Transcriptional Regulation of the Vitamin D_3 Receptor Gene by ZEB[1]

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Abstract

The hormone 1,25-dihydroxyvitamin D_3 influences the growth and differentiation of a number of cell types. The functions of 1,25-dihydroxyvitamin D_3 are mediated through the vitamin D_3 receptor (VDR); therefore, an understanding of the regulation of VDR expression is important when considering the molecular mechanisms of differentiation induced by vitamin D_3 and its analogues. ZEB, a Krüppel-type transcription factor known to repress the transcription of several genes, binds to two sites within the *VDR* promoter and activates the transcription of this receptor in a cell-specific manner. Transfection of *ZEB* into SW620 colon carcinoma cells results in an up-regulation of the expression of endogenous *VDR*, confirming the role of ZEB in the transcriptional activation of the *VDR* gene. The expression of VDR is also induced by c-MYB; thus, ZEB and c-MYB may modulate the levels of *VDR* expression during differentiation in embryonal development, as well as in cancer cells.

Introduction

VDR,[3] a member of the steroid-thyroid receptor family, mediates the action of 1,25-OH_2D_3 by binding to vitamin D-responsive elements and regulating gene transcription (1, 2). An understanding of the mechanisms regulating *VDR* gene expression is of importance because, *e.g.,* nonfunctional *VDR* signaling has adverse effects on early postnatal development (3–5). Furthermore, because the levels of *VDR* expression correlate with the degree of differentiation and/or inhibition of growth of several malignant cell lines (6–9), an understanding of the factors contributing to the expression of *VDR* is of particular importance to the possible therapeutic use of vitamin D_3 and its analogues as antiproliferative and/or differentiation agents.

The human protein ZEB is a transcription factor of the Krüppel type with an internal homeodomain and zinc finger motifs at its amino and COOH termini (10). Homologues of *ZEB* include the human variants *AREB6* (11) and *Nil-2a* (12), the murine *δEF1/MEB1* (13, 14), the rat *Zfhep* (15), the hamster *BZP* (16), the chicken *δEF1* (17), and the *Drosophila Zfh-1* (18, 19). Henceforth, we refer to these proteins as ZEB homologues. Alignment of the mouse, chicken, hamster, and human ZEB homologues reveals a high degree of conservation between all four species, with ≤99.5% being within the zinc finger domains and 85% being outside of these domains (14).

Histological analyses of chicken embryos established that the major sites of *ZEB* expression are the notochord, myotome, limb bud, and neural crest derivatives (17). In mouse embryos, the expression of ZEB is first detected in mesodermal tissues (*i.e.,* notochord, somite, and limb bud mesenchyme), as well as in neural crest derivatives (*i.e.,* dorsal root ganglia and cephalic ganglia), and in parts of the central nervous system (*i.e.,* hindbrain and motor neurons in the spinal cord; Ref. 20).

ZEB and its homologues bind to subsets of E-boxes (most frequently to the sequence CACCTG), as well as to other sites which are not E-boxes (10, 21). The E-boxes with the consensus sequence CANNTG are major target sites for basic helix-loop-helix proteins, which induce the transcription of a variety of genes (22). However, by binding to E-boxes, ZEB and its homologues have been shown to repress the transcription of several genes, including the chicken *δ-crystallin* gene (17), the *α4 integrin* gene (23), the *IL-2* gene (24), the *GATA-3* gene (25), the *CD4* gene (26), and others.

A role for ZEB in transcriptional activation has been suspected, based upon the presence of structural elements shown to be the active domains of some transcriptional activators (27–30). *E.g.*, ZEB has a long stretch of acidic amino acids (predominantly poly-Glu) at the COOH terminus and a domain rich in prolines in the middle (14, 17). To date, there are two reports that support the possible role of ZEB as a transcriptional activator. Watanabe *et al.* (11) demonstrated that *AREB6*, a human *ZEB* variant, activates transcription from the promoter of the rat *Na,K-ATPase α1 subunit* gene in a cell-specific manner; Chamberlain and Sanders (31) demonstrated that *δEF1* up-regulates expression from the chicken ovalbumin gene.

In this report, we describe the presence of two E-boxes within the *VDR* promoter to which ZEB binds *in vitro* and demonstrate that exogenous expression of ZEB in COS 7 cells results in a concentration-dependent up-regulation of VDR promoter activity. Optimal up-regulation of the *VDR* promoter by ZEB required its interaction with both binding sites. The transcriptional activation of the *VDR* promoter by

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[3] The abbreviations used are: VDR, vitamin D_3 receptor; 1,25-OH_2D_3, 1,25-dihydroxyvitamin D_3; CBP, CREB-binding protein; CtBP, C-terminal binding protein; Z1 and Z2, E-boxes in VDR promoter; FBS, fetal bovine serum; nt, nucleotide; GMSA, gel mobility shift assay.

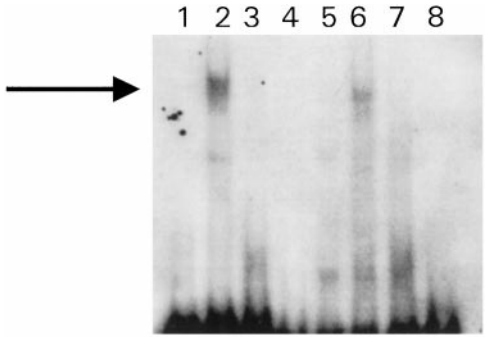

Fig. 1. Binding of ZEB to the two E-boxes present in the *VDR* promoter region. GMSAs were performed as described in "Materials and Methods" using digoxigenin-labeled probes containing the Z1 site (*Lanes 1–4*) or the Z2 site (*Lanes 5–8*). *Lanes 1* and *5,* the probes alone; *Lanes 2* and *6,* the probes incubated with recombinant ZEB protein; *Lanes 3* and *7,* the same binding reactions as in *Lanes 2* and *6,* except that a 100-fold molar excess of unlabeled probe was added; and *Lanes 4* and *8,* the binding reactions of probes with mutated E-boxes and recombinant ZEB protein. *Arrow*, the ZEB-binding complex. A representative gel of the GMSA is shown.

ZEB was not influenced by the presence of cofactors such as CtBP and CBP, although direct binding between ZEB and CtBP has been reported (32–34). We have also established that the *VDR* promoter is c-MYB inducible. Unlike other c-MYB-inducible promoters which can be repressed by ZEB (26, 35), the coexpression of ZEB and c-MYB in COS 7 cells induced the *VDR* reporter gene in an additive fashion.

