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Ligand-independent Regulation of the hairless Promoter by Vitamin D Receptor†

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Abstract

The characteristic alopecia associated with mutations in the hairless (*hr*) and vitamin D receptor (*VDR*) genes defines the resulting genetic disorders, known as atrichia and *VDRRIIa* rickets, as phenocopies. In both cases, the separation of the dermal papilla from the regressing hair follicle at the onset of the first catagen phase of the hair cycle and the development of dermal cysts and utricles subsequent to mutation of either gene suggests that their activities affect the same regulatory pathways. *VDR* functions as a hormonally activated transcription factor, and a role in transcription has been postulated for *Hr* due in part to its nuclear localization and homology with the GATA-1 zinc-finger domain. Therefore, we examined the hypothesis that *VDR* and *Hr* have a direct regulatory effect on each other *via* a transcriptional mechanism. Ectopic expression of the *VDR* repressed *hr* promoter activity in HaCaT cells and primary human keratinocytes (PHKs). While this repression occurs in the absence of 1,25 dihydroxyvitamin D3 (*D3*), the addition of ligand greatly augments the effect. However, we also demonstrate the rare phenomenon of ligand-independent promoter transactivation by *VDR*. We show that the full-length promoter is transactivated by *VDR* in a ligand-independent and cell type-specific manner, suggesting that direct transcriptional regulation of *hr* by the *VDR* accounts in part for the phenotypic overlap between atrichia and *VDRRIIa* rickets.

INTRODUCTION

The photosynthesis of vitamin D3 occurs largely in the basal keratinocytes of the epidermis and begins with the UVB-induced conversion of 7-dehydrocholesterol to vitamin D3 (1). Subsequent hydroxylations of vitamin D3 in the liver and kidney yield hormonally active 1,25 dihydroxyvitamin D3 (*D3*), which regulates the transactivation of gene expression in human keratinocytes through a canonical pathway of complex formation with its receptor, *VDR*, and complex binding to vitamin D responsive elements in the promoters of genes such as phospholipase D1 (2). *D3* regulates the proliferation and differentiation of keratinocytes, linking the regulation of keratinocyte homeostasis to UVB exposure. However, it has been suggested recently that *VDR* can activate transcription in keratinocytes in the absence of *D3* (3), uncoupling *VDR*-mediated transcriptional transactivation from photosynthetic activity. The alopecia sometimes associated with the rare, inherited recessive disorder, vitamin D-dependent rickets, type II (*VDDR II*, OMIM 277440) is ligand independent (4), suggesting that the loss of *D3*-independent transcriptional transactivation may play a role in *VDR* mutant phenotypes.

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Alopecia is manifested as a variety of phenotypes, each with a characteristic temporal onset, pattern of hair loss and histologically distinct features that correlate with their unique underlying genetic mechanism(s). Alopecia phenotypes can be separated into two general categories—those that arise through a failure of hair follicle (HF) morphogenesis and those that arise through a failure of HF cycling. Several genes have been identified whose mutation or knockout results in perturbations in HF cycling leading to hair loss. Among them are the hairless (hr) and VDR genes.

Mutations in the hr gene cause atrichia with papular lesions (APL, OMIM 209500) in humans (5) and the hr phenotype in mice (6). Mice with hr mutations are born with normal hairs, but undergo a cephalocaudal wave of hair shedding between days 16 and 21, corresponding to the induction of catagen in the first synchronous murine hair cycle (7). hr mutations in humans with APL, likewise, result in normal hairs that are shed shortly after birth (5,8,9). The resulting hair loss is permanent, due to the destruction of HF architecture following the onset of the first catagen. Still undetermined changes in cellular organization lead to the separation of the follicular signaling apparatus, the dermal papilla (DP), which becomes stranded in the dermis as the HF regresses during catagen. The disintegration of the HF is followed by the formation of sebum-filled dermal cysts, which along with the presence of utricles and the stranded DP are the defining morphological characteristics of the hr phenotype (7).

