10 Vitamin D and the Parathyroids

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Abstract The administration of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2$D] results in a dramatic decrease in PTH mRNA levels in rats with no change in serum calcium levels. The administration of 1,25$(OH)_2$D or its analogs has become an important part of the management of secondary hyperparathyroidism due to chronic renal failure. 1,25$(OH)_2$D acts to decrease PTH gene transcription. Two mechanisms may play a role in the transrepression. First, a VDRE has been described in the PTH promoter, which mediates PTH gene transrepression in transfected cells. In addition, an alternative E-box-like motif has been identified as another class of nVDRE in the human 1α-hydroxylase promoter. Negative regulation of the hPTH gene by liganded 1,25$(OH)_2$D receptor (VDR) is mediated by VDR-interacting repressor, the VDIR, directly binding to the E-box-type nVDRE at the promoter. Post-transcriptional mechanisms of PTH mRNA regulation in experimental kidney failure and after changes in dietary calcium and phosphorus are pertinent to the understanding of PTH expression. These involve the binding of *trans*-acting factors to a defined *cis*-acting instability element in the PTH mRNA 3′-untranslated region. The bone-derived hormone, fibroblast growth factor (FGF) 23 is increased by phosphate and 1,25$(OH)_2$D and acts on the kidney to cause a phosphaturia and decrease 1,25$(OH)_2$D synthesis. FGF23 acts on the parathyroid to decrease PTH expression.

Key Words: Parathyroid gland; parathyroid hormone; VDR; 1,25-dihydroxyvitamin D; VDRE; FGF23; PTH gene; calcium; phosphate; vitamin D analogs

1. INTRODUCTION

The action of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2$D] or its analogs to decrease PTH secretion is now a well-established axiom in clinical medicine for the suppression of the secondary hyperparathyroidism of patients with chronic kidney disease. So much so, that it is worthwhile to reflect upon its scientific basis. That is the purpose of the present review. There is ongoing academic and commercial activity in the development of drugs that may have more selective actions on the parathyroid while leading to less hypercalcemia. These attempts are of great clinical and pharmaceutical interest but still remain to be proven by rigorous scientific testing and therefore despite the extensive literature on the subject, the final word on the analogs awaits prospective outcome studies *(1, 2)* and are not discussed in detail in this chapter.

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2. THE PARATHYROID HORMONE GENE

2.1. The PTH Gene

The human parathyroid hormone (PTH) gene is localized on the short arm of chromosome 11 at 11p15 *(3, 4)*. The human and bovine genes have two functional TATA transcription start sites, and the rat only one. The two homologous TATA sequences flanking the human PTH gene direct the synthesis of two human PTH gene transcripts both in normal parathyroid glands and in parathyroid adenomas *(5)*. The PTH genes in all species that have been cloned have two introns or intervening sequences and three exons *(6)*. Strikingly, even though fish do not have discrete parathyroid glands, they do synthesize PTH using two distinct genes that share the same exon–intron pattern found in tetrapod PTH genes *(7, 8)*. The locations of the introns are identical in each case *(9)*. Intron A splits the 5′ untranslated sequence of the mRNA five nucleotides before the initiator methionine codon. Intron B splits the fourth codon of the region that codes for the prosequence of preProPTH. The three exons that result, thus, are roughly divided into three functional domains. Exon 1 contains the 5′ untranslated region. Exon 2 codes for the presequence or signal peptide and exon 3 codes for PTH as well as the 3′ untranslated region. It is interesting that the human gene is considerably longer in both intron A and the 3′ untranslated region of the cDNA compared to the bovine, rat, and mouse. The genes for PTH and PTHrP (PTH-related protein) are located in similar positions on sibling chromosomes 11 and 12. It is therefore likely that they arose from a common precursor by chromosomal duplication.

2.2. The PTH mRNA

Complementary DNA encoding for human *(10, 11)*, bovine *(12, 13)*, rat *(14)*, mouse *(15)*, pig *(14)*, chicken *(16, 17)*, dog *(18)*, cat *(19)*, horse *(20)*, macaca *(21)*, fugu fish *(7),* and zebrafish *(8)* PTH have all been cloned *(9)*. The PTH gene is a typical eukaryotic gene with consensus sequences for initiation of RNA synthesis, RNA splicing, and polyadenylation. The primary RNA transcript consists of RNA transcribed from both introns and exons, and then RNA sequences derived from the introns are spliced out. The product of this RNA processing, which represents the exons, is the mature PTH mRNA, which will then be translated into preproPTH. There is considerable identity among mammalian PTH genes, which is reflected in an 85% identity between human and bovine proteins and 75% identity between human and rat proteins. There is less identity in the 3′-noncoding region. A more extensive review of the structure and sequences of the PTH gene has been published elsewhere *(9)* in the book *Molecular Biology of the Parathyroid (22)*.

3. DEVELOPMENT OF THE PARATHYROID AND TISSUE-SPECIFIC EXPRESSION OF THE PTH GENE

The thymus, thyroid, and parathyroid glands in vertebrates develop from the pharyngeal region, with contributions both from pharyngeal endoderm and from neural crest cells in the pharyngeal arches. Studies of gene knockout mice have shown that the hoxa3, pax 1, pax 9, and Eya1 transcription factors are needed to form parathyroid

glands as well as many other pharyngeal pouch derivatives, such as the thymus. Glial cells missing2 (Gcm2), a mouse homologue of *Drosophila* Gcm, is a transcription factor whose expression is restricted to the parathyroid glands *(23)*. A human patient with a defective Gcm B gene, the human equivalent of Gcm-2, exhibited hypoparathyroidism and complete absence of PTH from the bloodstream *(24)*. The parathyroid gland of tetrapods and the gills of fish both express Gcm-2 and require this gene for their formation *(25)*. They also showed that the gill region expresses mRNA encoding the two PTH genes found in fish, as well as mRNA encoding the calcium-sensing receptor.

