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Dual Effect of 1α,25-Dihydroxyvitamin D_3 on *hsp28* and *PKC*β Gene Expression in Phorbol Ester-Resistant Human Myeloid HL-525 Leukemic Cells

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ABSTRACT. We investigated the effect of 1α-25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] on the expression of the 28-kDa heat shock protein gene (*hsp28*) and the protein kinase C beta gene (*PKC*β) in the human myeloid HL-60 leukemic cell variant HL-525, which is resistant to phorbol ester-induced macrophage differentiation. Northern and western blot analysis showed little or no *hsp28* gene expression in the HL-60 cell variant, HL-205, which is susceptible to such differentiation, while a relatively high basal level of *hsp28* gene expression was observed in the HL-525 cells. However, both cell lines demonstrated heat shock-induced expression of this gene. During treatment with 50–300 nM 1,25-$(OH)_2D_3$, a marked reduction of *hsp28* gene expression along with an induction of *PKC*β gene expression was observed in HL-525 cells. A gel mobility-shift assay demonstrated that the 1,25-$(OH)_2D_3$-induced alteration of *hsp28* gene expression was associated with decreased binding activity to the vitamin D_3 receptor–vitamin D_3 response element (VDR–VDRE), whereas binding to the heat shock transcription factor–heat shock element (HSF–HSE) was not altered. Our results suggest that the dual effect of 1,25-$(OH)_2D_3$ on *hsp28* and *PKC*β gene expression is due to the different sequence composition of the vitamin D response element in the promoter region as well as an accessory factor for each gene or that 1,25-$(OH)_2D_3$ increases *PKC*β gene expression, which, in turn, negatively regulates the expression of the *hsp28* gene or vice versa. BIOCHEM PHARMACOL 52;2:311–319, 1996.

KEY WORDS. 1α,25-dihydroxyvitamin D_3; HL-525; *hsp28*; *PKC*β

The human myeloid HL-60 leukemic cells undergo differentiation into cells with a macrophage phenotype when exposed to phorbol esters such as TPA‡. In contrast, some HL-60 cell variants including HL-525 (derived from an HL-60 cell line during long-term exposure to TPA) are resistant to phorbol ester-induced differentiation and display decreased *PKC*β expression relative to the HL-60 parent cells [1, 2]. Southern blot analysis indicates that the observed reduction in *PKC*β gene expression does not appear to be due to a gross deletion or rearrangement of the gene [3].

The involvement of *PKC*β in the phorbol ester-induced macrophage differentiation of HL-60 and variant HL-525 cells has been investigated extensively. The activation of *PKC*β is both necessary and sufficient for phorbol ester-induced differentiation. Restoration of *PKC*β isozyme deficiency in HL-525 cells by transfecting expression vectors containing either *PKC*β1 or *PKC*βII cDNA causes HL-525 cells to revert to a phenotype like that of the parental HL-60 cells, which is characterized by susceptibility to TPA-induced macrophage differentiation [4]. *PKC*β is considered one of the essential elements in the phorbol ester-induced signal transduction pathway, which leads to cell differentiation [4]. Cell adherence is also induced by the stimulation of actin polymerization, which is also dependent on *PKC*β [5].

Unlike TPA, 1,25-$(OH)_2D_3$ (calcitriol) induces monocytic differentiation in HL-525 cells [1]. Calcitriol also increases the expression of *PKC*β mRNA in HL-60 as well as in another phorbol ester-resistant HL-60 cell variant [2].

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‡ *Abbreviations:* TPA, 12-O-tetradecanoylphorbol-13-acetate; 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 25-$(OH)D_3$, 25-hydroxyvitamin D_3; HSP28, 28-kDa heat shock protein; *PKC*β, protein kinase C beta; HBSS, Hanks' balanced salt solution; TCA, trichloroacetic acid; PPO, 2,5-diphenyloxazole; VDR, vitamin D_3 receptor; VDRE, vitamin D_3 response element; HSF, heat shock transcription factor; HSE, heat shock element; and DTT, dithiothreitol.

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These observations suggest that 1,25-$(OH)_2D_3$ alters intracellular factor(s) related to TPA resistance in HL-525 cells. Recent studies demonstrate a relationship between HSP28 and resistance to cytotoxic agents [6]. Chinese hamster 023 cells that constitutively overexpress human HSP28 as a result of transfection with pHS2711 are resistant to antineoplastic drugs [6]. Taken together, we can contemplate (a) that HL-525 cells were selected during long-term exposure to TPA, because they contain a high level of HSP28, or (b) that *hsp28* gene expression is under the negative control of *PKC*β: namely, high levels of this isoenzyme cause a decrease in *hsp28* gene expression and low *PKC*β levels cause an increase in the *hsp28* gene expression. Our data demonstrated that in comparison with phorbol ester-sensitive HL-205 cells, HL-525 cells do indeed express a low level of *PKC*β but a high level of HSP28. Moreover, 1,25-$(OH)_2D_3$ decreases the level of HSP28 and increases the level of *PKC*β in HL-525 cells.

MATERIALS AND METHODS

Cell Culture

The human myeloid HL-60 cell variants HL-525 and HL-205 [1] were cultured in McCoy's 5a medium (Cellgro, Herndon, VA). The medium was supplemented with 26 mM sodium bicarbonate and 10% iron-supplemented calf serum (HyClone, Logan, UT). T-75 flasks containing cells were kept in a 37° humidified incubator with a mixture of 95% air and 5% CO_2.

