Expression of 25-Hydroxyvitamin D_3-24-Hydroxylase mRNA in Cultured Human Keratinocytes (43791)

MING L. CHEN,* GERHARD HEINRICH,† YOSH-IHIKO OHYAMA,‡ KYUICHIRO OKUDA,‡ JACK L. OMDAHL,§ TAI C. CHEN,* AND MICHAEL F. HOLICK*,[1]

Vitamin D, Bone, and Skin Research Laboratory, Endocrine Section, Department of Medicine, Boston University Medical Center; Section of Biomolecular Medicine,† Evans Department of Clinical Research, Department of Medicine, University Hospital, Boston, Massachusetts 02118; Department of Biochemistry,‡ Hiroshima University School of Dentistry, Hiroshima 734, Japan; and Department of Biochemistry,§ School of Medicine, University of New Mexico, Albuquerque, New Mexico 87131*

Abstract. **It is well documented that 1α,25-dihydroxyvitamin D_3 (1α,25[OH]$_2$D$_3$), the most active vitamin D metabolite, inhibits epidermal keratinocyte proliferation and promotes differentiation. 1α,25(OH)$_2$D$_3$ can be produced in keratinocytes from 25-hydroxyvitamin D_3 by the enzyme 25-hydroxyvitamin D_3-1α-hydroxylase (1-OHase). Hydroxylation of 1α,25(OH)$_2$D$_3$ by 25-hydroxyvitamin D_3-24-hydroxylase (24-OHase), the first step in the catabolic pathway of 1α,25(OH)$_2$D$_3$ could significantly reduce the intracellular concentration of 1α,25(OH)$_2$D$_3$. Therefore, the expression of 24-OHase could have a critical regulatory role in 1α,25(OH)$_2$D$_3$-dependent gene expression. As a first step to examine this possibility, the steady state level of 24-OHase mRNA in cultured human keratinocytes (CHK) was investigated. 24-OHase mRNA was not detected in control CHK. 1α,25(OH)$_2$D$_3$ caused a dose- and time-dependent increase in 24-OHase mRNA level. The highest accumulation of 24-OHase mRNA was observed in CHK treated with 0.1–1 μ*M* 1α,25(OH)$_2$D$_3$. The level of 24-OHase mRNA reached a plateau 12–24 hr after 1α,25(OH)$_2$D$_3$ treatment. 1β,25-dihydroxyvitamin D_3, the stereoisomer of 1α,25(OH)$_2$D$_3$, failed to induce 24-OHase mRNA expression significantly. In addition to 24-OHase mRNA, a 1.0-kb mRNA hybridized strongly with both rat and human 24-OHase cDNA probes. The origin of this 1.0-kb message is unknown at present, however, it was regulated by 1α,25(OH)$_2$D$_3$. These results demonstrate that 1α,25(OH)$_2$D$_3$ up-regulates the expression of 24-OHase mRNA, and this may be an important first step in the initiation of catabolism of 1α,25(OH)$_2$D$_3$ in human keratinocytes.** [P.S.E.B.M. 1994, Vol 207]

Epidermal keratinocytes differentiate from precursor cells in the basal layer of the epidermis. During differentiation, these cells begin to express in a spatially and temporally ordered pattern a set of genes that regulate the increasing differentiation and special functions of keratinocytes in the outer layers of the epidermis. Among the genes that are induced are transglutaminase, involucrin, and keratins (1–3). As they differentiate, keratinocytes eventually stop synthesizing DNA and become postmitotic.

1α,25-dihydroxyvitamin D_3 (1α,25[OH]$_2$D$_3$) has been shown to inhibit DNA synthesis in cultured human keratinocytes (CHK) and to increase levels of transglutaminase and cornified envelopes (4–7). These observations suggest an important function of 1α,25(OH)$_2$D$_3$ in keratinocyte proliferation and differentiation. Consistent with such a function is the presence of high-affinity intracellular vitamin D receptor (VDR) in keratinocytes (8, 9).

