5'-Flanking Region of the Parathyroid Hormone Gene Mediates Negative Regulation by 1,25-(OH)₂ Vitamin D₃*

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To study the effects of 1,25-(OH)₂ vitamin D₃ on the transcription of the human parathyroid hormone (PTH) gene, 684 base pairs of the 5'-flanking portion of the human PTH gene were fused to the bacterial neo gene. The fusion gene was transfected into rat pituitary cells, and mixed populations of colonies were selected using the neomycin analog, G418. The level of RNA initiated from the human PTH gene promoter region in these cells was suppressed by 1,25-(OH)₂ vitamin D₃. Synthesis of the same transcript under control of a viral promoter was not regulated by 1,25-(OH)₂ vitamin D₃. The effect of 1,25-(OH)₂ vitamin D₃ was detected within 24 h at physiologic doses of 1,25-(OH)₂ vitamin D₃, and was not influenced by addition of cycloheximide. Thus 1,25-(OH)₂ vitamin D₃ acts on the 5'-flanking portion of the PTH gene to decrease the rate of transcription by a mechanism that requires no new protein synthesis.

The level of ionized calcium in the extracellular fluid of terrestrial vertebrates is tightly regulated by the polypeptide, parathyroid hormone (PTH), and by the secosteroidal hormone, 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂ D₃), the active metabolite of vitamin D. PTH acts on bone and kidney to raise blood calcium; increases in blood calcium decrease the absorption of dietary calcium. The two calcium-regulating hormones each influence the biosynthesis of the other. PTH activates renal 25-hydroxyvitamin D₁ 1-hydroxylase, the last step in the synthesis of 1,25-(OH)₂ D₃ (1, 2). The regulators of PTH biosynthesis have been less well-defined, but recent studies using dispersed bovine parathyroid cells suggest that increased levels of calcium and 1,25-(OH)₂ D₃ lead to a fall in PTH mRNA levels (3, 4), and PTH gene transcription (5). The effects of 1,25-(OH)₂ D₃ on PTH mRNA levels and gene transcription rates (6) have also been demonstrated after administration of the hormone to rats in vivo.

In order to define gene sequences responsible for regulating PTH gene transcription, we have introduced portions of the human PTH (hPTH) gene into cultured cells (7). Since no established parathyroid cell lines have been available, rat pituitary GH₃C (GH₃) cells were used as host cells. These cells were chosen because the level of prolactin mRNA in these cells has been shown to be modulated by addition of calcium (8), or 1,25-(OH)₂ D₃ (8, 9) to the medium. Igarashi et al (7) showed that, in these cells, the PTH promoter on the plasmid pni101 (described in detail below) directs transcription that starts at the same sites used in human parathyroid glands. Here, we examine whether calcium and/or 1,25-(OH)₂ D₃ regulate PTH gene transcription in transfected GH₃ cells and explore the mechanism of this regulation.

EXPERIMENTAL PROCEDURES

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The abbreviations used are: PTH, human parathyroid hormone; hPTH, human parathyroid hormone; 1,25-(OH)₂ D₃, 1,25-dihydroxyvitamin D₃, 24,25-(OH)₂ D₃, 24,25-dihydroxyvitamin D₃, 25-OHD₃, 25-hydroxyvitamin D₃, hp, base pairs; kb, kilobase (pairs); LTR, long terminal repeat.

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by the method of Chirgwin et al. (12). The filter was hybridized with the neo probe (Fig. 1A) and then washed as in Northern blot analysis. Using the identical hybridization and washing conditions, we also used a prolactin probe (900 bp of PstI fragment of rat prolactin cDNA, pPRLl (15)), tubulin probe (1700 bp of PstI fragment of rat α-tubulin cDNA pPLoT1 (16)), and actin probe (2000 bp of HindIII fragment of chicken β-actin cDNA, pAI (17)). Three independent RNA preparations (each was isolated from two or three 100-cm² dishes) were analyzed for each condition of culture. RNA from each group was blotted twice. Dots were scanned with a densitometer (Helena Laboratories Quick Scan Jr., TX), and the height of each peak was measured.

