTCC at −449 to −444) [101]. However, these findings were obtained using South-western analysis and gel shift assays. Whether these sequences mediate transcriptional repression in response to $1,25(OH)_2D_3$ remains to be determined.

In addition to regulating calcium homeostasis, one of the other important roles of $1,25(OH)_2D_3$ is its effect on the immune system. Activation and proliferation of T-cells, resulting in the secretion of cytokines, are important steps in the initiation of the immune response. $1,25(OH)_2D_3$ inhibits T-lymphocyte proliferation and the expression of interleukin-2 [102]. Recent studies by Alroy et al. [103] provided a mechanism for the repression by $1,25(OH)_2D_3$ of interleukin-2. They reported that the VDR can bind to an important positive regulatory element of the interleukin-2 enhancer NFAT-1 (nuclear factor of activated T-cells), which is bound by the T-cell-specific transcription factor NFATp as well as by AP-1. Alroy et al. [103] showed that a VDR/retinoid X receptor (RXR) heterodimer can block NFATp–AP-1 complex-formation by inhibiting the interaction between NFATp and Jun/Fos and stably associating with the NFAT-1 element, providing for the first time a mechanism by which $1,25(OH)_2D_3$ can act as an immunosuppressive agent.

VDR HOMODIMERIZATION VERSUS HETERODIMERIZATION

A number of early reports indicated that, for the VDR to bind to DNA, a nuclear accessory factor is required [104–108]. The RXR has been reported to be closely related and perhaps identical to this nuclear accessory factor. It was reported that highly purified VDR derived from baculovirus or yeast systems [105,109] or *in vitro* translated VDR [104] was unable to interact directly with VDREs, suggesting that the VDR is unable to form natural homodimers. However, more recently Carlberg et al. [110], using MCF-7 breast cancer cells and SL-3 *Drosophila* cells, reported that the mouse osteopontin VDRE (a perfect direct repeat of the motif GGTTCA spaced by three nucleotides) is preferentially activated by VDR/RXR heterodimers, whereas the human osteocalcin VDRE, an AP-1 site followed by inexact direct repeats (GGTGACTCAccGGGTGAacgGGGGCA), was found to confer vitamin D inducibility by the VDR alone, possibly as a VDR homodimer. In addition, using gel shift assays, Carlberg et al. [110] reported that, for the osteopontin VDRE, a prominent shifted complex is observed in the presence of both VDRs and RXRs. In contrast, for the osteocalcin VDRE, VDR translated *in vitro* showed a shifted complex which was not enhanced by the addition of RXR. Thus the authors concluded that, unlike the results observed with the osteopontin VDRE, the RXR does not increase the affinity of the VDR for the osteocalcin VDRE. These findings are controversial, since Nishikawa et al. [111,112] reported, on using gel shift assays, that purified VDRs could bind to the osteopontin VDRE in the absence of RXRs but that RXRs were required for binding to the osteocalcin VDRE. Freedman et al. [113] also reported that VDR monomers can bind to the mouse osteopontin VDRE. Further studies from Freedman's lab [114] indicated that the regions within the VDR DNA-binding domain that confer selectivity for the osteopontin VDRE are at the tip of the first zinc finger module and at the N- and C-termini of the second zinc finger module. Thus, unlike the work of Carlberg et al. [110],

these studies suggest that the VDR alone can bind to the mouse osteopontin VDRE.

Additional controversy exists concerning the physiological relevance of VDR homodimers. In a recent study, Cheskis and Freedman [115] reported that the VDR exists as a monomer in solution and that homodimerization occurs upon binding to the osteopontin VDRE. $1,25(OH)_2D_3$ destabilizes homodimer formation, and VDR/RXR heterodimer formation is then favoured. 9-*cis*-Retinoic acid decreased heterodimer formation. It was suggested, as indicated previously by MacDonald et al. [116] using the osteocalcin VDRE, that 9-*cis*-retinoic acid drives the equilibrium from the VDR/RXR heterodimer to the RXR homodimer or the interaction of the RXR with other receptors. Thus the intracellular ligand concentration is important in regulating the response. The above results suggest that the heterodimer is the functional transactivating species. In recent studies by Nakajima et al. [117] a number of VDR mutants were constructed. None of the mutants lacking the capability to form a heterodimer with the RXR showed $1,25(OH)_2D_3$-dependent transcriptional activation, thus further suggesting the importance of heterodimerization for VDR transcriptional activation. The studies indicating the importance of heterodimerization were carried out using known VDREs. It is possible in future studies that VDR homodimers may be shown to have a functional role in VDR-mediated transcription of target genes yet to be identified.