The VDR is believed to regulate gene transcription

[1] To whom requests for reprints should be addressed at BUSM, 80 East Concord Street, M-1013, Boston, MA 02118.

Received September 15, 1993. [P.S.E.B.M. 1994, Vol 207]
Accepted May 4, 1994.

0037-9727/94/2071-0057$10.50/0

by interacting with regulatory elements in target genes (10). Since cellular differentiation at the molecular level is a well-ordered pattern of sequential activation and deactivation of gene transcription, the effects of $1\alpha,25(OH)_2D_3$ on keratinocyte differentiation are most likely mediated at the level of transcription and involves the VDR. These genomic actions require binding of $1\alpha,25(OH)_2D_3$ to the VDR, and this binding and the resulting effects on transcription are dose dependent (10).

A critical question is how intracellular levels of $1\alpha,25(OH)_2D_3$ are regulated during keratinocyte differentiation. $1\alpha,25(OH)_2D_3$ is probably available to keratinocytes via microvessels in the dermis. In addition, $1\alpha,25(OH)_2D_3$ can be produced locally by 25-hydroxyvitamin D_3-1α-hydroxylase (1-OHase) in keratinocytes (8, 11). An effective way to regulate the intracellular concentration of $1\alpha,25(OH)_2D_3$ is via the degradation pathway, which is initiated by 25-hydroxyvitamin D_3-24-hydroxylase (24-OHase) (11, 12). 24-OHase catalyzes the conversion of $1\alpha,25(OH)_2D_3$ and $25(OH)D_3$ to their respective 24-hydroxylated derivatives (12). Such a catabolic pathway may reduce the intracellular level of $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ and thus the biological action of $1\alpha,25(OH)_2D_3$ (13).

Expression of 24-OHase in epidermis could serve an effective regulatory role in keratinocytes differentiation by modulating the intracellular level of $1\alpha,25(OH)_2D_3$. We have therefore investigated whether this enzyme is expressed and regulated in keratinocytes. Our data show that 24-OHase mRNA and activity are expressed in keratinocytes and $1\alpha,25(OH)_2D_3$ is a potent inducer of 24-OHase. This observation suggests that a negative regulatory mechanism is operative in limiting $1\alpha,25(OH)_2D_3$ levels and the subsequent effects of $1\alpha,25(OH)_2D_3$ in epidermal keratinocytes.

Materials and Methods

Culture Conditions. CHK cultures were prepared from human newborn foreskin as described (5). Cells were maintained in culture medium consisting of MCDB 153 and supplemented with the following growth factors, EGF (25 ng/ml; Perpro Tech., Rocky Hill, NJ), insulin (5 μg/ml; Sigma Chemical Co., St. Louis, MO), PGE_1 (50 ng/ml, Sigma) and pituitary extract (30 μg/ml; Collaborative Research, Lexington, MA). The concentration of calcium in this medium was 0.15 m*M*.

To analyze the time-dependent induction of 24-OHase mRNA, subconfluent keratinocytes were fed with fresh medium containing 0.1 μ*M* $1\alpha,25(OH)_2D_3$. $1\alpha,25(OH)_2D_3$ was the gift of Dr. M. Uskokovic (Hoffmann-La Roche, Nutley, NJ). Control cells received vehicle (0.1% ethanol) only. Cells were collected at times indicated in the figure legends and stored at −80°C until analysis. In the dose-dependent experiments, CHK were treated with varying concentrations of $1\alpha,25(OH)_2D_3$ and analyzed 24 hr later. To determine the specificity of $1\alpha,25(OH)_2D_3$ action, parallel dishes were treated with either 0.1 μ*M* vitamin D_3 or $1\beta,25$-dihydroxyvitamin D_3 ($1\beta,25[OH]_2D_3$).

