Regulation of Parathyroid Hormone-like Peptide in Cultured Normal Human Keratinocytes

Effect of Growth Factors and 1,25 Dihydroxyvitamin D_3 on Gene Expression and Secretion

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Abstract

We have examined the expression and secretion of endogenous parathyroid hormone-like peptide (PLP) in primary cultures of normal human keratinocytes. In response to growth factors and fetal bovine serum, PLP mRNA expression and immunoreactive PLP release into conditioned medium was rapidly increased (within hours) whereas these effects were inhibited by 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$]. These early responses were not influenced by raising the medium calcium concentration from 0.15 to 1.0 mM. In contrast, increasing the medium calcium concentration to 1.0 mM, addition of $1,25(OH)_2D_3$, or a combination of both, resulted in a delayed augmentation (after several days) in PLP production which was associated with an increase in cellular differentiation as assessed by production of high molecular weight keratin. To investigate whether these factors were acting at the level of transcription of the PLP gene, a series of vectors were prepared by fusing segments of the 5′ flanking region of the rat PLP gene to a growth hormone reporter gene. Transient transfection of these constructs into cultured keratinocytes and measurement of immunoreactive growth hormone in the medium showed that a region stimulated by growth factors is located in a 1.9-kb fragment of the 5′ flanking region and that a PLP gene promoter region < 1.2 kb and > 0.3 kb upstream of the cap site contains *cis*-acting elements which respond positively to serum, and negatively to $1,25(OH)_2D_3$. These combined studies demonstrate that, in normal human keratinocytes, growth factors may acutely stimulate PLP mRNA levels and PLP release, whereas $1,25(OH)_2D_3$ inhibits these responses. At least part of these effects are at the level of gene transcription. Additionally, PLP synthesis and release are enhanced under conditions in which keratinocyte differentiation is induced. (*J. Clin. Invest.* 1991. 87:884–893.) Key words: calcium • epidermal growth factor • keratinocyte differentiation • transient transfection

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Received for publication 18 April 1990 and in revised form 27 September 1990.

J. Clin. Invest.
© The American Society for Clinical Investigation, Inc.
0021-9738/91/03/0884/10 $2.00
Volume 87, March 1991, 884–893

Introduction

Messenger RNAs and the gene encoding parathyroid hormone (PTH)-like peptide (PLP),[1] which was initially isolated from human tumors associated with hypercalcemia (1–3), have now been cloned and characterized in the human (4–9) and rat (10–12). PLP mimics many of the biological actions of PTH (13), but is also likely to have its own functions which are separate from its PTH-like effects. The distinct actions of PLP are at present unknown but may involve effects on cellular proliferation and/or differentiation (13–15). It is also likely that, whereas some features of the control of PLP and PTH gene transcription may be shared, others are not. PLP synthesis and/or secretion has been reported to be regulated by several different agents, including cyclic AMP, phorbol esters, growth factors, and steroid hormones in a number of tumor cell lines (16–19) and some of these effects appear to be initiated at the transcriptional level (18, 19). Although originally discovered in a variety of malignancies, mRNA encoding PLP has been identified in both normal and adenomatous tissue of several species. This includes normal human keratinocytes, stomach, normal islet cell, lactating mammary glands, normal and adenomatous parathyroid glands, brain, and fetal liver (20–22). In addition, cultured human keratinocytes secrete a material which stimulates adenylate cyclase activity in osteosarcoma cells (23) and may therefore represent a biologically active form of PLP. Primary cultures of keratinocytes proliferate in low calcium concentrations and terminally differentiate when calcium in the medium is raised to or above 1.0 mM (24). $1,25(OH)_2D_3$ receptors have been identified in skin and cultured keratinocytes (25, 26) and $1,25(OH)_2D_3$ has been shown to potentiate calcium-induced differentiation of keratinocytes in culture (27, 28). Furthermore, $1,25(OH)_2D_3$ can be produced locally from its substrate $25(OH)D_3$ in human keratinocytes (29) and may, therefore, regulate keratinocyte differentiation in an autocrine fashion. In addition, keratinocytes respond to growth factors such as epidermal growth factor (EGF; 30) and produce peptides such as transforming growth factor-α (31) which may therefore also exert a regulatory influence on these cells through autocrine or paracrine mechanisms. Taken

1. *Abbreviations used in this paper:* BPE, bovine pituitary extract; EGF, epidermal growth factor; GF, growth factor(s); hGH, human growth hormone; KBM, keratinocyte basal medium; KGM, keratinocyte growth medium; PLP, parathyroid hormone-like peptide; SRE, serum response element; VDRE, vitamin D–responsive element.

together, these characteristic features of human keratinocytes make them an excellent model for the study of the control of PLP expression by agents such as growth factors, calcium, and $1,25(OH)_2D$. We have therefore examined the effect of these potential regulatory factors on PLP mRNA expression and secretion. We also initiated a functional analysis of the PLP gene promoter by preparing chimeric constructs consisting of restriction fragments from the rat PLP gene 5′ flanking region fused to a human growth hormone (hGH) reporter gene and transfecting cultured human keratinocytes with these constructs.

Methods

Culture of normal human keratinocytes. Normal human keratinocytes were prepared, according to a previously published method (32), from skin tissue removed during breast reduction. The complete medium (KGM) for clonal growth of keratinocytes consists of keratinocyte basal medium (KBM, Clonetics Corp., San Diego, CA) supplemented with 10 ng/ml of EGF (Sigma Chemical Co., St. Louis, MO), 5 μg/ml of insulin (Sigma Chemical Co.), 0.5 μg/ml of hydrocortisone, 0.4% (wt/vol) bovine pituitary extract (BPE, Clonetics Corp.), and 0.15 mM calcium (Ca^{++}). Cells were grown to near confluency in 10-mm diameter wells in KGM, which provides maximal proliferation without differentiation. After a 24-h incubation in KBM with insulin, hydrocortisone, and 0.15 mM Ca^{++} (basal conditions), the medium was changed and replaced with KBM alone or combinations of KBM plus insulin and hydrocortisone, BPE, EGF, and 10% FBS. Calcium concentration was varied between 0.15 and 1.0 mM and verified by direct measurement using atomic absorption spectrophotometry. In some experiments the medium was supplemented with $1,25(OH)_2D_3$ (10^{-8} M). The medium was removed at timed intervals and frozen immediately for subsequent measurement of PLP by radioimmunoassay. Cells were trypsinized, an aliquot was counted, and the remaining cells were centrifuged at low speed (600 *g*), rinsed with phosphate-buffered saline, and lysed with a mixture of 4 M guanidinium thiocyanate, 25 mM trisodium citrate, 1 mM EDTA, and 0.1 M β-mercaptoethanol (GTC mixture). GTC extracts were stored at −70°C for subsequent RNA analysis by dot blot hybridization or Northern blot hybridization.