Due to the realization of the importance of the interaction between the VDR and the RXR, attempts have been made through site-directed mutagenesis to map specific regions of the VDR that are involved in heterodimerization. hVDR residues between Cys-403 and Ser-427 were found to be required for ligand binding and transactivation, but not heterodimerization [117]. Regions in the C-terminus between Lys-382 and Arg-402 were suggested to have important roles in heterodimerization [117], as were regions between Phe-244 and Leu-262 [118,119]. Site-directed mutagenesis studies suggested that Phe-244, Leu-254, Gln-259 and Leu-262, but not Lys-246, of the VDR interact with an RXR isoform on DNA [118]. These important studies are suggestive, at this time, of contact points. Complete understanding of the interaction of the VDR with the RXR will be obtained after crystallographic analysis of the VDR, which will allow the visualization of the three-dimensional contacts.

In addition to the RXR, it has been suggested that the RAR and the thyroid hormone receptor can also heterodimerize with the VDR [120–122]. Using the region of the human osteocalcin VDRE (positions -510 to -492; GGTGACTCAccGGGTGAac), Schrader et al. [121] reported binding of this element to a VDR/RAR heterodimer. However, MacDonald et al. [116], using the rat osteocalcin VDRE, showed that although RXRs can readily heterodimerize with VDRs, RARs do not. Concerning the possibility of VDR/thyroid hormone receptor heterodimerization, Schrader et al. [122] reported, using rat calbindin D_{9k} or mouse calbindin D_{28k} VDRE/thymidine kinase (tk) promoter/CAT reporter constructs transfected into human MCF-7 cells (which contain endogenous VDRs and thyroid hormone receptors), that either $1,25(OH)_2D_3$ or thyroid hormone could stimulate gene transcription. Stimulation by both hormones resulted in stimulation of CAT activity which was less than additive. In other studies, Schrader et al. [121] used the mouse osteopontin VDRE/tk/CAT construct or the -514 to -495 region of the human osteocalcin VDRE fused to tk/CAT co-transfected into *Drosophila* SL-3 cells transfected with VDRs and thyroid hormone receptors. They found that there was induction by thyroid hormone alone using both reporter constructs, and enhanced induction in the presence of both

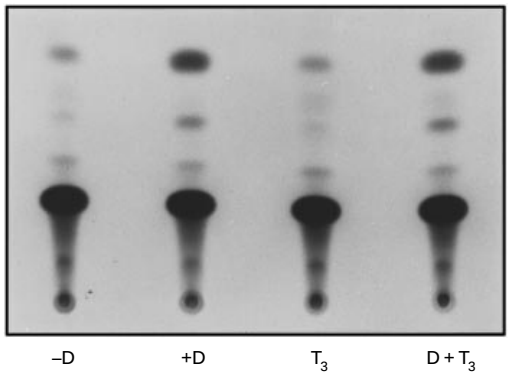

Figure 3 Activation of VDR-mediated transcription by $1,25(OH)_2D_3$ but not by 3,5,3′-tri-iodothyronine (T_3)

Human MCF-7 cells were transfected with a CAT reporter construct containing the rat osteocalcin VDRE [42] and treated with 10 nM $1,25(OH)_2D_3$ (D), 100 nM T_3 or a combination of $1,25(OH)_2D_3$ and T_3. CAT activity was assayed 16 h after stimulation with ligand(s). Three independent experiments showed similar results. Note that neither an activation with T_3 nor an enhanced response in the presence of both ligands was observed.

$1,25(OH)_2D_3$ and thyroid hormone using the -510 to -492 region of the human osteocalcin VDRE. These results suggested a VDR/thyroid hormone receptor signalling pathway. However, in MCF-7 cells transfected with natural calbindin D_{9k} promoter/CAT constructs containing nucleotides -1009 to $+61$ or -590 to $+61$ (both containing the calbindin D_{9k} VDRE; -490 to -472) (M. Raval-Pandya and S. Christakos, unpublished work) or the rat osteocalcin VDRE (-457 to -430)/tk/CAT construct (Figure 3), neither activation by thyroid hormone nor an enhanced response in the presence of both ligands was observed. Thus further work is needed to examine whether the RAR and the thyroid hormone receptor are indeed nuclear accessory factors for the VDR which may participate in activation of certain VDREs.