Hyperthermic Treatment

T-75 flasks containing cells were heated by total immersion in a circulating water bath (Heto, Birkerod, Denmark) maintained within ±0.05° of the desired temperature.

Drug Treatment

Vitamin D_3, 25-$(OH)D_3$, and 1,25-$(OH)_2D_3$ were obtained from Calbiochem, San Diego, CA. For drug treatment, cells were counted and added into the medium containing a drug.

Labeling, Two-Dimensional PAGE and Fluorography

Cells were labeled with 20 μCi/mL [^{3}H]leucine (sp. act. 160 Ci/mmol; Amersham, Arlington Heights, IL) in leucine-free medium for 8 hr. After labeling, cells were washed twice with cold HBSS. For two-dimensional PAGE, samples were solubilized in sample buffer containing 8 M urea, 1.7% NP-40, and 4.3% β-mercaptoethanol. Proteins were first separated in isoelectric focusing gels (pH 3.5 to 10). These gels were then laid across the top of a 10–18% linear gradient SDS polyacrylamide gel for two-dimensional analysis [7]. After electrophoresis, gels were fixed in 30% TCA for 30 min. For fluorography, gels were dehydrated by washing for 15 min in each of 25% acetic acid, 50% acetic acid, and glacial acetic acid, consecutively. After fixation, gels were placed in 125 mL PPO solution (20%, w/v, PPO in glacial acetic acid) for 2 hr. The PPO solution was removed, and the gel was shaken gently overnight in distilled water and dried for 2.5 hr at 60°. The gel was loaded into a casette with Kodak SB-5 X-ray film and placed in a −70° freezer. After optimum time exposure, the fluorograph film was developed with Kodak GBX developer and fixed with Kodak GBX fixer.

One-Dimensional PAGE and Western Blot

Samples were mixed with 2× Laemmli lysis buffer (1× = 2.4 M glycerol, 0.14 M Tris, pH 6.8, 0.21 M SDS, 0.3 mM bromphenol blue), and boiled for 10 min. Protein content was measured with BCA™ Protein Assay Reagent (Pierce, Rockford, IL). The samples were diluted with 1× lysis buffer containing 1.28 M β-mercaptoethanol, and an equal amount of protein (30 μg) was applied to a one-dimensional PAGE. Electrophoresis was carried out on a 10–18% linear gradient SDS–PAGE. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane and processed for immunoblotting with the HSP28 monoclonal antibody (StressGen, Vancouver, B.C.) or actin monoclonal antibody (ICN, Irvine, CA). The HSP28 antibody and actin antibody were diluted 1:10,000. Alkaine phosphatase-conjugated rabbit-mouse IgG (diluted 1:3000) for HSP28 antibody or streptavidin-horseradish peroxidase-conjugated sheep-mouse IgG (diluted 1:7500) for actin antibody was used to detect the primary antibody.

Northern Blot Analysis

*PKC*β and HSP28 mRNA levels were determined using the northern blot technique. Total cellular RNA was extracted by the LiCl-urea method of Tushinski *et al.* [8]. For RNA analysis, 30 μg of total RNA was electrophoresed in a 1% agarose-formaldehyde gel [9]. The RNA was blotted from the gels onto nitrocellulose membranes and baked at 80° for 2 hr in a vacuum oven. Membranes were prehybridized at 42° in 50% formamide, 1× Denhardt's solution, 25 mM KPO_4 (pH 7.4), 5× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate), and 50 μg/mL denatured and fragmented salmon sperm DNA. Hybridizations were at 42° in prehybridization solution containing 10% dextran sulfate and radiolabeled human *PKC*β cDNA probes (F. Collart, Argonne National Laboratory) or human *hsp28* cDNA probes (StressGen, Victoria, B.C.) at a concentration of 1.5 to 4 × 10^6 cpm/mL. For posthybridization, blots were washed twice in 2× SSC for 15 min at room temperature, washed once in 0.5× SSC and 0.1% SDS for 25 min at 50°, and washed twice in 0.2× SSC and 0.1% SDS for 1 hr at 50°. Blots were placed into a stainless steel cassette with an intensifying screen and autoradiographed.

Quantitation of HSF–HSE or VDR–VDRE Binding Activity

Conditions for the gel mobility-shift assay, a description of the ^{32}P-labeled HSE or VDRE oligonucleotide, and prepa-