The molecular mechanisms through which Hr regulates the hair cycle remain largely unknown. The presence of a GATA family homologous putative DNA-binding zinc-finger domain (6), pathogenetic mutations which occur in this domain (10), the presence of nuclear-receptor interacting LXXLL motifs (11), the nuclear localization of the hr gene product (11) and its tight association with the nuclear matrix (12) all suggest that Hr regulates HF activity through transcription.

The alopecia resulting from VDR mutation is a phenocopy of the atrichia which results from hr mutation (13,14). Patients with mutations in the VDR shed their hairs in a frontal to posterior wave beginning shortly after birth and subsequently develop dermal cysts. Mice in which the VDR is inactivated by ablation of its second zinc-finger domain develop a complete alopecia (15). VDR-associated alopecia exhibits the defining characteristics of a hr mutation. The DP separates from the HF matrix at the onset of the first catagen between days 15 and 19, club hair formation is impeded and dermal cysts develop from the HF remnants (16). Transgenic targeting of human VDR expression to the skin of VDR null mutant mice with the keratin 14 promoter rescues the alopecia phenotype (17,18).

The alopecic phenotype resulting from VDR knockout is not due to the secondary, compensatory effect of ligand overproduction (19). In fact, the epidermal phenotype of the VDR null mutant mouse is not dependent on receptor interaction with its ligand, D3, as alopecia in the VDR knockout mouse is rescued by transgenic expression of a mutant VDR containing an amino acid substitution which ablates hormone binding activity (4). However, mutations in the coactivator-binding AF2 domain do not completely rescue the phenotype.

The similarities between hr and VDR mutant phenotypes suggest that these nuclear proteins act cooperatively, or at least within the same pathway, in the regulation of catagen transition in the hair cycle. Coimmunoprecipitation studies have shown that the VDR can physically interact with the hr gene product in both Cos cells (20) and keratinocytes (21). Furthermore, both studies demonstrate that Hr expression inhibits the ability of the VDR to transactivate gene expression through its canonical mechanism which requires interaction with D3. In keratinocytes, at least, the interaction of Hr with the VDR is attenuated through ligand binding (21).

Neither the ability of Hr to interact with the VDR nor the necessity of their acting in concert, however, has been demonstrated to be essential to their activities during HF cycling. Indeed, several mechanisms could be postulated to account for the requirement of both factors. As VDR is a transcription factor which transactivates the expression of its downstream target genes through sequence-specific DNA binding, it is possible that it regulates the expression of the hr gene. Failure of VDR transactivational activity through mutation would result in the loss of hr gene expression resulting in the hr null phenotype. Conversely, Hr activity might upregulate the expression of the VDR, and the loss of functional Hr would result in the VDR null phenotype. Here, we examine the transcriptional activity of Hr and the VDR on each other, as possible mechanisms for the requirement of both factors in HF cycling.

MATERIALS AND METHODS

Plasmids

The phpf series of hr promoter 5' deletion reporter plasmids was constructed by cloning the upstream sequence of the hr gene from the upstream sites (see Fig. 1), to the *Bgl*III site at +102 into the polycloning site of the pGL3 basic luciferase reporter vector (Pro-mega). The vitamin D3-responsive reporter plasmid, p24OH-luc, which contains the D3 response elements from the 24-hydroxylase promoter (21), and the VDR mammalian expression vector, pCMV-VDR, were kindly provided by Dr. Daniel Bikle (University of California at San Francisco).

Cell culture

The human neuroblastoma cell line, SH-SY5Y, the human keratinocyte cell line, HaCaT, and the mouse fibroblast cell line, NIH3T3, were all maintained in DMEM (Invitrogen) supplemented with 2 mM L-glutamine, 50 U mL⁻¹ penicillin/streptomycin, and 10% fetal bovine serum (FBS). Primary human keratinocytes (PHKs), supplied by the Columbia University Skin Disease Research Center, were maintained in keratinocyte-serum free medium (K-SFM; Invitrogen).