Assay of PLP in the conditioned medium. Conditioned medium (1.5 ml per well), acidified to pH ~ 3 with 0.1% (vol/vol) trifluoroacetic acid was passed through cartridges of octadecylsilyl (ODS)-silica (C_{18} Sep-pak, Waters Associates, Mississauga, ON) washed with 10 ml of 0.1% trifluoroacetic acid, and eluted with 4 ml of 80% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid. Eluates evaporated to dryness in a Speed-Vac (Savant Instruments Inc., Hicksville, NY) were subsequently reconstituted in outdated blood bank plasma at the appropriate dilutions and radioimmunoassayed as described previously (33). Recovery of immunoreactive hPLP(1-34) by this method was 87±12%. Antiserum raised against synthetic hPLP(1-34) was used at a final dilution of 1:25,000 and synthetic hPLP(1-34) diluted in outdated blood bank plasma was used as a standard. The detection limit of the assay was 20 pg-equivalents of hPLP(1-34) per tube and the inter- and intraassay coefficients of variation were 18% and 7%, respectively. PLP immunoreactivity was undetectable in the FBS and BPE used in the medium and an equivalent amount of nonconditioned medium was treated in the same manner as conditioned medium to assess for nonspecific inhibition of binding by any other medium components. No such inhibition of binding was found. Results are expressed as picograms per 100,000 cells, which refers to picogram-equivalents per 100,000 cells relative to the hPLP(1-34) standard. Immunoreactive PLP secreted by keratinocytes into culture medium increased linearly over 96 h, the longest period of time during which collections were made, attesting to the relative stability of the secreted PLP.

To determine the relationship between PLP immunoreactivity and bioactivity, conditioned medium was collected, after a 48-h incubation, from six wells in which cells had been grown in complete medium (KGM). Aliquots were subjected to radioimmunoassay, as described, and to bioassay in an adenylate cyclase assay performed, as previously described, in UMR 106 osteosarcoma cells (34). In brief, cells were incubated with 1 μCi [2-^{3}H]adenine (16 Ci/mmol; New England Nuclear, Boston, MA) for 2 h in 1 ml of culture medium per well. Medium was then aspirated from the wells, cells were washed, and keratinocyte-conditioned medium to be assayed (diluted 1:1 with minimum essential medium-Hank's salts [Gibco Laboratories, Grand Island, NY]) was added. The final mixture contained 0.2% bovine serum albumin and 1 mM isobutyl methylxanthine (Sigma Chemical Co.), pH 7.5, and was incubated at 22°C for 10 min. The reaction was stopped by aspiration of the medium and addition of 0.5 ml of ice-cold 10% trichloroacetic acid. Approximately 3,000 cpm [^{14}C]cAMP (53.1 mCi/ml; New England Nuclear) were added to measure recovery. [^{3}H]cAMP was separated from other adenylated nucleotides by the method of Salomon et al. (35). Also assayed, in multiple dilutions, was hPLP(1-34) which was used as a standard in the assay. An excellent correlation was obtained between immunoreactive and bioactive PLP (Table I), albeit in unstimulated cultures collected after 48 h. The higher levels of immunoreactive than of bioactive hormone observed may relate to the fact that the standard used for quantitation in both assays was synthetic hPLP(1-34). If hPLP(1-34) is not the predominant species released into the culture medium, as seems likely, then it is possible that the native species does not react identically to hPLP(1-34) in both immunoassay and bioassay, i.e., this moiety may react relatively better in the immunoassay resulting in nominally higher immunoreactive concentrations. Nevertheless, in view of this correlation, in subsequent studies, to facilitate multiple measurements, only immunoreactive PLP was determined.

Immunocytochemistry. Cellular content of high molecular weight keratin was determined by immunocytochemistry. Cells were seeded in four chamber glass slides (Gibco Laboratories) at a density of 5,000 cells per chamber in KGM and 0.15 mM Ca^{++} until near confluency. After a 24-h incubation in KBM plus insulin and hydrocortisone, the medium was changed to KGM containing 0.15 or 1.0 mM Ca^{++} and with or without $1,25(OH)_2D_3$ (10^{-8} M) for 8 d. Medium was changed after 4 d. Cells were then fixed in 95% ethanol for 5 min, rinsed with distilled water, and stained with a rabbit antibody to high molecular weight keratin (Dakopatts, Dako Corp., Carpinteria, CA) using a modification of the three-layer peroxidase–antiperoxidase technique (36).

RNA analysis. For Northern blot analysis, GTC extracts were purified by cesium chloride gradient centrifugation (37) and 10 μg of total RNA was electrophoresed on a 1.1% agarose-formaldehyde gel. Ethidium bromide was added to each sample before electrophoresis to per-

Table I. Radioimmunoassay and Adenylate Cyclase Bioassay of PLP in Keratinocyte-conditioned Medium

Well	Immunoreactive PLP	Bioactive PLP
	*pg/10⁵ cells**	
1	381±27	211±2
2	336±51	242±11
3	422±58	296±20
4	542±41	331±15
5	209±23	170±10
6	504±41	313±31

Pearson's Correlation Coefficient equals 0.91 which is significant at $P < 0.02$.

* Each value represents the mean±SEM of triplicate determinations. Values were assayed as described in Methods.

mit detection by UV transillumination. RNA was transferred by blotting to a nylon membrane. For dot blot hybridization, samples were processed as described earlier (38). The filters were air-dried, baked at 80°C for 2 h, and then hybridized (39) with a 537-bp SacI, HindIII restriction fragment encoding exonIII (coding region) of the human PLP gene labeled with [^{32}P]dCTP by the random primer method. After incubation at 42°C for 24 h, filters were washed twice for 30 min each in 0.5× SSC, 0.1% SDS at 55°C (1× SSC is 0.15 M sodium chloride, 0.015 M trisodium citrate). Autoradiography of filters was carried out at −70°C using XAR films (Eastman Kodak Co., Rochester, NY) and two intensifying screens. The intensity of the dot blots was analyzed by laser densitometry (Ultroscan XL, LKB Instruments, Inc., Gaithersburg, MD).

Construction of rat PLP gene promoter–human growth hormone fusion genes. Restriction fragments encoding parts of the 5′ flanking region of the rat PLP gene were cloned into the plasmid pOGH (40) upstream of the reporter hGH gene. These fragments were derived from recombinant clone rPLPg10-2 (12), which contains rat genomic sequences representing the 5′ flanking region, exon I (encoding the 5′ noncoding region of the mRNA), intron I, exon II (encoding the prepro-precursor coding region), and intron II of the rat PLP gene. A HindIII/FspI fragment containing 1.9 kb and an EcoRI/FspI fragment containing 1.2 kb of the 5′ flanking sequence were blunt-end ligated into the BamHI site of pOGH to generate p1.9 GH and p1.2 GH, respectively. p1.9 GH was digested with HindIII and NaeI, the HindIII vector site blunt-ended and the vector religated to generate p 0.3 GH containing 0.3 kb of the 5′ flanking sequence. These fusion constructs were used to transform competent *Escherichia coli* DH5α and plasmid DNA was isolated and purified by pZ523 spin column chromotography (5 Prime-3 Prime Inc., West Chester, PA) and BioGel A 150M (Bio-Rad Laboratories, Mississauga, ON) chromotography. The orientation of insertion was verified by restriction mapping and nucleotide sequencing.