OTHERS FACTORS INVOLVED IN VDR-MEDIATED TRANSACTIVATION

Chicken ovalbumin upstream promoter transcription factor (COUP-TF)

COUP-TF, initially characterized by its binding to the chicken ovalbumin upstream promoter, is a member of the steroid/thyroid hormone nuclear receptor superfamily which is expressed ubiquitously [123]. COUP-TF shares sequence identity with other members of the steroid receptor family, including the presence of a double zinc finger DNA-binding domain. No ligand for COUP-TF has been identified, however. Thus it is classified as an orphan member of the family of steroid hormone receptors. Studies by Cooney et al. [124,125] have shown inhibition by COUP-TF of VDR-mediated transcription through the natural human osteocalcin VDRE. The mechanism of this repression was reported not to involve the formation of a functionally inactive VDR/COUP heterodimer, but rather to involve active silencing of transcription, direct competition with the VDR for binding to the VDRE and heterodimer formation with the RXR [125]. COUP-TF was also shown to repress thyroid hormone receptor- and RAR-mediated transcriptional activation [124,125]. The authors suggest that COUP-TF may play a master role in regulating transactivation by these steroid

receptors. They propose that the levels of both COUP-TF and the steroid receptor will determine the overall magnitude of hormone-induced gene expression.

TFIIB

Transcription factors including steroid hormone receptors that bind to specific response elements need to interact with the basal transcription complex in order for transcription to be initiated. Recent studies have begun to address the mechanisms involved in VDR-mediated transcription following binding of the VDR/RXR heterodimer to DNA [126,127]. Initiation of basal transcription involves binding of TFIID [TATA box binding protein and factors associated with it known as TAFs] to the TATA element followed by the association of other factors (TFIIA, TFIIB, TFIIE, TFIIF, TFIIH and RNA polymerase II) with the complex [128]. Blanco et al. [126] reported that co-transfection of the VDR and TFIIB in P19 embryonal carcinoma cells activated $1,25(OH)_2D_3$-dependent rat osteocalcin VDRE/CAT activity. In NIH3T3 cells, co-transfection with TFIIB resulted in repression of $1,25(OH)_2D_3$-mediated transcription using the rat osteocalcin VDRE, suggesting that TFIIB and the VDR interact but this interaction is modulated physically and functionally by cell-type-specific factors. Additional studies by MacDonald et al. [127], using a yeast two-hybrid protein interaction assay, showed that the C-terminal ligand-binding domain of the VDR interacts with the N-terminal domain in TFIIB. TFIIB did not interact with similar regions of the RXR. These studies suggest that the VDR contacts the preinitiation complex through TFIIB and that TFIIB may play a major role in mediating transcription following VDR/RXR heterodimerization.

Calreticulin

Recent preliminary results have indicated that calreticulin, a calcium-binding protein in the endoplasmic reticulum and the nucleus, inhibits the formation of the VDR/RXR heterodimeric complex and blocks $1,25(OH)_2D_3$-mediated transactivation of β_3 integrin [129] ($\alpha_v\beta_3$ integrin may mediate the attachment of osteoclasts to the bone matrix). These results suggested that calreticulin may be a modulator of $1,25(OH)_2D_3$-mediated actions on osteoclast function. It is likely that in future studies new advances will be made in the identification of additional accessory factors as well as the mechanisms involved in transcriptional regulation beyond VDR/RXR binding to the VDRE.

