The Vitamin D Response Element of the Involucrin Gene Mediates its Regulation by 1,25-Dihydroxyvitamin D_3

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Involucrin is a major protein of the cornified envelope of keratinocytes that provides much of the structural integrity of skin. Its expression is stimulated by a number of agents including calcium and 1,25-dihydroxyvitamin D_3 that promote the differentiation process in keratinocytes. Within the distal regulatory region of the involucrin promoter lies an AP-1 site and an element homologous to other vitamin D response elements. In previous studies mutation of the AP-1 site was found to reduce basal activity and block calcium stimulation of the involucrin promoter, whereas the vitamin D response element was not critical for calcium regulation. In this study both elements proved to be important for 1,25-dihydroxyvitamin D_3 stimulation of the involucrin promoter. Mutation of the AP-1 site reduced basal activity and blocked 1,25-dihydroxyvitamin D_3 stimulation of the involucrin promoter. In contrast, mutation of the vitamin D response element did not reduce basal expression of the involucrin promoter or prevent calcium stimulation of involucrin gene expression, but blocked 1,25-dihydroxyvitamin D_3 stimulation. The vitamin D response element from the involucrin gene bound the vitamin D receptor and the retinoid X receptor, but not the retinoic acid receptor, in a specific manner. We conclude that the AP-1 site and the vitamin D response element in the involucrin promoter play important roles in mediating the action of 1,25-dihydroxyvitamin D_3 on involucrin expression, but the vitamin D response element provides specificity for the 1,25-dihydroxyvitamin D_3 response lacking at the AP-1 site. ***Key words: AP-1/involucrin/keratinocytes/vitamin D receptor/vitamin D response element/PPARa/LXR/δP-1/DR3/PBS. J Invest Dermatol 119:1109–1113, 2002***

The observation that 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) induces keratinocyte differentiation was first made by Hosomi *et al* (1983) and provided a rationale for the previous and unexpected finding of $1,25(OH)_2D_3$ receptors in the epidermis (Stumpf *et al*, 1979). $1,25(OH)_2D_3$ is likely to be an autocrine or paracrine factor for epidermal differentiation as it is produced by the keratinocyte, but under normal circumstances keratinocyte production of $1,25(OH)_2D_3$ does not appear to contribute to circulating levels (Bikle *et al*, 1986). The receptors for and the production of $1,25(OH)_2D_3$ vary with differentiation (Pillai *et al*, 1988a) in a manner that suggests feedback regulation; both are reduced in the later stages of differentiation. The differentiation pathway that we have studied most directly is the formation of the cornified envelope, the structure within the keratinocyte that provides much of the structural integrity of the skin. The cornified envelope is composed of a number of proteins including involucrin that are cross-linked by the enzyme transglutaminase to form the cornified envelope (Simon and Green, 1984; Thacher and Rice, 1985; Hohl, 1990). $1,25(OH)_2D_3$ increases gene expression and the mRNA and protein levels for involucrin and transglutaminase, and promotes cornified envelope formation at subnanomolar concentrations in preconfluent keratinocytes (Hosomi *et al*, 1983; Smith *et al*, 1986; McLane *et al*, 1990; Pillai and Bikle, 1991; Su *et al*, 1994). The effects of $1,25(OH)_2D_3$ and calcium are additive and even synergistic in this effect (Su *et al*, 1994). The involucrin gene has been cloned (Eckert and Green, 1986), and the region responsible for its induction by calcium identified (Ng *et al*, 2000). The calcium-responsive region contains an AP-1 site that is essential not only for calcium responsiveness (Ng *et al*, 2000) but for induction of this gene by phorbol esters (Welter *et al*, 1995) and the activated nuclear hormone receptors PPARα (Hanley *et al*, 2000; Komuves *et al*, 2000) and LXR (Hanley *et al*, 2000). As shown in this study, the AP-1 site also is important for the ability of $1,25(OH)_2D_3$ to increase involucrin gene expression. 3′ of the AP-1 site, however, and separated from it by an SP-1 site, is a putative vitamin D response element (VDRE) containing two half sites separated by three nucleotides (DR3). In previous studies (Ng *et al*, 2000) this VDRE was shown not to be essential for calcium or phorbol ester stimulation of involucrin expression. In this study we demonstrate that this putative VDRE is functional and like other DR3 VDREs binds the vitamin D receptor (VDR) and retinoid X receptor (RXR). The close proximity of the VDRE and the AP-1 site may explain the synergism between calcium and $1,25(OH)_2D_3$ in the regulation of the involucrin gene.