RNA Isolation and Northern Hybridization. RNA was isolated according to Chomczynski and Sacchi (14) using RNAzol (Tel-Test B Inc., Friendswood, TX). Twenty-five micrograms of RNA from each sample were loaded and separated on 1% agarose-formaldehyde gel using standard procedures (15). Gels were routinely stained with ethidium bromide to check the integrity of RNA samples. RNA was transferred to a Zeta-probe membrane (Bio-rad, Rockville Center, NY) and hybridized with the rat kidney 24-OHase cDNA clone (p108) (16). A fragment of p108 (nt. 564–1864) containing the coding region of 24-OHase was extracted by digestion with AccI and KpnI. This cDNA fragment was labeled with ^{32}P-dCTP (Prime-it II kit; Stratgene, La Jolla, CA) and utilized as the hybridization probe. A human renal 24-OHase cDNA clone (ph24–1.8; J. Omdahl, unpublished result) was used as a hybridization probe in the experiment described in Figure 1 and found no species-specific effect. The blots were hybridized in buffer containing 6X SSPE, 5X Denhardt's solution, 1% SDS, 10% dextran sulfate and 100 μg/ml sonicated sperm DNA, at 60°C, overnight. Excess probe was removed by washing the blot twice in 1X SSC, 0.1% SDS at 65°C for 30 min each. The blots were exposed to X-ray film (Hyperfilm-MP; Amersham, Arlington Hts, IL) for 4 days. The autoradiographic images were quantified by densitometric scanning.

The blots were stripped and reprobed with a glyceraldehyde phosphate dehydrogenase (GAPDH) oligonucleotide (Oncogene Science, Uniondale, NY). The concentrations of 24-OHase mRNA were normalized to the GAPDH mRNA levels.

HPLC Analysis of Vitamin D_3 Metabolites. The conversion of $[^3H]$-$25(OH)D_3$ to $[^3H]$-$24,25(OH)_2D_3$ was analyzed in control cells and in CHK treated with 0.1 μ*M* $1\alpha,25(OH)_2D_3$ for 24 hr. Cells were incubated with 80,000 cpm (120 Ci/mmol) of $25(OH)$-$[^3H]D_3$ for 1 hr. The lipid phase of both cells and medium were extracted according to Bligh and Dyer (17). Vitamin D metabolites were separated using a Waters 501 HPLC system (Waters Associates, Milford, MA) equipped with a Zorbax Sil column (4.6 mm × 25 cm; DuPont, Wilmington, DE). The metabolites were eluted with a solvent of n-hexane-isopropanol-methanol (v/v, 88:6:6) and radioactivity was detected by Radiomatic Flo One HP radioactivity detector. The elution times for $24,25(OH)_2D_3$ and $1\alpha,25(OH)_2D_3$ were determined with authentic vitamin D metabolites applied in the same column.

Results

Two mRNA Species Are Detected with Rat and Human 24-OHase cDNA Probes. Figure 1 shows that the rat 24-OHase cDNA probe hybridized with two mRNA species of 1α,25(OH)$_2$D$_3$-treated CHK. The size (3.4 kb) of the slow migrating band is consistent with that of 24-OHase mRNA (15). The faster migrating band has an estimated size of 1.0 kb. The ratio of 3.4 kb/1.0 kb band intensity was 1.15 in CHK treated with 0.1 μ*M* 1α,25(OH)$_2$D$_3$ for 24 hr. The blot was reprobed with a human renal 24-OHase cDNA. There was no difference in the hybridization patterns and the ratio of 3.4 kb/1.0 kb observed with either rat or human probes.

1α,25(OH)$_2$D$_3$ Induced 24-OHase mRNA Expression. 24-OHase mRNA (3.4 kb) was not detectable in the control CHK cultures (Fig. 2). The level of 24-OHase mRNA increased significantly in CHK treated with 1α,25(OH)$_2$D$_3$ in a dose-dependent manner and plateaued at 1α,25(OH)$_2$D$_3$ concentrations of 0.1–1 μ*M*. The specificity of 1α,25(OH)$_2$D$_3$ to stimulate 24-OHase mRNA expression was confirmed since both 1β,25(OH)$_2$D$_3$ and vitamin D$_3$ (0.1 μ*M*) failed to induce 24-OHase mRNA accumulation significantly (Fig. 2). Time course studies indicated that the increase in 24-OHase mRNA was evident at 4 hr in CHK treated with 0.1 μ*M* 1α,25(OH)$_2$D$_3$ and reached a peak between 12 and 24 hr (Fig. 3). 24-OHase mRNA then declined gradually but remained elevated above the basal level at 48 hr. There was no significant change in GAPDH mRNA level over the same treatment period.