Cell Culture Conditions and Regulation Studies—Rat pituitary GH₄, cells were propagated in Dulbecco's modified Eagle's essential medium containing 10% calf serum, 50 units/ml of penicillin, and 50 µg/ml of streptomycin. The medium was changed every 3 days and the day before the preparation of RNA or transfection. GH₄ cells stably transfected with pnlOl (pnlOl-GH₄ cells) were grown in Dulbecco's modified Eagle's essential medium containing 10% calf serum and the neomycin analog, G418 (400 µg/ml). For regulation experiments, cells (5–6 x 10⁶ cells/100-cm² dish) were placed in chemically defined medium (11) containing no serum, 1 mM calcium, and no 1,25-(OH)₂D₃. Two days later medium was changed to chemically defined medium containing either no calcium or 1,25-(OH)₂D₃, 1 mM calcium and no 1,25-(OH)₂D₃, or 1 mM calcium and 1,25-(OH)₂D₃. The medium was changed again two days later (day 4). On day 5, cells were harvested and total RNA was extracted (12).

To examine the effects of cycloheximide on the action of 1,25-(OH)₃D₃, cycloheximide, 3 µg/ml, was added to the medium at the time of addition of 1,25-(OH)₃D₃, and cells were harvested 1 day later. In a second, longer experiment, cycloheximide, 1 µg/ml, was added to the medium at the time of addition of 1,25-(OH)₃D₃ and again at the time the medium was changed 2 days later. Cells were harvested on the third day. Dot blots of RNA from mixed pools of pnlOl-GH₄ cells were prepared and densitometric analyses were performed. In separate experiments, for the last 5 h of incubation, 10 µCi of [³H]leucine were added in the presence of cycloheximide. After the cells were rinsed with Dulbecco's modified Eagle's medium without serum, they were lysed with 1 ml of the buffer containing 0.15 M sodium chloride, 0.01 M Tris-Cl (pH 7.2), 1% Triton X-100, 0.1% sodium deoxycholate, 5 mM EDTA, 0.02% sodium azide, and 5% phenylethylsulfonyl fluoride. Then, cells were scraped with a rubber policeman and forced through a 20 gauge needle several times. After 10 µl of this lysate was put onto a Whatman No. 3MM filter disc, trichloroacetic acid-precipitable material was counted as described (20).

RESULTS
PnlOl-GH₄ Cells—Plasmid pnlOl (Fig. 1A) uses 684 bp of the promoter region of the hPTH gene to control expression of the neo gene from E. coli. The neo gene follows by the splicing and polyadenylation signals from pSV2neo (10), and a distal portion of the hPTH gene, which consists of exon 3 and 2 kb of 3'-flanking sequence (7, 18). This latter portion was included for convenience of plasmid construction. This plasmid was introduced into GH₄ cells by

![Fig. 1. Plasmid pnlOl](image-url)

The methods to construct pnlOl have been described (7). The 1-kb BglII-Acl fragment of pnlOl was nick-translated and used as a neo probe for Northern blot and RNA dot blot analysis. The 1700-bp BglII-HpaI fragment from plasmid pPTHgt168 (7) was also nick-translated and used as an exon 3 probe for Northern blot analysis. The 630-bp BglII-HindIII fragment was labeled with polynucleotide kinase, and single-stranded DNA (labeled at the BglII site) isolated from a strand-separation gel was used for S1 nuclease analysis (13). Only one of the six PstI sites is shown in the figure. This PstI site is just distal to the transcription initiation site (See Fig. 3). Plasmid pALTn101 is identical to pnlOl except in the region marked ASV (avian sarcoma virus). This region represents the avian sarcoma viral long terminal repeat, substituted for the PTH gene's promoter region. Neither plasmid is drawn precisely to scale.
electroporation (pnl101-GH4 cells) (7, 19). Individual colonies of cells surviving G418 selection (400 μg/ml), as well as mixed pools of 50–60 colonies, were studied.

Southern blot analysis (data not shown) demonstrated that, in individual clones, at least 10 rearranged copies of pnl101 were incorporated per diploid genome, several in tandem array. The copy number was similar (range 10–20 copies) in all clones; mixed pools of the colonies yielded a similar pattern. Moreover, these patterns were also seen after 20–30 passages of the cells.

**Regulation of RNA by 1,25-(OH)2D3 and Calcium**—To study the effects of 1,25-(OH)2D3 and calcium on the activity of the hPTH promoter, these modulators were added separately and together to pnl101-GH4 cells as described under “Experimental Procedures.” The ratio of total RNA or DNA to cell number was not affected by 1,25-(OH)2D3 or calcium. Plates of cells grown in medium containing calcium contained more cells at the end of the experiment than plates grown in the absence of calcium. Cells grown in the absence of calcium adhere to plates less well; presumably some cells were lost during the medium change on day 2 of the protocol. 1,25-(OH)2D3 administration did not affect cell number.