NEWLY IDENTIFIED VITAMIN D-DEPENDENT GENES IN CLASSICAL TARGET TISSUES

Effects of $1,25(OH)_2D_3$ in bone

Although $1,25(OH)_2D_3$ is known to affect bone growth and mineralization and to increase intestinal calcium and phosphate absorption, the detailed mechanism of action of $1,25(OH)_2D_3$ in these two classical target tissues has not been clearly understood. Evidence suggests that the anti-rachitic action of $1,25(OH)_2D_3$ is indirect and is achieved by the effect of the hormone on increased intestinal absorption of calcium and phosphorus, thus resulting in increased availability of these minerals for incorporation into bone [130]. However, *in vitro* studies have indicated that $1,25(OH)_2D_3$ can resorb bone [131]. Receptors for $1,25(OH)_2D_3$ are not present in the bone-resorbing osteoclasts, however, but rather in osteoblasts [2]. It has been suggested that $1,25(OH)_2D_3$ treatment may release a factor from osteoblasts that is responsible for stimulating osteoclast activity [132]. In addition, studies have indicated that immature progenitors can affect differentiation into osteoclasts in the presence of osteoblastic cells or bone marrow-derived stromal cells [133]. Thus the increase in osteoclasts in the presence of $1,25(OH)_2D_3$ may be an effect of the hormone on differentiation. Whether osteoblastic stromal cells are required for differentiation has been a matter of debate. Studies from Suda's group, which favours the involvement of accessory cells, have recently indicated that $1,25(OH)_2D_3$ can up-regulate the third component of complement (C3) in murine osteoblastic cells and in bone marrow-derived stromal cells [134,135], as well as in bone *in vivo* [136]. Thus a new target gene in bone, in addition to the osteoblast proteins osteopontin and osteocalcin, whose regulation by $1,25(OH)_2D_3$ has been studied in detail [76–86], has been identified. The up-regulation by $1,25(OH)_2D_3$ of C3 in bone was found to be tissue-specific. *In vitro* studies indicated that the regulation of C3 was at the transcriptional level [135]. An antibody against C3 inhibited osteoclast formation *in vitro*, suggesting the involvement of C3, an important factor in the immune response, in $1,25(OH)_2D_3$-mediated osteoclastic bone resorption.

In addition, coincident with the role of $1,25(OH)_2D_3$ in the differentiation of cells towards functional osteoclasts, Billecocq et al. [137] found that $1,25(OH)_2D_3$ increases the expression of carbonic anhydrase II protein and mRNA in bone marrow mononuclear cells. Carbonic anhydrase, which results in the formation of protons and bicarbonate from carbon dioxide and water, is expressed at high levels in the osteoclast and is involved in the process of bone resorption [138]. Billecocq et al. [137] also noted the need for accessory cells, since bone resorption in the presence of $1,25(OH)_2D_3$ was only observed with cultures of total bone marrow including stromal cells. In order to begin to address the mechanisms involved in the induction of carbonic anhydrase by $1,25(OH)_2D_3$, in preliminary studies binding sites on the chicken carbonic anhydrase II promoter were noted for c-Fos, JunD and EGR1 (early growth response gene). It was suggested that the up-regulation of carbonic anhydrase II transcription by $1,25(OH)_2D_3$ may be secondary to a primary effect of $1,25(OH)_2D_3$ on these transcription factors [139].

In addition to up-regulation by $1,25(OH)_2D_3$, down-regulation of the Id gene in osteoblastic (ROS 17/2.8) cells has been reported [140]. Id is a member of the helix–loop–helix family of proteins that regulate differentiation and is classified as a ubiquitous suppressor [141]. As indicated by nuclear run-on assays, $1,25(OH)_2D_3$ was found to down-regulate, by up to 80 %, the transcription of the Id gene. Whether a VDRE is present in the Id gene or whether similar findings are observed in other osteoblastic cells has not as yet been determined. The authors suggest that Id could be a major determinant in osteoblastic differentiation.

The regulation of C3, carbonic anhydrase and Id by $1,25(OH)_2D_3$ is of interest, since it suggests novel avenues of investigation related to the genomic effects of $1,25(OH)_2D_3$ on bone. The exact roles of C3, carbonic anhydrase and Id, the significance of their regulation in mediating the effects of $1,25(OH)_2D_3$ on bone and the mechanisms involved in $1,25(OH)_2D_3$-dependent regulation remain to be determined.