Transient transfection of human keratinocytes in culture. Primary cultures of human keratinocytes were grown in KGM containing 0.15 mM Ca^{++}. The near-confluent cells were then transfected with plasmid DNA using the cationic lipid, *N*-[1-(2,3-dioleyloxy) propyl]-*N,N,* N-trimethyl ammonium chloride (DOTMA) (41) (Lipofectin Reagent, Bethesda Research Laboratories, Gaithersburg, MD) according to the manufacturer's specifications using minor modifications. Briefly, 20 μg of Lipofectin Reagent and 10 μg of plasmid DNA were mixed in polystyrene tubes in a total volume of 75 μl, allowed to stand for 15 min at room temperature and then added drop-wise to the keratinocyte culture medium. After 20–22 h of incubation at 37°C, the medium was removed and replaced with medium containing various combinations of FBS (10%), BPE (0.4%), and EGF (10 ng/ml), and $1{,}25(OH)_2D_3$ (10^{-8} M), as described subsequently. These experiments were done in the presence of either low calcium (0.15 mM Ca^{++}) or high calcium (1.0 mM Ca^{++}). Samples were withdrawn from the incubation medium at 48 and 96 h and assayed for hGH using the Allegro hGH Immunoassay system (Nichols Institute Diagnostics, San Juan Capistrano, CA). Well-to-well variability was assessed in each experiment using pXGH5 (40) which contains the mMT-I promoter and was 21%.

To test for non-PLP promoter-mediated effects of FBS and $1{,}25(OH)_2D_3$ on hGH synthesis or secretion, keratinocytes were transfected with pONCGH which comprises pOGH with the rat oncomodulin promoter (42) upstream of the hGH reporter gene. Immunoassayable hGH levels in the medium of the transfected keratinocytes cultured in the presence of FBS, $1{,}25(OH)_2D_3$, or basal medium alone, did not differ significantly. Consequently effects observed in subsequent studies with the rat PLP gene promoter–hGH fusion genes are believed to be secondary to modulation of responsive sequences in the 5′ flanking region of the PLP gene resulting in altered transcription rather than to nongenomic effects such as effects on stability of mRNA encoding hGH or on hGH secretion.

Statistical analysis. Statistical analysis was by one way analysis of variance (ANOVA) or by Student's *t* test.

Results

Effect of GF and FBS on PLP secretion. Low concentrations of immunoreactive PLP were detected in the medium of keratinocytes maintained in KBM (basal levels) or in KBM plus insulin-hydrocortisone for 24 h (Fig. 1). Further studies with growth factors or serum were performed in the presence of insulin and hydrocortisone which did not alter PLP secretion. Addition of bovine pituitary extract (BPE, 0.4%) or EGF (10 ng/ml) produced small increases in PLP that failed to reach statistical significance. In contrast, addition of EGF plus BPE (GF) produced a twofold augmentation in immunoassayable PLP whereas 10% FBS produced greater than a fourfold increase during the same time period (Fig. 1).

Effect of extracellular calcium concentration on PLP secretion. When keratinocytes were incubated with 0.15, 0.5, or 1.0 mM Ca^{++}, no significant differences in PLP secretion were observed for up to 96 h. However, cells incubated in 1.0 mM Ca^{++} calcium demonstrated a marked increase in PLP secretion at 8 d (Fig. 2). This effect of 1.0 mM Ca^{++} was paralleled by an increase in production of high molecular weight keratin by the keratinocytes, indicative of cellular differentiation (43) (Fig. 3). No such keratinization was seen with 0.15 mM Ca^{++} (Fig. 3) and only slight keratinization was observed with the intermediate dose (0.5 mM Ca^{++}).

Effect of $1{,}25(OH)_2D_3$ on PLP secretion. Addition of $1{,}25(OH)_2D_3$ to the culture medium, in the presence of a low calcium concentration (0.15 mM) was found to inhibit the stimulatory effect of both GF and FBS at 24 h (Fig. 4). The time course of this effect of $1{,}25(OH)_2D_3$ on PLP secretion at low calcium concentration was then examined (Fig. 5). At 24 and

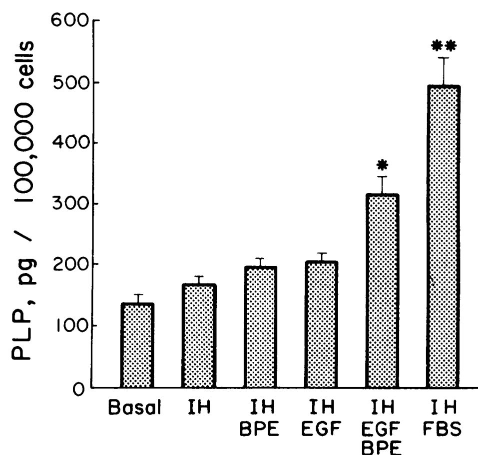


Figure 1. Effect of GF and FBS on PLP secretion. Normal human keratinocytes were grown as described in Methods. At time 0 the medium was removed and replaced with KBM alone (*Basal*) or combinations of KBM plus insulin-hydrocortisone (*IH*), 0.4% BPE, (10 ng/ml) EGF, and 10% FBS. Medium was collected at 24 h for measurements of PLP by radioimmunoassay. Cells were trypsinized and counted. PLP levels were expressed as picogram-equivalents of hPLP(1-34) per 100,000 cells (*PLP, pg/100,000 cells*). Each bar represents the mean±SEM of triplicate determinations and asterisks represent significant differences from basal at $P < 0.01$ (*) or $P < 0.001$ (**). The data shown are representative of three separate experiments.