Northern blot analysis (Fig. 2A) using the neo-coding sequence as a probe (Fig. 1A) revealed a prominent band of 2.4 kb, the predicted size for a transcript starting from the hPTH promoter and ending at the SV40 polyadenylation site. The start sites of this major mRNA have been previously defined in both S1 nuclease and primer extension assays as two clusters downstream from two closely spaced TATA homologies (7). Upon long exposure of the autoradiogram (Fig. 2B), four bands binding the neo probe were visualized. The two bands containing RNA larger than 2.4 kb in Fig. 2B probably represent transcripts not terminated by the SV40 polyadenylation signal; rehybridization of the same filter with a PTH gene exon 3 probe (Fig. 1A) revealed only these two bands (data not shown). The faint band containing the smallest RNA (Fig. 2B) may represent prematurely terminated transcript. The amount of the 2.4-kb transcript from cells exposed to no calcium or 1,25-(OH)2D3 (Fig. 2A, lane a) is greater than the amounts of transcript from cells grown in the other conditions (Fig. 2A, lanes b–d), suggesting that the presence of either calcium or 1,25-(OH)2D3 caused a fall in the amount of the hPTH promoter-driven transcript. Analysis using S1 nuclease provided similar data (not shown). Fig. 2B shows that all four neo transcripts showed similar change after treatment with calcium or 1,25-(OH)2D3. Thus, calcium and 1,25-(OH)2D3 affect the level of hPTH promoter-driven transcript without changing the start sites used or the size of the resultant mRNA. Effects of calcium and 1,25-(OH)2D3 were 20–40% of maximal in 1 day, and reached a maximum after 3–4 days (data not shown).

**Promoter Specificity of Gene Regulation**—The experiments described so far do not demonstrate that hPTH-specific sequences in pnl101 are responsible for the effects of 1,25-(OH)2D3 and calcium and cannot separate the effects of the nontranscribed hPTH promoter region from those of the small portion of the transcribed hPTH sequence fused to the neo gene. To make these distinctions, we transfected GH4 cells with the two control plasmids, pSV2neo (10) and pALTn101 (Fig. 1B), and mixed pools of the selected colonies were analyzed. Plasmid pSV2neo contains no hPTH gene sequences. The mRNA transcribed from pSV2neo is expected to be similar to that from pnl101, although the first portion of the mRNA comes from the SV40 early region instead of from the hPTH gene. The Northern blot shown in Fig. 3A confirms that neo mRNA from pSV2neo-transfected cells (lane e) is, as expected, slightly larger than mRNA from pnl101-GH4 cells. The second control plasmid, pALTn101, differs from pnl101 only in that it substitutes a retroviral avian sarcoma viral LTR promoter for the hPTH promoter. This promoter is followed by the same short transcribed sequence from human PTH gene exon 3. Northern blot analysis (Fig. 3A, lane d) shows that neo mRNA from pALTn101-GH4 cells comigrates with mRNA from pnl101-GH4 cells. S1 nuclease analysis was used to define more precisely the start sites of transcription in pnl101-GH4 and pALTn101-GH4 cells. A probe labeled at the BglII site in the PTH gene’s exon I was hybridized to human parathyroid gland mRNA. Lane h, Fig. 3B, shows that, as previously reported (7), PTH gene transcripts originate from two closely spaced clusters of sequences. Each cluster is found approximately 30 nucleotides downstream from a TATA homology. The micro heterogeneity within each cluster is found under a variety of digestion and hybridization conditions and is also revealed by primer extension analysis (7). Thus, this microheterogeneity may represent true microheterogeneity of start sites, or may represent artifacts of the methods of analysis. Lanes i and j of Fig. 3B show that, as previously reported (7), transcripts in pnl101-GH4 cells use the same start sites used by normal PTH mRNA. (The bands corresponding to mRNA cluster 2 are more clearly seen on a longer exposure of the autoradiogram). Lane k compares these transcripts with the transcripts in pALTn101-GH4 cells. Using a probe from pALTn101 that is labeled at the BglII site in the PTH gene’s exon I and extends back into the retroviral promoter region, the pALTn101 start site can be seen to start the same distance from the PTH gene’s BglII site as do PTH mRNA in cluster (2). Lane m, in which mRNA from pnl101-GH4 cells was hybridized to the pALTn101 probe, documents the extent of sequences in common (60 bases, from the PstI to BglII sites indicated in Fig. 1A) between pnl101-GH4 mRNA and pALTn101-GH4 mRNA. Thus, the pALTn101-GH4 mRNA is the same length as pnl101-GH4 mRNA and differs in sequence only in the first seven to eight bases.
Regulation of PTH Gene Transcription by 1,25-(OH)2D3