Results

Binding of ZEB to Sequences of the VDR Promoter. Analysis of the murine *VDR* promoter sequence (36) revealed the presence of two E-boxes, both with the consensus sequence CACCTG and shown to be the target for ZEB binding (10). The human *VDR* promoter sequence, which shows little sequence homology to the murine promoter region, also has two CACCTG E-boxes (37). The presence of potential ZEB binding sites in both species implies that an evolutionary conserved mechanism exists for regulation of the expression of the *VDR* gene. Therefore, as an initial experiment, we tested these two sites for their ability to bind to ZEB *in vitro*. Fig. 1, *Lanes 2* and *6* shows that both murine probes containing the two CACCTG boxes were capable of binding the COOH-terminal zinc fingers of ZEB. Competition with unlabeled probe abrogated the formation of a detectable DNA-protein complex (Fig. 1, *Lanes 3* and *7*), thereby confirming the specificity of the binding. In addition, probes in which the E-boxes were mutated did not bind to recombinant ZEB protein (Fig. 1, *Lanes 4* and *8*), indicating that the CACCTG sequence is the target site for ZEB.

Induction of VDR Promoter Activity by ZEB via the Two CACCTG E-Boxes. The effects of ZEB expression on the murine *VDR* reporter gene was measured in COS 7 cells. Cotransfection of the *VDR* reporter gene with increasing amounts of ZEB expression vector resulted in an essentially linear (≤5-fold) increase in *VDR* promoter activity (Fig. 2*A*).

To ascertain whether the two CACCTG E-boxes present in the *VDR* promoter were required to mediate the ability of ZEB to induce the expression of the *VDR* reporter gene, we mutated each of these sites. Mutated *VDR* promoter constructs, with a change in the first E-box at 1034–1039 nt (Z1), in the second E-box at 1129–1134 nt (Z2), or at both sites (Z1,2), were cotransfected with a fixed amount of ZEB expression plasmid in COS 7 cells and assayed for luciferase activity. Mutation Z1 resulted in an ~50% decrease in *VDR* reporter activity, whereas mutation Z2 produced an ~40% decrease (Fig. 2*B*). These results suggest that both E-boxes are involved in the induction produced by ZEB. This conclusion was further supported by the fact that when the double mutant Z1,Z2 was cotransfected with the ZEB expression vector, its activity was similar to that of the wild-type *VDR* reporter gene in the absence of *ZEB* expression (Fig. 2*B*).

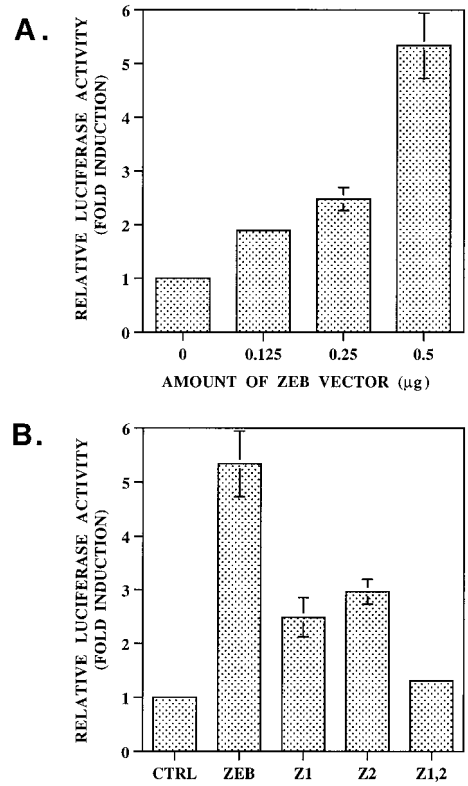

Fig. 2. Up-regulation of transcription from the murine *VDR* promoter induced by ZEB. In *A,* transcriptional activity of the *VDR* promoter fused to the luciferase gene in the pGL3Basic vector was assayed by cotransfection of this reporter gene (0.2 μg/well) with increasing amounts of ZEB expression vector (0.125, 0.25, and 0.5 μg) in COS 7 cells. *B,* requirement of CACCTG boxes in the *VDR* promoter region for the activation of the *VDR* promoter by ZEB in COS 7 cells. The activity of the wild-type *VDR* promoter reporter gene (0.2 μg/well) was measured in the presence of 0.5 μg of ZEB expression vector per well (*ZEB*) or the presence of 0.5 μg of control vector per well (*CTRL*) and compared with the activities of mutated *VDR* reporter genes with only one mutated E-box (*Z1 or Z2*) or with mutations in both E-boxes (*Z1,2*) in the presence of 0.5 μg of ZEB expression vector per well. Luciferase activities were normalized against the activities of the control vector pRL-TK. The average of three to seven independent transfections each with triplicate samples and SDs are shown. In some cases, the SDs were very low and do not show up as observable error bars.

The induction of the rat *Na,K-ATPase α1 subunit* gene by AREB6, a human ZEB variant, has been reported to be cell specific (11). To determine whether the induction of the *VDR* promoter by ZEB is cell specific, we performed *VDR* reporter

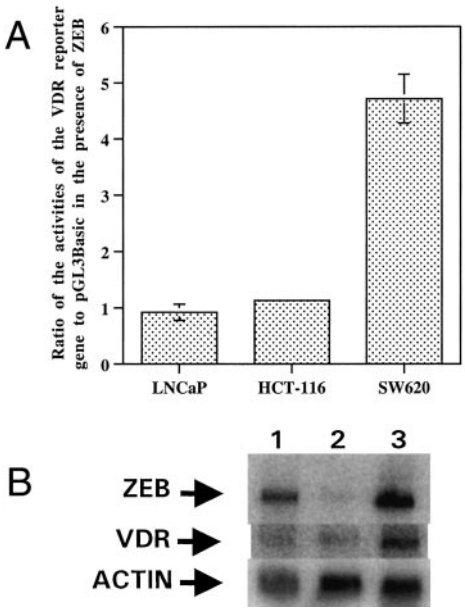

Fig. 3. Cell-specific activation of the VDR promoter by ZEB. In *A,* HCT-116, SW620, and LNCaP cells were transiently transfected with 0.2 μg/well of *VDR* reporter gene or control vector pGL3Basic in the absence or presence of 0.5 μg of ZEB expression vector. The results represent the change in transcriptional activity in the *VDR* reporter gene in the presence of ZEB normalized to that of the pGL3Basic control vector. The average of three independent transfections and SDs are shown. In *B,* Northern blot analyses for the expression of *ZEB, VDR,* and *actin* were performed as described in "Materials and Methods" for LNCaP (*Lane 1*), HCT-116 (*Lane 2*), and SW620 (*Lane 3*) cells.

gene/ZEB cotransfection experiments in human colon and prostate carcinoma cells in which 1,25-OH_2D_3 has been shown to inhibit proliferation (38–40). In the SW620 colon carcinoma cell line, exogenous *ZEB* expression induced *VDR* reporter gene activity by 2-fold; however, because the control pGL3Basic vector decreased activity in the presence of ZEB, the relative increase in *VDR* reporter gene activity was approximately 4.7 $\pm$ 0.4-fold (Fig. 3*A*). In LNCaP prostate cancer cells, the expression of exogenous ZEB had no significant effect on both the pGL3Basic and *VDR* reporter gene, whereas in HCT-116 colon carcinoma cells, the cotransfection with ZEB resulted in an ~2-fold increase in both pGL3Basic and *VDR* reporter gene transcriptional activities; therefore, the ratio of these two activities was close to 1.0 (Fig. 3*A*). Among the cell lines used, SW620 cells exhibited the highest endogenous steady-state levels of *ZEB* and *VDR* mRNA expression (Fig. 3*B*); therefore, it is most probable that cell type characteristics, rather than *ZEB* expression levels, contribute to the observed differences in ZEB transcriptional activity on the *VDR* reporter gene.