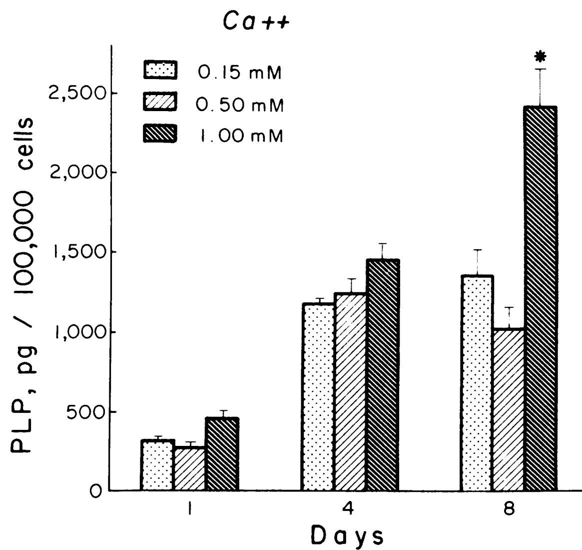


Figure 2. Effect of calcium on PLP secretion. Normal human keratinocytes were incubated as described in Methods in KGM to near confluency, then in KBM with insulin, hydrocortisone, and 0.15 mMCa^{++} for 24 h. The medium was then changed to KGM containing either 0.15, 0.5, or 1.0 mM calcium (Ca^{++}). PLP in the medium was determined over 1, 4, and 8 d of incubation. Each bar represents the mean±SEM of triplicate determinations and the asterisk indicates a significant difference from PLP in medium containing 1.0 mM calcium after 8 d of incubation ($P < 0.001$). The data shown are representative of triplicate experiments.

48 h there was inhibition of PLP secretion by $1,25(OH)_2D_3$. PLP accumulation in the medium during this time was also inhibited by $1,25(OH)_2D_3$ at medium calcium concentrations of 0.5 and 1.0 mM (Fig. 6). In contrast, at later time points, $1,25(OH)_2D_3$ stimulated PLP secretion, stimulation being augmented by increasing the medium Ca^{++} concentration from 0.15 to 1.0 mM (Fig. 6). This late effect was also paralleled by an increase in immunostaining of keratinocytes for high molecular weight keratin, suggesting a delayed effect of $1,25(OH)_2D_3$ on cellular differentiation (Fig. 3).

Effect of GF, FBS, and $1,25(OH)_2D$ on PLP mRNA levels. Dot blot analysis of total RNA in GTC extracts of keratinocytes incubated with GF in complete medium and low calcium concentration (0.15 mM) showed a rapid and transient increase of PLP mRNA, which peaked at 1 h and returned to baseline levels by 24 h (Fig. 7 *A*). A similar early increase in PLP mRNA was also seen after incubation of keratinocytes with 10% FBS. Northern blot analysis revealed the presence of a PLP transcript of 1.4 kb (Fig. 7 *B*). Addition of GF to keratinocytes increased expression of this transcript at 1 h, but mRNA levels returned to basal levels by 120 h (Fig. 7 *B*). Addition of $1,25(OH)_2D_3$ inhibited this early increase in PLP mRNA (at 1 h). In contrast, $1,25(OH)_2D_3$ increased PLP mRNA at later times (120 h), when effects on cellular differentiation were also seen.

Effect of FBS and $1,25(OH)_2D_3$ on PLP gene promoter activity. In preliminary experiments, the effects of FBS and of $1,25(OH)_2D_3$ on hGH release by keratinocytes transfected with the construct pONCGH was assessed (Table II). No stimulation or inhibition of basal activity was observed, thus indicating the absence of nonspecific effects of these agents in the hGH transient transfection assay.

Chimeric constructs containing 0–1.9 kb of the 5′ flanking region of the rat PLP gene fused to the hGH reporter gene were used to transiently transfect primary cultures of human keratinocytes (Fig. 8). Stimulation of immunoreactive hGH release by 10% FBS was observed in conditioned medium from cultures of these keratinocytes transfected with constructs containing 1.9 and 1.2 kb of the 5′ flanking region of the gene (Fig. 9). Immunoreactive hGH release was increased at 48 h and reached maximal levels at 96 h. Much reduced (although still significant) hGH concentrations were measured in the conditioned medium of cells transfected with p0.3GH, containing only the first 300 bp of the promoter. This therefore suggested that there are enhancer elements located between 300 and 1,200 bp upstream of the cap site that are responsive to FBS.

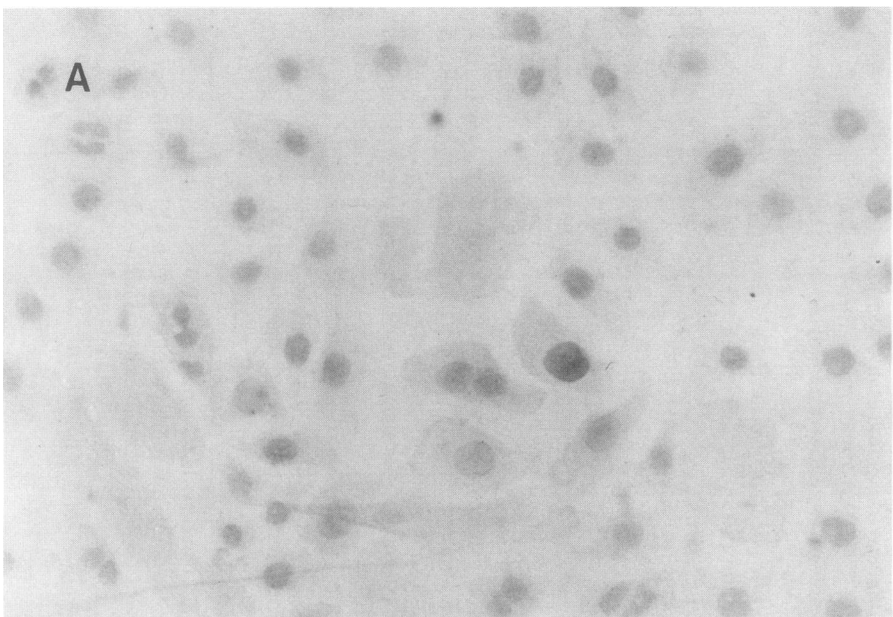
When transfection studies were repeated in the presence of $1,25(OH)_2D_3$ (10^{-8} M), no effect of this sterol was noted with either pOGH or p0.3GH in either the presence or absence of 10% FBS. However, $1,25(OH)_2D_3$ reduced by ~ 50% the stimulation by FBS in cells transfected with p1.2GH or p1.9GH (Fig. 9). This reduction was observed after 48 and 96 h of incubation.