For transfection, 4×10^5 SY5Y, 5×10^5 HaCaT, 5×10^5 NIH 3T3 cells or 5×10^5 PHKs were seeded in triplicate 60 mm dishes 16–20 h prior to transfection. Cells were transfected with a total of 6 μ g DNA. In double transfection experiments, 3 μ g of each plasmid was used. In triple transfection experiments, 2 μ g of each plasmid was used, and enough pbluescript plasmid was added to normalize the amount of DNA in each sample to 6 μ g. Each transfection included an additional 1 μ g of pSV- β gal as a transfection efficiency control. Cells were transfected with 10 μ L of Lipofectamine (Invitrogen) according to the manufacturer's protocol. At the time of transfection, the cells were washed once in PBS without Ca²⁺ and Mg²⁺, and maintenance medium was replaced with DMEM supplemented with 2 mM L-glutamine, 50 U mL⁻¹ penicillin/streptomycin and 10% hormone-depleted FBS, except for PHK cultures which remained in K-SFM. Serum was depleted of hormone with AG 1-X8 Resin (Bio-Rad) according to the manufacturer's protocol. 1,25 dihydroxyvitamin D3 (Sigma) was solubilized in ethanol immediately prior to its addition to cultures at 24 h posttransfection at a final concentration of 10⁻⁷ M as indicated.

Luciferase assay

Cells were washed in PBS without Ca²⁺ and Mg²⁺ and harvested at 48 h posttransfection in 400 μ L of lysis buffer (Luciferase Assay System, Promega). Luciferase production was assayed by the addition of 10–20 μ L of lysate to 100 μ L of Luciferase Reagent and recording the activity with a luminometer. Luciferase assays were normalized with β -galactosidase activity which was assayed by chlorophenolred- β -D-galactopyranoside (CPRG) conversion.

siRNA knockdown

HaCaT cells grown in six-well plates at 30% confluence were transfected using oligofectamine (Invitrogen) with pooled siRNA oligonucleotide molecules (Dharmacon) targeting hr mRNA sequence or nontargeting sequence (siCONTROL Non-Targeting siRNA #2) as a control, according to the manufacturer's protocol. At 48 h posttransfection, RNA was harvested by spin column (RNeasy Mini Kit; Qiagen). Equal amounts of RNA were reverse transcribed with random oligonucleotide primers and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Primers used with the Power SYBR Green PCR Master Mix (Applied Biosystems) to determine the levels of hr, VDR and the endogenous control for normalization, β -actin, by real-time PCR were: 5'hr: CAAGCACCTGCTCAGTGGTT, 3'hr: AAGAGT-CCATGGTGGCAACG, 5'VDR: CGTACTGCTGAAGTCAAG-TGC, 3'VDR: CTCCTCCTCATGCAAGTTCAG, 5' β actin: CCCAG-CACAATGAAGATCAA and 3' β -actin: GTGTAACGCAACTAA-GTCAT. mRNA levels were quantified by the relative quantification default program on an ABI7300 real-time PCR system (ABI).

RESULTS

We previously demonstrated that the thyroid-responsive element (TRE) in the hr promoter, through which hr expression is regulated in the developing rat cerebellum (22), also regulates the human hr promoter in neuronal cells. Unexpectedly, the hr promoter was unresponsive to thyroid hormone (T3) in keratinocytes (23). In order to extend our investigation into other factors that regulate hr expression, we searched the sequence of the promoter between the TRE and the mRNA cap site for other transcription factor binding sites known to play a role in the skin. We found 14 putative mammalian VDR half-sites, with the most 5' located at -2699 and the most 3' located at -665 relative to the start site of transcription, distributed throughout the promoter with half of them clustering at sites between positions -1047 and -665 (data not shown). Although none of the sites were organized into the canonical direct repeats (DR) separated by several nucleotides that comprise a vitamin D-responsive element, one of the half sites fell within the DR+4 binding site of the thyroid hormone receptor. Therefore, we engineered a series of deletion constructs (Fig. 1) in which 5' deletions of the upstream region of the hr promoter were made on average every 500 bp, and these hr promoter fragments (hpf) were cloned into the pGL-3 luciferase reporter vector.