ration of whole-cell extracts or nuclear extracts were as published previously [10–12]. A double-stranded HSE (upper strand 5′-CTT AAC GAG **AGA AGG TTC** CAG **ATG** AGG GCT GAA-3′, [13]) or VDRE (upper strand 5′-TTA ACG AGA GAA **GGT TCC** AGA TGA GGG **CTG AAC** CCT C-3′, [13]) oligonucleotide of the human *hsp28* gene promoter was used. Bold nucleotides represent essential sites for HSF or VDR binding. Binding reactions with 20 μg of whole-cell extracts for HSE or 5 μg of nuclear extracts for VDRE were performed for 15 min at 25° in a final volume of 25 μL of binding buffer (10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM DTT for HSF; 10 mM HEPES, pH 8.0, 15% glycerol, 2 mM EDTA, 0.5 mM spermidine, 20 mM NaCl, 4 mM $MgCl_2$, 2 mM DTT for VDR) containing about 0.5 ng of radiolabeled probe, 10 μg of yeast tRNA (Boehringer Mannheim, Indianapolis, IN), 1 μg of *Escherichia coli* DNA (Sigma Chemical Co., St. Louis, MO), 2 μg of poly(d[I-C]) (Pharmacia LKB, Piscataway, NJ), 50 μg BSA (Sigma Chemical Co.). Samples were electrophoresed on a nondenaturing 4.5% polyacrylamide gel for 2.5 hr at 140 V. After electrophoresis, gels were fixed with 7.5% acetic acid for 15 min and rinsed with water for 3 min. For autoradiography, gels were dried in a slab gel dryer (model 483, Bio-Rad, Richmond, CA) for 1.5 hr at 80° and placed into a stainless steel cassette with an intensifying screen. Gels were autoradiographed on Fuji RX X-ray film. After an exposure of 2–4 days at −70°, autoradiographic film was developed with Kodak GBX developer and fixed with Kodak GBX fixer.

RESULTS

Comparison of hsp28 *Gene Expression in HL-205 and HL-525 Cells*

The data presented in Fig. 1A demonstrate no detectable level of HSP28 mRNA in unheated HL-205 cells, which are known to be sensitive to differentiation induction by phorbol esters. In contrast, a marked amount of HSP28 mRNA was detected in extracts from unheated HL-525 cells, which are resistant to such differentiation [1]. The level of HSP28 mRNA increased after heat shock at 45° for 10 min in both cell lines. The level of HSP28 mRNA in HL-525 cells was approximately 2-fold higher than that in HL-205 cells as determined by densitometer. Similarly, higher levels of HSP28 protein were observed by western blot analysis in HL-525 cells (Fig. 2). The presence of HSP28 protein was detected in unheated HL-525 cells but not in HL-205 cells. The protein level increased and reached its maximum value within 6 hr after heat shock at 45° for 10 min in HL-205 cells. Unlike HL-205 cells, heat shock did not alter the level of HSP28 protein in HL-525 cells. This may be due to the high intrinsic level of HSP28 protein in HL-525 cells.

Effect of $1,25\text{-}(OH)_2D_3$ *on the Expression of* PKCβ *and* hsp28 *Genes*

Northern blots in Fig. 3 illustrate the effect of various concentrations (1–50 nM in Fig. 3A and 50–300 nM in Fig. 3B) of $1,25\text{-}(OH)_2D_3$ treatment for 3 days on the levels of *PKCβ* and HSP28 mRNA in HL-525 cells. The level of *PKCβ* mRNA increased during $1,25\text{-}(OH)_2D_3$ treatment, whereas the level of HSP28 mRNA decreased during this treatment. The level of alteration was dependent upon $1,25\text{-}(OH)_2D_3$ concentration. The significant alterations occurred at drug concentrations equal to or above 50 nM. These results are consistent with those of Obeid *et al.* [14] and correspond to concentrations of $1,25\text{-}(OH)_2D_3$ that induce maximal differentiation of HL-60 cells. Data from western blot analysis also clearly demonstrated that treatment with 50–300 nM $1,25\text{-}(OH)_2D_3$ markedly suppressed the level of HSP28 in HL-525 cells (Fig. 4). Similar results were obtained in the experiments for the kinetics of treatment with a single concentration (50 nM) of 1,25-

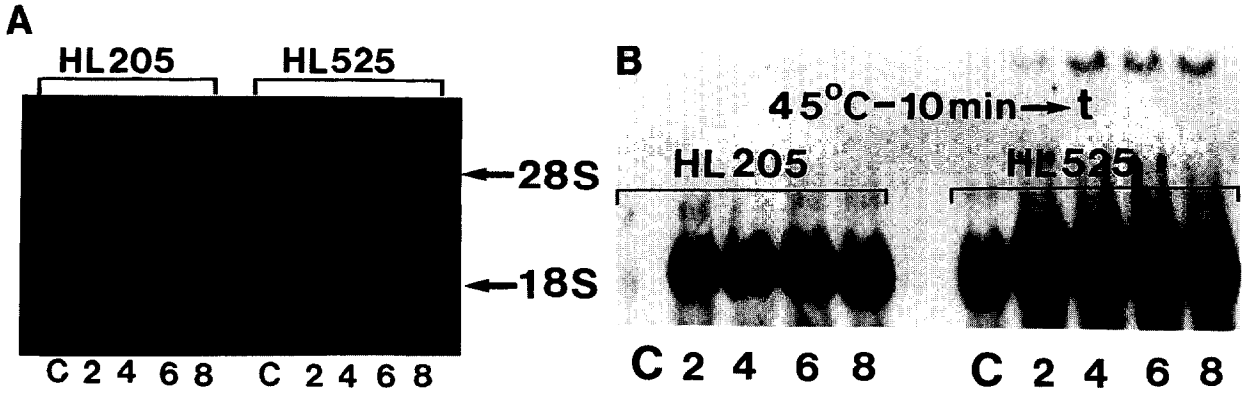

FIG. 1. Northern blot analysis of HSP28 mRNA from heated HL-205 and HL-525 cells. Cells were heated at 45° for 10 min and incubated at 37° for the times (2–8 hr) indicated at the bottom of each lane. Cells were harvested, and RNA was isolated. An equal amount of RNA (30 μg) was loaded onto each lane of an agarose-formaldehyde gel for separation (panel A). After separation, RNA was blotted onto a nitrocellulose membrane and hybridized with a ^{32}P-labeled probe for HSP28 mRNA (panel B). C = unheated control cells. The arrows in panel A indicate the position of the 28S and 18S rRNAs.