We wished to determine whether the endogenous *VDR* gene is up-regulated by ZEB overexpression in SW620 colon carcinoma cells. Attempts to stably transfect SW620 cells with a ZEB expression vector were unsuccessful, and no ZEB-expressing colonies were obtained from G418-resistant cells (data not shown), possibly because ZEB overexpression selects against cell proliferation. Therefore, SW620 cells were transiently transfected with a ZEB expression vector.

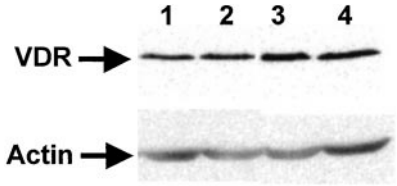

Fig. 4. Endogenous VDR expression in SW620 cells transfected with ZEB expression vector. SW620 cells were transiently transfected with 6 μg of control vector (*Lanes 1* and *2*) or 6 μg of ZEB expression vector (*Lanes 3* and *4*). Cells were harvested at 48 h after transfection. Total protein extraction and Western blot analyses were performed as indicated in "Materials and Methods." The experiment was repeated twice with duplicate samples, and a representative Western blot is shown.

This transfection resulted in an ~50% increase in endogenous VDR protein steady-state levels as estimated by Western blot analyses (Fig. 4). The observed up-regulation of VDR protein by ZEB in transiently transfected SW620 cells was derived from a cell pool in which only a fraction of the total cell number was successfully transfected. To establish the fraction of SW620 cells transfected, we used a β–galactosidase expression vector (CMV-Gal) to evaluate transient transfection efficiency ("Materials and Methods") and observed that 11.6 $\pm$ 0.8% of the SW620 cell population was transfected with CMV-Gal. Assuming a similar transfection efficiency with the *ZEB* expression vector, these findings suggest that transfection of ~12% of SW620 cells by the *ZEB* expression vector resulted in the observed 50% up-regulation of endogenous VDR protein steady-state levels (Fig. 4) and that the up-regulation of VDR protein levels in those cells transfected with *ZEB* is higher than the 50% increase determined from the analysis of total cell lysates.

Induction of VDR Promoter Activity by c-MYB. ZEB has been reported to negatively regulate both myogenesis and hematopoiesis by repressing genes that control these differentiation processes (35). These authors proposed that activation of hematopoietic genes in the presence of ZEB is achieved by an unidentified mechanism requiring the presence of both the c-MYB and Ets transcription factors. The activation of the *CD4* promoter in the presence of ZEB has also been reported to require both c-MYB and Ets (26). Analysis of the sequence of the mouse *VDR* promoter revealed that there are five potential c-MYB binding sites. Cotransfection of COS 7 cells with *VDR* reporter gene construct and increasing amounts of *c-MYB* expression construct resulted in a linear ($\leq$15-fold) increase in *VDR* promoter activity (Fig. 5*A*). This finding suggests that c-MYB is another transcription factor with the potential to regulate *VDR* promoter activity. In addition, we confirmed that CBP is a coactivator with c-MYB (41) in the context of the *VDR* promoter (Fig. 5*B*). The up-regulation of endogenous *VDR* expression by c-MYB was confirmed by transfection experiments in WEHI-3B D+ murine myelomonocytic leukemia cells, U-937 human histiocytic lymphoma cells, and HL-60 human promyelocytic leukemia cells. In all of these cell lines, exogenous *c-MYB* expression resulted in an ~50% increase in endogenous VDR protein steady-state levels (Fig. 6), thereby providing further evidence that the *VDR* gene is regulated by c-MYB.

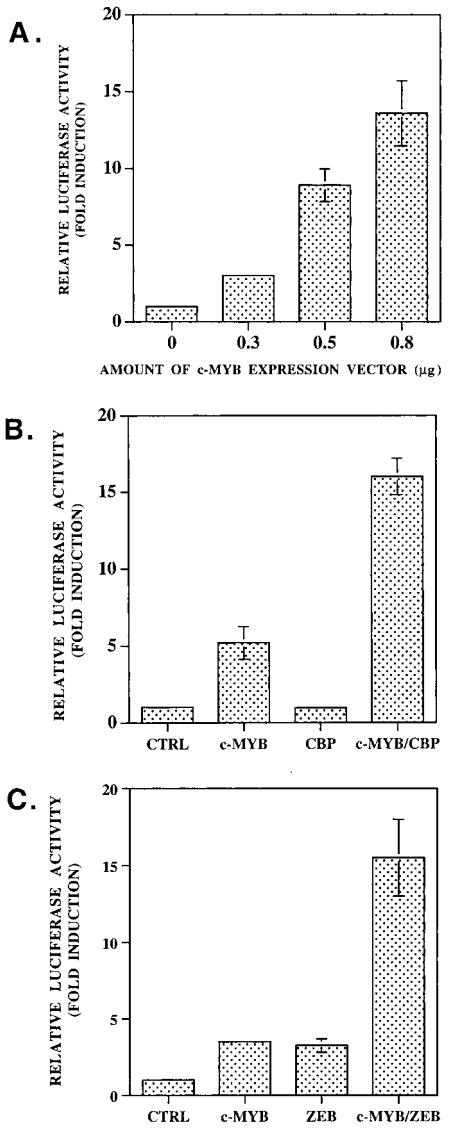

Fig. 5. Transcription from the *VDR* reporter gene induced by c-MYB. In *A,* COS 7 cells were transiently transfected with 0.2 μg of *VDR* reporter gene construct and with increasing amounts of c-MYB expression construct (0.3, 0.5, and 0.8 μg). Luciferase activities were normalized against the activities of the control vector pRL-null. *B,* coactivation of the induction of the *VDR* promoter by c-MYB and CBP. COS 7 cells were transiently transfected with 0.2 μg of *VDR* reporter gene construct and c-MYB (0.3 μg), CBP (0.3 μg), or the combination of c-MYB and CBP expression vectors. Luciferase activity was assayed at 48 h after transfection. Luciferase activities of the *VDR* reporter construct were normalized against the activities of the pGL3Basic vector alone, because CBP has an inducing effect on the control itself. *C,* additive induction of the transcription from the VDR promoter by c-MYB and ZEB. COS 7 cells were transiently transfected with 0.2 μg of VDR reporter gene construct and c-MYB (0.3 μg), ZEB (0.3 μg), or the combination of c-MYB and ZEB expression vectors. Luciferase activity was assayed at 48 h after transfection. Luciferase activities were normalized against the activities of the control vector pRL-null. The average of three independent transfections and SDs are shown. Experiments with very small SDs do not show observable error bars.

