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PTHrP Gene Expression in Cancer: Do All Paths Lead to Ets?

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Abstract

Parathyroid hormone-related protein (PTHrP) came to the attention of the scientific community in the mid-1980s because of its association with the paraneoplastic syndrome of humoral hypercalcemia of malignancy. Recently, a crucial role for the peptide has been identified in the metastatic growth of cancer cells in bone. Efforts to understand the peptide's role in these pathological processes have evolved into the study of PTHrP gene expression. Currently, regulation of the third PTHrP promoter is beginning to be understood in the context of activation of certain signaling pathways involved in the growth and progression of specific neoplasms. In addition, factors that modulate the entire PTHrP-transcriptional unit, as well as the stability of the mRNA, are being elucidated at the level of *cis*-acting sequences.

Keywords

bone metastasis; hypercalcemia; Ras-MapK; HTLV-1; TGF- β ; vitamin D

I. INTRODUCTION

Parathyroid hormone-related protein (PTHrP) came to the attention of the scientific community in the mid-1980s because of its association with the paraneoplastic syndrome of humoral hypercalcemia of malignancy (HHM). Once PTHrP had been identified, it was discovered that many cancers produced PTHrP, but only a limited subset led to the development of HHM. Over the past 15 years, the attempts to understand the basis of overproduction of PTHrP in HHM, coupled with efforts to identify pharmaceuticals capable of reducing its expression in hypercalcemic patients, have evolved into the study of PTHrP gene expression.

Since PTHrP was identified, it has been recognized as a central player in multiple pathological and developmental scenarios that have captured the attention of the scientific community. Of particular importance to the field has been the discovery that the expression of PTHrP by tumor cells is crucial to the growth of metastatic cancer cells in bone. PTHrP has also been implicated in two developmental processes that have tremendous psychosocial impact in humans: long bone growth and the hair cycle. In both cases, control of PTHrP gene expression is a key event in permitting the ordered as well as continued growth of these structures.^{1–3} Finally, PTHrP signaling appears to be central to the process of smooth muscle relaxation in a number of organs.⁴ These findings have provided an impetus to investigate PTHrP gene expression and lead to a better understanding of how the gene functions and is regulated.

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II. PTHRP GENE STRUCTURE

The PTHrP gene shares remarkable homology to the PTH gene. In humans, the gene for PTHrP is localized on chromosome *12p11*, whereas the gene-encoding PTH is in an analogous region on chromosome *11p15*. These segments of the chromosomes have similar banding patterns and contain related genes such as the A and B isoforms of lactate dehydrogenase, Sox 5 and 6, and the *Hand K-ras* genes.⁵ It is believed that both chromosomes originated from a single ancestor and were generated as distinct entities through tetraploidization events.⁶ Eight of the first 13 N-terminal acids of PTHrP are identical to PTH and the three-dimensional structure of residues 13–34 are strikingly similar, which is responsible for the binding and activation of their common receptor, the PTH/PTHrP receptor.⁷

The human PTHrP gene is a complex transcriptional unit composed of nine exons spanning more than 15 kilobases of genomic DNA. The 5'-end of the gene contains transcriptional regulatory regions consisting of three distinct promoters identified as P1, P2, and P3, respectively.^{8–12} The 3'-end of the gene contains three exons that may be alternatively spliced to encode carboxyl termini of three distinct mature PTHrP-protein isoforms.^{9,11} Exons 1, 2, 3, and 4 encode the 5'-untranslated regions in the mature mRNA; exon 5 encodes for the “prepro” region; and the exon 6 product contains the majority of the coding region. Exons 7, 8, and 9 contain isoform-specific 3'-coding sequences to produce proteins of 139 (1-139), 141 (1-141), and 173 (1-173) amino acids,^{9,13–15} respectively, as well as unique 3'-untranslated regions. Depending on the specific promoter used, as well as 5' and 3' alternative splicing, 15 different mRNA species can be produced¹¹; however, the products of exons 5 and 6 are present in all transcripts.^{16–18}

III. Alternative Promoter Usage

Transcription of the PTHrP gene arises from three distinct promoters: P1, P2, and P3. A TATA-containing promoter, P3, is located in an intron 35 bp upstream to exon 4[–494 to –486 bp and the transcription start site is –464 relative to the ATG translation start site in exon 5].⁹ A second TATA promoter, P1, is located 25 bp 5' of exon 1 and ~2.7 kb upstream of P3[–3250 to –3242 and the transcription start site is –3221].¹⁹ P2 is a GC-rich regulatory region located upstream of exon 3 and the transcription initiation site is 11 bp upstream of the exon 3 splice acceptor site [–726].¹²