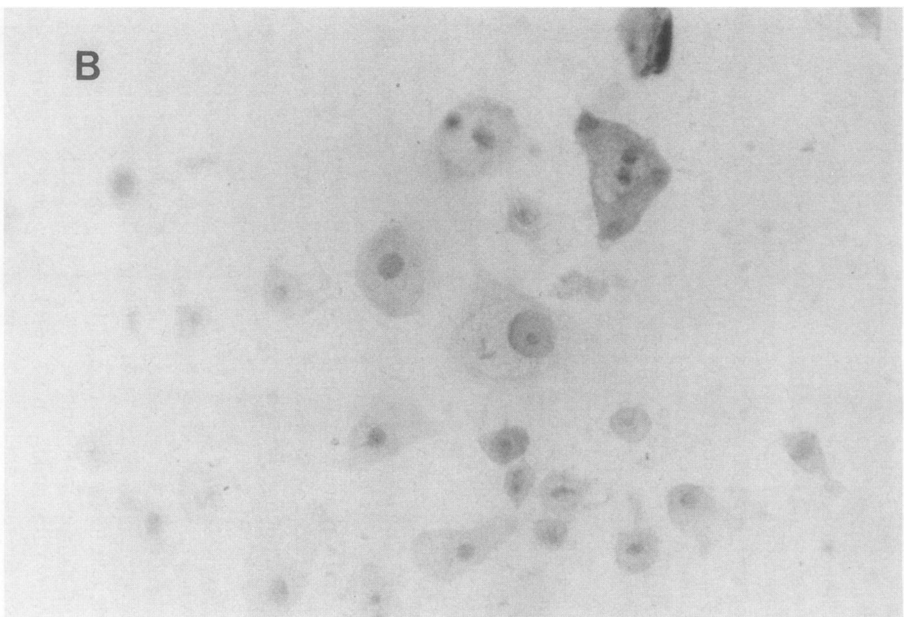
Effect of GF and $1,25(OH)_2D_3$ on PLP gene promoter activity. When construct p1.9GH was transfected into cultured human keratinocytes, addition of GF caused a fourfold increase in the immunoassayable hGH in the medium relative to non-GF-treated (control) cells (Fig. 10). This contrasted with the 10-fold increase in immunoreactive hGH levels observed when cells were stimulated with 10% FBS. Addition of $1,25(OH)_2D_3$ reduced the response by ~ 50% in GF- as well as FBS-treated cells.

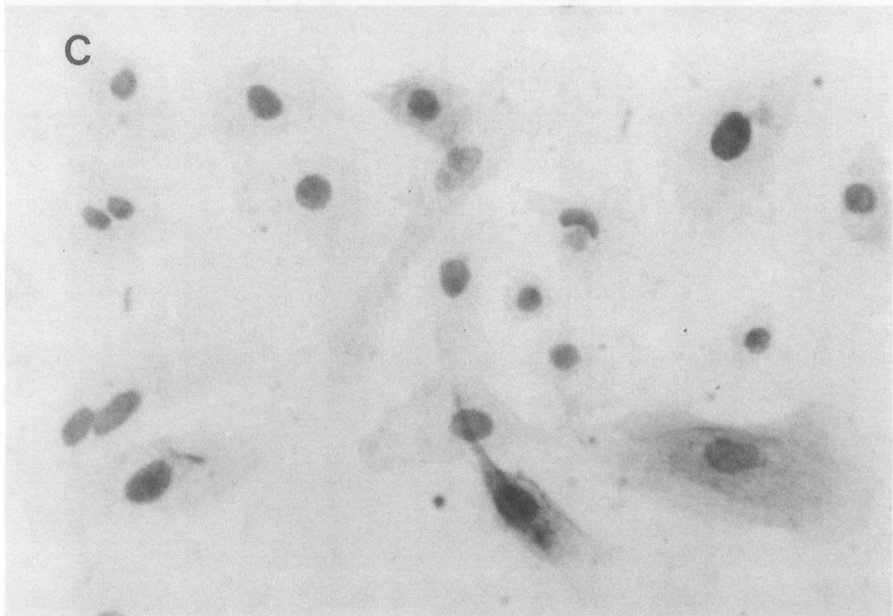
Influence of calcium concentration on effects of GF, FBS, and $1,25(OH)_2D_3$ on PLP gene promoter activity. Table III shows the immunoassayable hGH levels in the medium of cells transfected with construct p1.9GH and cultured in either low (0.15 mM) or high (1.0 mM) Ca^{++}. Varying the calcium concentration did not influence the stimulatory effect of growth factors or serum or the inhibitory effects of $1,25(OH)_2D_3$ on PLP gene promoter activity.

Discussion

PLP, initially discovered in association with malignancies, has now been shown, as well, to be expressed in a number of normal tissues, including keratinocytes (20–22). Although previous studies have reported regulation of PLP gene expression in tumor cell lines (18, 19), in this study we have examined regulation of expression and release of the peptide from primary cultures of normal human keratinocytes. GF, such as transforming growth factor-α, are known to be synthesized by keratinocytes which also appear capable of responding to these agents (30, 31). Although EGF per se slightly increased PLP accumulation in conditioned medium, this was not a statistically significant effect perhaps owing to the relatively small number of replicates performed. More substantial increases were observed with GF mixtures. We therefore assessed the influence of GF mixtures and of serum on PLP mRNA expression and on PLP secretion into conditioned medium. These positive regulators rapidly enhanced PLP mRNA expression (by one hour) and concomitantly increased PLP secretion.







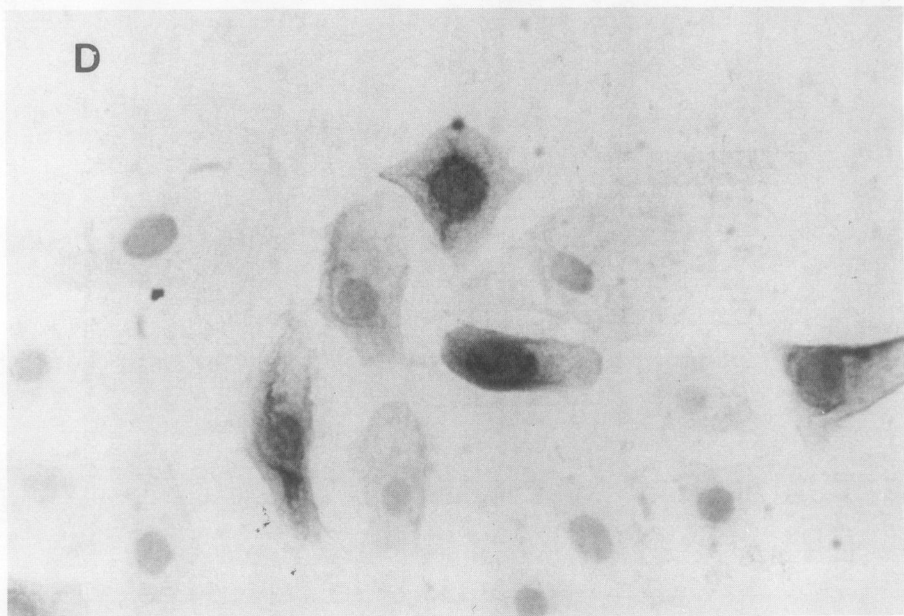


Figure 3. Immunocytochemical stain of cultured normal human keratinocytes for high molecular weight keratin. Keratinocytes were fixed, permeabilized, and processed for immunocytochemistry as described in Methods. No counterstain was used so that the dark staining immunoperoxidase reaction is evident. Cells were cultured in (*A*) 0.15 mM Ca^{++}, (*B*) in 0.15 mM Ca^{++} and $1,25(OH)_2D_3$, (*C*) in 1.0 mM Ca^{++}, and (*D*) in 1.0 mM Ca^{++} and $1,25(OH)_2D_3$. Control incubations with nonimmune serum or an irrelevant immune serum did not stain. ×100.