The presence of 24-OHase mRNA in CHK treated with 1α,25(OH)$_2$D$_3$ is consistent with the increased 24-OHase enzyme activity in these cells. In CHK treated with 0.1 μ*M* 1α,25(OH)$_2$D$_3$ for 24 hr, the conversion of ^{3}H-25(OH)D$_3$ to ^{3}H-24,25(OH)$_2$D$_3$ was 7.2 ± 0.9 *p*mol/mg protein/hr (n = 3). We were unable to detect 24-OHase activity in control CHK.

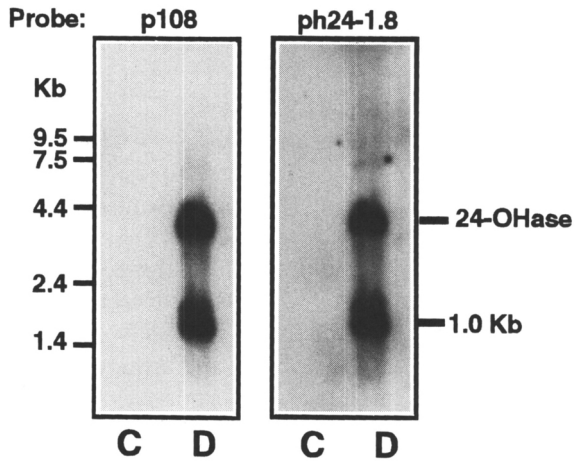

Figure 1. Northern analysis of 24-OHase and 1.0-kb mRNAs. Poly A(+) mRNA (5 μg), isolated from CHK treated with (D) or without (C) 0.1 μ*M* 1α,25(OH)$_2$D$_3$ for 24 hr, was analyzed by Northern blot. The nylon membrane was probed with rat 24-OHase cDNA (p108). It was then stripped and reprobed with human 24-OHase cDNA (ph24–1.8). The blot was exposed to X-ray film for 48 hr. Numbers on the left indicate MW marker in kb.