The initial studies of PTHrP promoter expression suggest the possibility of tissue-specific promoter usage.^{9,16–20} Southby of the Melbourne group published a series of RT-PCR-based studies reporting alternative promoter usage in cell lines as well as in breast, lung, parathyroid, and renal neoplasms.^{18,21} These authors concluded that P3-derived transcripts were present in all samples, whereas combined P2/P3 usage was prevalent in malignant breast and bone tumor-derived lines. Substantial P1 usage was infrequent and generally limited to cell lines and tumors of squamous cell origin. Bouizar et al.²² examined the promoter usage in a retrospective study of 74 primary breast cancer samples by semiquantitative RT-PCR. In these samples, P2- and P3-derived transcripts were much more abundant than those derived from P1. In addition, higher levels of P3-derived transcripts were observed in samples from patients that later developed bone metastases. Also, estrogen receptor-positive samples had more abundant P2-derived transcripts than tumors that lacked this receptor. Overall, these reports emphasize the importance of P3 in transcriptional regulation of PTHrP gene expression in various cancers and derived cell lines; however, more extensive studies using quantitative techniques will be necessary to establish a clear image of alternative promoter usage in primary and metastatic cancer.

IV. TRANSCRIPTIONAL REGULATION OF P3

A. HTLV-1 Tax-Ets

The study of hypercalcemia associated with adult T-cell leukemia/lymphoma (ATLL) has led to the identification of the first nonpromoter cis-acting sequences that contribute to the transcriptional regulation of PTHrP. ATLL is an aggressive and often fatal malignancy of helper/inducer T-lymphocytes (CD4⁺) caused by infection with a complex retrovirus: human T-cell lymphotropic virus type 1 (HTLV-1).²³ Hypercalcemia is frequently observed in acute ATLL patients and represents a life-threatening complication of this disease.²⁴ The virus contains the structural *gag*, *pol*, and *env* genes, as well as a regulatory gene region (pX) that encodes several proteins from four open reading frames, including Tax.^{25,26} Tax is a 40 kD nuclear phosphoprotein that increases viral transcription from the HTLV-1 LTR as well as from many cellular genes, including interleukin-2 (IL-2) and the IL-2 receptor (IL-2R) α chain.²⁷ Finally, HTLV-1-mediated cellular transformation requires the expression of Tax.²⁸

The association between Tax and PTHrP was first made by Wantanabe and colleagues when they found a correlation between the intensity of HTLV-1 genome-reactive bands in Southern blots from peripheral blood mononuclear cells of ATLL patients and PTHrP mRNA levels from those samples as measured by RT-PCR.²⁹ These investigators then cotransfected a Tax expression vector and a large P2/P3-containing human PTHrP-chloramphenicol acetyltransferase (CAT) reporter gene and observed a 15-fold increase in CAT activity. This finding was extended by Dittmer and associates, who used a series of classic molecular approaches to define a Tax-responsive region immediately upstream of the P3 promoter.^{30–32}

Nearly 90% identity is shared among a 90-base region immediately upstream of the human P3 promoter [–490 to –582] and the single rat and mouse PTHrP TATA promoter.³³ This region is probably part of the P3 core promoter and the *cis*-acting elements within this sequence probably serve to recruit the TATA binding protein-containing TFIID initiation complex to the TATA box.³⁴ Within this region are two overlapping Ets factor-binding sites (EBS) (EBS I and EBS II in the opposite direction) and one inverted Sp1 DNA-binding consensus site. These were identified because of their high homology with the Tax-responsive element-2 located within the HTLV-1 long terminal repeat (LTR).³⁵ Both physical and functional interactions were characterized between the PTHrP EBS I (5'-TCCGGAAGC-3' [–539 to –532]), EBS II (52-TCCGGAAAG-3' [–540 to –534]) and Sp1 (52-CCCACC-3' [–524 to –519]) sites with HTLV-1 Tax, Ets-1, and Sp1 transcription factors in a series of studies.³¹ In Dittmer's first report, a marked stimulation of various P2/P3-, as well as isolated P3-driven, human PTHrP reporter constructs was observed after cotransfection with Tax in Jurkat T cells.³¹ The basal and Tax-stimulated activity of truncated constructs eliminating the EBS I sequence was decreased ~80–90%. Furthermore, electrophoretic mobility shift assays (EMSA) provided evidence of physical and functional DNA/protein complex formation between recombinant Ets-1 and the P3 EBS I. In a subsequent report, this group found that mutation or truncation of EBS I or mutation of the Sp1 sequence could reduce basal CAT activity from P3 reporter genes in Jurkat T cells by 75–80%.³⁰ Interestingly, truncation of the EBS II element restored most of basal CAT activity, suggesting that EBS II may act as a negative regulatory element.³⁰ Complex formation between recombinant Ets-1 and/or Sp1 proteins and the upstream P3 sequence was demonstrated by DNase I footprinting and EMSA using either purified Sp1 or HeLa nuclear extracts.³² Cotransfection of *Drosophila* Schneider cells with a P2/P3-CAT construct and vectors expressing Ets-1 and Sp1 showed a cooperative induction of reporter-gene activity sevenfold over that stimulated by Ets-1 alone.³² Finally, ternary complex formation between Tax, Ets-1, and Sp1 was indicated using a yeast two-hybrid system.³²

The studies by Dittmer and Brady were seminal in identifying Ets factors as the first class of transcription factors likely to play a major role in the regulation of PTHrP gene expression.