4. PROMOTER SEQUENCES

Regions upstream of the transcribed structural gene often determine tissue specificity and contain many of the regulatory sequences for the gene. For PTH, analysis of this region has been hampered by the lack of a parathyroid cell line. It has been shown that the 5 kb of DNA upstream of the start site of the human PTH gene was able to direct parathyroid gland-specific expression in transgenic mice *(26)*. Analysis of the human PTH promoter region identified a number of consensus sequences by computer analysis *(27)*. These included a sequence resembling the canonical cAMP-responsive element 5′-TGACGTCA-3′ at position –81 with a single residue deviation. This element was fused to a reporter gene (CAT) and then transfected into different cell lines. Pharmacological agents that increase cAMP led to an increased expression of the CAT gene, suggesting a functional role for the cAMP-responsive element (CRE). Specificity protein (Sp) and the nuclear factor-Y (NF-Y) complex are thought to be ubiquitously expressed transcription factors associated with basal expression of a host of gene products. Sp family members and NF-Y can cooperatively enhance transcription of a target gene. There is a highly conserved Sp1 DNA element present in mammalian PTH promoters *(28)*. Coexpression of Sp proteins and NF-Y complex leads to synergistic transactivation of the hPTH promoter, with alignment of the Sp1 DNA element essential for full activation *(28)*. The presence of a proximal NF-Y-binding site in the hPTH promoter highlights the potential for synergism between distal and proximal NF-Y DNA elements to strongly enhance transcription *(29)*.

Several groups have identified DNA sequences that might mediate the negative regulation of PTH gene transcription by 1,25-dihydroxyvitamin D [1,25$(OH)_2$D]. Demay et al. *(30)* identified DNA sequences in the human PTH gene that bind the 1,25$(OH)_2$D receptor. Nuclear extracts containing the 1,25$(OH)_2$D receptor were examined for binding to sequences in the 5′-flanking region of the hPTH gene. A 25-bp oligonucleotide containing sequences from –125 to –101 from the start of exon 1 bound nuclear proteins that were recognized by monoclonal antibodies against the 1,25$(OH)_2$D receptor. The sequences in this region contained a single copy of a motif (AGGTTCA) that is homologous to the motifs repeated in the upregulatory 1,25$(OH)_2$D response element of the osteocalcin gene. When placed upstream to a heterologous viral promoter, the sequences contained in this 25-bp oligonucleotide mediated transcriptional repression in response to 1,25$(OH)_2$D in GH4C1 cells but not in ROS 17/2.8 cells. Therefore, this downregulatory element differs from upregulatory elements both in sequence composition and in the requirement for particular cellular factors other than the 1,25$(OH)_2$D receptor (VDR) for repressing PTH transcription *(30)*. Russell et al. *(31)* have shown that there

are two negative VDREs in the rat PTH gene. One is situated at –793 to –779 and bound a VDR/RXR heterodimer with high affinity and the other at –60 to –746 bound the heterodimer with a lower affinity. Transfection studies with VDRE-CAT constructs showed that they had an additive effect. Liu et al. *(32)* have identified such sequences in the chicken PTH gene and demonstrated their functionality after transfection into the opossum kidney (OK) cell line. They converted the negative activity imparted by the PTH VDRE to a positive transcriptional response through selective mutations introduced into the element. They showed that there was a p160 protein that specifically interacted with a heterodimer complex bound to the wild-type VDRE, but was absent from complexes bound to response elements associated with positive transcriptional activity. Thus, the sequence of the individual VDRE appears to play an active role in dictating transcriptional responses that may be mediated by altering the ability of a VDR/RXR heterodimer to interact with accessory factor proteins. Further work is needed to demonstrate that any of these differing negative VDREs function in this fashion in parathyroid cells.

The transrepression by $1,25(OH)_2D$ has also been shown to be dependent upon another promoter element. Kato's laboratory have identified an E-box (CANNTG)-like motif as another class of nVDRE in the human 1α-hydroxylase promoter *(33, 34)*. In sharp contrast to the previously reported DR3-like motif in the hPTH gene promoter, a basic helix-loop-helix factor, designated VDR interacting repressor (VDIR), transactivates through direct binding to this E-box-type element (1nVDRE). However, the VDIR transactivation function is transrepressed through ligand-induced protein–protein interaction of VDIR with VDR/RXR. In the absence of $1,25(OH)_2D$, VDIR appears to bind to 1nVDRE for transactivation through the histone acetylase (HAT) coactivator, p300/CBP. Binding of $1,25(OH)_2D$ to VDR induces interaction with VDIR and dissociation of the HAT coactivator, resulting in recruitment of histone deacetylase (HDAC) corepressor for ligand-induced transrepression *(34)*. They have also characterized the functions of VDIR and E-box motifs in the human (h) PTH and hPTHrP gene promoters *(35)*. They identified E-box-type elements acting as nVDREs in both the hPTH promoter (hPTHnVDRE –87 to –60 bp) and in the hPTHrP promoter (hPTHrPnVDRE –850 to –600 bp, –463 to –104 bp) in a mouse renal tubule cell line. The hPTHnVDRE alone was enough to direct ligand-induced transrepression mediated through VDR/retinoid X receptor and VDIR. Direct DNA binding of hPTHnVDRE to VDIR, but not VDR/retinoid X receptor, was observed and ligand-induced transrepression was coupled with recruitment of VDR and histone deacetylase 2 (HDAC2) to the hPTH promoter. They concluded that negative regulation of the hPTH gene by liganded VDR is mediated by VDIR directly binding to the E-box-type nVDRE at the promoter, together with recruitment of an HDAC corepressor for ligand-induced transrepression *(35)*. These studies were specific to a mouse proximal tubule cell line and await the development of a parathyroid cell line to confirm them in a homologous cell system.

5. REGULATION OF PTH GENE EXPRESSION

5.1. 1,25-Dihydroxyvitamin D

PTH regulates serum concentrations of calcium and phosphate, which, in turn, regulate the synthesis and secretion of PTH. $1,25(OH)_2D$ has independent effects on

calcium and phosphate levels and also participates in a well-defined feedback loop between $1,25(OH)_2D$ and PTH *(36)*.