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Abbreviations: $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; KGM, keratinocyte growth medium; RXR, retinoid X receptor; VDR, vitamin D receptor; VDRE, vitamin D response element.

EXPERIMENTAL PROCEDURES

Cell culture Normal human keratinocytes were isolated from neonatal human foreskins and grown in serum-free keratinocyte growth medium (KGM, Clonetics, San Diego, CA) as described previously (Pillai *et al*,

1988). Briefly, keratinocytes were isolated from newborn human foreskins by trypsinization (0.25% trypsin, 4°C overnight), and primary cultures were established in KGM containing 0.07 mM calcium. After 2 d the calcium concentration was changed to 0.03 mM calcium, and unless otherwise stated the first and second passage keratinocytes were grown in this medium. All experiments used second passage keratinocytes. Details regarding the addition of calcium and $1,25(OH)_2D_3$ are found in the figure legends and/or text for each experiment.

Construction of vectors The 3.7 kb fragment of the human involucrin promoter (gift from Dr. Lorne B. Taichman) (−2461 to +1228 bp of the involucrin gene, numbered with transcription start site as +1) was subcloned into a pGL-3 basic vector (Promega, Madison, WI) (Carroll *et al*, 1993), linking it to the luciferase gene as previously described (Ng *et al*, 2000). The AP-1 site at −2114 to −2108 bp was mutated at base −2110 (TGAGTCA to TGAGcCA) using polymerase chain reaction (PCR) based mutagenesis as previously described (Ng *et al*, 2000). In similar fashion the putative VDRE (−2097 to −2083) was mutated at 10 base pairs in both half sites in a manner that created a BamHI site for screening purposes (GGCAGATCTGGCAGA to caggacTCTGGatcc). Correct orientation of the inserts with respect to the luciferase sequence was verified by restriction enzyme analysis. All sequences were verified by automated nucleotide sequencing using fluorescence dye terminators.

Transfection, selection, and luciferase assay Second passage keratinocytes plated in 60 mm culture dishes were cotransfected with 2 μg of the various involucrin–luciferase promoter constructs and 0.2 μg of pRSV β-gal or pRL-TK using a polybrene method (Jiang *et al*, 1991) modified as previously described (Ng *et al*, 2000) or Superfect (Qiagen, Valencia, CA). pRSV β-gal is a β-galactosidase expression vector that contains a β-galactosidase gene that is driven by a Rous sarcoma virus promoter and enhancer, whereas pRL-TK is a renilla luciferase construct driven by a thymidine kinase promoter. These constructs were used as internal controls to normalize for transfection efficiency. Following the addition of calcium as calcium chloride (to a final concentration of 1.2 mM) and/or $1,25(OH)_2D_3$ the cells were lysed 24–48 h later, and the cell extracts were assayed for luciferase activity using the Luciferase Assay System (Promega, Madison, WI) and β-galactosidase activity using the Galacto-Light™ kit (TROPIX, Bedford, MA). Every experiment was done in triplicate and was repeated at least three times with different keratinocyte preparations.

Northern analysis Total RNA was isolated from the keratinocytes using the STAT-60 kit™ (Tel.Test 'B', Friendswood, TX), according to the procedures recommended by the manufacturer. The isolated RNA (30 μg per lane) was electrophoresed through a 0.8% agarose-formaldehyde gel, transferred to a nylon membrane (Hybond-N+; Amersham, Arlington Heights, IL) using PosiBlot 30–30 Pressure Blotter (Stratagene, La Jolla, CA), and immobilized by baking the membrane at 80°C for 2 h. The human involucrin cDNA probe (gift from Dr. Robert Rice) was labeled with ^{32}P-dCTP (Amersham, Arlington Heights, IL) by Random Prime-IT II labeling kit (Stratagene) and purified by NucTrap Probe Purification Columns (Stratagene, La Jolla, CA). The membrane was prehybridized and hybridized in 5 × sodium citrate/chloride buffer (SSC), 5 × Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 20 μg per ml salmon sperm DNA with the ^{32}P-labeled human involucrin cDNA. Following hybridization at 65°C overnight, the membrane was washed in solutions with decreasing ionic strength and increasing temperature, to a final stringency of 0.1 × SSC, 0.1% SDS, at 65°C. The [^{32}P]cDNA-mRNA hybrids were visualized by exposing to X-ray film. The human involucrin mRNA was normalized to the levels of 18S ribosomal RNA in the same RNA blots as determined by rehybridization of the filter with a ^{32}P-end-labeled cDNA for 18S RNA.