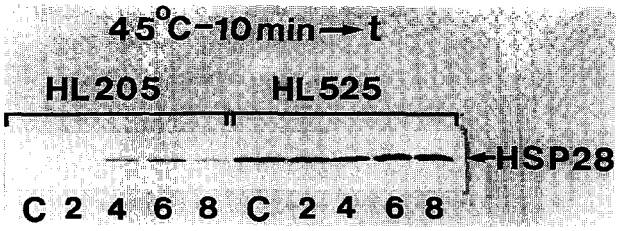

FIG. 2. Heat-induced HSP28 synthesis in HL-205 and HL-525 cells. Cells were heated at 45° for 10 min and incubated at 37° for the times (2–8 hr) indicated at the bottom of each lane. Equal amounts of protein (30 μg) from cell lysates were separated by SDS–PAGE, transferred onto a nitrocellulose membrane, and processed for immunoblotting with HSP28 antibody. C = unheated control cells.

$(OH)_2D_3$ (Figs. 5 and 6). Figure 5 shows that the level of HSP28 mRNA markedly decreased within 1 day and reached minimal value within 2 days during the treatment with the drug in HL-525 cells. In contrast, the level of *PKC*β increased rapidly and reached maximal value within 2 days during the treatment with the drug. Unlike HSP28 mRNA, the level of HSP28 protein decreased gradually during 3 days of the drug treatment (Fig. 6). This discrepancy is probably due to differences in the half-lives of HSP28 mRNA and its protein: short half-life of HSP28 mRNA versus long half-life of HSP28 protein.

Comparison between the Effect of Vitamin D_3 and That of Its Metabolites on the Level of HSP28

To examine whether vitamin D_3 and its metabolites have a similar effect on the level of HSP28, we chose vitamin D_3 and its metabolites, 25-$(OH)D_3$ and 1,25-$(OH)_2D_3$. Figure 7 shows that various concentrations of vitamin D_3 (1 nM–100 μM) and 25-$(OH)D_3$ (1 nM–1 μM) did not affect markedly the level of HSP28, whereas 1,25-$(OH)_2D_3$ (1–300 nM) reduced the level of HSP28 in HL-525 cells. The level of reduction was dependent upon the drug concentrations. Higher concentrations of 1,25-$(OH)_2D_3$ (50–300 nM) significantly reduced the level of HSP28.