$1,25(OH)_2D$ potently decreases transcription of the PTH gene (Fig. 2). This action was first demonstrated in vitro in bovine parathyroid cells in primary culture, where $1,25(OH)_2D$ led to a marked decrease in PTH mRNA levels *(37, 38)* and a consequent decrease in PTH secretion *(39–42)*. The physiological relevance of these findings was established by in vivo studies in rats *(43)*. The localization of $1,25(OH)_2D$ receptor mRNA (VDR mRNA) to parathyroids was demonstrated by in situ hybridization studies of the thyroparathyroid and duodenum (Fig. 1). VDR mRNA was localized to the parathyroids in the same concentration as in the duodenum, the classic target organ of $1,25(OH)_2D$ *(44)*. Rats injected with amounts of $1,25(OH)_2D$ that did not increase serum calcium had marked decreases in PTH mRNA levels, reaching <4% of control at 48 h. This effect was shown to be transcriptional both in in vivo studies in rats *(43)* and in in vitro studies with primary cultures of bovine parathyroid cells *(45)*. When 684 bp of the 5′-flanking region of the human PTH gene was linked to a reporter gene and transfected into a rat pituitary cell line (GH4C1), gene expression was lowered by

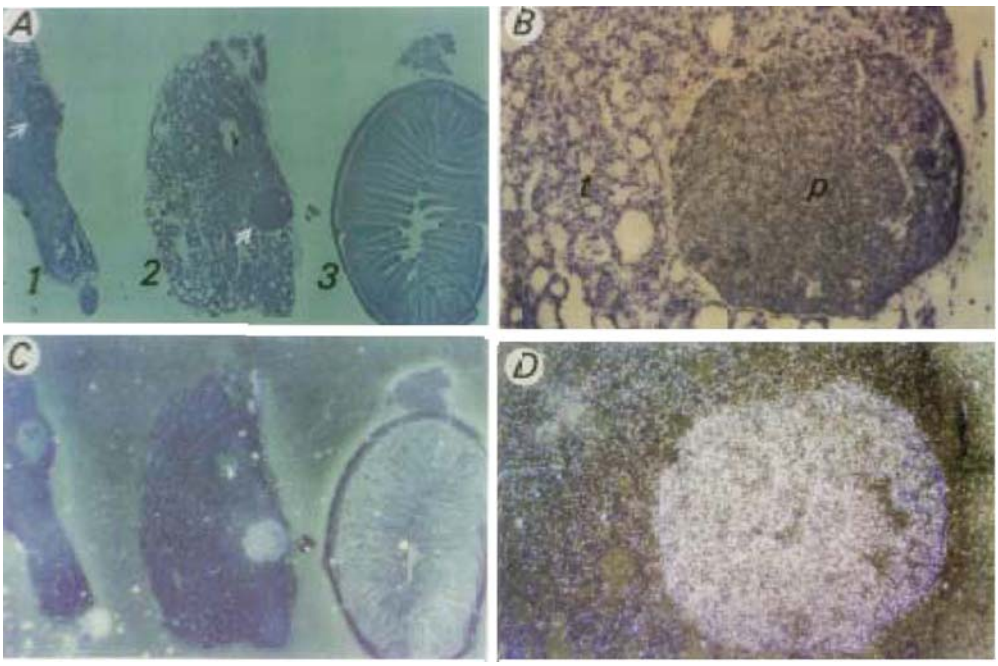

Fig. 1. The $1,25(OH)_2D$ receptor (VDR) is localized to the parathyroid in a similar concentration to that found in the duodenum, indicating that the parathyroid is a physiological target organ for $1,25(OH)_2D$. In situ hybridization with the VDR probe in rat parathyroid–thyroid sections. (**a**) and (**c**) Parathyroid–thyroid tissue from a control rat (*left section*) and from a $1,25(OH)_2D$-treated rat (100 pmol at 24 h) (*middle preparation*). Duodenum from a $1,25(OH)_2D$-treated rat. *White arrows* point to parathyroid glands. (**b**) and (**d**) Higher power view of the parathryoids in (**a**) and (**c**). *Top figures* were photographed under bright-field illumination, whereas *bottom figures* show dark-field illumination of the same sections. Reproduced with permission from Naveh-Many et al. *(44)*.

$1,25(OH)_2D$ *(46)*. These studies suggest that $1,25(OH)_2D$ decreases PTH transcription by acting on the 5′-flanking region of the PTH gene, probably at least partly through interactions with the vitamin D receptor-binding sequences and/or the E box that binds VDIR noted earlier. The effect of $1,25(OH)_2D$ may involve heterodimerization with the retinoid acid receptor. This is because 9 *cis*-retinoic acid, which binds to the retinoic acid receptor, when added to bovine parathyroid cells in primary culture, led to a decrease in PTH mRNA levels *(47)*. Moreover, combined treatment with 1×10^{-6} M retinoic acid and 1×10^{-8} M $1,25(OH)_2D$ decreased PTH secretion and preproPTH mRNA more effectively than either compound alone *(47)*. Alternatively, retinoic acid receptors might synergize with VDRs through actions on distinct sequences.

A further level at which $1,25(OH)_2D$ might regulate the PTH gene would be at the level of the $1,25(OH)_2D$ receptor. $1,25(OH)_2D$ acts on its target tissues by binding to the $1,25(OH)_2D$ receptor, which regulates the transcription of genes with the appropriate recognition sequences. Concentration of the VDR in $1,25(OH)_2D$ target sites could allow a modulation of the $1,25(OH)_2D$ effect, with an increase in receptor concentration leading to an amplification of its effect and a decrease in receptor concentration dampening the $1,25(OH)_2D$ effect. Naveh-Many et al. *(44)* injected $1,25(OH)_2D$ into rats and measured the levels of VDR mRNA and PTH mRNA in the parathyroid tissue. They showed that $1,25(OH)_2D$ in physiologically relevant doses led to an increase in VDR mRNA levels in the parathyroid glands in contrast to the decrease in PTH mRNA levels. Weanling rats fed a diet deficient in calcium were markedly hypocalcemic at 3 weeks and had very high serum $1,25(OH)_2D$ levels. Despite the chronically high serum $1,25(OH)_2D$ levels, there was no increase in VDR mRNA levels. Furthermore, PTH mRNA levels did not fall and were increased markedly. The low calcium in the bloodstream may have prevented the increase in parathyroid VDR levels, which may partially explain PTH mRNA suppression.