Because the *VDR* promoter is up-regulated by ZEB, we reasoned that a possible role for ZEB in repressing *VDR* promoter activity might be detected only if ZEB competes with another transcriptional activator of *VDR* promoter activity, such as c-MYB. However, cotransfection experiments with *c-MYB* and *ZEB* in COS 7 cells resulted in a supra-additive up-regulation of *VDR* promoter expression (Fig. 5*C*).

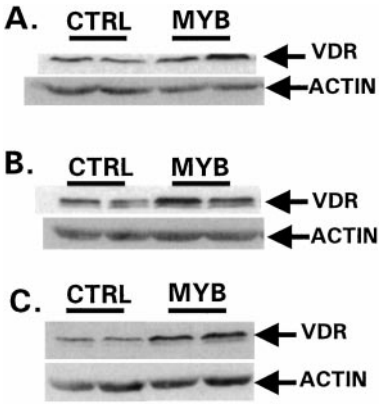

Fig. 6. Up-regulation of the endogenous expression of VDR by c-MYB. WEHI-3B D+ (*A*), U-937 (*B*), and HL-60 (*C*) cells were transiently transfected with 4 μg of control vector (*CTRL*) or with 4 μg of c-MYB expression vector (*MYB*). Cells were harvested at 48 h, and Western blot analyses were performed as described in "Materials and Methods." The transfections were repeated twice with duplicate samples; representative Western blots are shown.

Discussion

The VDR Is a Ligand-dependent Transcription Factor that Mediates the Regulation of Gene Expression by 1,25-OH_2D_3. A major function of 1,25-OH_2D_3 is the maintenance of physiological levels of calcium and phosphate in the plasma (42). However, new functions for 1,25-OH_2D_3 have been considered after the VDR was localized in a variety of cell types and after 1,25-OH_2D_3 was identified as a factor which influences cellular proliferation and differentiation (7, 43, 44). The regulation of the expression of the *VDR* gene is of particular interest because a correlation has been established between steady-state levels of VDR and the ability of 1,25-OH_2D_3 to influence cell growth and differentiation (6–9). In addition, by enhancing the stability of the VDR protein, 1,25-OH_2D_3 can initiate a positive feedback loop which may enhance differentiation (45, 46). The ability of the Sp1 and WT1 transcription factors to up-regulate the expression of the *VDR* gene has been demonstrated (47, 48).

In the present study, we report that another transcriptional factor, ZEB, up-regulates the activity of the *VDR* promoter by binding to two E-boxes within this promoter. Although it is possible that ZEB enhances *VDR* promoter activity indirectly, through enhanced expression of transcriptional activators of the *VDR* gene or by blocking the binding of a transcriptional repressor(s) to the *VDR* promoter, our data are most consistent with a direct effect of ZEB on *VDR* promoter activity. The concept of direct activation of the *VDR* gene by ZEB is supported by the ability of ZEB to bind specifically to two E-box-containing *VDR* promoter sequences *in vitro* (Fig. 1). However, is it possible that ZEB blocks repression of the *VDR* promoter, instead of directly activating it? A *VDR* promoter construct encompassing the first 500 bp upstream of the transcriptional start site exhibits expression levels higher

than that of the construct with an additional 1000 bp upstream (36). One possible interpretation of this finding is that a transcriptional repressor targets sequences close to the two ZEB (Z1 and Z2) sites we have examined. If such a repressor exists, ZEB may block its binding and, therefore, its repressive function. Direct competition between ZEB and a putative repressor for binding to the same E-boxes is unlikely; otherwise, we would have observed similar levels of expression from the short (0.5 kb) *VDR* construct (which lacks both the Z1 and Z2 E-boxes) and from the mutant Z1,2 *VDR* construct (1.5 kb). Rather, the mutant Z1,2 *VDR* construct (1.5 kb) is expressed at levels similar to that of the wild-type 1.5-kb *VDR* construct in the absence of ZEB coexpression (Fig. 2*B*).

It has been reported that ZEB represses the transcription of some promoters through interaction with the corepressor CtBP (33, 34). Our findings establish that chicken ZEB can also activate gene expression, and because the role of ZEB in transcriptional activation is cell specific, we reasoned that the dual role of ZEB in repression and activation may depend not only upon the promoter sequences but also upon the association of ZEB with different cofactors. The corepressor CtBP has been shown to interact with a wide variety of transcription factors with dual roles, such as BKLF (32, 49), AREB6 (11), and Evi-1 (50). However, CtBP1 and CtBP2 cotransfection experiments with ZEB in COS 7 cells did not produce a change in the up-regulation of *VDR* promoter activity by ZEB (data not shown). Therefore, the activating function of ZEB on the *VDR* promoter is not attributable to a lack of or low steady-state level of CtBP in COS 7 cells. We also have examined whether the activation of the *VDR* promoter by ZEB is dependent upon the presence of the transcriptional coactivator CBP. Cotransfection of ZEB and CBP resulted in an additive up-regulation of the *VDR* reporter gene; however, transfection with CBP alone increased transcription from the *VDR* reporter gene, as well as from the control pGL3Basic vector (data not shown). Therefore, the specificity of the CBP/ZEB coactivation was not confirmed.

Unlike *c-MYB* inducible promoters whose expression is down-regulated by ZEB (26), we have found that expression from the *VDR* promoter is up-regulated in an additive fashion by c-MYB and ZEB in COS 7 cells (Fig. 5*C*). c-MYB transfections also induced endogenous *VDR* gene expression (Fig. 6) in several cell types, including WEHI-3B D+ myelomonocytic leukemia cells that can differentiate along the granulocytic pathway (51) or the monocytic pathway (52), U-937 histiocytic lymphoma cells that can be induced to differentiate into monocyte-like cells (53), and HL-60 promyelocytic leukemia cells that can differentiate into monocyte- (54) or neutrophil-like cells (55, 56). The increase in the steady-state levels of the VDR protein in c-MYB transfected cells and the ability of c-MYB to induce the *VDR* reporter gene in transient transfections argue that c-MYB is a transcriptional regulator of the *VDR* gene. The precise mechanism (direct or indirect) by which c-MYB influences *VDR* gene expression is the subject of future experimental work.

The activation of *VDR* gene expression by c-MYB is not surprising because, in addition to the role of c-MYB in the proliferation of immature hematopoietic cells (57, 58), c-MYB has been also implicated in several differentiation pathways (59, 60). The contrast between the reported opposing effects of ZEB and c-MYB on transcriptional activity (26) and our observation of cooperativity between ZEB and c-MYB (Fig. 5*C*) is most likely related to the role of ZEB as a transcriptional activator, rather than as a repressor, in our system. Therefore, instead of the requirement for c-MYB and/or Ets to overcome ZEB-induced transcriptional repression (26, 35) in our system, both ZEB and c-MYB positively stimulate *VDR* promoter activity and supra-additively enhance expression. These different interactions of ZEB and c-MYB may be promoter and cell-type specific and may be important in modulating the expression of genes involved in decisions of proliferation *versus* differentiation.