Effects of $1,25(OH)_2D_3$ in the intestine

One of the most extensively studied effects of $1,25(OH)_2D_3$ is the stimulation of intestinal calcium absorption [142,143]. However, the exact mechanisms involved in this process have still not been definitively determined. It has been suggested that the intestinal calcium absorptive process is affected by $1,25(OH)_2D_3$ in three phases. The first phase involves calcium transfer into the cell, and may not be dependent on genomic actions of $1,25(OH)_2D_3$. The second phase responds more slowly to $1,25(OH)_2D_3$ and involves

the movement of calcium through the cell interior. It has been suggested that the vitamin D-induced calcium-binding protein, calbindin, reported to be a facilitator of calcium diffusion [144], has a role in this process [145,146]. The third phase involves calcium extrusion from the cell and involves calcium transport against a concentration gradient. The intestinal plasma membrane calcium pump protein and mRNA in vitamin D-deficient rats and chicks have been shown to be stimulated by $1,25(OH)_2D_3$, suggesting that the effect of $1,25(OH)_2D_3$ on intestinal calcium absorption may be mediated, at least in part, by a direct effect on calcium pump expression [95,147–150]. Although $1,25(OH)_2D_3$ has been reported to affect the transcription of the intestinal plasma membrane calcium pump gene [146], the mechanism involved remains to be determined. Studies concerning the inter-relationship between the vitamin D endocrine system and the calcium pump will be an important area of future research which will contribute to a better understanding of the process of $1,25(OH)_2D_3$-mediated intestinal calcium absorption.

EFFECTS OF $1,25(OH)_2D_3$ IN NON-CLASSICAL TARGET TISSUES

Besides bone and intestine, as indicated in the Introduction section $1,25(OH)_2D_3$ affects numerous other systems. Since $1,25(OH)_2D_3$ receptors have been identified in these non-classical target tissues, it has been suggested that the actions of $1,25(OH)_2D_3$ are mediated, at least in part, by genomic mechanisms. A well characterized action of $1,25(OH)_2D_3$ in a number of normal and malignant cells is its ability to inhibit proliferation and to stimulate differentiation [2,3]. The effect of $1,25(OH)_2D_3$ on the inhibition of proliferation and the stimulation of differentiation of keratinocytes is of interest, and has been related to the effective treatment of skin lesions found in psoriasis, a disease of abnormal growth of the epidermis, with $1,25(OH)_2D_3$ or its side-chain analogue MC903 [13,14,151,152]. However, the molecular basis of the effect of $1,25(OH)_2D_3$ on keratinocyte differentiation, which may involve an interaction with calcium [153,154], is not clearly understood.

In addition to its effects on the differentiation of keratinocytes, $1,25(OH)_2D_3$ has been found to inhibit proliferation and induce differentiation of leukaemia cells [3] and to inhibit the proliferation of a number of other malignant cells, including breast, prostate and colon cancer cells [155–158]. A very active area of current research is the development of analogues of $1,25(OH)_2D_3$ which inhibit growth and induce differentiation but do not affect serum calcium [18]. New insight concerning the mechanism involved in the effect of $1,25(OH)_2D_3$ on the differentiation of leukaemic cells into monocytes/macrophages was recently provided by Liu et al. [159]. They found that the cyclin-dependent kinase inhibitor p21, which is involved in blocking cell cycle progression, is transcriptionally induced in the myelomonocytic U937 cell line. A functional VDRE was identified in the human p21 promoter (AGGGAGattGGTTCA; -779 to -765). Transient overexpression of p21 in U937 cells in the absence of $1,25(OH)_2D_3$ resulted in the expression of monocyte/macrophage specific markers, suggesting a key role for p21 in the $1,25(OH)_2D_3$-mediated differentiation of leukaemic cells.

$1,25(OH)_2D_3$ has also been reported to affect the secretion of a number of hormones. It is known that $1,25(OH)_2D_3$ inhibits the secretion and synthesis of parathyroid hormone [1]. As indicated previously in this review, a negative VDRE has been identified in the promoter of the parathyroid hormone gene which may mediate the transcriptional repression in response to $1,25(OH)_2D_3$ [100]. In the pituitary, $1,25(OH)_2D_3$ has been