Western analysis Keratinocytes were washed twice with phosphate-buffered saline (PBS) and then incubated in RIPA lysis buffer (50 mM HEPES pH 7.4, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 20 μg per ml phenylmethylsulfonyl fluoride, 1 μg per ml leupeptin, 1 μg per ml pepstatin A, and 2 μg per ml aprotinin) for 5 min. Cells were scraped into microfuge tubes, incubated on ice for 15 min, and pelleted by centrifugation. The supernatant was collected. The protein concentration of the lysate was measured with the BCA Protein Assay Kit (Pierce, Rockford, IL). Equal amounts of protein (50 μg) were then electrophoresed through 7.5% polyacrylamide gels at 200 V for 30 min and electroblotted onto polyvinylidene difluoride membranes (0.2 μm, Bio-Rad Laboratories, Hercules, CA) in an electroblotting buffer (25 mM Tris, 192 mM glycine, 5% methanol) at 130 V for 2 h. Following incubation in blocking buffer (100 mM Tris base, 150 mM NaCl, 5% nonfat milk, and 0.5% Tween-20), the blot was incubated with a polyclonal mouse antihuman involucrin antibody (Sigma), dilution 1:2000, overnight at 4°C. After washes in the blocking buffer, the membranes were incubated for 1 h with antimouse IgG secondary antibody conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL) diluted 1:5000 in the blocking buffer. Following a second series of washes, bound antibody complexes were visualized using the SuperSignal ULTRA Chemiluminescent Kit (Pierce, Rockford, IL) and subsequent exposure to X-ray film. The specific bands on the autoradiograms were quantitated by densitometry.

DNA mobility shift assay The nuclear extracts were made from keratinocytes according to the method described by Abmayr and Workman (1994). The synthetic oligonucleotide used for the DNA mobility shift assay contained the wild-type VDRE sequence, part of the SP-1 sequence, but not the AP-1 sequence (5′-GCGGGAGGCAGATCTGGCAGATACTGA-3′) and was end-labeled by T4 polynucleotide kinase. Competing oligonucleotides included the wild-type sequence used as probe, the mutated VDRE sequence (5′-GCGGGAcaggacTCTGGatccTACTGA-3′), and the VDRE at region −152 to −172 in the human 24-hydroxylase gene. The DNA-protein reactions were performed in a total of 17 μl: nuclear extracts (12 μg protein) were incubated with 2 μg of poly(dI:dC) (Pharmacia) and 10,000 cpm of ^{32}P-labeled probe in 10 μl binding buffer (20 mM HEPES, pH 7.9, 20% glycerol, 50 mM KCl, 0.5 mM dithiothreitol) at 30°C for 25 min. Unlabeled competitors were added in 100 molar excess before incubation. In the super gel-shift reaction, a polyclonal antiVDR antibody, polyclonal retinoic acid receptor γ1 antibody, or polyclonal RXRα antibody (Affinity Bioreagents) was added to the DNA–protein reaction and incubated for an additional 25 min. Protein–DNA complexes were electrophoresed in a 6% nondenaturing polyacrylamide gel in 1 × gel-shift running buffer (50 mM Tris, 380 mM glycerin, 2 mM ethylenediamine tetraacetic acid, pH 8.5), and the bands were identified by autoradiography.

RESULTS

The ability of $1,25(OH)_2D_3$ to increase the mRNA and protein levels of involucrin is shown in **Fig 1**. In this experiment $1,25(OH)_2D_3$ from 10^{-11} to 10^{-8} M was added to preconfluent keratinocytes 24 h (for mRNA) or 48 h (for protein) before the cells were harvested for mRNA and protein assessment of involucrin levels. As shown, $1,25(OH)_2D_3$ has a dose-dependent ability to increase the mRNA and protein levels of involucrin.