The expression of ZEB in embryogenesis suggests that this transcription factor regulates VDR expression levels starting in early development when initial differentiation takes place, a concept which is consistent with a role for 1,25-OH_2D_3 in differentiation. The beginning of ZEB expression coincides with the beginning of organogenesis (17). ZEB is primarily expressed in the mesoderm (17, 20), a layer of the postgastrulation embryo that gives rise to cartilage, bone, fibrous tissue, muscle cells, and parts of the urogenital system, as well as the vascular system, including blood cells. Coincidentally, 1,25-OH_2D_3 has been demonstrated to play a role in the formation of bone and cartilage (61–63), in the induction of immature myeloid cells toward monocyte/macrophages, and in the differentiation of small intestine (64). Therefore, the expression of ZEB may well be necessary for the function of 1,25-OH_2D_3 in the differentiation of several cell types.

We hypothesize that ZEB is a transcriptional regulator of the *VDR* gene *in vivo*, a concept supported by similarities in the phenotypes of *ZEB* and *VDR* knockout mice. Both *ZEB* null mutant mice (20) and *VDR* null mutant mice (65) exhibit signs of growth retardation and abnormalities in skeletal elements. On the basis of the findings described in the present report, we speculate that the lack of ZEB expression in *ZEB* knockout mice results in relatively low steady-state levels of the VDR at particular developmental stages and thus contributes to the skeletal deformities observed in these animals. The fact that *ZEB* null mutants exhibit much more severe bone abnormalities and that the onset of growth retardation is earlier than that in *VDR* knockout mice further suggests that ZEB regulates the expression of not only the *VDR* but also of other genes which contribute to skeletal development in embryogenesis. *E.g.*, we have found that the activity of the murine *osteocalcin 2* promoter is also modulated by ZEB.[4]

In summary, taking into account our findings, as well as those in previous reports, that ZEB functions as either a transcriptional repressor (17, 23–26) or as a transcriptional activator (11, 31) in the context of different promoters, we conclude that the role of ZEB as a transcriptional factor is promoter and cell-type dependent. Requirements for particular structural elements of the promoter region, the presence of different protein partners for ZEB, the presence of transcriptional partners competing with ZEB for binding sites,

[4] D. L. Lazarova *et al.*, unpublished data.

and/or the possibility that different posttranscriptional modifications of ZEB exist are all factors which may determine whether ZEB acts in a repressive or activating mode for gene transcription.

Materials and Methods

Cell Culture. COS 7 cells were maintained in DMEM containing 10% FBS. SW620 and HCT-116 human colon carcinoma cells, as well as LNCaP human prostate cancer cells, were cultured in minimal essential medium and supplemented with 10% FBS. WEHI-3B murine myelomonocytic leukemia cells were maintained in McCoy's modified 5A medium with 15% FBS, HL-60 human promyelocytic leukemia cells were maintained in RPMI 1640 with 20% FBS, and U-937 human histiocytic lymphoma cells were maintained in RPMI 1640 with 10% FBS. All cells were grown in a humidified incubator at 37°C in a 5% carbon dioxide/95% air atmosphere.

Plasmid Constructs. The *VDR* promoter region was obtained by PCR with Taq Polymerase (Boehringer Mannheim, Indianapolis, IN) using 200 ng of genomic DNA isolated from WEHI-3B D− murine myelomonocytic leukemia cells. The forward primer was VDR 96: TCCCTCCTGTGCTTTTCTTC, and the reverse primer was VDR 1551: CCGCAC CCCGATCCGC (bp numbers are according to the published mouse *VDR* promoter sequence in Ref. 36). The PCR product of 1456 bp was cloned into pCR2.1, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The identity of the clone was confirmed by sequencing and comparison to the published mouse *VDR* promoter region (36). The VDR reporter construct was obtained by subcloning the *VDR* promoter region in pGL3-Basic vector (Promega Corp., Madison, WI). The following expression constructs were kind gifts from different researchers: pCINeo-ZEB (Dr. D. Dean, Washington University School of Medicine, St. Louis, MO); RSV-CBP (Dr. T. Kouzarides, Wellcome/CRC Institute, Cambridge, UK); CtBP1 (Dr. G. Chinnadurai, Institute for Molecular Virology, St. Louis University Medical Center, St. Louis, MO); pcDNA3-CtBP2 (Dr. M. Crossley, University of Sydney, Sydney, Australia); pMCEF-c-myb (Dr. K. Weston, Institute of Cancer Research, Chester Beatty Laboratories, London, UK); pGEX2X, encoding the COOH-terminal zinc fingers of ZEB (Dr. T. Genetta, University of Pennsylvania School of Medicine, Philadelphia, PA); and pLUC-OG2 (Dr. G. Karsenty, Baylor College of Medicine, Houston, TX). To increase expression levels, the *ZEB* cDNA sequence was subcloned downstream of the *EF-1a* promoter (kindly provided by Dr. N. Shirafuji, University of Tokyo, Tokyo, Japan), which normally drives the transcription of *polypeptide chain elongation factor 1a* in eukaryotes (66). Mutations in the two ZEB binding sites of the *VDR* promoter were introduced with the QuikChange site-directed mutagenesis kit of Stratagene (La Jolla, CA). The E-box CACCTGA (1034–1040 nts) was mutated by deletion to CAA; the second E-box (1129–1134 nt) was mutated by a substitution to CACCAA.

Transfection, Luciferase Assay, and Determination of Transfection Efficiency. Transfections were performed with cells plated in 24-well dishes at 72 h before transfection (unless specified otherwise) using the GenePorter transfection reagent, according to the protocol of the manufacturer (Gene Therapy Systems, Inc., San Diego, CA). Briefly, reporter gene constructs were cotransfected with indicated amounts of various expression vectors. After diluting DNA and transfection reagent with serum-free medium, the two solutions were combined, and complexes were allowed to form for 45 min at room temperature. Cells were incubated with DNA-GenePorter mixture for 5 h before the addition of medium containing double the regular concentration of FBS (as described in the "Materials and Methods Cell Culture" section). Luciferase reporter assays were performed at 24 or 48 h after transfection, following the protocol of Promega Corp. To normalize for transfection efficiency, pRL-TK or pRL-null vectors (Promega Corp.) were cotransfected. However, because the expression of certain proteins influences the expression of pRL-TK, and even that of pRL-null in some transfections, the protein concentration, determined by the method of Bradford (67), was used to normalize the values for luciferase activity. To determine the transfection efficiency of the GenePorter-SW620 cell system, SW620 cells were transfected as described above with either 1 μg of CMV-Gal or pCINeo (Promega Corp.); three wells of cells were transfected with each plasmid. Cells were then processed for β-galactosidase staining with the PanVera (Madison, WI) β–galactosidase Staining Kit, according to manufacturer's protocols. A total of 300 cells/well were counted, and the percentage of blue cells was determined.