Fig. 3. Analysis of RNA generated in GH4 cells transfected with the three plasmids. A. Northern blot analysis. 30 µg (lanes a, b, c, and e) and 5 µg (lane d) of total RNA were blotted, and nick-translated neo probe was used for hybridization. Lane a, one clone of GH4 cells transfected with pn101. Lane b, GH4 cells without transfected DNA. Mixed pools of GH4 cells transfected with pn101 (lane c), pALTn101 (lane d), and pSV2neo (lane e) are also shown. B. S1 nuclease analysis. The end-labeled single-stranded probes in lanes f-j and lanes k-m are described in the adjacent drawing. Lane f, GH4 cells without transfected DNA; lane g, mixed pool of pSV2neo-GH4 cells; lane h, human parathyroid adenoma poly(A) RNA; lane i, one clone of pn101-GH4 cells; lane j, mixed pool of pn101-GH4 cells; lane k, mixed pool of pALTn101-GH4 cells; lane l, GH4 cells without transfected DNA; lane m, mixed pool of pn101-GH4 cells. The single (lanes f-j) and double (lanes k-m) black triangles indicate the undigested probes, respectively. In lanes i, f, g, j, k, and m, 30 µg of total RNA was loaded. In lane h, 5 µg of total RNA was used. In lane h, 1 µg of poly(A) RNA was used. C, schematic representation of probes and RNA analyzed in S1 nuclease assay shown in B. Asterisks indicate ends of fragments labeled with 32P. The short, dotted region at the beginning of mRNA 4 indicates the short portion of PTH mRNA not hybridizing to the pALTn101 probe.

Fig. 4. Regulation of transfected genes by 1,25-(OH)2D3 and calcium. RNA samples were denatured and blotted onto a nylon membrane filter. The filter was hybridized with the neo probe and then washed in the same condition as in Northern hybridization. 15 µg of total RNA from the mixed pool of pn101-GH4 cells (left), pSV2neo-GH4 cells (middle), and pALTn101-GH4 cells (right) were blotted onto each dot. Dots analyzed were shown to give signals within a linear dose-response interval. The peak height of the conditions of no calcium or 1,25-(OH)2D3 vitamin D3 was arbitrarily considered to be 100. The numbers represent mean ± S.E.

Fig. 4 presents the results of quantitative dot blot analyses of calcium-1,25-(OH)2D3 experiments, using GH4 cells transfected with pn101, pSV2neo, and pALTn101. Three independently isolated RNA preparations were analyzed for each condition; each RNA preparation was blotted twice. Exposure to 1,25-(OH)2D3 or calcium led to an approximately 2-fold fall in hPTH promoter-driven neo transcript, confirming the results of Fig. 2. RNA from GH4 cells with no transfected DNA gave no detectable signal (not shown). The calcium experiments using the cells transfected with the control plasmids led to surprising results. 1 mM of added calcium suppressed the level of neo mRNA, even when the neo gene was driven by the viral promoters and the mRNA contained no sequences from the PTH gene (pSV2neo/GH4). This result means that the effect of calcium on neo mRNA levels in these transfected GH4 cells is unrelated to any specific effect on the hPTH gene or its transcript. In contrast, the suppressive effect of 1,25-(OH)2D3 was only seen when the neo gene was driven by the hPTH promoter. When the neo mRNA was driven by the viral promoters, 1,25-(OH)2D3 had no effect. Therefore, we can conclude that the inhibitory effect of 1,25-(OH)2D3 requires the presence of the hPTH promoter region. Furthermore, because the transcripts derived from pn101 and pALTn101 differ only in the first few bases, we can also conclude that 1,25-(OH)2D3 acts on the 684-bp promoter region at the level of primary gene transcription, rather than on mRNA stability or mRNA processing.

The effect of 1,25-(OH)2D3 on the hPTH promoter occurred over a range of doses typical of those used in other studies of cultured cells. Doses as low as 10^-11 M of 1,25-(OH)2D3 showed
some inhibitory effect (30% inhibition), and this effect reached a maximum at $10^{-10}$ M. On the other hand, neither 24,25-(OH)$_3$D$_3$ or 25-OHD$_3$ showed any significant inhibition even at the dose of $10^{-8}$ M (data not shown).