To examine more closely the direct effect of $1,25(OH)_2D_3$ on gene expression we transfected keratinocytes with a promoter construct of the involucrin gene coupled to a luciferase reporter (**Fig 2**). This construct contained the 5′ promoter, the noncoding

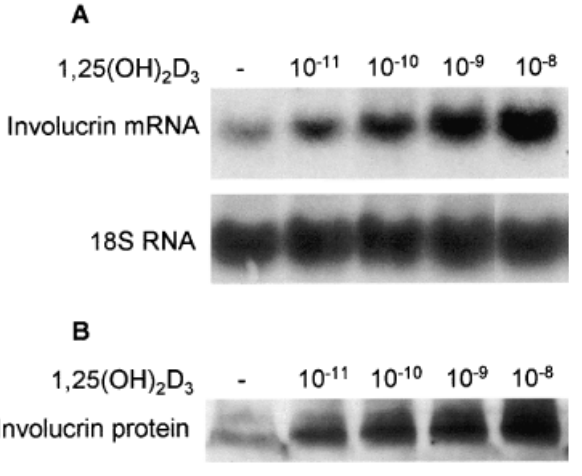

Figure 1. $1,25(OH)_2D_3$ increases involucrin mRNA and protein levels. Second passage keratinocytes were grown in 0.03 mM calcium for 4 d at which point the indicated concentrations of $1,25(OH)_2D_3$ or vehicle (1% ethanol) were added for an additional 24 h (mRNA) or 48 h (protein) incubation. The involucrin mRNA levels (*top panel*) were determined by northern analysis; the involucrin protein levels (*bottom panel*) were determined by Western analysis.

exon 1, and the intron. The keratinocytes were incubated in 0.03 or 1.2 mM calcium to which 10^{-9} M $1,25(OH)_2D_3$ or vehicle (1% ethanol) was added at the time of the calcium switch. The cells were harvested 48 h later. Calcium has a major stimulatory effect on the involucrin promoter, increasing its expression 15-fold in this experiment. $1,25(OH)_2D_3$ further increases involucrin promoter activity 2-fold at both calcium concentrations, however (**Fig 2**).

In examining a series of deletion mutants generated during our previous search for the calcium response region (Ng *et al*, 2000), it became clear that the effect of $1,25(OH)_2D_3$ was mediated by the 5′ regulatory region of the promoter (−2461 to −1881 bp, data not shown). In previous studies this region of the involucrin promoter has been shown to be important for its regulation by a number of agents including calcium and phorbol esters. As shown in **Fig 3** this region contains in close proximity an AP-1 site, an SP-1 site, and a previously overlooked putative VDRE. When we tested the responsiveness of an involucrin promoter construct containing a point mutation in the AP-1 site, the basal levels of promoter activity fell to 20% of the wild-type levels, and $1,25(OH)_2D_3$ failed to increase the activity of the mutated promoter (**Fig 4**). This same mutation blocks calcium and phorbol ester stimulation of the involucrin promoter as we have previously reported (Ng *et al*, 2000). These results suggest that the AP-1 site is required for $1,25(OH)_2D_3$ stimulation of the involucrin promoter, but the requirement may be indirect.

The adjacent VDRE suggested a more direct means by which $1,25(OH)_2D_3$ could stimulate the involucrin gene. Therefore, we mutated the VDRE within the context of the 3.7 kb involucrin promoter construct and evaluated its response to $1,25(OH)_2D_3$. The results are shown in **Fig 5**. In this experiment the cells transfected with the wild-type or VDRE mutant (DR3 mutant) were incubated in 0.03 mM calcium to which 10^{-8} M $1,25(OH)_2D_3$ or vehicle was added for 24 h before harvesting the cells for the luciferase assay. Unlike the AP-1 mutation, basal levels of involucrin promoter activity were not affected; however, the ability of $1,25(OH)_2D_3$ to increase involucrin promoter activity was completely lost. In contrast, calcium and phorbol ester responsiveness was retained. In this experiment we used a suboptimal dose of phorbol myristate acetate (10 M) to enhance our chances of seeing an effect of this mutation on responsiveness, but no alteration was observed. Although the data in **Fig 5** do indicate a partial reduction in calcium responsiveness caused by the VDRE mutation, this has not been a consistent observation as shown by our previous results that the VDRE is not required for calcium stimulation of the involucrin promoter (Ng *et al*, 2000).