The founding member of the Ets family is the E26 avian retrovirus transformation-specific (Ets) gene.³⁶ In mammals, about 30 members of this family of nuclear transcription factors have now been identified.^{37,38} All members of the family have a conserved 85 amino acid Ets domain, which is a winged helix-turn-helix motif that binds DNA at a GGAA/T consensus site.^{37,38} These factors are downstream nuclear targets of many signal transduction kinase cascades, and phosphorylation of the proteins alters DNA binding, transcriptional activation, association with other transcription factors, subcellular localization, and protein turnover.³⁷ Ets factors can both activate and repress gene expression in cooperation with other transcription factors, coactivators, and core-repressors.^{37,39–41} Some Ets factors are expressed in a tissue-specific manner, whereas others are ubiquitously expressed.^{37,42} Ets-1 plays a central role in the development and differentiation of the T-cell lineage³⁷ and is expressed by ATLL cells; therefore, Ets-1 appears to be the major factor in the regulation of PTHrP gene expression in this circumstance.

Although the Tax-transactivation studies led to the first identification of transcription factor-binding sequences within the PTHrP gene, questions have arisen as to the relevance of this viral protein to ATLL-associated hypercalcemia. Most of the work describes interactions between PTHrP gene sequences and purified Tax, Sp1, and Ets proteins *in vitro*. Recent surveys of HTLV-1 Tax mRNA expression from human-patient samples suggest that exceedingly low levels of this nuclear factor are present in ATLL cells.^{43,44} A model of ATLL-induced hypercalcemia using SCID/beige mice has recently been developed, and leukemia cells purified from hypercalcemic animals contain undetectable levels of Tax mRNA or protein.⁴⁵ Finally, a Q-RT-PCR-based survey of six HTLV-1 transformed cell lines demonstrated an inverse relationship between Tax and PTHrP expression.⁴⁶ Taken together, these findings suggest that the ability of Tax to stimulate P3-containing PTHrP-CAT constructs in transient transfection assays may not correspond to a central role for this nuclear factor in ATLL-induced hypercalcemia.

B. Smad-Ets

The study of bone metastasis has opened up a new area in which the study of PTHrP gene expression has become an integral part. Although much of this work has been done in the context of breast cancer, which has a high incidence of bone metastases, it is likely that the underlying pathobiology pertains to other types of cancer that produce osteolytic metastases. Growth of metastatic cancer in bone depends on tumor cell interactions with the resident cells of the bone microenvironment.⁴⁷ Production of PTHrP by the cancer cell in the bone microenvironment stimulates the PTH receptor-bearing cells of the osteoblast lineage.⁴⁷ This sets in motion the natural bone resorptive process, including the expression of several factors, such as RANK-ligand, that lead to the formation of active bone-resorbing osteoclasts.^{48,49} As a result of osteoclast-mediated destruction of the mineral and protein components of bone, growth factors embedded in matrix are released.^{47,49} These factors stimulate survival and proliferation of cancer cells at sites of bone metastasis. Thus a vicious cycle results where bone resorption, initially stimulated by the metastatic cancer cells, then results in increased tumor growth and further destruction of bone.^{47,49,50} If this scenario accurately describes this disease process, a specific blockade of PTHrP gene expression in cancer patients with metastatic cancers in bone may provide some relief from the devastating consequences of the growth of cancer cells in this organ.

Breast cancer metastasis to bone is often modeled *in vivo* by injecting human tumor cells into the central arterial circulation of immunocompromised animals.⁵¹ Ultimately, some of the cancer cells leave the circulation and proliferate in the bone marrow microenvironment.⁵¹ Among the frequently studied breast cancer cell lines, the MDA-MB-231 cells efficiently colonize bone; therefore, this cell line has become a focal point for bone metastasis research.

⁵² The central role for PTHrP in the process of bone metastasis was established by injecting neutralizing antibodies to PTHrP into immunocompromised mice and inhibiting the growth of MDA-MB-231 cells at sites of bone metastasis.⁵²

Transforming growth factor- β (TGF- β) is one of the most abundant growth factors stored in bone and is released in its active form during osteoclastic bone resorption.⁵³ TGF- β was originally reported to stimulate PTHrP gene expression in squamous cell carcinoma cell lines⁵⁴ and proved to be one of a few growth factors contained in bone matrix capable of increasing PTHrP expression in the MDA-MB-231 cell lines.⁵⁰ Using the intracardiac injection procedure, Guise and coworkers reported that expression of dominant negative or constitutively active TGF- β receptors could modulate the growth of MDA-MB-231 cells in bone.⁵⁰