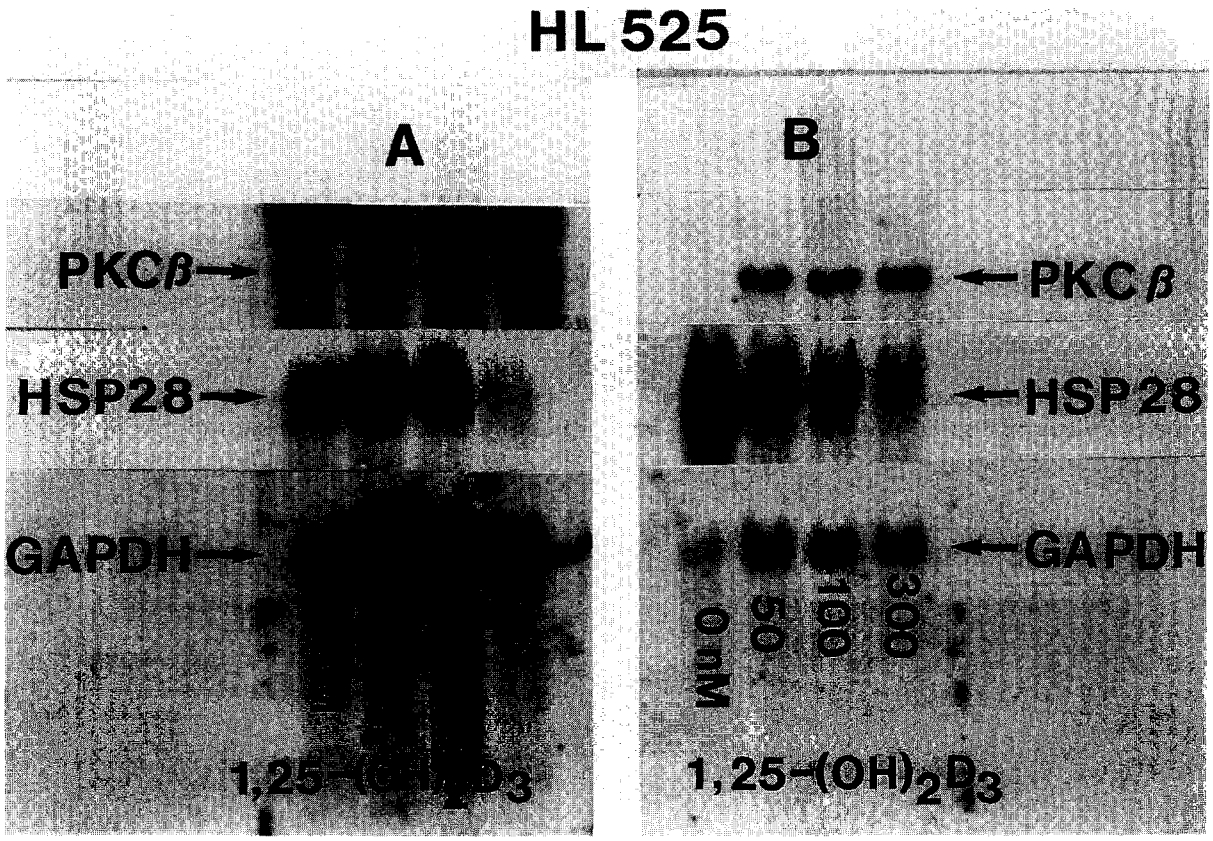

FIG. 3. Alteration of *PKC*β and *HSP28* gene expression by treatment with various concentrations of 1,25-$(OH)_2D_3$ in HL-525 cells. Cells were treated for 3 days with various concentrations of 1,25-$(OH)_2D_3$ indicated at the bottom of each lane. Cells were harvested, and RNA was isolated and separated as described in Fig. 1. After separation, RNA was blotted onto a nitrocellulose membrane and hybridized with ^{32}P-labeled probes for *PKC*β, HSP28, and GAPDH mRNA. (A) 0–50 nM treated cells. (B) 0–300 nM treated cells. GAPDH: GAPDH probe was used to verify the equivalent amounts and integrity of RNAs loaded in each lane. The apparent reduction of GAPDH mRNA in lane 0 in panel B was the result of underloading of the sample. Autoradiograms were from the same blot, which was stripped and rehybridized with different probes.

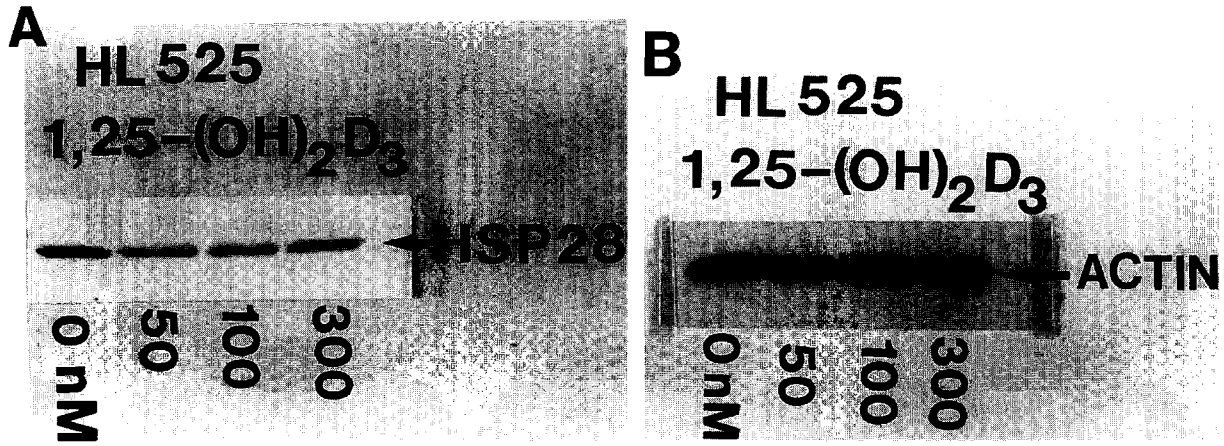

FIG. 4. Effect of various concentrations of $1,25\text{-}(OH)_2D_3$ on the level of HSP28 (panel A) in HL-525 cells. Cells were treated for 3 days with 0–300 nM, $1,25\text{-}(OH)_2D_3$, as indicated at the bottom of each lane. Western blot analysis was performed as described in Fig. 2. Actin (panel B) was shown as an internal standard.

Effect of $1,25\text{-}(OH)_2D_3$ on VDR–VDRE or HSF–HSE Binding Activity

Our previous results (Figs. 3 and 5) indicated that the effect of $1,25\text{-}(OH)_2D_3$ on *hsp28* gene expression occurred at the transcriptional level. To determine whether the suppression of *hsp28* gene expression was due to altered upstream regulation of transcription, VDR–VDRE or HSF–HSE binding activity was measured by gel mobility-shift assay. It is known that binding of the VDR to various VDRE sequences can either negatively or positively regulate the expression of target genes [11, 15]. It is also known that the binding of HSF to HSE is necessary for transcriptional activation of eukaryotic heat shock genes [16–18]. Gel mobility-shift analysis of nuclear extracts from untreated control cells showed the formation of VDR–VDRE complex (C in Fig. 8). In contrast, VDR binding activity was not detected in extracts from cells treated with 50 nM $1,25\text{-}(OH)_2D_3$ (V in Fig. 8). Gel mobility-shift analysis of whole cell extracts from heated cells showed the formation of HSF–HSE complex (H in Fig. 9). Although the levels of HSP28 mRNA and its protein were prominent in HL-525 cells (Figs. 1–7), gel mobility-shift assay failed to detect the formation of HSF–HSE complex [$1,25\text{-}(OH)_2D_3$ in Fig. 9]. Moreover, no HSF binding activity was detected in extracts from cells treated with various concentrations of $1,25\text{-}(OH)_2D_3$ (1–50 nM).