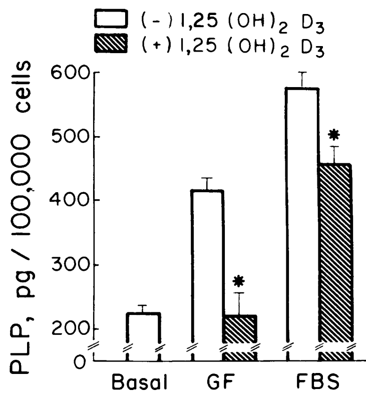


Figure 4. Effect of $1,25(OH)_2D_3$ on PLP secretion in the presence of GF and FBS. Normal human keratinocytes were incubated for 24 h in KBM plus insulin-hydrocortisone and 0.15 mM Ca^{++} (*Basal*); or in the same medium with 10% FBS or with bovine pituitary extract (0.4%) plus EGF (10 ng/ml) (*GF*) without (*open bars*) or with (*hatched bars*) $1,25(OH)_2D_3$ (10^{-8} M). PLP in the medium was determined by radioimmunoassay at 24 h. Each bar represents the mean±SEM of triplicate determinations. Asterisks represent significant differences from corresponding incubations performed in the absence of $1,25(OH)_2D_3$ at $P < 0.01$. The data shown are representative of four separate experiments.

Although multiple species of PLP mRNA have been reported in extracts of skin, one dominant transcript and occasionally one additional transcript has been reported in primary cultures of keratinocytes (20, 21). Furthermore, single PLP mRNA transcripts have also been reported in analyses performed with other cultured cells (22, 44). Whether the appearance of single or dual transcripts in cultured keratinocytes relates to differences in origin of the human keratinocytes, differences in preparation or differences in culture conditions is uncertain. Nevertheless, multiple PLP transcripts may not al-

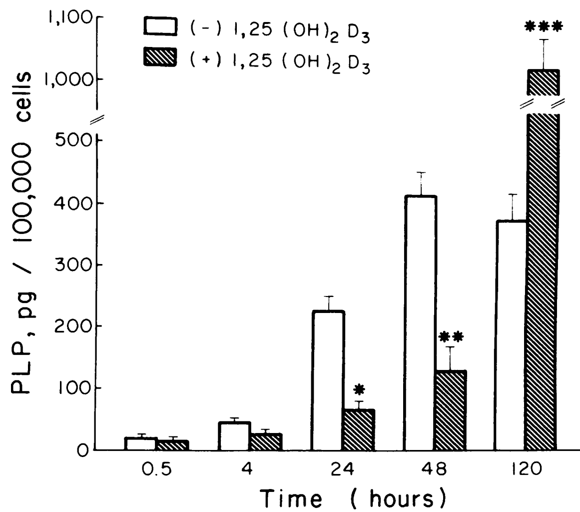


Figure 5. Time course of PLP secretion in the presence of GF, with or without $1,25(OH)_2D_3$. Normal human keratinocytes were incubated in KGM and 0.15 mM Ca^{++} without (*open bars*) or with (*hatched bars*) $1,25(OH)_2D_3$ as described in Methods. Medium was changed after 72 h and replaced with KGM and 0.15 mM Ca^{++} without or with $1,25(OH)_2D_3$. Aliquots of medium were removed at timed intervals for PLP measurements. Each bar is the mean±SEM of triplicate determinations. The asterisks indicate significant differences in PLP concentrations from incubations without $1,25(OH)_2D_3$ over the same time interval at $P < 0.01$ (*), $P < 0.001$ (**), and $P < 0.0001$ (***). The data shown are representative of triplicate experiments.

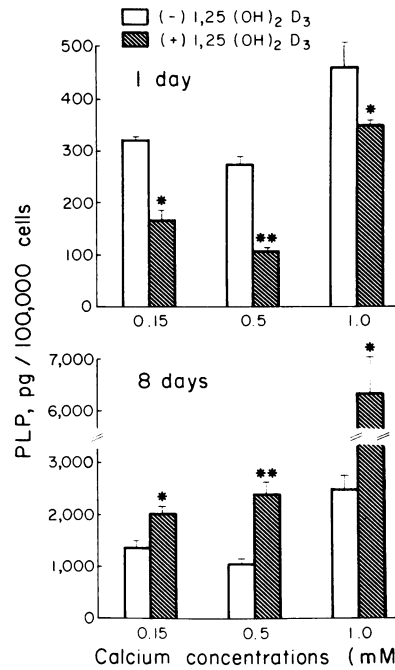


Figure 6. Effect of calcium and $1,25(OH)_2D_3$ on PLP secretion. Normal human keratinocytes were incubated in KGM and increasing calcium concentrations without (*open bars*) or with (*hatched bars*) $1,25(OH)_2D_3$ (10^{-8} M) for 1 or 8 d. PLP was measured by radioimmunoassay as described in Methods. Each bar is the mean±SEM of triplicate determinations. The asterisks indicate significant differences in PLP concentrations from incubations at the same calcium concentration but without $1,25(OH)_2D_3$ at $P < 0.005$ (*) and $P < 0.001$ (**). The results are representative of three separate experiments.