TGF- β signaling results from ligand-induced dimerization of its type I and type II receptors, resulting in a transphosphorylation event that activates the serine/threonine kinase of the type I receptor.⁵⁵ This receptor initiates signaling mainly through phosphorylation of Smad2 and Smad3 but has been reported to activate other second messenger pathways.⁵⁵ Phosphorylated Smad2 and Smad3 associate with Smad4 and this complex is then translocated from the cytoplasm to the nucleus.⁵⁵ The Smad complex activates transcription by binding to AGAC-based motifs in promoter regions.⁵⁵

Recently, Dittmer's group identified a functional Smad-binding site (AGACAGAC [-509 to 498]) located 34 bp upstream of the PTHrP P3 initiation site.⁵⁶ Using the MDA-MB-231 line, they found that the TGF- β -induced activation of PTHrP gene expression was mediated exclusively by increased transcription from P3.⁵⁶ The Smad site is 23 bases downstream from the EBS1 site, suggesting that physical and functional interactions between Ets factors and Smads may be involved in regulating the P3 promoter. Individual mutations in the Smad, Sp1, or EBS1 sequences resulted in a remarkable decrease in both basal and TGF- β -stimulated PTHrP reporter-gene activity.⁵⁶ The addition of Ets-1 synergistically increased TGF- β -stimulated PTHrP reporter-gene activity and EMSA suggested that increased levels of Smad 3 and 4 may facilitate increased binding of Ets factors to the P3 promoter EBS.⁵⁶

Again, Ets-1 appears to be the primary Ets factor that facilitates activation of PTHrP gene expression in breast cancer cell lines. Mammary epithelium does not express Ets-1, but invasive carcinomas and more aggressive cell lines acquire expression of this transcription factor.⁵⁷ Only Ets-1 was found to cooperate with Smad 3 to activate PTHrP reporter-gene activity in MDA-MB-231 cells, whereas other Ets factors found in breast cancer cell lines, such as Ets2, Ets-1, and Elf-1, failed to do so.⁵⁶ However, ectopic Ets-1 expression by itself is not sufficient to activate reporter genes in low PTHrP-expressing breast cancer and breast epithelial cell lines.^{56,59} Ets-1-mediated induction of P3 transcriptional activity appears to be dependent on certain activated second-messenger pathways, such as the PKC α in MDA-MB-231 cells.⁶⁰ This kinase increased Ets-1 gene expression as well as activated the transcription factor by phosphorylation.⁶⁰ In MCF-7 cells, PTHrP gene expression is activated by the combined activities of Ets-2 and PKC ϵ .⁶¹ Therefore, it appears that TGF- β -induced activation of high levels of PTHrP gene expression in breast cancer cell lines depend upon the presence of a specific Ets factor and activation of other signaling pathways.

C. Ras-MapK-Ets

The association between an activated ras-mitogen activated protein kinase (MapK) pathway and high levels of PTHrP gene expression was suggested by the early surveys that identified squamous cell carcinomas as the tumor type most frequently associated with HHM. It has long been known that many squamous cell carcinomas have mutations that activate the H-ras gene.⁶² Ras stands at the crossroads of cellular signaling, relaying stimuli from growth-factor

receptors at the plasma membrane to cytoplasmic kinase cascades that ultimately activate transcription factors. Ras proteins are monomeric GTPases that signal transiently when bound to GTP.⁶³ Activation of ras proteins requires farnesylation of a cysteine residue near the c-terminus of the protein to appropriately target the signal transducer to the plasma membrane.⁶³ Ras proteins can transduce signals to at least seven independent signaling cascades; however, the MapK cascades are considered the prototypical downstream consequence of ras signaling.⁶³ The MapK cascades are actually three parallel signaling pathways, comprised of three kinases, that serially transduce signals through the cytoplasm (see Fig. 2).⁶⁴ Each of these pathways terminates with a MapK—namely extracellular signal-regulated kinase (Erk), p38, or Jun N-terminal kinase (Jnk)—that phosphorylates various transcription factors, including Ets factors (Fig. 2).⁶⁵ The upstream kinases are confusingly referred to as mitogen-activated kinase kinase (MapKK) and mitogen-activated kinase kinase kinase (MapKKK) (Fig. 2). Growth factor signals are typically transduced from ras through raf (MapKKK) to MEK (MapKK), terminating in ERK (MapK) (Fig. 2).⁶⁴ The cascades that terminate in p38 or JNK are typically activated by stress or cytokines, but can be activated through ras by PI3 kinase and rac/cd42 (Fig. 2).⁶⁴

The direct connection between ras, the MapK cascade, and PTHrP gene expression was established in the mid-90s by Rabbani, Goltzman, and colleagues. Using a series of rodent cell lines transformed with activated ras or oncogenic forms of receptor tyrosine kinases, they observed that PTHrP protein and mRNA levels were decreased by ras farnesylation inhibitors.^{66–68} The ras inhibitors decreased serum calcium levels in animals with tumors derived from oncogene-transformed cells as well as the rat Leydig tumor-derived cell line H-500.^{66–68} Ultimately, through the use of small molecule inhibitors as well as dominant-negative and constitutively active forms of signaling intermediates downstream of ras, PTHrP gene expression was found to be regulated by the Erk and Jnk arms of the ras-MapK pathway.⁶⁹ Although these studies did not directly address the *cis*-acting sequences that mediated activation of PTHrP gene expression, the authors speculated that an EBS site upstream of the P3-like rat PTHrP promoter was the probable downstream target of ERK activation.⁶⁹ A recent study of EGF receptor-mediated activation of PTHrP gene expression in human keratinocytes found that the EBS site was crucial for full ras-ERK-mediated activation of the P3 promoter by this receptor tyrosine kinase.⁷⁰