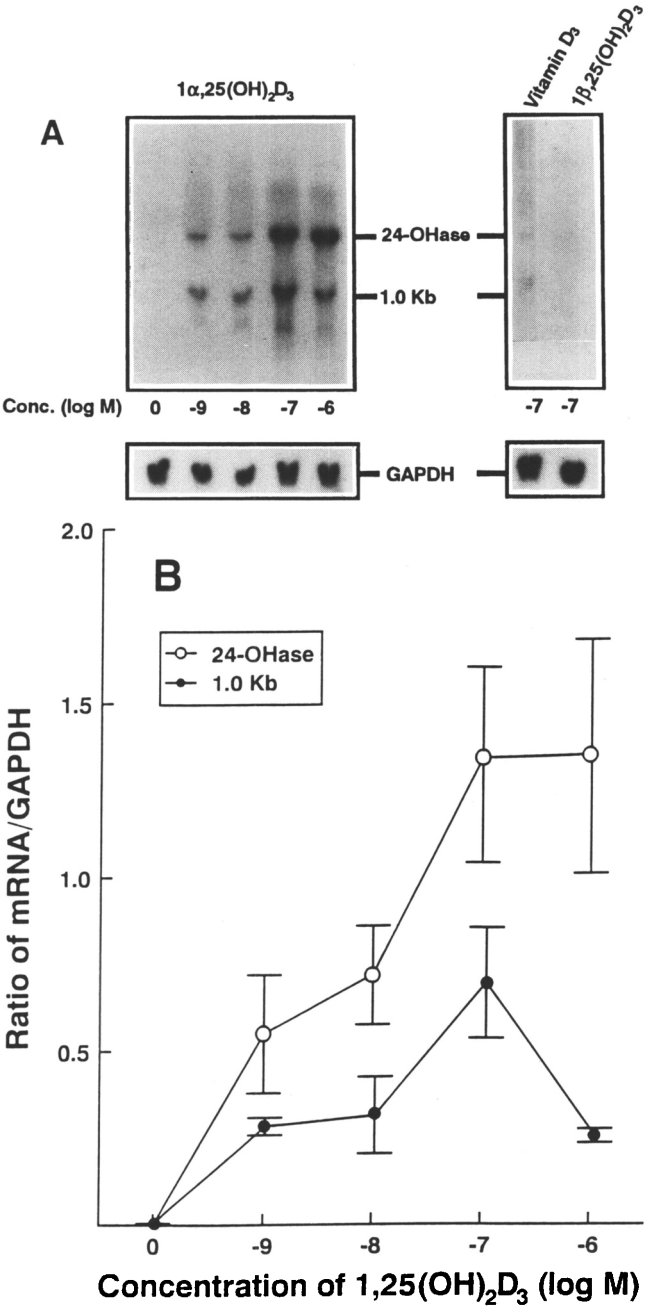

Figure 2. Concentration-Dependent induction of 24-OHase and 1.0-kb mRNAs by 1α,25(OH)$_2$D$_3$. Keratinocytes were treated with varying concentrations of 1α,25(OH)$_2$D$_3$ for 24 hr. Some dishes received 0.1 μ*M* vitamin D$_3$ or 1β,25(OH)$_2$D$_3$. Control cells received vehicle (0.1% ethanol) only. Twenty-five micrograms total RNA from each sample were analyzed. (A) Autoradiograph of a representative experiment. The blot was exposed for 4 days. The positions of 24-OHase and 1.0-kb mRNAs are indicated. The bottom panel shows GAPDH mRNA. (B) Densitometric data of scanned autoradiographs. The levels of 24-OHase and 1.0-kb mRNAs (mean ± SD, n = 3) were normalized to those of GAPDH levels.