We have previously demonstrated that HaCaT cells are representative of hr promoter activity in epidermal keratinocytes (23). In order to determine whether the VDR regulates hr promoter activity in keratinocytes, we transiently transfected HaCaT cells with our series of hpf deletion-luciferase fusion constructs in the presence or absence of a cotransfected VDR expression plasmid. At 24 h posttransfection the cells were treated with ethanol as a vehicle control or D3 was added to the cultures to a final concentration of 10^{-7} M. Lysates of the cultures were harvested at 48 h and assayed for luciferase activity (Fig. 2).

Cotransfection of the VDR expression plasmid along with the hpf constructs resulted in inhibition of hr promoter activity in the five largest promoter fragments ranging from 21% inhibition (hpf4) to approximately 60% inhibition (hpf2). Inhibition of the full-length hpf6 construct was 43%, and the average inhibition across all repressed constructs measured $43.4 \pm 16.1\%$. The addition of 10^{-7} M D3 to the culture medium was responsible for repression of promoter activity in the presence and absence of exogenously expressed VDR. In cells which were not cotransfected, D3 repressed luciferase activity averaging $60.3 \pm 9.8\%$, compared to untransfected cells. In cells cotransfected with VDR, D3 repressed luciferase activity averaging $47.76 \pm 10.5\%$, compared to cotransfected cells that were not treated with D3. Cultures that were cotransfected with VDR and treated with D3 experienced the greatest

repression, averaging $70.0 \pm 12.4\%$. In general, however, the magnitude of inhibition by D3 was independent of whether or not VDR was cotransfected along with the reporter construct, when cotransfected and non-cotransfected cells are analyzed separately. For example, D3-mediated inhibition of the hpf6 and hpf5 constructs was 57.9% and 45.9%, respectively, when compared with cultures that were not treated with D3 and not transfected with VDR. The D3-mediated inhibition of the same constructs in cells cotransfected with VDR was 58.8% and 44.6%, respectively, when compared with cultures that were not treated with D3 but also transfected with VDR. This result suggests that while the relative inhibition resulting from D3 treatment is constant, the total inhibition is relative to the amount of available VDR in the cells. Additionally, hpf6 contains a TRE that is absent in hpf5, demonstrating that the TRE, which itself contains a VDR half-site, plays no role in the repression of the hr promoter by VDR.

Interestingly, the p24OHase reporter vector, a control for ligand-dependent transactivational activity through the VDR, was also repressed by approximately 60% by cotransfection of VDR, consistent with models of downregulation of gene activity by VDR in the absence of its ligand. The addition of ligand, however, transactivated luciferase activity by approximately 4.5-fold in the presence or absence of cotransfected VDR, demonstrating that the effect on hr activity is promoter specific.

Surprisingly, hpf1, the smallest promoter fragment which comprises just 285 bp of hr upstream sequence is not regulated by D3. In the presence of D3, its activity remains unchanged. However, the activity of hpf1 is transactivated by cotransfection of VDR. The smallest hpf is upregulated three- to 3.3-fold by the cotransfection of VDR, irrespective of whether or not D3 is added to the culture. While the basal activity of the hpf1 fragment is relatively small compared with the largest fragment (whose basal expression is 40 times greater), it is comparable to the expression level of the 24OHase construct in keratinocytes. Furthermore, the upregulation of hpf1 demonstrates that the hr promoter contains elements capable of being transactivated by the VDR in a ligand-independent manner. The empty pGL3 basic vector resulted in negligible activity which remained unchanged in all conditions (data not shown).

Because CMV vectors are known to inhibit the promoter activity of some genes, we examined the dose response of promoter activity to VDR transfection compared to the parent CMV expression vector, pCDNA3 (Fig. 3). HaCaT cells were transfected with 3 μ g of hpf6 and a total of 2 μ g of pCDNA3, pCMV-VDR or a combination of the two in varying concentrations. Increases in the amount of the VDR plasmid relative to the vector resulted in increased repression of the hr promoter construct. Complete replacement of the empty vector with the VDR expression plasmid resulted in 59.25% inhibition of hr promoter activity. In all cases, the addition of D3 resulted in the enhancement of inhibition.