To determine that the putative VDRE in the involucrin promoter interacted directly with the VDR, the DNA mobility shift assay with the VDRE as probe was performed as shown in **Fig 6**. This probe contained the VDRE but not the AP-1 site and only a portion of the SP-1 site. Nuclear extracts from the keratinocytes showed a prominent retarded band that was competed out with the unlabeled wild-type oligonucleotide and an oligonucleotide containing the VDRE from the human 24-hydroxylase gene, but was unaffected by an oligonucleotide containing the mutated VDRE. When the nuclear extract was incubated with an antibody to VDR or RXRα, the band was completely shifted to a higher molecular size consistent with VDR and RXRα binding to the VDRE. In contrast, no supershift was observed when an antibody to retinoic acid receptor γ was employed.

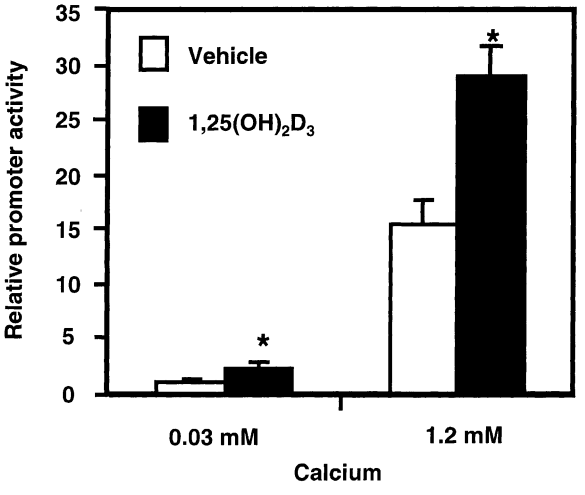

Figure 2. $1,25(OH)_2D_3$ stimulates involucrin promoter activity. The keratinocytes were grown for 3 d in 0.03 mM calcium and then transfected with the 3.7 kb involucrin promoter-luciferase construct and pRSV β-gal construct using the polybrene/glycerol method as described in *Experimental Procedures*. Twenty-four hours later the cells were either switched to 1.2 mM calcium or maintained at 0.03 mM calcium to which 10^{-9} M $1,25(OH)_2D_3$ or vehicle (1% ethanol) was added for an additional 48 h. The results of the luciferase assay were normalized to galactosidase activity. The error bars enclose mean ± SD of triplicate determinations. *$p < 0.01$ compared to vehicle.

DISCUSSION

Involucrin is a major component of the cornified envelope, and thus plays an important role in the differentiated function of the epidermis. Not surprisingly its expression in the epidermis is tightly linked to the differentiation status of the keratinocyte. Involucrin expression increases as the keratinocyte leaves the stratum basale to enter the differentiation pathway leading ultimately to its death and incorporation into the stratum corneum. As such involucrin is a widely used marker for keratinocyte differentiation, and its expression is modulated by agents that regulate keratinocyte differentiation. A number of these agents including the best studied — calcium and phorbol esters — have

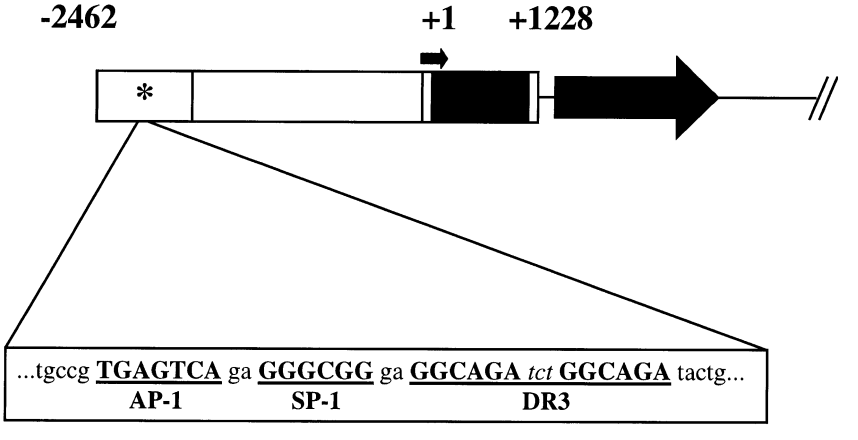

Figure 3. The sequence of the involucrin promoter containing the distal AP-1 site, the adjacent SP-1 site, and the putative VDRE.