The ras-MapK pathway likely represents a common mechanism by which certain stimuli activate PTHrP gene expression. Many of the well established activators of PTHrP gene expression, such as growth factors, angiotensin, serum, cytokines, phorbol esters, and physical stretching, all signal through components of the ras-MapK pathway.¹⁵ The ras-MapK pathway may also activate PTHrP gene expression through stimuli that are not generally recognized to be linked to this signal transduction pathway. For example, activation of PTHrP gene expression by the calcium receptor in prostate cancer cells and human embryonic kidney lines is mediated in part by ras-MapK.⁷¹ This activation is somewhat circuitous, involving calcium receptor-induced G protein-mediated activation of matrix metallo-proteinases, which presumably release plasma membrane-tethered EGF-like ligand HB-EGF that, in turn, activates the EGF-receptor.^{71–73} Finally, emerging evidence suggests that EBS-dependent activation of PTHrP-P3 promoters in HTLV-1-transformed cell lines is mediated by T-cell receptor induction of the ERK arm of the ras-MapK pathway.⁴⁶ A portion of TGF- β -induced activation of PTHrP gene expression has been attributed to activation of the p38 arm of the ras-MapK cascade, based in large part on the use of the p38 inhibitors SB 203580 and SB202190.⁷⁴ This finding has been disputed by Dittmer in a report that suggests that the SB P38 inhibitors target the TGF- β type I receptor kinase.⁷⁵ Putting this controversy aside, it is clear that diverse stimuli can induce signaling by each of the three arms of the ras-MapK pathway, resulting in activation of PTHrP gene expression.

At this point, the precise mechanism by which Ets factors activate PTHrP gene expression in response to ras-MapK signaling has not been elucidated. Cotransfection of both the Ets-1 and Ets-2 expression vectors ~was able to activate P3-containing reporter genes in human keratinocytes.⁷⁰ Ras-MapK induction of MMP gene expression is considered prototypical and is mediated by an Ets-1/Jun cooperative interaction at an EBS that is six to seven bases upstream of an Ap-1 site.^{37,65,76} The closest Ap-1 site in the region near the PTHrP-P3 EBS is 35bp downstream of the transcription initiation site [-430 to -422]. Also, mutation of the surrounding GAS/EBS2 [-541 to -534], Sp-1, and Smad sites failed to reduce ras-MapK-activated P3 reporter-gene activity in human keratinocytes or T cells.^{46,70} Thus the precise transcription factors that cooperate with Ets factors to mediate ras-MapK induction of PTHrP gene expression remain to be identified (Fig. 3).

Although not thoroughly investigated at this point, the activation of the P3 PTHrP promoter by Ets, Sp1, and Smad implies that there is interaction with the basal transcription complex associated with the TATA box. Interactions of this nature are generally mediated by coactivators such as CBP/p300.^{37,39} In a study that used a generic coactivator comprised of the adenoviral E1a 13S with deleted repressor domains, activation of mouse PTHrP reporter genes was found to be dependent on an intact EBS-1 site.⁷⁷ This finding implies that Ets proteins activate the mouse P3-like PTHrP promoter through interactions with coactivators. The role of coactivators and chromatin structure represent the next stage of study of the PTHrP P3 promoter.

V. REGULATION OF P1 AND P2

P1 is the most restricted of the promoters, but transcription from this promoter is a major contributor to high levels of PTHrP expression in hypercalcemia-inducing squamous carcinoma cell lines.^{21,78} To date, a single regulatory element has been identified in the region upstream of P1. Chilco et al. identified a cAMP-responsive element (CRE) located -80bp [-3302 to -3295] upstream of the start of exon 1, suggesting that this motif may be part of the core promoter.⁷⁹ This element was able to mediate the stimulation of a P1 reporter gene by calcitonin and forskolin. They found that P1 was remarkably more sensitive to cAMP-mediated activation than P3, suggesting that P1 may be the primary responsive promoter to the cAMP/PKA/CREB pathway in the BEN lung cancer cell line.⁷⁹ Except for this study, P1 reporter activity has been infrequently investigated, in part because of the fact that squamous carcinoma cell lines, in which this promoter is active, are difficult to transfect. It is likely that understanding of the regulation of the PTHrP P1 promoter will need to emerge before hypercalcemia associated with squamous cell carcinomas is understood at a more fundamental level.