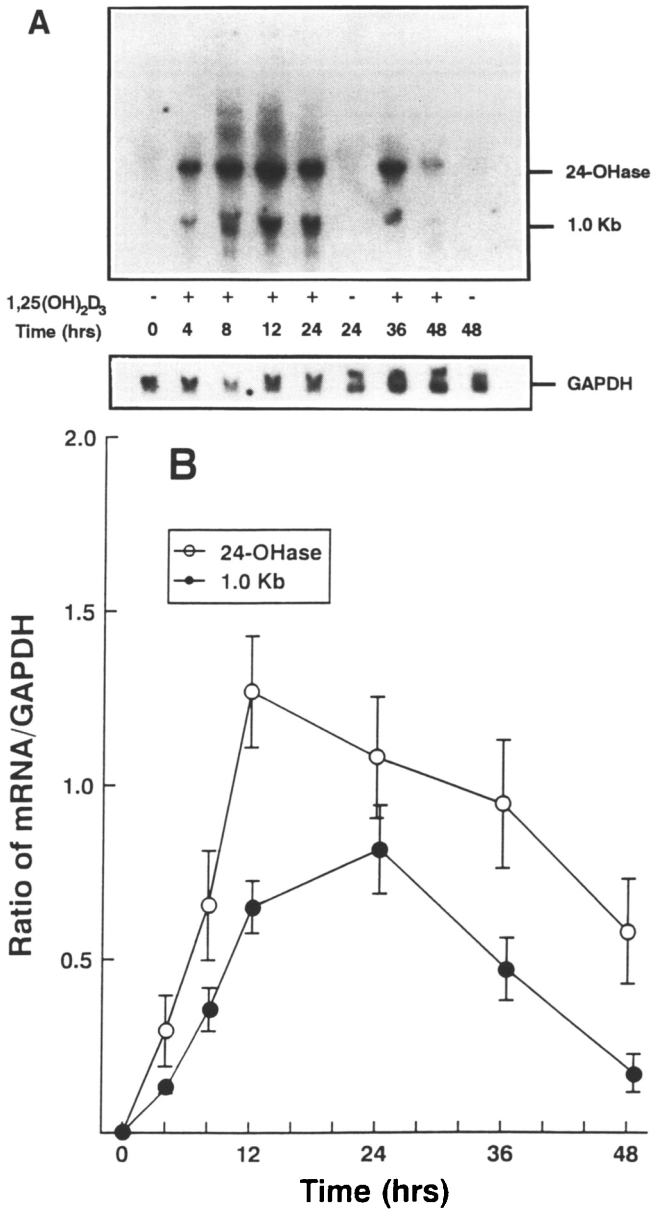

Figure 3. Time-Dependent induction of 24-OHase and 1.0-kb mRNAs by $1\alpha,25(OH)_2D_3$. Keratinocytes were treated with 0.1 μM $1\alpha,25(OH)_2D_3$ for indicated time. Samples were then processed as described in the legend to Figure 2. (A) Autoradiograph of a representative experiment. (B) Densitometric values of 24-OHase and 1.0-kb mRNAs (mean ± SE, n = 2).

$1\alpha,25(OH)_2D_3$ Increases the 1.0-kb mRNA Level. The primary sequence of this 1.0-kb message is not known at present, and, therefore, we do not yet understand its precise relationship to the 24-OHase mRNA. Interestingly, it was regulated by $1\alpha,25(OH)_2D_3$. The dose-response experiments (Fig. 2) showed that the 1.0-kb mRNA was induced by 1 nM of $1\alpha,25(OH)_2D_3$ and reached a plateau at 0.1 μM. In contrast to the 24-OHase mRNA, the 1.0-kb mRNA did not further increase but rather decreased at a higher concentration of $1\alpha,25(OH)_2D_3$ (1 μM). In CHK treated with 0.1 μM $1\alpha,25(OH)_2D_3$, this 1.0-kb mRNA accumulated up to 24 hr, in parallel with 24-OHase mRNA (Fig. 3).

Discussion

We demonstrated that 24-OHase mRNA in CHK was up-regulated specifically by $1\alpha,25(OH)_2D_3$ in a time- and dose-dependent manner (Fig. 2 and 3). $1\beta,25(OH)_2D_3$, a biologically inert isomer of $1\alpha,25(OH)_2D_3$ (5), failed to induce 24-OHase mRNA expression substantially (Fig. 2). This increase could be due to increased gene transcription or mRNA stabilization or both. Enhanced transcription probably accounts for most of the initial accumulation of 24-OHase mRNA (prior to 4 hr), since 24-OHase mRNA in CHK was not detected under basal conditions. Moreover, 24-OHase message accumulated rapidly in the first 12 hr after $1\alpha,25(OH)_2D_3$ administration (Fig. 3), suggesting a $1\alpha,25(OH)_2D_3$/VDR-mediated up-regulation of 24-OHase gene transcription.

After 24 hr of continuous accumulation, 24-OHase mRNA level in CHK began to decline (Fig. 3). In addition to the normal turnover of mRNA, this decline could be partially due to the depletion of $1\alpha,25(OH)_2D_3$ in culture medium. We and others (11, 18) have observed that the concentration of $1\alpha,25(OH)_2D_3$ in CHK reduces to the background level after 16–24 hr of incubation. The decrease in the $1\alpha,25(OH)_2D_3$ concentration coincides with the increase of 24-hydroxylated vitamin D metabolites. This suggests a high degree of sensitivity of 24-OHase expression to cellular $1\alpha,25(OH)_2D_3$ levels.