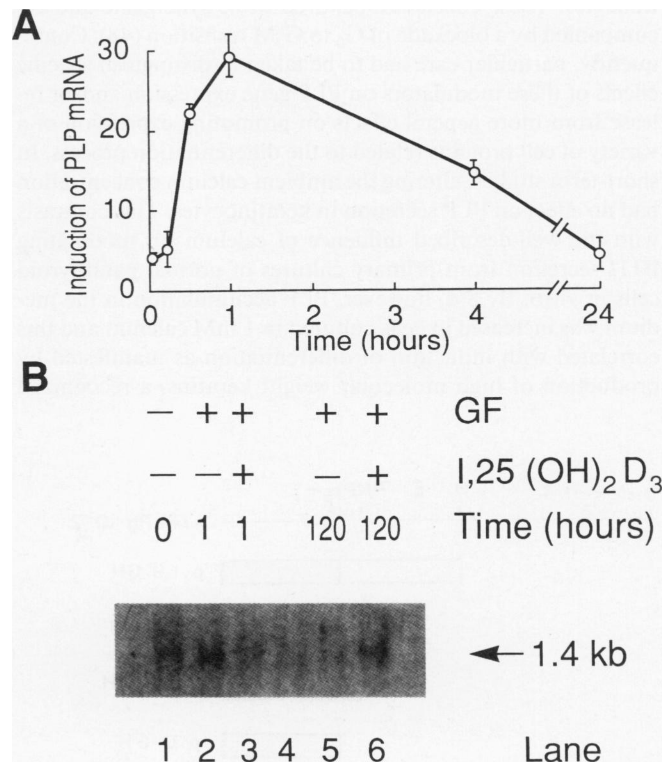


Figure 7. Influence of GF and $1,25(OH)_2D_3$ on PLP gene expression in human keratinocytes. (*A*) The influence of GF on the time course of PLP mRNA expression in keratinocytes was first assessed by dot blot analysis as described in Methods. (*B*) The influence of GF and $1,25(OH)_2D_3$ on PLP mRNA expression was then determined at 1 and 120 h of incubation, by Northern blot analysis, as described in Methods. Each lane contained 10 μg of total cellular RNA. The results are representative of three separate experiments.

Table II. Influence of Serum and Vitamin D on Control Promoter Activity in the hGH Transient Transfection Assay

Medium	hGH
	ng/ml
Basal	26.7±5
FBS	32.3±3
FBS + 1,25 $(OH)_2D_3$	33.3±3

Immunoassayable hGH was measured in medium of keratinocytes transfected with pONCGH. Basal indicates levels obtained in the absence of 10% FBS and 10^{-8} M $1,25(OH)_2D_3$. Each value is the mean±SEM of triplicate determinations. No significant differences in measured concentrations of hGH were observed.

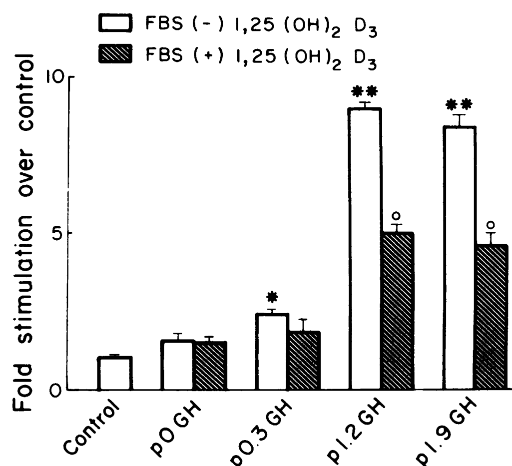


Figure 9. Stimulation of rat PLP gene promoter activity by FBS and inhibition by $1,25(OH)_2D_3$. Cultured human keratinocytes were transfected with the constructs described in Fig. 8, and were incubated in basal medium with insulin-hydrocortisone and 0.15 mM Ca^{++} and either 10% FBS alone (*open bars*) or 10% FBS plus 10^{-8} M $1,25(OH)_2D_3$ (*hatched bars*), as described in Methods. At 96 h, aliquots of the medium were taken and hGH levels measured by radioimmunoassay. Control refers to hGH levels in the medium of cells not transfected with the constructs and was unmeasurable. Control incubations were assigned the detection limits of the assay (20 pg/ml) and a value of 1. Concentrations of hGH in the medium of cells transfected with p0.3GH, p1.2GH, and p.1.9 GH and incubated in basal medium with insulin-hydrocortisone and 0.15 mM Ca^{++} were equivalent to those shown for pOGH. The results shown are the mean±SEM of triplicate determinations. Asterisks indicate a significant difference from control value: $*P < 0.05$; $**P < 0.001$. Open circle indicates a significant difference from incubations performed in the absence of $1,25(OH)_2D_3$: $^{\circ}P < 0.001$. The results are representative of four separate experiments.

ways be observed despite the possibility of alternative splicing during PLP gene expression.

In view of the fact that calcium and $1,25(OH)_2D_3$ are known to influence the regulation of synthesis of the related peptide, PTH, (45–47) and that calcium is the major modulator of PTH secretion, we also assessed the influence of calcium and $1,25(OH)_2D_3$ on PLP production and release in keratinocytes. Both the calcium ion and $1,25(OH)_2D_3$ are also known to have profound effects on the control of differentiation of keratinocytes. These effects are believed to be synergistic and accompanied by a blockade of G_0 to G_2M transition (48). Consequently, particular care had to be taken to distinguish specific effects of these modulators on PLP gene expression and/or release from more general effects on promoting expression of a variety of cell proteins related to the differentiation process. In short-term studies, altering the ambient calcium concentration had no effect on PLP secretion in keratinocytes. This contrasts with the well-described influence of calcium on modulating PTH secretion from primary cultures of normal parathyroid cells in vitro. By 8 d, however, PLP accumulation in the medium was increased in cells cultured in 1 mM calcium and this correlated with induction of differentiation as manifested by production of high molecular weight keratins, a recognized marker of keratinocyte differentiation (43). Long-term culture with $1,25(OH)_2D_3$ also enhanced PLP mRNA levels and accumulation in conditioned medium. This again appeared to correlate with the more generalized influence of the sterol on inducing keratinocyte differentiation, and this effect was augmented by incubation in 1 mM calcium. Consequently, these delayed augmentations of PLP release may have reflected a more generalized increase in the stimulation of production of a variety of keratinocyte proteins. Whether this delayed PLP mRNA increase is due to alterations in gene transcription or stability awaits further study. Nevertheless, these findings suggest that in these primary cultures of normal keratinocytes enhanced PLP production is associated with the differentiation process.

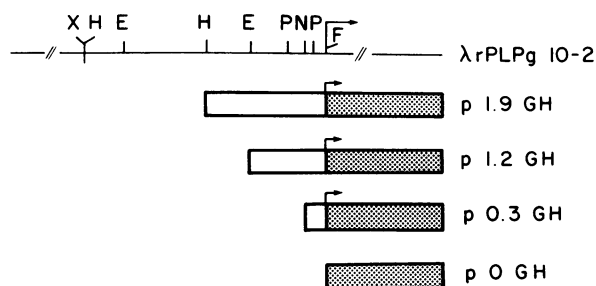


Figure 8. Chimeric constructs of rat PLP gene 5' flanking sequences and the hGH reporter gene used to transfect cultured keratinocytes. The open bars indicate the portions of the 5' flanking sequence of the rat PLP gene from recombinant clone rPLPg10-2 which were fused to the hGH structural gene (*hatched bar*) in plasmid pOGH as described in Methods. The orientation of the inserted restriction fragment is indicated by the arrow. Restriction enzyme sites: Xba I, X; Hind III, H; Eco RI, E; Pvu II, P; Nae I, N; and Fsp I, F.