Although our initial studies were performed in HaCaT cells to represent epidermal keratinocytes, we repeated our experiments with selected hpfs in PHKs to examine the effects in the context of normal skin (Fig. 4). Several differences between the activity of the promoter fragments in HaCaT cells and PHKs were notable. Similar to our results in HaCaT cells, the 24OHase promoter was transactivated 4.7-fold in the absence of ectopic VDR expression and 11.1-fold in its presence. Also, the addition of D3 downregulated the expression of hpf6 by 42.0%, of hpf2 by 32.2% and of hpf1 by 61.0%. Cotransfection of VDR, however, resulted in almost complete inhibition of all promoter fragments tested, regardless of whether or not the cells were treated with D3. In the presence of D3, hpf6 activity was repressed by 95.1%. In its absence, repression was still almost complete at 96.9%. Likewise, hpf2 activity was repressed 87.8% and 89.4% in the presence and absence of D3, respectively. Finally, although the smallest promoter fragment, hpf1, was

transactivated by the VDR in a ligand-independent manner in HaCaT cells, it was transrepressed in PHKs. The expression of VDR in PHK cells inhibits the expression of *hpf1* by over 95% in the presence and absence of D3.

As noted above, we previously demonstrated that T3 transactivated *hr* in neuroblastoma cells, but the promoter was unresponsive in epidermal keratinocytes. To determine whether the inhibition of *hr* activity was cell type specific, as is transactivation, we repeated the transient transfections and luciferase assays in the human neuroblastoma cell line, SY5Y (Fig. 5). With the exception of *hpf3*, whose activity is downregulated by cotransfection of the VDR, the promoter fragments are uniformly unaffected by either the cotransfection of VDR, the addition of D3 to the medium or both conditions. Unexpectedly, in SY5Y cells, the 24OHase control was not transactivated by the addition of D3 to the cultures, even in the presence of exogenously expressed VDR, demonstrating that the inhibition of *hr* promoter activity is cell type specific and suggesting that neuroblastoma cells lack important cofactors for VDR activity.

Because 24OHase, our positive control for D3-regulated transactivation was unresponsive in SY5Y cells, we examined the activity of the *hr* promoter in murine NIH3T3 cells, in which we knew that the 24OHase reporter was transactivated by D3 (Fig. 6). Indeed, although the basal transcription of the 24OHase luciferase fusion construct was downregulated 53.6% by the cotransfection of the VDR, the addition of D3 to the cultures elicited a 2.9-fold induction of transcription in the absence of exogenous VDR and a 2.4-fold induction in its presence. Without exception, the *hpfs* did not respond to the addition of D3 alone. All of the fragments, surprisingly, were transactivated by the cotransfection of VDR. Even though 3T3 cells demonstrated the capability of mounting a response to D3 with the p24OHase reporter, the addition of D3 to cells cotransfected with VDR had no significant effect on the transactivation of *hr* promoter activity, demonstrating that transactivation of *hr* by the VDR is ligand independent and promoter specific. In the absence of D3, the upregulation of *hr* promoter activity had a mean of 6.5 ± 2.5 -fold and ranged from 3.5-fold with *hpf2* to 10.3-fold with *hpf4*. With the addition of D3, promoter fragments were transactivated averaging 7.8 ± 4.7 -fold and ranged from 3.4-fold with *hpf2* to 15.7-fold with *hpf4*. Individually, differences between the raw values of luciferase activity between D3-treated and untreated for each promoter fragment were within the standard deviation.

To address a converse transcriptional relationship between the VDR and *Hr*, we analyzed the RNA expression level of VDR in HaCaT cells transfected with *hr* targeting siRNAs by quantitative real-time PCR (Fig. 7). The inhibition of *hr* expression by specific *hr*-targeting siRNAs was 81.3% of mock-transfected cells, or 71.8% inhibition compared to the nonspecific background effect. Within the same cultures, the effect of *hr* knockdown on VDR mRNA levels was not significant. VDR mRNA levels remained relatively unchanged with *hr*-targeting siRNAs resulting in VDR expression at 73.4% of mock-transfected levels and at 86.5% compared to background nontargeting siRNA levels. These data suggest that the effect of *hr* expression on VDR expression in epidermal keratinocytes is minimal.