GMSA. The GMSAs were performed using the DIG Gel Shift Kit of Roche Diagnostics (Indianapolis, IN). The probes were double-stranded oligonucleotides of the mouse VDR promoter region encompassing the two E-boxes. The Z1 probe included the sequence CTGAGCGCCCTGCAGGAGAAACTCACCTGAG-GTTCCC (1011–1047 nt) and its complimentary strand; the Z2 probe included the sequence CTCAGGTACGGGTGACACACCTGGGGGAGGCGTTTAC (1112–1148 nt) and its complimentary strand. Double-stranded oligonucleotides in which the E-boxes were mutated were also used for GMSAs (upper strand for Z1: CTGCAGGAGAAACTC-AAGGTTCCCCATCCG; for Z2: CTCAGGTACGGGTGACACACCGGGGAGGCGTTTAC). Probes were labeled with digoxigenin-11-ddUTP using terminal transferase, according to the protocol of Roche Diagnostics. Binding reaction mixes (20 μl) contained 30 fmol of probe, 20 mM HEPES (pH 7.9), 50 mM KCl, 2 mM $MgCl_2$, 0.1 mM $ZnSO_4$, 3 mM DTT, 7% glycerol, 0.1 μg/μl BSA, 1 μg of poly(dA-dT), 0.1 μg of poly L-lysine, and ~60 ng of glutathione S-transferase fusion protein. For competition experiments, a 100-fold molar excess of unlabeled probe was used. After incubation for 15 min at room temperature, reaction mixtures were loaded onto 5% acrylamide (80:1 acrylamide:bisacrylamide) gels and run for 2 h at 200 V and 4°C in 0.25 × Tris-borate-EDTA buffer [10 × concentration: 890 mM Tris, 890 mM boric acid, and 20 mM EDTA (pH 8.0)]. After overnight capillary transfer of gels onto nylon membranes, the signals were detected by chemiluminescence.

Western Blot Analyses. Cells were lysed as described by Ikegaki *et al.* (68), and equal amounts of total protein were separated on SDS-polyacrylamide gels, transferred to nitrocellulose, and immunostained with different primary and secondary antibodies. Antibodies included anti-actin mouse

monoclonal antibody, derived from clone C4 (Boehringer Mannheim), anti-VDR rat monoclonal antibody, derived from clone 9A7 (Biomol Res. Laboratories, Plymouth Meeting, PA), antimouse-HRP antibody, derived from clone NA 931 (Pharmacia Biotech, Piscataway, NJ), and antirat-HRP antibody (#5795; Sigma Chemical Co., St. Louis, MO). Signals were detected by chemiluminescence (Amersham Biotech, Piscataway, NJ).

Northern Blot Analyses. Total RNA was isolated with Trizol reagent (Life Technologies, Inc., Gaithersburg, MD), according to the protocol of the manufacturer, and poly(A) RNA was selected with Oligotex mRNA kit (Qiagen, Valencia, CA). For Northern blot analyses, 1.5 μg of poly(A) RNA were separated on 1% formaldehyde denaturing gel and transferred to nylon membranes. Probes for ZEB, VDR, and actin were prepared from the cDNAs for chicken ZEB, human VDR, and chicken actin, respectively. Probes were labeled with the Random Primers DNA labeling system of Life Technologies, Inc. using [α-^{32}P]-dCTP. Prehybridizations and hybridizations were carried out using Rapid-hyb buffer (Amersham Pharmacia Biotech, Piscataway, NJ).

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References

1. Pike J. W. Intracellular receptors mediate the biologic action of 1,25-dihydroxyvitamin D_3. Nutr. Rev., *43:* 161–168, 1985.

2. Carlberg, C., Bendik, I., Wyss, A., Meier, I., Sturzenbecker, L. J., Grippo, J. F., and Hunziker, W. Two nuclear signaling pathways for vitamin D. Nature (Lond.), *361:* 657–660, 1993.

3. Henry H. L., and Normal, A. W. Vitamin D: two dihydroxylated metabolites are required for normal chicken egg hatchability. Science (Wash. DC), *201:* 835–837, 1978.

4. Ornoy, A., Goodwin, D., Noff, D., and Edelstein, S. 24,25-Dihydroxyvitamin D is a metabolite of the vitamin D essential for bone formation. Nature (Lond.), *276:* 517–519, 1978.

5. Sunde, M. L., and Turk, C. M. The essentiality of vitamin D metabolites for embryonic chick development. Science (Wash. DC), *200:* 1067–1069, 1978.

6. Hewison M., Dabrowski, M., Faulkner, L., Hughson, E., Vadher, S., Rut, A., Brickell, P. M., O'Riordan, J. L. H., and Katz, D. R. Transfection of vitamin D receptor cDNA into the monoblastoid cell line U937: the role of vitamin D_3 in the homotypic macrophage adhesion. J. Immunol., *153:* 5709–5719, 1994.

7. Shabahang, M., Buffan A. E., Nolla, J. M., Schumaker, L. M., Brenner, R. V., Buras, R. R., Nauta, R. J., and Evans, S. R. The effect of 1,25-dihydroxyvitamin D_3 on the growth of soft-tissue sarcoma cells as mediated by the vitamin D receptor. Ann. Surg. Oncol., *3:* 144–149, 1996.

8. Song L. N. Demonstration of vitamin D receptor expression in a human megakaryoblastic leukemia cell line: regulation of vitamin D receptor mRNA expression and responsiveness by forskolin. J. Steroid Biochem. Mol. Biol., *57:* 265–274, 1996.

9. Davoust, N., Wion, D., Chevalier, G., Garabedian, M., Brachet, P., and Couez, D. Vitamin D receptor stable transfection restores the susceptibility to 1,25-dihydroxyvitamin D_3 cytotoxicity in a rat glioma resistant clone. J. Neurosci. Res., *52*: 210–219, 1998.

10. Genetta, T., Ruezinsky, D., and Kadesch, T. Displacement of an E-box-binding repressor by basic helix-loop-helix proteins: implications for B-cell specificity of the immunoglobulin heavy-chain enhancer. Mol. Cell. Biol., *14:* 6153–6163, 1994.

11. Watanabe, Y., Kawakami, K., Hirayama, Y., and Nagano, K. Transcription factors positively and negatively regulating the Na, K-ATPase α1 subunit gene. J. Biochem. (Tokyo), *114:* 849–855, 1993.

12. Williams, T. M., Moolton, D., Burlein, J., Romano, J., Bhaerman, R., Godillot, A., Mellon, M., Rauscher, F., and Kant, J. A. Identification of zinc-finger protein that inhibits IL-2 expression. Science (Wash. DC), *254:* 1791–1794, 1991.

13. Sekido, R., Takagi, T., Okanami, M., Moribe, H., Yamamura, M., Higashi, Y., and Kondoh, H. Organization of the gene encoding transcriptional repressor δEF1 and cross-species conservation of its domains. Gene, *173:* 227–232, 1996.

14. Genetta, T., and Kadesch, T. Cloning of a cDNA encoding a mouse transcriptional repressor displaying striking conservation across vertebrates. Gene, *169:* 289–290, 1996.

15. Cabanillas, A. M., and Darling, D. S. Alternative splicing gives rise to two isoforms of Zfhep, a zinc finger/homeodomain protein that binds T3-response element. DNA Cell Biol., *15:* 643–651, 1996.