PTHrP P2 was originally identified in 1993 and yet little is known regarding the transcriptional mechanisms involving potential *cis* regulatory sequences within this promoter. GC-rich *cis* regulatory regions do not appear to be limited to a particular class of genes. They have been located in housekeeping, viral, receptor, ion-channel, cytokine, structural protein, and DNA-binding protein genes.⁸⁰ They are frequently located in the 5' region of genes lacking typical TATA- or CCAAT-consensus elements. GC-rich DNA regulatory sequences generally contain numerous Sp1 response elements for binding of Sp1 and Sp3 transcription factors.⁸⁰ These factors have also been shown to functionally bind to other transcription factor DNA response elements such as NF- κ B. Sp1 positively regulates transcription alone or in cooperation with NF- κ B, E2F, p53, RB, STAT-1, or GATA-1 transcription factors.⁸⁰ In contrast, Sp3 has inhibitory action; therefore, the ratio of Sp1/Sp3 in a specific cellular context will determine the activation or repression of transcription regulated by GC-rich *cis* elements containing Sp1 or Sp1-like sites.⁸¹ Using a software screening library (MatInspector, Genomatrix, Hamburg, Germany), we identified a VDRE, three overlapping AP-2 sites, three overlapping Sp1 sites,

and two distinct NF- κ B sites within 250 bp [−1013 to −763] upstream of the P2 transcription-initiation site.⁸² We observed constitutive and specific binding of the NF- κ B subunit p50 from primary ATLL cell nuclear extracts to an inversed NF- κ B-like response element (ctcggggctCCCCtc) located from −125 to −110 [−918 to −904] bp upstream of the P2 transcription initiation site (VR, TJR, unpublished observation). Although P2 appears to be expressed in a wide variety of cell lines and tumors, quantitative approaches will be required to determine if this promoter makes a substantial contribution to PTHrP gene expression in physiological or pathological circumstances.

VI. GLOBAL REGULATION OF THE PTHrP GENE

A. Vitamin D

Vitamin D and its various normocalcemic derivatives have been proposed as potential therapeutic agents that could control hypercalcemia induced by excessive PTHrP gene secretion as well as its production by breast and prostate cancers that have metastasized to the bone. The inhibitory effect of these compounds on PTHrP gene expression has been reported in a variety of human cell lines, including ras-transformed keratinocytes, several human squamous cell carcinomas, prostate cancer, fibrosarcoma, pancreatic cancer, HTLV-1 transformed cells, and a thyroid carcinoma cell line.^{83–90} This sterol represses both basal and growth factor-induced PTHrP gene expression at the level of transcription.^{87,91} It is speculated that this regulation mirrors the 1,25-(OH)₂D₃-mediated repression of PTH gene expression in the parathyroid gland that serves as a feedback loop to control calcium homeostasis.⁸⁸ The human PTHrP gene has an atypical vitamin D-responsive element (VDRE) located 517–546 [−3849 to −3840] bp upstream of P1 that is homologous to a similar negative element found in the upstream region of the human PTH gene.⁸⁸ Classic transcriptional regulation by 1,25-(OH)₂D₃ involves the diffusion of the sterol to the nucleus and the binding of its receptor (VDR), which may or may not be associated with the VDRE.⁹² In this situation, the complex of 1,25-(OH)₂D₃/VDR recruits the retinoid-X receptor to form a functionally active heterodimer at the VDRE present in the promoters of genes activated by the sterol.⁹³ In contrast, vitamin D-mediated regulation of the human PTH and PTHrP genes does not involve RXR, and the unliganded VDR is clearly bound to the chromatinized atypical VDRE. Stimulation with 1,25-(OH)₂D₃ dissociates the PTHrP VDRE/VDR complex.^{88,94} A recent report demonstrated that Ku regulatory subunits p70/p86 were constitutively associated with the VDR on the chromatinized PTHrP VDRE.⁹⁴ Addition of 1,25-(OH)₂D₃ recruited the catalytic subunit of DNA-dependent protein kinase that phosphorylates the VDR, causing it to dissociate from the VDRE.⁹⁴ On the basis of this evidence, it appears that the dissociation of the VDR from the region upstream of P1 leads to repression of gene expression from this promoter.^{88, 94}

The report of 1,25-(OH)₂D₃-induced repression of PTHrP gene expression in diverse groups of cell lines suggests that this sterol can repress all three promoters. P1 is universally active in squamous carcinoma cell lines that express high levels of PTHrP and in the case of the NCI H520 and FA-6 lines, 1,25-(OH)₂D₃, as well as various analogues, repressed this promoter by approximately 70%.^{85,89} Also, the PTHrP P3 promoter was substantially repressed by 1,25-(OH)₂D₃ in the FA-6 line.⁸⁹ The analysis of the atypical VDRE upstream of P1 was performed in the HTLV-1-transformed line MT2 that expresses PTHrP transcripts primarily from P3.^{46, 94} What has yet to be clarified is whether the VDRE upstream of P1 mediates repression from both this proximal promoter and from P3 that is 2755 bp downstream. This issue is further complicated by reports of 1,25-(OH)₂D₃-induced regulation of the rat PTHrP gene, which contains three negative VDRE sequences, −1121, −805 and −726, relative to the transcription start site of its P3-like promoter.^{95,96} Of these three elements, the one at −1121 appears also to bind RXR, whereas the −726 site is homologous to the human P1-PTHrP and PTH nVDRE.