The active metabolite, $1,25(OH)_2D_3$, has previously been reported to decrease PLP gene transcription in a human medullary thyroid carcinoma cell line (18) but to have no effect in a human carcinoid cell line (19). We have shown that, in short-term studies, $1,25(OH)_2D_3$ reduces PLP mRNA levels and PLP accumulation in conditioned medium of primary cultures of normal human keratinocytes. This effect is, therefore, analogous to the effect of $1,25(OH)_2D_3$ on PTH mRNA expression in primary cultures of normal parathyroid cells (38). These acute inhibitory effects of $1,25(OH)_2D_3$ in keratinocytes were not altered by changes in the ambient calcium concentration. Therefore, effects of $1,25(OH)_2D_3$ on PLP regulation may

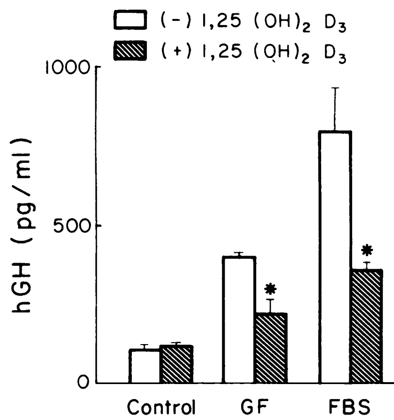


Figure 10. Effect of GF and FBS on rat PLP gene promoter activity in the absence and presence of $1,25(OH)_2D_3$. Cultured human keratinocytes were transfected with construct p1.9GH and treated, from left to right with either no FBS or GF (*Control, open bar*); $1,25(OH)_2D_3$, 10^{-8} M, alone (*Control, hatched bar*), GF alone (*open bar*); GF with $1,25(OH)_2D_3$ (*hatched bar*); 10% FBS alone (*open bar*); or 10% FBS with $1,25(OH)_2D_3$, 10^{-8} M (*hatched bar*); as described in Methods. After 96 h, aliquots of the medium were taken and hGH levels were measured by radioimmunoassay. Each bar is the mean±SEM of triplicate determinations. Asterisks indicate a significant difference between $1,25(OH)_2D_3$-treated and nontreated cells ($P < 0.001$). The results are representative of four separate experiments.

differ depending on the duration of exposure to the sterol and its influence on cell cycle–related events.

Our transient transfection analysis demonstrated that both GF and serum enhanced PLP promoter activity indicating that at least part of the stimulatory effect of these modulators on PLP production in keratinocytes resides at the level of augmenting gene expression. The difference in the apparent kinetics of the effect of growth factors and serum on enhancing endogenous PLP production by keratinocytes and on increasing hGH release during transient transfection assays may relate, at least in part, to the delay involved in handling a foreign protein, i.e., hGH by the keratinocytes. Our studies are consonant with others using the hGH transient transfection system who have shown that GH secretion is only detected after 24 h and is maximally increased at 48–96 h (40).

Table III. Influence of Calcium Concentration on the Stimulatory Effect of Growth Factors and Serum and on the Inhibitory Effect of Vitamin D on PLP Gene Promoter Activity

Medium	hGH	
	0.15 mM Ca^{++}	1.0 mM Ca^{++}
	pg/ml	
Control*	92±13*	115±25
GF‡	410±20	400±25
GF + $1,25(OH)_2D_3$‡	220±50§	146±35‖
FBS‡	620±70	503±35
FBS + $1,25(OH)_2D_3$	353±22¶	363±35¶

* Immunoassayable hGH was measured in medium of keratinocytes transfected with p1.9 GH. Control indicates levels obtained in the absence of GF and FBS. Each value is the mean±SEM of triplicate determinations.
‡ Concentrations of GF, $1,25(OH)_2D_3$ and FBS were as described in Methods.
Values of cells with significant differences from cells incubated in the same calcium concentration with GF or FBS but without $1,25(OH)_2D_3$ are indicated at $P < 0.05$ (¶), $P < 0.01$ (§) and $P < 0.001$ (‖).

Our results indicate that PLP gene expression by serum is controlled in keratinocytes by elements lying < 1.2 kb 5′ to the cap site. *Cis*-acting elements responsive to serum, such as the short, conserved, enhancer-like element of c-*fos* or β-actin referred to as the serum response element (SRE) are known to regulate the transcription of these eukaryotic genes (49). We have sequenced 950 bp upstream of the cap site of the rat PLP gene (12) and, in this sequence, we do not find a consensus sequence for an SRE of the c-*fos*/β-actin type. Therefore, the SRE of the rat PLP gene may lie further upstream, or alternatively, may be unique. Additional studies with deleted portions of the rat PLP gene will be required to more precisely define this SRE.

Our studies also show that the PLP promoter contains a *cis*-acting element responsive to $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ is known to act on various genes in a manner similar to that of other steroid hormones. It is thought that the $1,25(OH)_2D_3$–receptor complex directly regulates gene transcription as a consequence of binding to genomic sequences that act as classic enhancers. A vitamin D responsive element (VDRE) has recently been identified in the human osteocalcin gene (50–52) and is related to sequences which are responsive to other members of the steroid hormone family of receptors. However, $1,25(OH)_2D_3$ stimulates the expression of the osteocalcin gene, whereas we have shown that the $1,25(OH)_2D_3$–receptor complex acts in an opposite way on PLP gene promoter activity when transfected in normal human keratinocytes. Therefore, the nature of the VDRE may be potentially quite distinct from the positive *cis*-acting element identified in the osteocalcin gene. Alternatively, since $1,25(OH)_2D_3$ may, in a time- and/or differentiation-dependent manner, exert either negative or positive effects on PLP gene expression in keratinocytes, two distinct VDREs may exist or a single VDRE may be present and function differently in different contexts by interacting with other transcription factors which confer either a positive or a negative responsiveness to the VDRE. Further analyses will be required to distinguish these possibilities.

We have, therefore, demonstrated that PLP is controlled in a well-regulated fashion in normal keratinocytes by both positive and negative modulators. We also demonstrated that the PLP gene promoter contains *cis*-acting elements capable of responding to these modulators. Identification of these regulatory factors, both extrinsic to the gene and within the PLP gene sequence, can now be used to explore potential sites of dysregulation associated with the overproduction of PLP. This may provide insights into the mechanism of excess PLP production which can occur during malignant transformation of squamous cells, such as keratinocytes.

Acknowledgments

We thank the Biotechnology Research Institute for accommodation to A.K., and Dr. R. Sawka for the immunocytochemical studies. We also thank Michael Sebag for his expert assistance in part of this work. We are grateful to D. Allen and W. Campbell for secretarial assistance.

This work was supported by a grant from the National Cancer Institute of Canada and by grants MT-5775 and MA-9315 from the Medical Research Council of Canada. R. Kremer is the recipient of a fellowship and G. N. Hendy of a scholarship from the Medical Research Council of Canada. J. Henderson is the recipient of a studentship from the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche de Québec (FCAR).

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