5.1.1. Calreticulin and the Action of $1,25(OH)_2D$ on the PTH Gene

Calreticulin is a calcium-binding protein present in the endoplasmic reticulum of the cell and may also have a nuclear function. It regulates gene transcription via its ability to bind a protein motif in the DNA-binding domain of nuclear hormone receptors of sterol hormones. It has been shown to prevent vitamin D's binding and action on the osteocalcin gene in vitro *(48)*. Sela-Brown et al. *(49)* showed that calreticulin might inhibit the action of vitamin D on the PTH gene. Both rat and chicken VDRE sequences of the PTH gene were incubated with recombinant VDR and retinoic acid receptor (RXR) proteins in a gel retardation assay and showed a clear retarded band. Purified calreticulin inhibited binding of the VDR–RXR complex to the VDREs in gel retardation assays. This inhibition was due to direct protein–protein interactions between VDR and calreticulin. OK cells were transiently cotransfected with calreticulin expression vectors and either rat or chicken PTH gene promoter-CAT constructs. The cells were then assayed for $1,25(OH)_2D$-induced CAT gene expression. $1,25(OH)_2D$ decreased PTH promoter-CAT transcription. Cotransfection with sense calreticulin, which increases calreticulin protein levels, completely inhibited the effect of $1,25(OH)_2D$ on the PTH promoters of

both rat and chicken. Cotransfection with the antisense calreticulin construct did not interfere with the effect of vitamin D on PTH gene transcription. Calreticulin expression had no effect on basal CAT mRNA levels. In order to determine a physiological role for calreticulin in regulation of the PTH gene, levels of calreticulin protein were determined in the nuclear fraction of rat parathyroids. The rats were fed either a control diet or a low calcium diet, which leads to increased PTH mRNA levels, despite high serum $1,25(OH)_2D$ levels that would be expected to inhibit PTH gene transcription *(49)*. It was postulated that high calreticulin levels in the nuclear fraction would prevent the effect of $1,25(OH)_2D$ on the PTH gene. In fact, hypocalcemic rats had increased levels of calreticulin protein, as measured by Western blots, in their parathyroid nuclear faction. This may help explain why hypocalcemia leads to increased PTH gene expression, despite high serum $1,25(OH)_2D$ levels, and may also be relevant to the refractoriness of the secondary hyperparathyroidism of many chronic renal failure patients to $1,25(OH)_2D$ treatment. These studies, therefore, indicate a role for calreticulin in regulating the effect of vitamin D on the PTH gene and suggest a physiological relevance to these studies *(49)*.

Russell et al. *(50)* studied the parathyroids of chicks with vitamin D deficiency and confirmed that $1,25(OH)_2D$ regulates PTH and VDR gene expression in the avian parathyroid gland. Brown et al. *(51)* studied vitamin D-deficient rats and confirmed that $1,25(OH)_2D$ upregulated parathyroid VDR mRNA. Rodriguez et al. *(52)* showed that administration of the calcimimetic R-568 resulted in increased VDR expression in parathyroid tissue. In vitro studies of the effect of R-568 on VDR mRNA and protein were conducted in cultures of whole rat parathyroid glands. Incubation of rat parathyroid glands in vitro with R-568 resulted in a dose-dependent decrease in PTH secretion and an increase in VDR expression. Together with previous work on the effect of extracellular calcium to increase parathyroid VDR mRNA in vitro *(53)*, they concluded that activation of the CaR upregulates the parathyroid VDR mRNA.

All these studies show that $1,25(OH)_2D$, and calcium in certain circumstances, increases the expression of the VDR gene in the parathyroid gland, which would result in increased VDR protein synthesis and increased binding of $1,25(OH)_2D$. This ligand-dependent receptor upregulation would lead to an amplified effect of $1,25(OH)_2D$ on the PTH gene and might help explain the dramatic effect of $1,25(OH)_2D$ on the PTH gene.

Vitamin D may also amplify its effect on the parathyroid by increasing the activity of the calcium receptor (CaR). Canaff et al. *(54)* showed that in fact there are VDREs in the human CaR's promoter. The calcium-sensing receptor (CaR), expressed in parathyroid chief cells, thyroid C-cells, and cells of the kidney tubule, is essential for maintenance of calcium homeostasis. They showed that parathyroid, thyroid, and kidney CaR mRNA levels increased twofold at 15 h after intraperitoneal injection of $1,25(OH)_2D$ in rats. Functional VDREs have been identified in the CaR gene and probably provide the mechanism whereby $1,25(OH)_2D$ upregulates parathyroid, thyroid C-cell, and kidney CaSR expression.

The use of $1,25(OH)_2D$ is limited by its hypercalcemic effect, and therefore a number of $1,25(OH)_2D$ analogs have been synthesized that are biologically active but are less

hypercalcemic than $1,25(OH)_2D$ *(55)*. These analogs usually involve modifications of the $1,25(OH)_2D$ side chain, such as 22-oxa-$1,25(OH)_2D$, which is the chemical modification in oxacacitriol *(56)*, or a cyclopropyl group at the end of the side chain in calcipotriol *(57, 58)*. Brown et al. *(59)* showed that oxacalcitriol in vitro decreased PTH secretion from primary cultures of bovine parathyroid cells with a similar dose response to that of $1,25(OH)_2D$. In vivo the injection of both vitamin D compounds led to a decrease in rat parathyroid PTH mRNA levels *(59)*. However, detailed in vivo dose–response studies showed that in vivo $1,25(OH)_2D$ is the most effective analog for decreasing PTH mRNA levels, even at doses that do not cause hypercalcemia *(60)*. Oxacalcalcitriol and calcipotriol are less effective for decreasing PTH RNA levels but have a wider dose range at which they do not cause hypercalcemia. This property might be useful clinically. Paricalcitol was shown to be effective at reducing PTH concentrations without causing significant hypercalcemia or hyperphosphatemia as compared to placebo. Paricalcitol treatment was shown to reduce PTH concentrations more rapidly with fewer sustained episodes of hypercalcemia and increased Ca × P product than $1,25(OH)_2D$ therapy *(61)*. The marked activity of $1,25(OH)_2D$ analogs in vitro as compared to their modest hypercalcemic actions in vivo probably reflects their rapid clearance from the circulation *(62)*. Despite the great interest in the development and marketing of new $1,25(OH)_2D$ analogs to decrease PTH gene expression and serum PTH levels without causing hypercalcemia, there have been few rigorous comparisons of their biological effects compared to those of $1,25(OH)_2D$ itself *(63, 64)*. There has been a lot of debate about the relative advantages of the so-called "nonhypercalcemic" vitamin D analogs over $1,25(OH)_2$ vitamin D_3. Drueke *(1)* has analyzed the clinical trials that have been performed and concluded that all clinical studies were retrospective in nature and suffered from the limitations of retrospective data analysis. The question is still open.