The $1\alpha,25(OH)_2D_3$-induced 24-OHase expression in CHK was dose-dependent (Fig. 2). Two possible mechanisms may account for the dose-dependence. First, binding of $1\alpha,25(OH)_2D_3$ to the VDR enhances the stimulation of transcription of genes whose promoters contain VDR-response elements (VDREs) (19). Several potential VDREs have been identified in the 24-OHase genomic DNA (20). In the absence of ligand, the VDR binds to the VDREs with low affinity (19). Thus, there is a dose relationship between occupancy of the VDR and transcription activity of $1\alpha,25(OH)_2D_3$ target genes. Another mechanism appears to involve the stabilization of the VDR against degradation by its ligand (21). Whatever the mechanisms, our data add to the increasing body of evidence that suggests that the availability and intracellular concentration of $1\alpha,25(OH)_2D_3$ determine the extent of VDR-regulated transcription responses.

Keratinocytes contain both 24-OHase and 1-OHase (8, 11). The relative activities of these hydroxylases may control the intracellular concentration of $1\alpha,25(OH)_2D_3$ in keratinocytes. Interestingly, it has been shown that 24-OHase activity, in a reciprocal relationship to 1-OHase, is higher in the differentiated keratinocytes compared with the proliferating cells (8).

We showed that 24-OHase mRNA expression and enzyme activity are induced by its own substrate. These observations suggest that 24-OHase, together with 1-OHase, could be an important determinant in $1\alpha,25(OH)_2D_3$/VDR-mediated keratinocyte differentiation.

In addition to 24-OHase mRNA (3.4 kb), a 1.0-kb mRNA was detected in CHK. This 1.0-kb mRNA appears to be polyadeylated based on its presence in the pool of oligo-dT selected mRNA (Fig. 1). The 1.0-kb mRNA was not reported in rat kidney and intestine (22, 23), or HL-60 promyelocytes (24). It is possible that the 1.0-kb mRNA is encoded by a different gene and cross-hybridized with rat 24-OHase cDNA probe. A recent report described a 6.0-kb mRNA in human colon carcinoma HT-29 cells was cross-hybridized to rat 24-OHase cDNA probe (25). Another possibility is that this 1.0-kb mRNA is derived from the same transcription unit as the 3.4-kb mRNA. The coregulation of 3.4-kb and 1.0-kb mRNAs by $1\alpha,25(OH)_2D_3$ supports this possibility.

We are thankful to Kelly Persons for his assistance with keratinocyte culture. This study was supported in part by the National Institute of Health Grants DK43690, AR36963, and RR00533, and the postdoctoral fellowship T32DK07201 (M. L. C.).