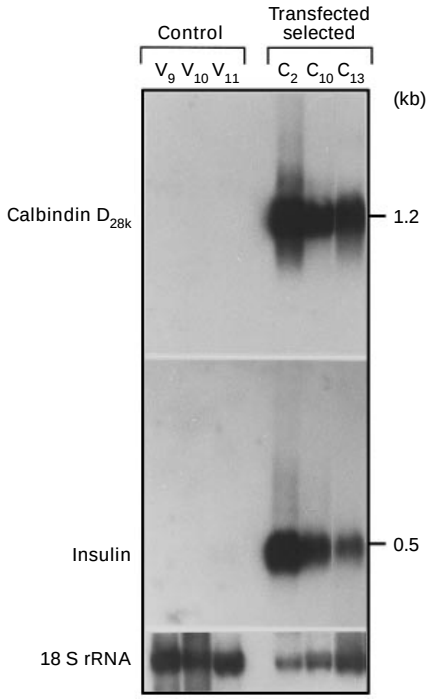

Figure 4 Northern analysis of islet β-cell calbindin D_{28k} and insulin mRNA in control and calbindin D_{28k}-transfected cells

Control cells were transfected with vector (V) alone. Results with positive clones C_2, C_{10} and C_{13} of calbindin D_{28k}-transfected cells are shown. Insulin mRNA is enhanced when calbindin D_{28k} is transfected and overexpressed in the islet β-cell line RIN 1046-38.

reported to enhance the agonist-induced secretion of both prolactin and thyroid-stimulating hormone [160,161]. In addition to affecting hormone secretion from the parathyroid and pituitary glands, $1,25(OH)_2D_3$ has also been reported to enhance pancreatic insulin secretion [162–164]. The pancreas was the first non-classical target tissue reported to possess receptors for $1,25(OH)_2D_3$ [165]. Although $1,25(OH)_2D_3$ has been reported to improve insulin secretion in vitamin D-deficient animals, it has been suggested that impairment of islet secretory function in such animals may be due to the hypocalcaemia, poor growth and inanition characteristic of these animals [163,166,167]. This is still a matter of debate, however. It is possible that $1,25(OH)_2D_3$ may act together with calcium to control β-cell function. A role for the vitamin D-dependent calcium-binding protein calbindin D_{28k} (which is present in islets in addition to kidney, intestine and brain) in insulin secretion and expression has been suggested [168]. When calbindin D_{28k} was transfected and overexpressed in the islet β-cell line RIN 1046-38, which contains receptors for $1,25(OH)_2D_3$, a marked induction (6–35-fold) of insulin secretion and mRNA expression was observed (Figure 4), suggesting a direct role for calbindin in insulin biosynthesis and secretion. The inter-relationship between $1,25(OH)_2D_3$, calbindin and insulin secretion remains to be resolved.

$1,25(OH)_2D_3$ has also been reported to have effects on the immune system. It suppresses T-cell proliferation and the expression of cytokines such as interleukin-2, as mentioned earlier, and interferon-γ [8,102,169]. This effect is in contrast with the effect of $1,25(OH)_2D_3$ to enhance macrophage phagocytic activity [169]. The effects of $1,25(OH)_2D_3$ on the immune system may be subtle, since vitamin D deficiency is not associated with major