The ability of $1,25(OH)_2D$ to decrease PTH gene transcription is used therapeutically in the management of patients with chronic renal failure. They are treated with $1,25(OH)_2D$ or its prodrug 1(OH)D in order to prevent the secondary hyperparathyroidism of chronic renal failure. The poor response in some patients who do not respond may well result from poor control of serum phosphate, decreased VDR concentration in the patients' parathyroids *(65)*, an inhibitory effect of a uremic toxin(s) on VDR-VDRE binding *(66)*, or tertiary hyperparathyroidism with monoclonal parathyroid tumors *(67)*. The development of calcimimetic drugs, which act to directly activate the CaR has provided a significant advance in the treatment that we can offer patients with secondary hyperparathyroidism and in clinical use these drugs are frequently used in combination with $1,25(OH)_2D$ or its analogs.

5.1.2. Studies on Mice with VDR Gene Deletion

The VDR total knockout phenotype is characterized by high PTH levels, hypocalcemia, hypophosphatemia, bone malformations, rickets, and alopecia *(68, 69)*. Most, but not all of the phenotypes, were reversed by correcting the serum calcium concentration with a high calcium–lactose diet *(70)*. In order to investigate PTH regulation by the VDR in as close as possible to the physiological conditions, we have ongoing work where we have generated parathyroid-specific VDR knockout mice (*PT-VDR*$^{-/-}$),

by crossing PTH promoter-Cre mice with total body floxed-VDR mice. VDR expression was decreased specifically in the parathyroid glands of the *PT-VDR*$^{-/-}$ mice. These mice had a normal phenotype but their serum PTH levels were significantly increased with no change in serum calcium or phosphorus. The sensitivity of the parathyroid glands of the *PT-VDR*$^{-/-}$ mice to calcium was intact as measured by serum PTH levels after changes in serum calcium. Serum type I collagen C-telopeptides (CTX), a marker of bone resorption, was increased ($\times$2.5) in the *PT-VDR*$^{-/-}$ mice with no change in the bone formation marker, serum osteocalcin, consistent with a resorptive effect of the increased serum PTH levels in the *PT-VDR*$^{-/-}$ mice. Therefore, deletion of the VDR specifically in the parathyroid decreases parathyroid CaR expression and only moderately increases basal PTH levels, suggesting that the VDR has a limited role in parathyrodid physiology *(71)*.

5.2. Calcium

A remarkable characteristic of the parathyroid is its sensitivity to small changes in serum calcium, which leads to large changes in PTH secretion. This remarkable sensitivity of the parathyroid to increase hormone secretion after small decreases in serum calcium levels is unique to the parathyroid. All other endocrine glands increase hormone secretion after exposure to a high extracellular calcium. This calcium sensing is also expressed at the levels of PTH gene expression and parathyroid cell proliferation (Fig. 2).

Calcium and phosphate both have marked effects on the levels of PTH mRNA in vivo *(72)*. The major effect is for low calcium to increase PTH mRNA levels and low phosphate to decrease PTH mRNA levels. Naveh-Many et al. *(73)* studied rats in vivo. They showed that a small decrease in serum calcium from 2.6 to 2.1 mmol/l led to large increases in PTH mRNA levels, reaching threefold that of controls at 1 and 6 h. A high serum calcium had no effect on PTH mRNA levels even at concentrations as high as 6.0 mmol/l. Yamamoto et al. *(74)* also studied the in vivo effect of calcium on PTH mRNA levels in rats. They showed that hypocalcemia induced by a calcitonin infusion for 48 h led to a sevenfold increase in PTH mRNA levels. Rats made hypercalcemic (2.9–3.4 mM) for 48 h had the same PTH mRNA levels as controls that had received no infusion (2.5 mM). Therefore, hypercalcemia in vivo has a limited effect to decrease PTH mRNA levels. These results emphasize that the gland is geared to respond to hypocalcemia and not hypercalcemia.

5.2.1. Mechanisms of Regulation of PTH mRNA by Caclium

The mechanism whereby calcium regulates PTH gene expression is particularly interesting. Changes in extracellular calcium are sensed by a calcium sensor that then regulates PTH secretion *(75, 76)*. Signal transduction from the CaSR involves activation of phospholipase C, D, and A_2 enzymes *(77)*. The response to changes in serum calcium involves the protein phosphatase type 2B, calcineurin *(78)*. In vivo and in vitro studies demonstrated that inhibition of calcineurin by genetic manipulation or pharmacologic agents affected the response of PTH mRNA levels to changes in extracellular calcium *(78)*.