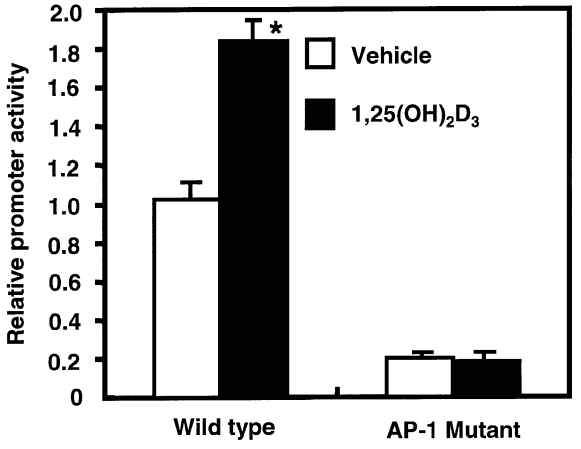

Figure 4. Mutation of the distal AP-1 site lowers basal activity and blocks $1,25(OH)_2D_3$ stimulation of the involucrin promoter. Keratinocytes were grown in 0.03 mM calcium and then transfected with the wild-type involucrin promoter–luciferase construct (WT) or the comparable construct in which a point mutation in the distal AP-1 site was made (mut AP-1). In each case pRSV β-gal was used for normalization as described in the legend to **Fig 2**. Twenty-four hours after transfection the cells were incubated with 10^{-9} M $1,25(OH)_2D_3$ or vehicle (1% ethanol) for an additional 24 h before assessing luciferase activity. The error bars enclose mean ± SD of triplicate determinations. *$p < 0.01$ compared to vehicle.

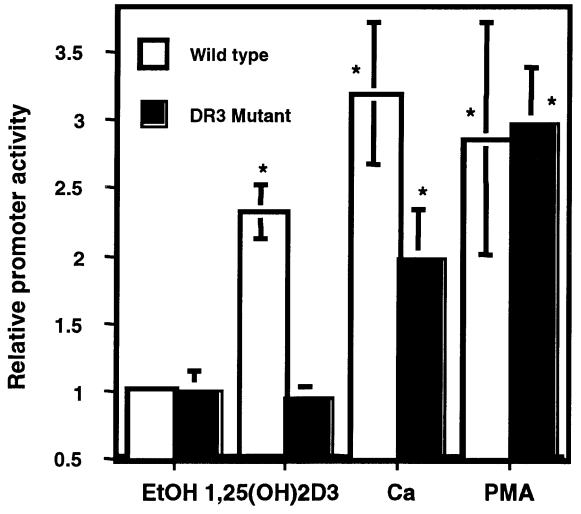

Figure 5. Mutation of the VDRE blocks $1,25(OH)_2D_3$ stimulation of the involucrin promoter but not that of calcium or phorbol esters without affecting basal activity. Keratinocytes were grown in 0.03 mM calcium and then transfected using Superfect with the wild-type involucrin promoter-luciferase construct (wild-type) or the comparable construct in which a 10 bp mutation in the putative VDRE was made (DR3 mutation). In each case pRL-TK was used for normalization as described in *Experimental Procedures*. Twenty-four hours after transfection the cells were incubated with 10^{-8} M $1,25(OH)_2D_3$ or vehicle (1% ethanol) in 0.03 mM calcium, 1.2 mM calcium (Ca), or 10 M phorbol myristate acetate (PMA) in 0.03 mM calcium for an additional 24 h before assessing luciferase activity. The error bars enclose mean ± SD of triplicate determinations. *$p < 0.01$ compared to vehicle.