DISCUSSION

Both the *hr* and VDR genes are essential for progression through the catagen phase of the hair cycle. The phenotype that results from the homozygous disruption of either gene, including a pattern of hair loss and morphologic changes that accompany the catagen transition as well as histologic features of remnant hair follicles, suggests that the two genes function within the same pathway.

Several mechanisms can be postulated to account for the requirement of both genes within a single pathway. A “cooperative” mechanism would require both Hr and VDR to properly regulate the hair cycle, and postulates a direct interaction between the two gene products which would regulate transcription of downstream genes. In support of this hypothesis, a physical interaction between the VDR and the Hr protein has been demonstrated through coimmunoprecipitation studies (20,21,24). Furthermore, this interaction has been demonstrated to strongly repress D3-dependent VDR-mediated transactivation. The exact mechanism of this repression remains undetermined, as various reports suggest that the Hr–VDR interaction remains stable upon the addition of D3, which would require the recruitment of unique repressive cofactors to the Hr–VDR complex, and that the presence of D3 displaces Hr from a VDR complex, which suggests that Hr represses VDR activity by competition for ligand-complex interaction. Finally, it has been demonstrated that most, but not all, pathogenic hr mutations disrupt the repressive activity of Hr on VDR transactivation of the 24-hydroxylase promoter in cultured monkey kidney cells (24), suggesting that the repression of VDR activity by Hr in the HF is the predominant pathogenetic mechanism of most hr mutants. However, it remains unclear how the repression of ligand-dependent gene activation regulates HF homeostasis, as the role of VDR in HF cycling is ligand independent (19). Furthermore, antisense-mediated inhibition of hr gene expression in normal human keratinocytes resulted in no change in the expression of sentinel genes for D3 regulation, involucrin, transglutaminase and phospholipase C- γ , in the absence of D3 as compared to vector-transfected or hr-overexpressing cells (21). Finally, in the same manner as the studies outlined above, we have been able to coimmunoprecipitate Hr and VDR in Cos-1 cells that exogenously express both proteins; however, we failed to detect an interaction between the two using yeast two-hybrid and mammalian two-hybrid systems, as well as by overlay assay (data not shown).

A second “convergent” mechanism, which cannot yet be ruled out, would postulate that both genes act independently on the activity of a third gene or set of genes. For example, as the biochemical function of Hr protein has yet to be identified, it might potentiate the activity of a downstream promoter by altering the conformation of DNA through its putative zinc-finger, by regulating the interaction of DNA with the nuclear scaffolding with which it is tightly associated, or through regulating a biochemical interaction such as phosphorylation, acetylation or methylation. Any of these activities could be required in advance of ligand-independent transcriptional induction by the VDR. For example, hr has sequence homology to the JmjC domain of a growing family of proteins which have recently been shown to possess demethylase activity ([25]; reviewed in Takeuchi *et al.* [26]). The JmjC domain-containing protein, JHDM3A, has been demonstrated to act as a histone lysine demethylase (27). Loss of JHDM3A activity upregulates the expression of its target gene, suggesting that proteins containing the JmjC domain regulate transcription at the level of chromatin structure. These activities are consistent with our previous demonstration that Hr is tightly bound to the nuclear matrix (28). A demethylase activity inherent in the Hr protein would support the “cooperative” or the “convergent” mechanisms depending on the requirement of VDR for demethylation. Furthermore, the regulation of the wnt pathway, which is required for HF morphogenesis (29), might serve as a model for the convergent hypothesis, as it has been demonstrated that VDR regulates wnt signaling in keratinocytes at the level of β -catenin–Lef1 interaction (30) and that Hr regulates wnt signaling in the HF through wise, which acts at the level of receptor interaction (31).

Finally, a “linear” mechanism postulates a relationship between the two genes in which the expression of one gene is directly upregulated by the expression of the other. Loss of activity of the upstream gene in this example would result in the loss of expression of the downstream gene. Mutation in either gene, therefore, would abrogate all downstream activity in the pathway. While we did not detect any indication that the expression of the

VDR was affected by the siRNA-mediated knockdown of hr gene expression in HaCaT cells (Fig. 7), we also examined the hypothesis that vitamin D3 and its nuclear receptor regulate the expression of the hr gene through its promoter.