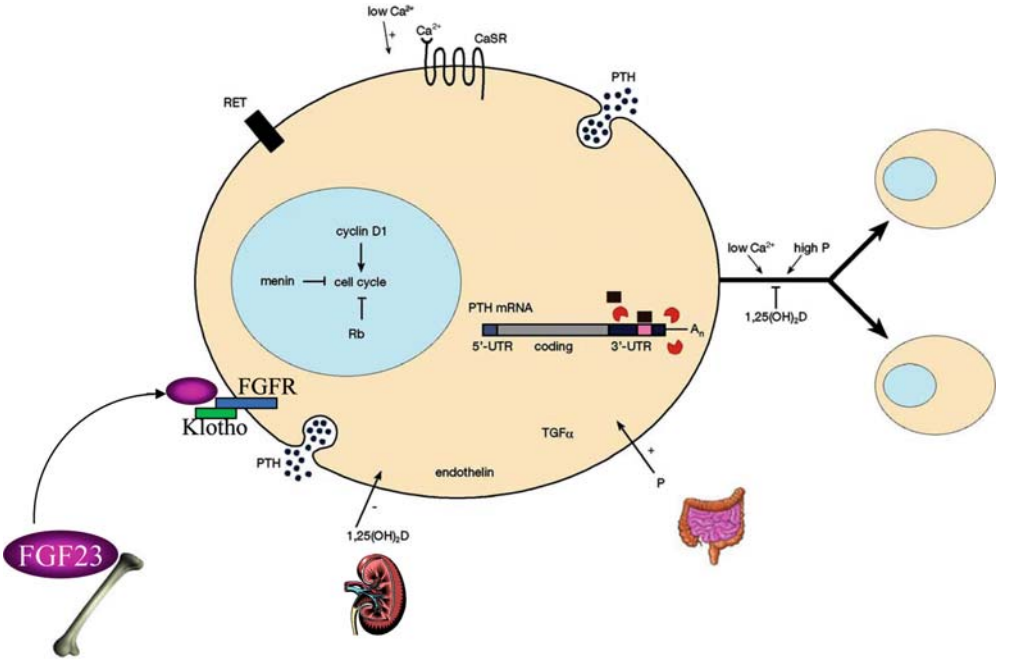

Fig. 2. Regulation of parathyroid proliferation, gene expression, and secretion. Cyclin D1 driven by the PTH promoter and activating mutations of the menin gene are known to cause parathyroid adenomas. Germ-line mutations of the latter cause MEN 1. The very rare parathyroid carcinomas show lack of expression of the retinoblastoma protein (pRb). Activating mutations of the RET proto-oncogene result in MEN 2a. A low serum calcium leads to a decreased activation of the CaR and results in increased PTH secretion, PTH mRNA stability, and parathyroid cell proliferation. A high serum phosphate leads to similar changes in all these parameters. $1,25(OH)_2D$ decreases PTH gene transcription markedly and decreases parathyroid cell proliferation. A high serum phosphate and chronic kidney disease lead to an increase in FGF23 secretion by osteocytes, which then acts on the kidney to decrease phosphate reabsorption and inhibit the synthesis of $1,25(OH)_2D$ and on the parathyroid to decrease PTH expression. Endothelin and TGFα are increased in the parathyroids of proliferating parathyroid glands. PTH mRNA stability is regulated by parathyroid cytosolic proteins (*trans* factors) binding to a short defined *cis* sequence in the PTH mRNA 3′-UTR and preventing degradation by ribonucleases in a process involving KSRP recruitment of the exosome. The defined protective proteins are AUF1 and Unr. In hypocalcemia there is more binding of the stabilizing *trans* factors to the *cis* sequence leading to a more stable transcript. A low serum phosphate leads to much less binding and a rapidly degraded PTH transcript. (Modified from Silver with permission *(99)*).

Moallem et al. *(79)* have performed in vivo studies on the effect of hypocalcemia on PTH gene expression. The effect is post-transcriptional in vivo and involves protein–RNA interactions at the 3′-untranslated region of the PTH mRNA *(79)*. A similar mechanism is involved in the effect of phosphate on PTH gene expression, so the mechanisms involved will be discussed after the independent effect of phosphate on the PT is considered.

5.3. Phosphate

5.3.1. Phosphate Regulates the Parathyroid Independently of Calcium and 1,25(OH)$_2$D

The demonstration of a direct effect of high phosphate on the parathyroid in vivo has been difficult. One of the reasons is that the various maneuvers used to increase or decrease serum phosphate invariably leads to a change in the ionized calcium concentration. A number of careful clinical and experimental studies suggested that the effect of phosphate on serum PTH levels was independent of changes in both serum calcium and 1,25(OH)$_2$D levels. In dogs with experimental chronic renal failure, Lopez-Hilker et al. *(80)* have shown that phosphate restriction corrected their secondary hyperparathyroidism independent of changes in serum calcium and 1,25(OH)$_2$D levels. They did this by placing the uremic dogs on diets deficient in both calcium and phosphate. This led to lower levels of serum phosphate and calcium, with no increase in the low levels of serum 1,25(OH)$_2$D. Despite this, there was a 70% decrease in PTH levels. This study suggested that, at least in chronic renal failure, phosphate affected the parathyroid cell by a mechanism independent of its effect on serum 1,25(OH)$_2$D and calcium levels *(80)*. Therefore, phosphate plays a central role in the pathogenesis of secondary hyperparathyroidism, both by its effect on serum 1,25(OH)$_2$D and calcium levels and possibly independently. A raised serum phosphate also stimulates the secretion of FGF23 which in turn decreases PTH gene expression and serum PTH levels *(81, 82)*. This effect would act as a counterbalance to the stimulatory effect of phosphate on the parathyroid and is discussed separately in this chapter.

Kilav et al. *(83)* bred second-generation vitamin D-deficient rats and then placed the weanling vitamin D-deficient rats on a diet with no vitamin D, low calcium, and low phosphate. After one night of this diet, serum phosphate had decreased markedly with no changes in serum calcium or 1,25(OH)$_2$D. These rats with isolated hypophosphatemia had marked decreases in PTH mRNA levels and serum PTH. However, the very low serum phosphates in these in vivo studies may have no direct relevance to possible direct effects of high phosphate in renal failure. It is necessary to separate nonspecific effects of very low phosphate from true physiologic regulation. To establish that the effect of serum phosphate on the parathyroid was indeed a direct effect, in vitro confirmation was needed, which was provided by three groups. Rodriguez was the first to show that increased phosphate levels increased PTH secretion from isolated parathyroid glands in vitro. The effect required maintainance of tissue architecture *(84–86)*. The effect was found in whole glands or tissue slices but not in isolated cells. The requirement for intact tissue suggests either that the sensing mechanism for phosphate is damaged during the preparation of isolated cells or that the intact gland structure is important to the phosphate response.