16. Franklin, A. J., Jelton, T. L., Shelton, K. D., and Magnuson, M. A. BZP, a novel serum-responsive zinc finger protein that inhibits gene transcription. Mol. Cell. Biol., *14:* 6773–6788, 1994.

17. Funahashi, J-I., Kamachi, Y., Goto, K., and Kondoh, H. δ-Crystallin enhancer binding protein δEF1 is a zinc finger homeodomain protein implicated in postgastrulation embryogenesis. Development, *119:* 433–446, 1993.

18. Fortini, M. E., Lai, Z., and Rubin, G. M. The Drosophila *zfh-1* and *zfh-2* genes encode novel proteins containing both zinc-finger and homeodomain motifs. Mech. Dev., *34:* 113–122, 1991.

19. Lai, Z., Fortini, M. E., and Rubin, G. M. The embryonic expression patterns of *zfh-1* and *zfh-2*, two Drosophila genes encoding novel zinc-finger homeodomain proteins. Mech. Dev., *34:* 123–134, 1991.

20. Takagi, T., Moribe, H., Kondoh, H., and Higashi, Y. dEF1, a zinc finger and homeodomain transcription factor, is required for skeleton patterning in multiple lineages. Development, *125:* 21–31, 1998.

21. Sekido, R., Murai, K., Funahashi, J., Kamachi, Y., Fujisawa-Sehara, A., Nabeshima, Y., and Kondoh, H. The d-crystallin enhancer-binding protein δEF1 is a repressor of E2-box-mediated gene activation. Mol. Cell. Biol., *14:* 5692–5700, 1994.

22. Murre, C., Bain, G., vanDijk, M. A., Engel, I., Furnari, B. A., Massari, M. E., Matthews, J. R., Quong, M. W., Rivera, R. R., and Stuiver, M. H. Structure and function of helix-loop-helix proteins. Biochim. Biophys. Acta, *1218:* 129–135, 1994.

23. Postigo, A. A., and Dean, D. C. ZEB, a vertebrate homolog of Drosophila Zfh-1, is a negative regulator of muscle differentiation. EMBO J., *16:* 3935–3943, 1997.

24. Yasui, D. H., Genetta, T., Kadesch, T., Williams, T. M., Swain, S. L., Tsui, L. V., and Huber, B. T. Transcriptional repression of the *IL-2* gene in Th cells by ZEB. J Immunol., *160:* 4433–4440, 1998.

25. Gregoire J. M., and Romeo, P. H. T-Cell expression of the human *GATA-3* gene is regulated by a non-lineage-specific silencer. J. Biol. Chem., *274:* 6567–6578, 1999.

26. Brabletz, T., Jung, A., Hlubek, F., Lohberg, C., Meiler, J., Suchy, U., and Kirchner, T. Negative regulation of CD4 expression in T cells by the transcriptional repressor ZEB. Int. Immunol., *11:* 1701–1708, 1999.

27. Gill, G., and Ptashne, M. Mutants of Gal4 protein altered in activation formation. Cell, *51:* 121–126, 1987.

28. Hope, I. A., Mahadevan, S., and Struhl, K. Structural and functional characterization of the short acidic transcriptional activation domain of yeast GCN4 protein. Nature (Lond.), *333:* 635–640, 1988.

29. Mitchell, P. J., and Tjian, R. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science (Wash. DC), *245:* 371–378, 1989.

30. Seipel, K., Georgiev, O., and Schaffner, W. Different activation domains stimulate transcription from remote ("enhancer") and proximal ("promoter") positions. EMBO J., *11:* 4961–4968, 1992.

31. Chamberlain, E. M., and Sanders M. M. Identification of the novel player δEF1 in estrogen transcriptional cascades. Mol. Cell. Biol., *19:* 3600–3606, 1999.

32. Turner, J., and Crossley, M. Cloning and characterization of mCtBP2, a co-repressor that associates with basic Krüppel-like factor and other mammalian transcriptional regulators. EMBO J., *17:* 5129–5140, 1998.

33. Postigo, A. A., and Dean, D. C. ZEB represses transcription through interactions with the corepressor CtBP. Proc. Natl. Acad. Sci. USA, *96:* 6683–6688, 1999.

34. Furusawa T., Moribe, H., Kondoh, H., and Higashi, Y. Identification of CtBP1 and CtBP2 as corepressors of zinc finger-homeodomain factor dEF1. Mol. Cell. Biol., *19:* 8581–8590, 1999.

35. Postigo, A. A., Sheppard, A. M., Mucenski, M. L., and Dean, D. C. c-Myb and Ets proteins synergize to overcome transcriptional repression by ZEB. EMBO J., *16:* 3924–3934, 1997.

36. Jehan, F., and DeLuca, H. Cloning and characterization of the mouse vitamin D receptor promoter. Proc. Natl. Acad. Sci. USA, *94:* 10138–10143, 1997.

37. Miyamoto, K-I., Kesterson, R. A., Yamamoto, H., Taketani, Y., Nishiwaki, E., Tatsumi, S., Inoue, Y., Morita, K., Takeda, E., and Pike, J. W. Structural organization of the human vitamin D receptor chromosomal gene and its promoter. Mol. Endocrinol., *11:* 1165–1179, 1997.

38. Tanaka, Y., Bush, K. K., Klauck, T. M., and Higgings, P. J. Enhancement of butyrate-induced differentiation of HT-29 human colon carcinoma cells by 1,25- dihydroxyvitamin D_3. Biochem. Pharmacol., *38:* 3859–3865, 1989.

39. Giuliano, A. R., Franceschi, R. T., and Wood, R. J. Characterization of the vitamin D receptor from the Caco-2 human colon carcinoma cell line: effect of cellular differentiation. Arch. Biochem. Biophys., *285:* 261–269, 1991.

40. Fife, R. S., Sledge, G. W., Jr., and Proctor, C. Effects of vitamin D_3 on proliferation of cancer cells *in vitro*. Cancer Lett., *120:* 65–69, 1997.

41. Dai, P., Akimaru, H., Tanaka, Y., Hou, D. X., Yasukawa, T., Kanei-Ishii, C., Takahashi, T., and Ishii, S. CBP as a transcriptional activator of c-Myb. Genes Dev., *10:* 528–540, 1996.

42. Jones, G., Strugnell, S. A., and DeLuca, H. F. Current understanding of the molecular actions of vitamin D. Physiol. Rev., *78:* 1193–1231, 1998.

43. Buras, R. R., Schumaker, L. M., Davoodi, F., Brenner, R. V., Shabahang, M., Nauta, R. J., and Evans, S. R. Vitamin D receptors in breast cancer cells. Breast Cancer Res. Treat., *31:* 191–202, 1994.

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

45. Santiso-Mere, D., Sone, T., Hilliard, G. M., Pike, J. W., and McDonnell, D. P. Positive regulation of the vitamin D receptor by its cognate ligand in heterologous expression systems. Mol. Endocrinol., *7:* 833–839, 1993.