1. Green H. Terminal differentiation of cultured human epidermal cells. Cell **11**:405–416, 1977.
2. Dover R, Watt FM. Measurement of the rate of epidermal terminal differentiation: Expression of involucrin by S-phase keratinocytes in culture and in psoriatic plaques. J Invest Dermatol **89**:349–352, 1987.
3. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R. The catalog of human cytokeratins: Patterns of expression in normal epithelia, tumors, and cultured cells. Cell **31**:11–24, 1982.
4. Hosomi J, Hosoi J, Abe E, Suda T, Kuroki T. Regulation of terminal differentiation of cultured mouse epidermal cells by 1,25-dihydroxyvitamin D_3. Endocrinology **113**:1950–1957, 1983.
5. Smith EL, Walworth NC, Holick MF. Effect of 1,25-dihydroxyvitamin D_3 on the morphologic and biochemical differentiation of cultured human epidermal keratinocytes grown in serum-free conditions. J Invest Dermatol **86**:709–714, 1986.
6. Matsumoto K, Hashimoto K, Nishida Y, Hashiro M, Yoshikawa K. Growth-inhibitory effect of 1,25-dihydroxyvitamin D_3 on normal human keratinocytes cultured in serum-free medium. Biochem Biophys Res Comm **166**:916–923, 1990.
7. Pillai S, Bikle D. Role of intracellular-free calcium in the cornified envelope formation of keratinocytes: Differences in the mode of action of extracellular calcium and 1,25-dihydroxyvitamin D_3. J Cell Physiol **146**:94–100, 1991.
8. Pillai S, Bikle DD, Elias P. 1,25-dihydroxyvitamin D production and receptor binding in human keratinocytes varies with differentiation. J Biol Chem **263**:5390–5395, 1988.
9. Milde P, Hauser U, Simon T, Mall G, Ernst V, Haussler MR, Frosch P, Rauterberg EW. Expression of 1,25-dihydroxyvitamin D_3 receptors in normal and psoriatic skin. J Invest Dermatol **97**:230–239, 1991.
10. Pike JW. Vitamin D_3 receptors: Structure and function in transcription. Annu Rev Nutr **11**:189–216, 1991.
11. Bikle DD, Nemanic MK, Gee E, Elias P. 1,25-dihydroxyvitamin D_3 production by human keratinocytes. J Clin Invest **78**:557–566, 1986.
12. Inaba M, Burgos-Trinidad M, DeLuca HF. Characteristics of the 25-hydroxyvitamin D_3- and 1,25-dihydroxyvitamin D_3-24-hydroxylase(s) from HL-60 cells. Arch Biochem Biophys **284**:257–263, 1991.
13. Reinhardt TA, Horst RL. Self-induction of 1,25-dihydroxyvitamin D_3 metabolism limits receptor occupancy and target tissue responsiveness. J Biol Chem **264**:15917–15921, 1989.
14. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem **162**:156–159, 1987.
15. Sambrook J, Fritsch EE, Maniatis T. Molecular Cloning (2nd ed). New York: Cold Spring Harbor Laboratory Press, 1989.
16. Ohyama Y, Noshiro M, Okuda K. Cloning and expression of cDNA encoding 25-hydroxyvitamin D_3 24-hydroxylase. FEBS Lett **278**:195–198, 1991.
17. Bligh EG, Dyer WG. A rapid method of total lipid retraction and purification. Can J Biochem Physiol **37**:911–917.
18. Ray R, Ray S, Holick MF. Metabolism of ^{3}H-1α,25-dihydroxyvitamin D_3 in cultured human keratinocytes. J Bone Min Res Suppl **8**:S2122, 1993.
19. Sone T, Kerner S, Pike JW. Vitamin D receptor interaction with specific DNA. J Biol Chem **266**:23296–23305, 1991.
20. Ohyama Y, Noshiro M, Eggerstsen G, Gotoh O, Kato Y, Bjorkhem I, Okuda K. Structural characterization of the gene encoding the 25-hydroxyvitamin D_3 24-hydroxylase. Biochemistry **32**:76–82, 1993.
21. Wiese RJ, Uhland-Smith A, Ross TK, Prahl JM, DeLuca HF. Up-Regulation of the vitamin D receptor in response to 1,25-dihydroxyvitamin D_3 results from ligand-induced stabilization. J Biol Chem **267**:20082–20086, 1992.
22. Armbrecht HJ, Boltz MA. Expression of 25-hydroxyvitamin D 24-hydroxylase cytochrome P450 in kidney and intestine. Effect of 1,25-dihydroxyvitamin D and age. FEBS Lett **292**:17–20, 1991.
23. Shinki T, Jin CH, Nishimura A, Nagai Y, Ohyama Y, Noshiro M, Okuda K, Suda T. Parathyroid hormone inhibits 25-hydroxyvitamin D_3-24-hydroxylase mRNA expression stimulated by 1,25-dihydroxyvitamin D_3 in rat kidney but not in intestine. J Biol Chem **267**:13757–13762, 1992.
24. Chen K-S, Prahl JM, DeLuca HF. Isolation and expression of human 1,25-dihydroxyvitamin D_3 24-hydroxylase cDNA. Proc Natl Acad Sci USA **90**:4543–4547, 1993.
25. Zhao X, Feldman D. Regulation of vitamin D receptor abundance and responsiveness during differentiation of HT-29 human colon cancer cells. Endocrinology **132**:1808–1814, 1993.