Parathyroid responds to changes in serum phosphate at the level of secretion, gene expression, and cell proliferation, although the mechanism of these effects is unknown (Fig. 2). The effect of high phosphate to increase PTH secretion may be mediated by phospholipase A$_2$-activated signal transduction. Bourdeau et al. *(87, 88)* showed that arachidonic acid and its metabolites inhibit PTH secretion. Almaden *(89)* showed

in vitro that a high phosphate medium increased PTH secretion, which was prevented by the addition of arachidonic acid.

5.4. Protein–PTH mRNA Interactions Determine the Regulation of PTH Gene Expression by Serum Calcium and Phosphate

The clearest rat in vivo models for effects of calcium and phosphate on PTH gene expression are diet-induced hypocalcemia with a large increase in PTH mRNA levels and diet-induced hypophosphatemia with a large decrease in PTH mRNA levels. In both instances the effect was post-transcriptional, as shown by nuclear transcript run-on experiments *(72)*. Parathyroid cytosolic proteins were found to bind in vitro transcribed PTH mRNA *(79)*. Interestingly, this binding was increased with parathyroid proteins from hypocalcemic rats (with increased PTH mRNA levels) and decreased with parathyroid proteins from hypophosphatemic rats (with decreased PTH mRNA levels). Proteins from other tissues bound to PTH mRNA, but this binding is regulated by calcium and phosphate only with parathyroid proteins. Intriguingly, binding requires the presence of the terminal 60 nucleotides of the PTH transcript.

Naveh-Many and colleagues utilized an in vitro degradation assay to study the effects of hypocalcemic and hypophosphatemic parathyroid proteins on PTH mRNA stability *(79)*. This assay reproduced the differences in PTH mRNA levels observed in vivo. Moreover, the difference in RNA stability by the parathyroid extracts was totally dependent on an intact 3′-untranslated region (UTR) and, in particular, on the terminal 60 nucleotides. Proteins from other tissues in these rats were not regulated by calcium or phosphate. Therefore, calcium and phosphate change the properties of parathyroid cytosolic proteins, which bind specifically to the PTH mRNA 3′-UTR and determine its stability (Fig. 2). What are these proteins?

5.4.1. Identification of the PTH mRNA 3′-UTR-Binding Proteins that Determine PTH mRNA Stability

5.4.1.1. AU-rich Binding Factor (AUF1). Sela-Brown et al. and Dinur et al. have utilized affinity chromatography using the PTH RNA 3′-UTR to isolate these RNA-binding proteins *(90, 91)*. The proteins, which bind the PTH mRNA, are also present in other tissues, such as brain, but only in the parathyroid their binding is regulated by calcium and phosphate. A major band from the eluate of a PTH 3′-region RNA affinity chromatography was identical to AU-rich binding factor (AUF1) *(91)*. Recombinant AUF1 bound the full-length PTH mRNA and the 3′-UTR. Added recombinant AUF1 also stabilized the PTH transcript in the in vitro degradation assay. These results showed that AUF1 is a protein that binds to the PTH mRNA 3′-UTR and stabilizes the PTH transcript.

The regulation of protein–PTH mRNA binding involves post-translational modification of AUF1. AUF1 levels are not regulated in PT extracts from rats fed calcium and phosphorus depleted diets. However, two-dimensional gels showed post-translational modification of AUF1 that included phosphorylation *(78)*. There is no parathyroid cell

line, but a PTH mRNA *cis* acting 63-nt element *(92)* is recognized in HEK 293 cells as an instability element. RNA interference for AUF1 decreased human PTH secretion in cotransfection experiments *(78)*.

Most patients with chronic kidney disease develop secondary hyperparathyroidism with disabling systemic complications. Calcimimetic agents are effective tools in the management of secondary hyperparathyroidism, acting through allosteric modification of the calcium-sensing receptor (CaR) on the parathyroid gland to decrease PTH secretion and parathyroid cell proliferation. R-568 decreased both PTH mRNA and serum PTH levels in adenine high phosphorus-induced chronic kidney disease *(93)*. The effect of the calcimimetic on PTH gene expression was post-transcriptional and correlated with differences in protein–RNA binding and post-translational modifications of the *trans*-acting factor AUF1 in the parathyroid. The AUF1 modifications as a result of uremia were reversed to those of normal rats by treatment with R-568. Therefore, uremia and activation of the CaR mediated by calcimimetics modify AUF1 post-translationally. These modifications in AUF1 correlate with changes in protein–PTH mRNA binding and PTH mRNA levels *(93)*.

5.4.1.2. Unr (Upstream of N-*Ras*). A second parathyroid cytosolic protein which is part of the stabilizing PTH mRNA 3′-UTR-binding complex was shown to be Unr by affinity chromatography *(90)*. Depletion of Unr by small interfering RNA decreased PTH mRNA levels in HEK293 cells transiently cotransfected with the human PTH gene. Overexpression of Unr increased the rat full-length PTH mRNA levels but not a PTH mRNA lacking the terminal 60-nucleotide *cis*-acting protein-binding region. Therefore, Unr binds to the PTH *cis* element and increases PTH mRNA levels, as does AUF1. Unr, together with the other proteins in the RNA-binding complex, determines PTH mRNA stability *(90)*. Recent findings have identified an additional decay promoting protein, KSRP, that differentially interacts with PTH mRNA to recruit the degradatory machinery *(94)*. The balance between the stabilizing and destabilizing proteins determines PTH mRNA levels in response to physiological stimuli *(95)*.