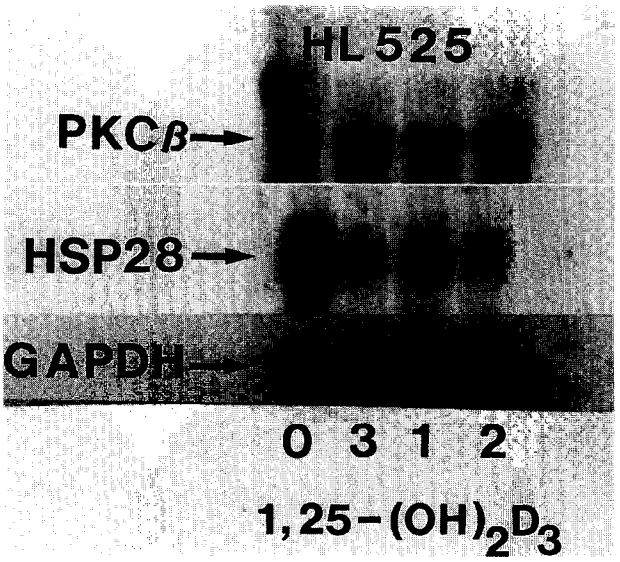

FIG. 5. Duration of $1,25\text{-}(OH)_2D_3$ effect of expression of *PKCβ* and *hsp28* genes in HL-525 cells. Cells were treated with 50 nM for 0–3 days, as indicated at the bottom of each lane. Northern blot analysis was performed as described in Fig. 1. GAPDH: GAPDH probe hybridized to the same blot to verify RNA uniformity.

DISCUSSION

Several conclusions can be drawn from the data presented. Unlike HL-205 cells, phorbol ester-resistant HL-525 cells contain a relatively high level of HSP28 mRNA and protein. While *hsp28* gene expression was suppressed during treatment with $1,25\text{-}(OH)_2D_3$ in HL-525 cells, *PKCβ* gene expression was stimulated. These results are consistent with previous reports that demonstrate the induction of *PKCβ* gene expression by treatment with $1,25\text{-}(OH)_2D_3$ [2, 14, 19]. The alteration of *hsp28* gene expression resulted from treatment with $1,25\text{-}(OH)_2D_3$, the most active natural metabolite of vitamin D_3, but not with $25\text{-}(OH)D_3$ or vitamin D_3 itself. This alteration was negatively related to the formation of the VDR–VDRE complex but not the HSF–HSE complex within the promoter of the *hsp28* gene.

The low molecular weight heat shock protein HSP28 consists of several isoforms, including unphosphorylated (HSP28a in Fig. 6) and phosphorylated (HSP28b in Fig. 6) protein. It can be phosphorylated following stimulation with heat shock, as well as treatment with the Ca^{2+} ionophore, addition of serum to medium, sodium arsenite, cycloheximide, or cytokines [20–25]. Data from Fig. 6 show little or no presence of phosphorylated HSP28b with or