Cotransfection of the VDR along with a full-length hr promoter-luciferase gene fusion construct into HaCaT cells in hormone-depleted medium demonstrated that VDR repressed hr gene expression in a ligand-independent manner (Figs. 2 and 3). The addition of the VDR ligand, D3, further repressed hr promoter activity, demonstrating that VDR regulates hr expression through both ligand-dependent and -independent mechanisms. The repression of hr expression was promoter specific and not due to the autocrine production of D3, as the D3-responsive 24OHase promoter was activated by the addition of hormone in the same cell type. Surprisingly, identical results obtained with a 5' deletion of the full-length promoter construct, which eliminates the TRE from the promoter, show that the VDR does not mediate repression through the TRE, which contains a VDR half-site. We have previously demonstrated that T3 does not regulate hr expression in keratinocytes (23) and that a pathogenic mutation in the T3-interacting domain of Hr does not alter *in vitro* interactions of Hr and thyroid hormone receptor (28). In fact, in all of our experiments, repression or transactivation occurred similarly in all deletion constructs, suggesting that our smallest construct, hpf1, contains a unique VDR-responsive element through which transactivation or transrepression occurs in a cell typespecific manner. Studies to determine the nature of this putative VDRE are ongoing.

In PHKs, which were included to represent normal epidermis, the repression of hr gene expression by ligand-independent action of the VDR is nearly complete and is not enhanced by the addition of D3 (Fig. 4). While we observe that hr promoter activity is affected similarly in both models of epidermal keratinocytes, the repression of hr promoter activity is inconsistent with the hypothesis that VDR is required for hr expression, and thus for HF cycling.

Although we would be tempted to rule out the relevance of hr gene regulation by VDR because of this inconsistency, we were surprised to discover that the hr promoter can be transactivated by the VDR in a ligand-independent manner (Fig. 6). Unlike previous demonstrations of ligand-independent VDR-mediated transcriptional transactivation, which include regulation of the osteocalcin VDRE in CV1 cells (32) and the prolactin promoter in HeLa cells (33), transactivation of the hr gene did not require the addition of exogenous factors and was not enhanced by the addition of D3. The ligand-independent activity of VDR was promoter-specific and not due to autocrine production of hormone, as the 24OHase promoter required D3 for transactivation in NIH3T3 cells. Our demonstration that the full-length hr promoter can be transactivated by the VDR in NIH3T3 cells without the addition of D3 is consistent with two requirements imposed on the validation of the linear hypothesis. One requirement is the ability of hr gene expression to be regulated by the VDR. The second requirement is that this regulation be D3 independent, as the mutant VDR hair loss phenotype is ligand independent (18). A third requirement would be for this activity to occur in the HF, which raises the question of the validity of cell culture models for Hr and VDR activity in catagen transition. Most studies on the relationship of Hr and VDR to HF cycling, including our own, have been carried out in monkey kidney cells, HaCaT cells or PHKs derived from postnatal skin devoid of HFs, because of technological limitations on the ability to culture the diverse repertoire of keratinocytes that comprise the HF. Which of these cells contains the contextual milieu that best represents the HF during catagen is an open question. We have demonstrated that the hr promoter is capable of transactivation by unliganded VDR in a defined cellular context, which is consistent with both the ligand independence of a Hr-VDR interaction and the requirement of both factors to maintain HF cycling. Therefore, we propose a model in which the direct transactivation of the hr

promoter by the VDR defines another mechanism through which HF homeostasis is maintained.

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Figure 1. Schematic representation of hr promoter fragment constructs. The 5' boundary of each hr promoter fragment inserted into the pGL3 luciferase reporter vector is indicated relative to the start site of transcription. The position of the thyroid-responsive element (TRE) is indicated by a closed box. The start site of transcription of the hairless gene is indicated by an arrow.

Figure 2.