5.4.1.3. KSRP (K-homology Splicing Regulator Protein). We have shown that mRNA decay promoting protein KSRP binds to PTH mRNA in intact parathyroid glands and in transfected cells *(94)*. This binding of KSRP is decreased in glands from calcium depleted or experimental chronic kidney failure rats where PTH mRNA is more stable, compared to parathyroid glands from control and phosphorus depleted rats where PTH mRNA is less stable. The differences in KSRP-PTH mRNA binding counter those of AUF1. PTH mRNA decay depends on the KSRP-recruited exosome in parathyroid extracts. In transfected cells, KSRP over-expression and knockdown experiments show that KSRP decreases PTH mRNA stability and steady-state levels through the PTH mRNA ARE. Over-expression of isoform p45 of the PTH mRNA stabilizing protein AUF1 blocks KSRP-PTH mRNA binding and partially prevents the KSRP mediated decrease in PTH mRNA levels. Therefore, calcium or phosphorus depletion, as well as chronic kidney failure, regulate the interaction of KSRP and AUF1 with PTH mRNA and its half-life. The balance between the stabilizing and destabilizing proteins determines PTH mRNA levels in response to physiological stimuli.

5.4.2. A Conserved Sequence in the PTH mRNA 3′-UTR Binds Parathyroid Cytosolic Proteins and Determines mRNA Stability in Response to Changes in Calcium and Phosphate

We have identified the minimal sequence for protein binding in the PTH mRNA 3′-UTR and determined its functionality *(92)*. A minimum sequence of 26 nucleotides was sufficient for PTH RNA–protein binding and competition (Fig. 2). Significantly, this sequence was preserved among species *(9)*. To study the functionality of the sequence in the context of another RNA, a 63-bp cDNA PTH sequence consisting of the 26 nucleotide and flanking regions was fused to growth hormone (GH) cDNA. The conserved PTH RNA protein-binding region was necessary and sufficient for responsiveness to calcium and phosphate and determines PTH mRNA stability and levels *(92)*.

The PTH mRNA 3′-UTR-binding element is AU rich and is a type III AU-rich element (ARE). Sequence analysis of the PTH mRNA 3′-UTR of different species revealed a preservation of the 26-nt protein-binding element in rat, murine, human, macaque, feline, and canine 3′-UTRs *(9)*. In contrast to protein-coding sequences that are highly conserved, UTRs are less conserved. The conservation of the protein-binding element in the PTH mRNA 3′-UTR suggests that this element represents a functional unit that has been evolutionarily conserved. The *cis*-acting element is at the 3′-distal end in all species where it is expressed.

6. FIBROBLAST GROWTH FACTOR 23 AND THE PARATHYROID

Phosphate homeostasis is maintained by a counterbalance between efflux from the kidney and influx from intestine and bone. Fibroblast growth factor-23 (FGF23) is a bone-derived phosphaturic hormone that acts on the kidney to increase phosphate excretion and suppress biosynthesis of $1,25(OH)_2$vitamin D. FGF23 signals through fibroblast growth factor receptors (FGFR) bound by the transmembrane protein Klotho *(96)*. Since most tissues express FGFRs, expression of Klotho virtually determines FGF23 target organs. Takeshita et al. *(97)* were the first to show that Klotho protein is expressed not only in the kidney but also in the parathyroid, pituitary, and sino-atrial node. In addition, Urakawa et al. *(98)* injected rats with FGF23 and demonstrated increased Egr-1 (early growth response gene-1) mRNA levels in the parathyroid, suggesting that the parathyroid may be a further FGF23 target organ. Phosphate homeostasis is maintained by a counterbalance between efflux from the kidney and influx from intestine and bone. FGF23 is a bone-derived phosphaturic hormone that acts on the kidney to increase phosphate excretion and suppress biosynthesis of vitamin D. FGF23 signals with highest efficacy through several FGF receptors (FGFRs) bound by the transmembrane protein Klotho as a coreceptor. Since most tissues express FGFR, expression of Klotho determines FGF23 target organs. We have identified the parathyroid as a target organ for FGF23 in rats *(81)*. We showed that the parathyroid gland expressed Klotho and two FGFRs. The administration of recombinant FGF23 led to an increase in parathyroid Klotho levels. In addition, FGF23 activated the MAPK pathway in the parathyroid through ERK1/2 phosphorylation and increased early growth response 1 mRNA levels. Using both rats and in vitro rat parathyroid cultures, we showed that FGF23 suppressed both parathyroid hormone (PTH) secretion and PTH gene expression.

The FGF23-induced decrease in PTH secretion was prevented by a MAPK inhibitor. These data indicate that FGF23 acts directly on the parathyroid through the MAPK pathway to decrease serum PTH (Fig. 2). This bone–parathyroid endocrine axis adds a new dimension to the understanding of mineral homeostasis. Krajisnik et al. *(82)* showed similar results using bovine parathyroid cells in primary culture. Interestingly, they also showed that FGF23 led to a dose-dependent increase in the expression of the 1α-(OH)ase enzyme in the parathyroid. The increased $1,25(OH)_2D$ might then act autocrinely to decrease PTH gene transcription.

7. SUMMARY

The PTH gene is regulated by a number of factors (Fig. 2). $1,25(OH)_2D$ acts on the PTH gene to decrease its transcription, and this action is used in the management of patients with chronic kidney disease. The major effect of calcium on PTH gene expression in vivo is for hypocalcemia to increase PTH mRNA levels, and this is mainly post-transcriptional. Phosphate also regulates PTH gene expression in vivo, and this effect appears to be independent of the effect of phosphate on serum calcium and $1,25(OH)_2D$. The effect of phosphate is also post-transcriptional. *Trans*-acting parathyroid cytosolic proteins bind to a defined *cis* element in the PTH mRNA 3′-UTR. This binding determines the degradation of PTH mRNA by degrading enzymes and thereby PTH mRNA half-life. The post-transcriptional effects of calcium and phosphate are the result of changes in the balance of these stabilizing and degrading factors on PTH mRNA. These interactions also regulate PTH mRNA levels in experimental uremia. In diseases such as chronic renal failure, secondary hyperparathyroidism involves abnormalities in PTH secretion and synthesis. FGF23 also acts on its receptors, the Klotho-FGFR1, 2c, and 3c to decrease PTH mRNA levels and secretion. An understanding of how the parathyroid is regulated at each level will help devise rational therapy for the management of such conditions, as well as treatment for diseases, such as osteoporosis, in which alterations in PTH may have a role.

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