^{88,96} Computer analysis of 4.5 kb of sequence upstream of the translation start site of the human PTHrP gene suggests that there are three other potential VDR consensus sites, one of which is about 60 [-822] bases upstream of P2. Finally, in primary prostate epithelial cells and foreskin keratinocytes, 1,25-(OH)2D3 failed to repress PTHrP gene expression,^{97,98} raising the possibility that vitamin D may regulate PTHrP differently in non-transformed cells.

B. Methylation

Methylation of cytosine residues within CpG dinucleotide sequences is an important mechanism of transcriptional silencing in both normal as well as cancerous tissues.⁹⁹ The PTHrP gene has a large CpG island and many individual CpG sites, both within the GC-rich P2 promoter and in the 2200bp between P1 and P2 [-1405 to -593] (Fig. 1). A CpG island is defined as a DNA region ranging from 500 bp to 5 Kb, containing more than 60% G+C content, and with a ratio of CpG to GpC of at least 0.6.¹⁰⁰ In normal tissues, CpG islands are mostly unmethylated, whereas methylation of cytosine residues in CpG dinucleotides frequently occurs in cancer and is associated with gene silencing.

A connection between methylation and PTHrP gene expression was first identified by the Broadus and Philbrick group in a panel of 12 renal carcinoma cell lines that exhibited an “on or off” pattern of PTHrP expression.¹⁰¹ The authors showed that CpGs within the PTHrP CpG island were unmethylated in expressing as well as non-expressing cell lines. However, four CpG dinucleotides in the intron between exons 2 and 3 [-2030, -1890, -1510, -1510] (see Fig. 1) were methylated in all PTHrP-negative cell lines and unmethylated in all the cell lines expressing PTHrP. The CpG sites located in the P3 promoter region were unmethylated in all cases, whereas a CpG site in the P1 region was methylated in all 12 cell lines examined. Also, treatment of PTHrP-negative cell lines with 5'-aza-cytidine, a demethylating agent, was able to restore PTHrP mRNA expression. These findings suggest that methylation of residues upstream of the CpG island may negatively regulate PTHrP gene expression.

The influence of DNA methylation on PTHrP gene expression has also been investigated in a second set of studies focusing primarily on lung cancers. Ganderton et al. showed that CpG residues in the intron upstream of the CpG island (overlapping those in the region analyzed in the renal carcinomas) were extensively methylated in the high PTHrP-expressing lung carcinoma cell line BEN, whereas methylation of these residues was reduced in lung fibroblasts that express little PTHrP mRNA.^{102,103} An RT-PCR-based evaluation of matched sets of squamous cell carcinomas of the lung and normal lung samples indicated that the tumor samples expressed substantially higher levels of PTHrP mRNA, but methylation of the CpG residues of the second intron in the matched sets was identical or increased in the carcinomas as compared to their normal counterparts.¹⁰⁴ The authors concluded that methylation of the 52 end of the PTHrP gene had no influence on its expression in lung cancer.¹⁰⁴

In order to reconcile the conclusions from the investigations using renal carcinoma cell lines with those using primary lung tumors, it may be useful to consider that the impact of methylation of upstream regions of the PTHrP gene could be cell type- or promoter-dependent. At this time, little is known about how methylation influences PTHrP gene expression in normal cells and how this may in turn influence gene expression in cancers derived from specific cell types. Advances in the understanding of chromatin modifications are likely to have a major impact on future PTHrP gene expression studies.

C. Messenger RNA Stability

Alternative splicing of the 3'-end of the human PTHrP gene produces three protein isoforms (Fig. 1): 1-139, 1-173, and 1-141. Exons 7-9 contain unique 3'-untranslated regions (UTR), each of which contains AUUUA regions thought to be important in the regulation of mRNA

turnover in general.^{105–107} The half-life of the PTHrP mRNA isoforms range from 30 minutes to over 4 hours. PTHrP has a high transcription rate, but steady-state levels of its mRNA are low because of a rapid mRNA turnover.^{86,91,108} Southby and colleagues showed that PTHrP 1–141 mRNA had the shortest half-life (approximately 30 minutes) among the three isoforms, whereas 1–173 mRNA was the most stable, with a half-life of up to several hours.²¹ The half-life of PTHrP mRNA can also be increased in keratinocytes and squamous carcinoma cell lines by stimulation with TGF- β 109–111 and epidermal growth factor (EGF).¹¹² In HTLV-1-infected cell lines, interleukin-2 (IL-2)¹¹³ also stabilized PTHrP mRNA.