Inhibition of hr promoter activity by vitamin D3. Triplicate cultures of HaCaT cells were transfected with the indicated human hairless promoter-luciferase fusion constructs or p24OHase-luciferase, a positive control for 1,25 dihydroxyvitamin D3 induction. The pCMV-VDR expression plasmid was cotransfected as indicated or an equal quantity of pbluescript was cotransfected to normalize the amount of total DNA transfected into the cells. At 24 h posttransfection EtOH or 1,25 dihydroxyvitamin D3 in EtOH was added to a final concentration of 10^{-7} M to the depleted serum cultures as indicated. Cell lysates were harvested at 48 h, and luciferase activity was assayed. Bars represent the average luciferase activity on an arbitrary scale $\pm$ standard deviation. Error bars smaller than the symbols do not appear. Graph is representative of multiple trials.

Figure 3.

Dose response of VDR transfection to hpf6 inhibition. Triplicate cultures of HaCaT cells were cotransfected with the hpf6 hairless promoter-luciferase fusion construct and the indicated combination of the pCDNA3 expression vector and pCMV-VDR. At 24 h posttransfection EtOH or 1,25 dihydroxyvitamin D3 in EtOH was added to a final concentration of 10^{-7} M to the depleted serum cultures as indicated. Cell lysates were harvested at 48 h, and luciferase activity was assayed. Bars represent the average luciferase activity on an arbitrary scale $\pm$ standard deviation. Error bars smaller than the symbols do not appear. Graph is representative of multiple trials.

Figure 4.

VDR represses the hr promoter in primary keratinocytes. Duplicate cultures of PHKs were transfected with the indicated human hairless promoter-luciferase fusion constructs or p24OHase-luciferase. The pCMV-VDR expression plasmid or an equal quantity of pbluescript was cotransfected as in Fig. 2. At 24 h posttransfection 1,25 dihydroxyvitamin D3 in EtOH was added to a final concentration of 10^{-7} M to the serum-free cultures as indicated. Cell lysates were harvested at 48 h, and luciferase activity was assayed. Bars represent the mean luciferase activity on an arbitrary scale $\pm$ standard deviation. Error bars smaller than the symbols do not appear. Graph is representative of multiple trials.

Figure 5.

Cell type specificity of hr promoter regulation by vitamin D3. Triplicate cultures of SY5Y cells were transfected with the indicated human hr promoter-luciferase fusion constructs or p24OHase-luciferase. The pCMV-VDR expression plasmid or an equal quantity of pbluescript was cotransfected as in Fig. 2. At 24 h posttransfection 1,25 dihydroxyvitamin D3 in EtOH was added to a final concentration of 10^{-7} M to the depleted serum cultures as indicated. Cell lysates were harvested at 48 h, and luciferase activity was assayed. Bars represent the mean luciferase activity on an arbitrary scale $\pm$ standard deviation. Error bars smaller than the symbols do not appear. Graph is representative of multiple trials.

Figure 6.

Transactivation of hr promoter by VDR. Triplicate cultures of NIH3T3 cells were transfected with the indicated human hr promoter-luciferase fusion constructs or p24OHase-luciferase. The pCMV-VDR expression plasmid or an equal quantity of pbluescript was cotransfected as in Fig. 2. At 24 h posttransfection 1,25 dihydroxyvitamin D3 in EtOH was added to a final concentration of 10^{-7} M to the depleted serum cultures as indicated. Cell lysates were harvested at 48 h, and luciferase activity was assayed. Bars represent the mean luciferase activity on an arbitrary scale $\pm$ standard deviation. Error bars smaller than the symbols do not appear. Graph is representative of multiple trials.

HaCaT cells

Figure 7.

Effect of hr-targeting siRNAs in keratinocytes. Triplicate plates of HaCaT cells were mock transfected (control) or transfected with hr-targeting siRNAs. RNAs from the cultures were harvested at 48 h posttransfection and used as templates in quantitative real-time RT-PCR reactions using hr or VDR-specific primers as indicated. Histograms represent the relative quantitation of six individual samples of hr or VDR RNA, compared to the mock-transfected cultures (100%).