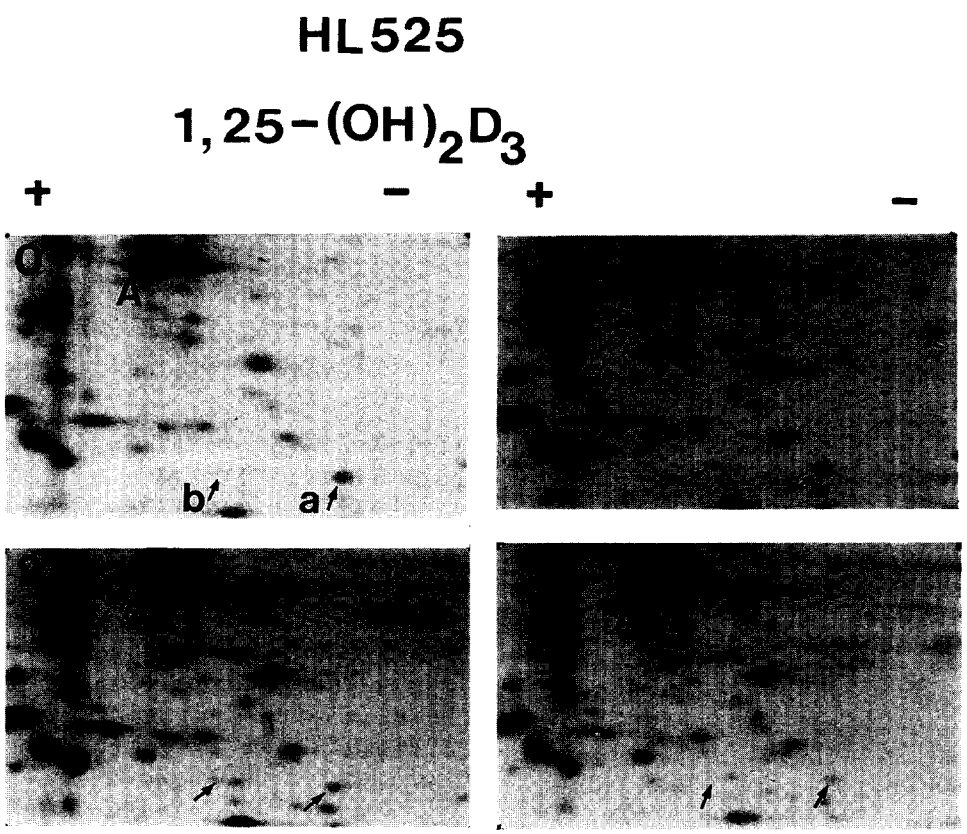

FIG. 6. Two-dimensional SDS-polyacrylamide gel electrophoretic analysis of proteins. HL-525 cells were treated with 50 nM 1,25-$(OH)_2D_3$ for 0–3 days, as indicated at the top of each panel. Cells were then labeled with 20 μCi/mL [^{3}H]leucine for 8 hr in leucine-free medium. Lysates from cells were analyzed, and ^{3}H-labeled proteins were detected by fluorography. Only a section of the fluorograph is shown. The locations of HSP28a (a), HSP28b (b), and actin (A) are identified. The numbers in the panels indicate the length of the drug treatment in days.

without 1,25-$(OH)_2D_3$ treatment. HSP28 is known to be involved in the development of thermotolerance [26] and drug-resistance [6]. This protein can function as a molecular chaperone [27]. Data from Fig. 1 show the presence of HSP28 in HL-525 cells but not in HL-205 cells. Taken together, these observations suggest that expression of *hsp28* gene is associated with phorbol ester resistance in HL-525 cells.

It is well established that the transcriptional induction of heat shock genes in eukaryotes is mediated by HSF [17, 18, 28–33]. This protein can be activated upon heat shock [17, 18, 34, 35]. The activated HSF binds to the promoters which contain the HSE [16] and then stimulates transcription [17, 18, 34]. Our data in Figs. 1 and 9 confirm that *hsp28* gene expression was accompanied by the binding of HSF to HSE. However, our data also illustrate that constitutive expression of *hsp28* gene occurred without the formation of the HSF–HSE complex in HL-525 cells. These

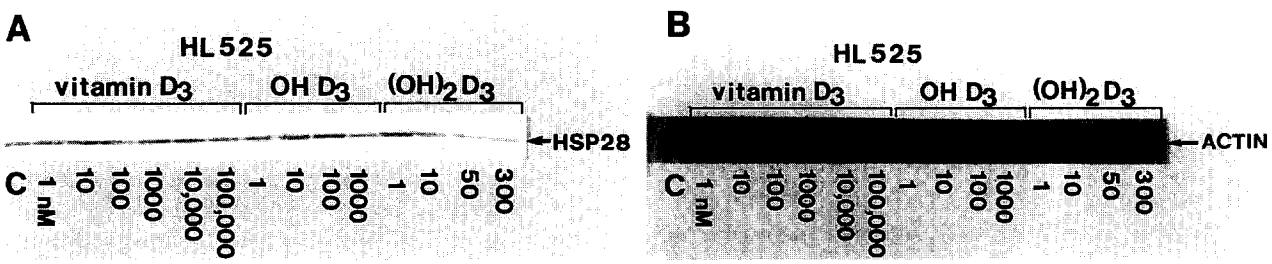

FIG. 7. Differential effect of 1,25-$(OH)_2D_3$ and its metabolites on the level of HSP28 (panel A) in HL-525 cells. Cells were treated for 3 days with the concentrations (nM) of vitamin D_3, 25-hydroxyvitamin D_3 [OH D_3], or 1α,25-dihydroxyvitamin D_3 [$(OH)_2D_3$] indicated at the bottom of each lane. Western blot analysis was performed as described in Fig. 2. Actin (panel B) was used as an internal standard.

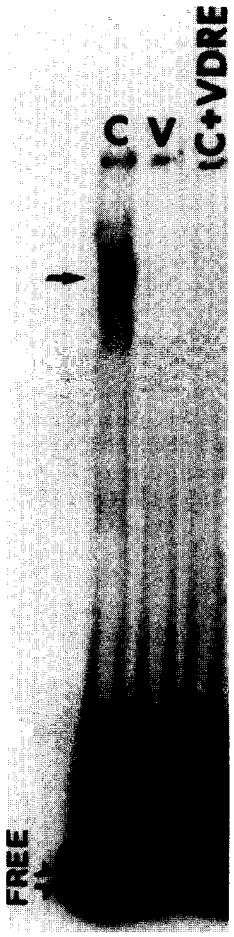

FIG. 8. Detection of a vitamin D_3 receptor (VDR)–vitamin D_3 response element (VDRE) complex in HL-525 cells. The gel mobility-shift assay was performed with a ^{32}P-labeled VDRE, and nuclear extracts (5 μg protein) were prepared from untreated control cells or 1,25-$(OH)_2D_3$-treated cells. C: untreated control cells. V: cells treated for 3 days with 50 nM 1,25-$(OH)_2D_3$. C + VDRE: competition assays were performed by adding a 200-fold molar excess of a nonlabeled VDRE oligonucleotide into nuclear extract from untreated control cells. The closed arrow indicates the position of the VDR–VDRE complex. The open arrow indicates a free ^{32}P-labeled oligonucleotide fragment (FREE).

results suggest that another transcription factor(s) is(are) also involved in the regulation of *hsp28* gene expression in HL-525 cells.