**Effect of Cycloheximide on the Regulation of 1,25-(OH)$_2$D$_3$—**
The effect of 1,25-(OH)$_2$D$_3$ on the hPTH promoter could be a direct action of the hormone and its receptor on the hPTH promoter; alternatively, 1,25-(OH)$_2$D$_3$ could stimulate the synthesis of a protein(s) which then would regulate the hPTH promoter. To ask whether the 1,25-(OH)$_2$D$_3$ effect requires new protein synthesis, cycloheximide, 3 µg/ml (for 1 day) or 1 µg/ml (for 3 days), was added just before the addition of 1,25-(OH)$_2$D$_3$ (Fig. 5). Neither the basal amount of the mRNA at each time point nor the inhibitory effect of 1,25-(OH)$_2$D$_3$ was affected by cycloheximide, even though protein synthesis was inhibited by 85 to 95%. Thus, the effect of 1,25-(OH)$_2$D$_3$ on hPTH promoter-derived transcription requires no new protein synthesis.

**DISCUSSION**

Russell et al. (3) and Silver et al. (4) have shown that increases in the level of extracellular calcium lead to decreased PTH gene transcription and decreased PTH mRNA levels in dispersed bovine parathyroid cells. Here, we show that calcium has only a nonspecific suppressive effect on transcripts of pnlOl in GH$_4$ cells. Calcium administration led to a fall in neo mRNA levels in GH$_4$ cells transfected with plasmids analogous to pnlOl but missing the hPTH promoter (pSV2neo and pALTn101). The endogenous mRNA encoding tubulin and actin also fell after calcium addition (data not shown). The rather general suppressive effect of calcium may make it difficult to detect any further specific response of the PTH gene to changes in extracellular calcium in these cells. The intracellular response to changes in extracellular calcium may well differ in GH$_4$ cells and parathyroid cells. For example, changes in extracellular calcium lead to greater changes in intracellular calcium in parathyroid cells (20, 21) than in rat pituitary cells. It is also possible that portions of the PTH gene missing from pnlOl may be required for a specific response to calcium. In any case, we can only conclude that GH$_4$ cells transfected with pnlOl do not demonstrate specific effects of calcium on PTH gene expression.

In contrast, the effect of 1,25-(OH)$_2$D$_3$ on hPTH promoter-driven transcription is demonstrable in GH$_4$ cells transfected with pnlOl. In physiologically relevant doses, 1,25-(OH)$_2$D$_3$ administration leads to a fall in hPTH promoter-driven transcription; this effect occurs in the absence of new protein synthesis. The effect is a quantitatively modest one, when contrasted to the more dramatic fall in PTH mRNA levels found in intact rats given 1,25-(OH)$_2$D$_3$ (6). The 2-fold effects found here do correspond roughly to those found by Silver et al. (4), when they added 1,25-(OH)$_2$D$_3$ to dispersed bovine parathyroid cells. It is possible that the multiple copies of the PTH gene in GH$_4$ cells may not all be regulatable or may titrate out important regulatory molecules. Alternatively, GH$_4$ cells, which may contain fewer receptors for 1,25-(OH)$_2$D$_3$ than parathyroid cells (9, 22) may not be able to respond to 1,25-(OH)$_2$D$_3$ as vigorously as parathyroid cells. Further, pnlOl may be missing portions of the PTH gene necessary for directing a quantitatively more vigorous response to 1,25-(OH)$_2$D$_3$.

Recently, 1,25-(OH)$_2$D$_3$ has been shown to increase the rate of transcription of the rat osteocalcin gene in osteosarcoma cells (23) and to increase dramatically the level of mRNA encoding calcium-binding protein in the small intestine after administration in vivo (24). On the other hand, 1,25-(OH)$_2$D$_3$ is known to decrease the steady-state level of the c-myc gene transcript in human leukemic cells (25). 1,25-(OH)$_2$D$_3$ binds to an intracellular receptor analogous in structure to other steroid hormone receptors (1, 2); presumably, this receptor then acts by binding to specific genomic sequences. A working hypothesis consistent with our data is that the 1,25-(OH)$_2$D$_3$ receptor binds to sequences in the 684-bp promoter region of the hPTH gene and that this binding leads to a decrease in transcription of the gene. Future studies are needed to define more precisely the specific DNA sequences responsive to 1,25-(OH)$_2$D$_3$ to determine whether the 1,25-(OH)$_2$D$_3$ receptor binds to these sequences, and to determine the mechanism of down-regulation of transcription.

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Regulation of PTH Gene Transcription by 1,25-(OH)₂D₃