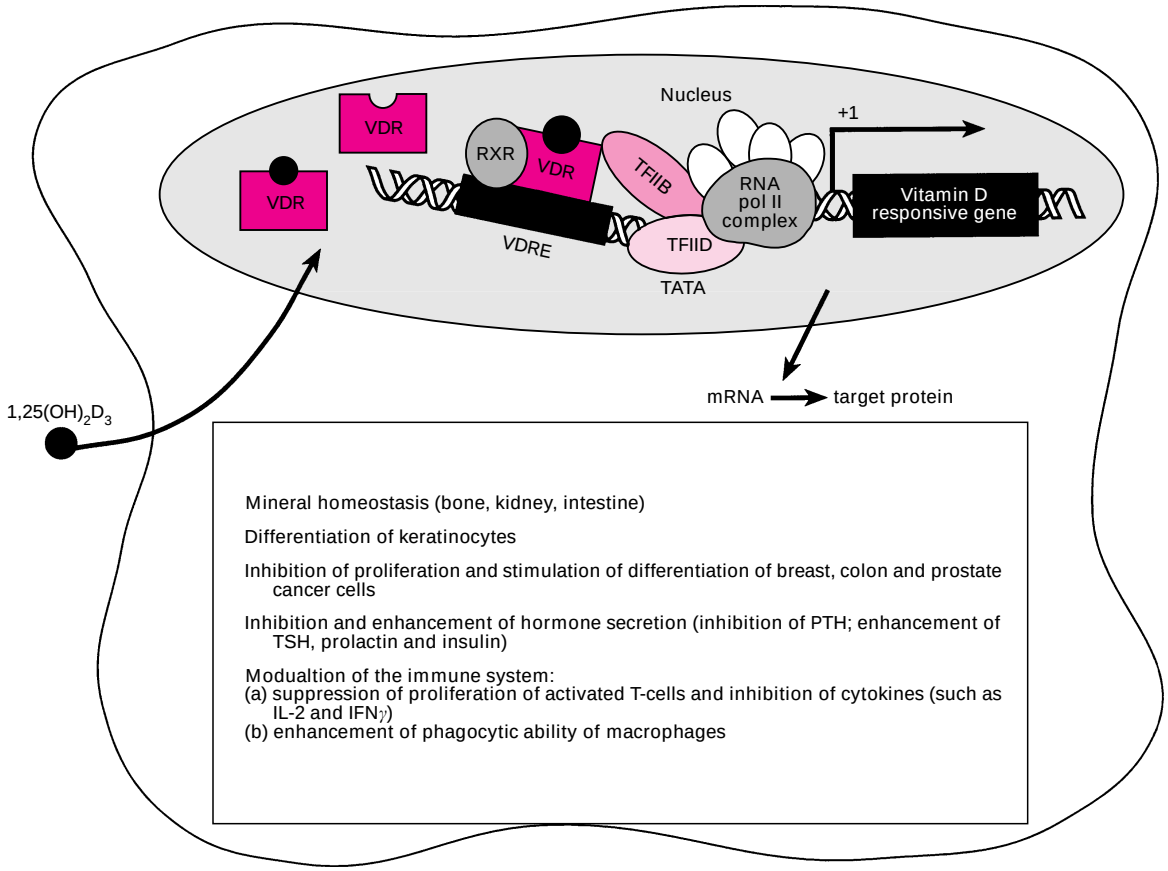

Figure 5 Genomic mechanism of action of $1,25(OH)_2D_3$ in target cells

See the text for discussion of VDR/RXR heterodimerization and of the suggested interaction of TFIIB and the VDR. $1,25(OH)_2D_3$ is known to affect mineral homeostasis, to promote differentiation of keratinocytes and leukaemia cells and to affect the immune system. Suggested roles for $1,25(OH)_2D_3$ in the enhancement of hormone secretion and for differentiation of other malignant cells are also included in the model. Abbreviations: pol, polymerase; PTH, parathyroid hormone; TSH, thyroid-stimulating hormone; IL-2, interleukin-2; IFNγ, interferon γ.

deficiencies in the immune system. It has been suggested, however, that analogues of $1,25(OH)_2D_3$ may have therapeutic potential when given in combination with classical immunosuppressive drugs.

NEW DIRECTIONS OF VITAMIN D RESEARCH

Exactly how $1,25(OH)_2D_3$ acts to produce these effects on differentiation, proliferation, hormone secretion and the immune system is not known. The elucidation of the mechanisms involved will be an active area of further research. $1,25(OH)_2D_3$ may mediate these effects, at least in part, by genomic mechanisms (Figure 5). New target genes and novel VDREs will undoubtedly be identified which should expand our understanding of the sequences involved in vitamin D-mediated genomic mechanisms. It is becoming increasingly evident that sequences divergent from the consensus response element may be physiologically important. New insight may also be obtained concerning different transcription factors which are involved in mediating these diverse biological responses. Although studies concerning vitamin D regulation have focused on transcriptional mechanisms, it is likely that post-transcriptional regulation by $1,25(OH)_2D_3$ will be an important mechanism of control of a number of the newly identified target genes.

In addition to studies related to responsive elements and transcription factors, which have been a major focus of research in the vitamin D field in the past 5 years, studies related to the functional significance of target proteins in different systems are needed in the future. The use of transfected cells, knockout mice or transgenic mice that overexpress a particular vitamin D-dependent protein should result in a better understanding of the physiological significance of target proteins. It will be of interest, for example, to examine physiological abnormalities in VDR, calbindin, osteocalcin and 24-hydroxylase knockout mice. Such *in vivo* studies, combined with continuing studies concerning the basic molecular mechanism of $1,25(OH)_2D_3$ action, will provide new insight into the role of $1,25(OH)_2D_3$ in calciotropic and non-calciotropic target tissues.

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