1,25-$(OH)_2D_3$ has several cellular functions. The drug produces a stimulation of calcium influx through activation of voltage-gated Ca^{2+} channels and an involvement of cyclic AMP-dependent protein kinase A and protein kinase C [36]. The drug also has an important role in cell growth and differentiation. 1,25-$(OH)_2D_3$ affects cell differentiation by direct interaction with the cell membrane and by the transduction of the signal to the genome. The direct effects of the drug on the cell membrane may be mediated by stimulation of phosphoinositide metabolism [37] and activation of *PKC*β [14]. 1,25-$(OH)_2D_3$ receptor (VDR), which is known as a 55-kDa nucleoprotein, may play a principal role in signal transduction [38]. The VDR becomes phosphorylated upon treatment with 1,25-$(OH)_2D_3$ [39]. Activated Raf-1 and mitogen-activated protein kinase may be involved in this phosphorylation [40]. The phosphorylation plays a central role in the transcriptional activity of the VDR [39]. The VDR interacts with the VDRE in the promoter region of a number of target genes, either as a homodimer or a heterodimer with retinoid or orphan receptors. The binding of the VDR to the VDRE either stimulates the expression of the target genes such as calbindin-D 9-kDa [15, 41], osteocalcin [42], and 24-hydroxylase [43] or suppresses transcription of the genes encoding Id [44] and parathyroid hormone [11]. The binding of the receptor to the response element does not require

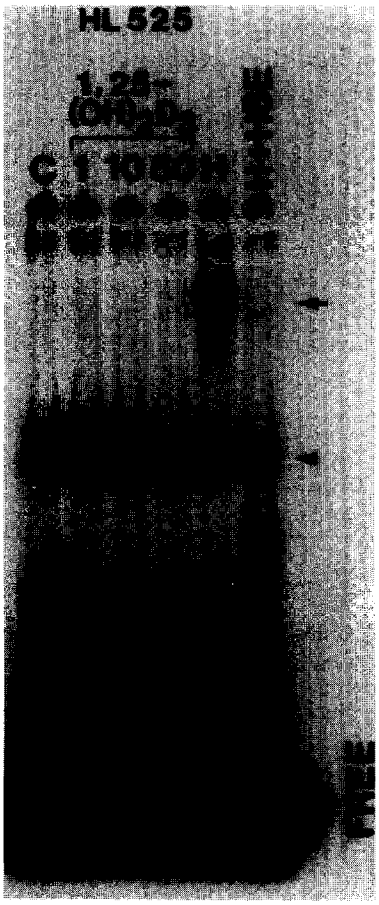

FIG. 9. Detection of a heat shock element (HSE)–binding factor (HSF) complex in HL-525 cells. The gel mobility-shift assay was performed with a ^{32}P-labeled HSP28 HSE, and whole-cell extracts (20 μg protein) were prepared from untreated control cells, 1,25-$(OH)_2D_3$-treated cells, or heated cells. C: untreated unheated control cells. 1,25-$(OH)_2D_3$: cells treated for 3 days with the various concentrations (nM) or 1,25-$(OH)_2D_3$ indicated at the top of each lane. H: cells heated at 45° for 15 min. H + HSE: competition assays were performed by adding a 200-fold molar excess of a nonlabeled HSE oligonucleotide. The closed arrow indicates the position of the HSF–HSE complex. The closed arrowhead indicates a non-heat-specific interaction. The open arrow indicates a free ^{32}P-labeled oligonucleotide fragment (FREE).

the ligand but does require a protein, termed an accessory factor, that has a molecular weight of 59–64 kDa [45]. Unlike previous observations, our data show that VDR constitutively binds to the VDRE in the promoter region of *hsp28* gene and activates its gene transcription. Interestingly, 1,25-$(OH)_2D_3$ treatment dissociates VDR–VDRE complex and inactivates *hsp28* gene expression (Fig. 8). Previous studies show that differential effects of 1,25-$(OH)_2D_3$ on various target genes may be due to differences in VDRE sequence composition and the requirements for particular cellular factors rather than VDR itself [11]. What remains unknown is how 1,25-$(OH)_2D_3$ differentially alters *hsp28* gene and *PKCβ* gene expression in HL-525 cells. At the present time, only speculations can be made concerning mechanisms. Since 1,25-$(OH)_2D_3$ has up- and down-regulatory effects by binding to VDRE, it suggests that the sequence composition of VDRE in the promoter region of the *hsp28* gene and the accessory factor differ from that for the *PKCβ* gene. However, other possibilities such as an indirect effect(s) of 1,25-$(OH)_2D_3$ on the regulation of either *hsp28* or *PKCβ* gene expression should be considered. A most plausible indirect effect may involve the up-regulation of *PKCβ* gene expression, which, in turn, negatively regulates the expression of the *hsp28* gene or vice versa. Obviously, further studies at the cellular and molecular levels are necessary to understand the differential mechanism involved in the regulation of these genes by 1,25-$(OH)_2D_3$ in HL-525 cells.

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