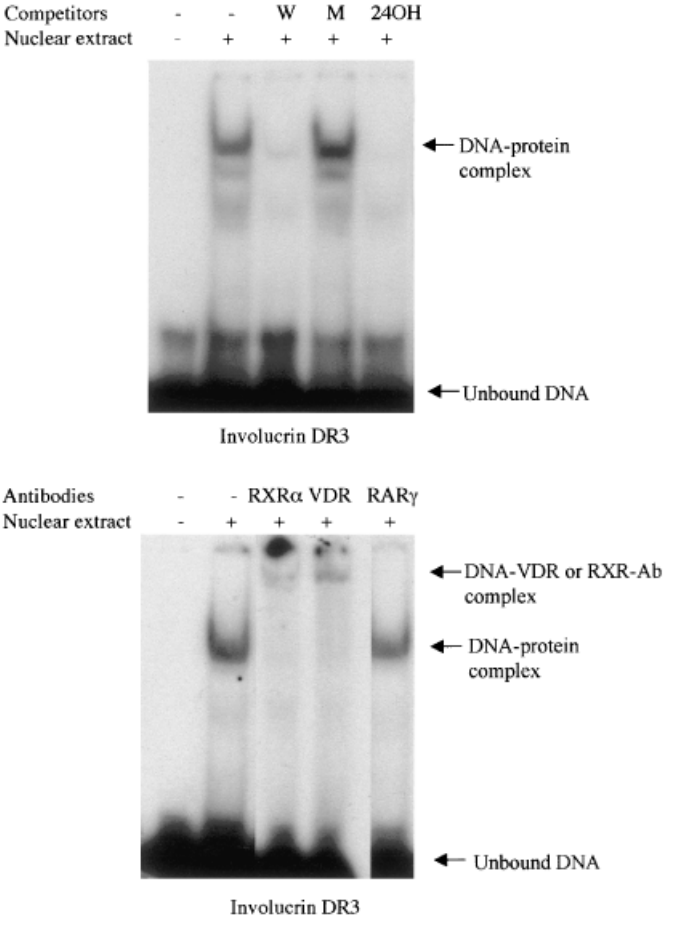

Figure 6. DNA mobility shift assays showing the specific binding of VDR and RXR to the VDRE of the involucrin gene. The sequence of the probe is found in *Experimental Procedures* and included the VDRE and part of the SP-1 site, but excluded the AP-1 site. A dominant retarded band is observed with nuclear extracts from keratinocytes (*top panel*). 100 molar excess of the unlabeled wild-type sequence (W) eliminated binding as did the VDRE sequence from the 24-hydroxylase gene (24OH), but the sequence containing the mutated VDRE (M) did not. Antibodies to RXRα and VDR supershifted the binding, but anti-retinoic acid receptor γ had no effect.

been shown to act at least in part through the distal AP-1 site in the involucrin promoter (Welter *et al*, 1995; Hanley *et al*, 2000b; Komuves *et al*, 2000; Ng *et al*, 2000). The involucrin promoter has five AP-1 sites, but only the proximal and distal sites respond to phorbol esters (Welter *et al*, 1995), and only the distal site responds to calcium (Ng *et al*, 2000). Mutations in the distal AP-1 site reduce basal activity and block the ability of calcium, phorbol esters, PPARα agonists, and LXR agonists to stimulate involucrin gene expression (Welter *et al*, 1995; Hanley *et al*, 2000; Komuves *et al*, 2000; Ng *et al*, 2000). Thus the distal AP-1 site is clearly important for involucrin gene expression. Adjacent to the distal AP-1 site is an SP-1 site that appears to increase AP-1 factor binding to the AP-1 site and enhance its function (Banks *et al*, 1998), although the SP-1 site in and of itself is not sufficient to drive involucrin gene expression. Both calcium and phorbol esters alter the levels of AP-1 factors and their binding to the distal involucrin AP-1 site in keratinocytes (Welter *et al*, 1995; Ng *et al*, 2000). Furthermore, the regulation of the involucrin gene by calcium and phorbol esters is blocked by inhibitors of protein kinase C that would be expected to block these changes in the AP-1 factors (Denning *et al*, 1995; Welter *et al*, 1995; Ng *et al*, 2000).