46. Arbour, N. C., Prahl, J. M., and DeLuca H. F. Stabilization of the vitamin D receptor in rat osteosarcoma cells through the action of 1,25-dihydroxyvitamin D_3. Mol. Endocrinol., *7:* 1307–1312, 1993.

47. Jehan, F., and DeLuca H. F. The mouse vitamin D receptor is mainly expressed through an Sp1-driven promoter *in vivo*. Arch. Biochem. Biophys., *377:* 273–283, 2000.

48. Maurer, U., Jehan, F., Englert, C., Hubinger, G., Weidmann, E., DeLuca, H. F., and Bergmann, L. The Wilms' tumor gene product (WT1) modulates the response to 1,25-dihydroxyvitmin D_3 by induction of the vitamin D receptor (VDR). J. Biol. Chem., *276:* 3727–3732, 2000.

49. Crossley, M., Whitelaw, E., Perkins, A., Williams, G., Fujiwara, Y., and Orkin, S. H. Isolation and characterization of the cDNA encoding BKLF/TEF-2, a major CACCC-box-binding protein in erythroid cells and selected other cells. Mol. Cell. Biol., *16:* 1695–1705, 1996.

50. Tanaka, T., Mitani, K., Kurokawa, M., Ogawa, S., Tanaka, K., Nishida, J., Yazaki, Y., Shibata, Y., and Hirai, H. Dual functions of the AML1/Evi-1 chimeric protein in the mechanism of leukemogenesis in t(3;21) leukemias. Mol. Cell. Biol., *15:* 2383–2392, 1995.

51. Burgess, A. W., and Metcalf, D. Characterization of a serum factor stimulating the differentiation of myelomonocytic leukemic cells. Int. J. Cancer, *26:* 647–654, 1980.

52. Abe, J., Moriya, Y., Saito, M., Sugawara, Y., Suda, T., and Nishii, Y. Modulation of cell growth, differentiation, and production of interleukin-3 by 1-alpha, 25-Dihydroxyvitamin D_3 in the murine myelomonocytic leukemia cell line WEHI-3. Cancer Res., *46:* 6316–6321, 1986.

53. Amento, E. P., Bhalla, A. K., Kurnick, J. T., Kradin, R. L., Clemens, T. L., Holick, S. A., Holick, M. F., and Krane, S. M. 1-alpha, 25-Dihydroxyvitamin D_3 induces maturation of the monocyte cell line U937, and, in association with a factor from human T lymphocytes, augments production of the monokine, mononuclear cell factor. J. Clin. Investig., *73:* 731–739, 1984.

54. Mangelsdorf, D. J., Koeffler, H. P., Donaldson, C. A., Pike, J. W., and Haussler, M. R. 1,25-Dihydroxyvitamin D_3-induced differentiation in a human promyelocytic leukemia cell line (HL-60): receptor-mediated maturation to macrophage-like cells. J. Cell Biol., *98:* 391–398, 1984.

55. Miyara, C., Abe, E., Suda, T., and Kiroki, T. Alternative differentiation of human promyelocytic leukemia cells (HL-60) induced selectively by retinoic acid and 1α,25-dihydroxyvitamin D_3. Cancer Res., *45:* 4244–4248, 1985.

56. Brown, G., Bunce, C. M., Rowland, D. C., and Williams, G. R. All-*trans*-Retinoic acid and 1-alpha,25-dihydroxyvitamin D_3 cooperate to promote differentiation of the human promyeloid leukemia cell line HL-60 to monocytes. Leukemia, *8:* 806–815, 1994.

57. Westin, E. H., Gallo, R. C., Arya, S. K., Eva, A., Souza, L. M., Baluda, M. A., Aaronson, S. A., and Wong-Staal, F. Differential expression of the *amv* gene in human hematopoietic cells. Proc. Natl. Acad. Sci. USA, *79:* 2194–2198, 1982.

58. Gonda, T. J., and Metcalf, D. Expression of myb, myc, and fos proto-oncogenes during the differentiation of a murine myeloid leukemia. Nature (Lond.), *310:* 249–251, 1984.

59. Ferrari, S., Donelli, A., Manfredini, R., Sarti, M., Roncaglia, R., Tagliafico, E., Rossi, E., Torelli, G., and Torelli, U. Differential effects of c-myb and c-fes antisense oligodeoxynucleotides on granulocytic differentiation of human myeloid leukemia HL60 cells. Cell Growth Differ., *1:* 543–548, 1990.

60. Arsura, M., Luchetti, M. M., Erba, E., Golay, J., Rambaldi, A., and Introna, M. Dissociation between p93B-myb and p75c-myb expression during the proliferation and differentiation of human myeloid cell lines. Blood, *83:* 1778–1790, 1994.

61. Owen, T. A., Aronow, M. S., Barone, L. M., Bettencourt, B., Stein, G. S., and Lian, J. B. Pleiotropic effects of vitamin D on osteoblast gene expression are related to the proliferative and differentiated state of the bone cell phenotype: dependency upon basal levels of gene expression, duration of exposure, and bone matrix competency in normal rat osteoblast cultures. Endocrinology, *128:* 1496–1504, 1991.

62. Tsonis, P. A., Sargent, M. T., Del Rio-Tsonis, K., and Jung, J. C. 9-cis Retinoic acid antagonizes the stimulatory effect of 1,25-dihydroxyvitamin D_3 on chondrogenesis of chick limb bud mesenchymal cells: interactions of their receptors. Int. J. Dev. Biol., *40:* 1053–1059, 1996.

63. Takeda, S., Yoshizawa, T., Nagai, Y., Yamato H., Fukumoto, S., Sekine, K., Kato, S., Matsumoto, T., and Fujita T. Stimulation of osteoclast formation by 1,25-dihydroxyvitamin D requires its binding to vitamin D receptor (VDR) in osteoblastic cells: studies using VDR knockout mice. Endocrinology, *140:* 1005–1008, 1999.

64. van Leeuwen, P. T. M., and Pols, H. A. P. Vitamin D: anticancer and differentiation. *In:* D. Feldman, F. H. Glorieux, and J. W. Pike (eds.), Vitamin D, pp. 1089–1105. New York: Academic Press, 1997.

65. Yoshizawa, T., Handa, Y., Uematsu, Y., Takeda, S., Sekine, K., Yoshihara, Y., Kawakami, T., Arioka, K., Sato, H., Uchiyama, Y., Masushige, S., Fukamizu, A., Matsumoto, T., and Kato, S. Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. Nat. Genet., *16:* 391–396, 1997.

66. Mizushima, S., and Nagata, S. pEF-BOS, a powerful mammalian expression vector. Nucleic Acids Res., *18:* 5322–5323, 1990.

67. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein dye binding. Anal. Biochem., *72:* 248–254, 1976.

68. Ikegaki, N., Bukovsky, J., and Kennett, R. H. Identification and characterization of the *NMYC* gene product in human neuroblastoma cells by monoclonal antibodies with defined specificities. Proc. Natl. Acad. Sci. USA, *83:* 5929–5933, 1986.