$1,25(OH)_2D_3$ induces differentiation in keratinocytes (Bikle and Pillai, 1993). These cells not only contain the VDR (Pillai *et al*, 1988a), but are prodigious producers of this hormone (Bikle *et al*, 1986). Among the genes induced by $1,25(OH)_2D_3$ in keratinocytes is involucrin (Su *et al*, 1994). The mechanism for its induction by $1,25(OH)_2D_3$ has remained obscure, however. As $1,25(OH)_2D_3$ increases the intracellular concentration of calcium within the keratinocyte (Pillai and Bikle, 1991), one possibility is that the stimulation of involucrin expression is mediated by

calcium. The effect of $1,25(OH)_2D_3$ on intracellular calcium levels could readily be explained by the ability of $1,25(OH)_2D_3$ to increase the levels of the calcium sensing receptor (Ratnam *et al*, 1999). Furthermore, $1,25(OH)_2D_3$ induces members of the phospholipase C family (Pillai *et al*, 1995), which by hydrolyzing phosphatidyl inositol bis phosphate increase inositol tris phosphate and diacyl glycerol leading to release of calcium from intracellular stores and activation of protein kinase C, respectively. Either event would be expected to stimulate involucrin gene expression and be blocked by mutations in the AP-1 site. Our observation that mutation of the distal AP-1 blocks $1,25(OH)_2D_3$ stimulation of the involucrin promoter is consistent with these mechanisms of action.

A more appealing mechanism, however, emanates from our observation of a putative VDRE in the involucrin gene proximal to the AP-1 site. This VDRE is a perfect direct repeat (GGCAGA for each half site) with a three nucleotide intervening sequence making it a DR3. It is not identical to any other functional VDRE to our knowledge, but known functional VDREs show substantial variation (Haussler *et al*, 1998). The involucrin VDRE has the sequence A/GGnnnA found for each half site in most VDREs described to date, and these specific nucleotides may be important for the binding to the VDR and RXR. This VDRE is clearly functional within the context of the involucrin promoter in that mutations in the half sites block $1,25(OH)_2D_3$ responsiveness. Furthermore, both VDR and RXR bind to this VDRE as expected for a functional VDRE of the DR3 class (Haussler *et al*, 1998). Unlike distal AP-1 site mutations, mutation of the VDRE does not alter basal activity or prevent calcium and phorbol ester responsiveness of the involucrin promoter. As we previously showed (Ng *et al*, 2000), the VDRE is not required for calcium stimulated binding of AP-1 factors to the AP-1 site; as we show in this report the AP-1 site is not required for binding of VDR and RXR to the VDRE. These results are consistent with the concept that the distal AP-1 site is required for basal activity and the regulation of involucrin promoter activity by a variety of factors, whereas the VDRE serves the more specific purpose of modulation of involucrin gene expression by $1,25(OH)_2D_3$.

Whether the VDRE and the AP-1 and/or SP-1 site interact to promote involucrin gene expression is not clear at this stage. As shown in this report, $1,25(OH)_2D_3$ can enhance the ability of calcium to stimulate involucrin promoter activity. The degree of enhancement is modest (2–3-fold) compared to the stimulation generally observed by calcium, but is additive to the regulation by calcium suggesting an independent mechanism. This result is consistent with nuclear run on results directly assessing calcium and $1,25(OH)_2D_3$ regulated transcription of the involucrin gene reported earlier (Su *et al*, 1994). This degree of stimulation of transcription by $1,25(OH)_2D_3$ is less than expected based on the increases in mRNA and protein levels following $1,25(OH)_2D_3$ administration (**Fig 1**). These results imply both transcriptional and post-transcriptional control, a situation also observed for $1,25(OH)_2D_3$ induction of calbindins (Christakos *et al*, 1992). In fact we (Su *et al*, 1994) previously reported that both calcium and $1,25(OH)_2D_3$ altered the half-life of the mRNA suggesting at least one post-transcriptional mechanism at work. In that report (Su *et al*, 1994), we also noted a synergism between calcium and $1,25(OH)_2D_3$ on involucrin transcription as well as mRNA levels within the first 24 h after the calcium switch. Directly testing this interaction may be difficult because alterations in the AP-1 site have such profound effects on involucrin gene expression. Nevertheless, our results indicate that the VDRE in the involucrin promoter is functional and is likely to contribute to the regulation of the involucrin gene by $1,25(OH)_2D_3$.

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