There have been a series of studies on the mechanism of growth factor induced-increased PTHrP mRNA stability. Studies examining the effect of EGF on PTHrP mRNA stability demonstrated stabilization of PTHrP mRNA containing exon 7 (PTHrP 139), but not 9 (PTHrP 141), in human immortalized keratinocytes (HaCaT).¹¹² Sellers et al. also showed that when the PTHrP 1–141 UTR was removed, TGF- β treatment was able to dramatically increase the stability of the transcript in squamous carcinoma cells in an *in vitro* system.¹¹¹ The authors attributed the effect to the decreased binding of various proteins (18 to 33 kDa) to a *cis*-element present within the PTHrP terminal coding region.

Numerous 3' UTR binding proteins associated with mRNA degradation have been described and include AUF-1 family members, tristetraprolin (TTP), K homology-type splicing regulatory protein (KSRP), hnRNPA1, hnRNPC, and AU-A, AU-B, AU-C.¹¹⁴ TGF- β did not appear to alter the proteins binding in the 3' UTR; however, the short half-life of the PTHrP 1–139 and 1–141 mRNAs suggests that protein binding the 3' UTR may promote rapid degradation.¹¹⁵ The 3' UTRs of both the 139 and 141 isoforms have binding proteins with molecular weights ranging from 37–40 kD,¹¹⁵ which is consistent with p37^{AUF-1} and p40^{AUF-1} forms that promote mRNA degradation.¹¹⁶ Sellers showed that several unique proteins bound to the PTHrP 141 and 139 3' UTRs;¹¹⁵ however, the 1–141 mRNA bound an 80kD protein that is similar to the molecular weight of KSRP, which is believed recruit the exosome for mRNA degradation.¹¹⁷ In conclusion, the rapid turnover of PTHrP mRNA is similar to that observed in early response genes, such as *c-fos*, GM-CSF and IL-2, whose rapid turnover may be due to AU-rich instability elements in the 3' UTR. Modulation of PTHrP mRNA stability by agents such as TGF- β may be mediated by altered protein binding to *cis* elements in the coding region.

VIII. HORMONES

Many reports suggest PTHrP expression is modulated by multiple hormonal stimuli. In cancers such as breast and prostate in which PTHrP production is believed to play a significant role in progression of the disease, it is well established that receptors for steroid hormones also have great influence. This suggests that there may be a regulatory relationship between hormonal signaling and PTHrP gene expression. Estradiol, tamoxifen, and hydroxyl-tamoxifen produced a 2-fold stimulation of PTHrP mRNA expression and secretion in the breast cancer cell line MCF-7. Estradiol also increased PTHrP mRNA and secretion in uterine tissue,^{118–120} and a pituitary cell line¹²¹ was used to determine that the effect of estradiol on PTHrP expression was attributable to transcriptional events and not to increased mRNA stability. In contrast, Sugimoto¹²² reported that freshly isolated breast cancer cells treated with estradiol failed to increase in PTHrP mRNA expression, but medroxyprogesterone repressed transcript levels. Androgens also have been reported to induce downregulation of PTHrP mRNA expression in the androgen receptor-negative prostate cancer line PC-3 that had been permanently transfected with an androgen receptor expression vector.¹²³ Administration of the androgen receptor antagonist, flutamide, to this line reversed the repression, suggesting that the effect was at least in part mediated by transcriptional regulation of the androgen receptor.¹²³ The inhibitory effect on PTHrP gene expression was also observed in Leydig cells.¹²⁴ Testosterone-activated PTHrP

gene expression in the human prostate cancer cell line, LNCAP, which is androgen-receptor positive.¹²⁵ Taken together, it is difficult to evaluate the precise relationship between sex steroid hormones and PTHrP gene expression; however, the findings may suggest that the influence of these hormones may be dependent on specific oncogenic events that give rise to individual breast or prostate cancers.

Several studies demonstrated a potent inhibitory effect of glucocorticoids on PTHrP expression in squamous carcinoma cell lines,^{126–128} Leydig cell tumors¹²⁴ and a thyroid carcinoma cell line.⁸⁶ The effect was attributable to transcriptional events affecting the transactivation of all three promoters.¹²⁷ There are numerous potential glucocorticoid response elements in the P1 and P2 promoter regions, but their functional activity has not been tested.¹²⁹ It should also be noted that there have been several reports that glucocorticoids do not decrease PTHrP concentrations in animals with hypercalcemia,^{130,131} raising questions about the relevance of the *in vitro* studies.

The stimulatory effect of prolactin on PTHrP secretion by mammary cells during lactation has been reported.^{132,133} However, the effect of prolactin on PTHrP gene regulation in breast cancer has been difficult to establish.¹³⁴ Interestingly, prolactin is now believed to serve as a significant growth factor in breast cancer, using an autocrine/paracrine loop that is involved in cellular transformation.¹³⁵ In addition, prolactin and its receptor are expressed in 95% of breast cancers.¹³⁶

Clarification of the effects of sex hormones on PTHrP gene expression will likely provide insight into health issues beyond cancer. Estrogen and PTHrP appear to have opposing effects on the growth plate, and it is speculated that estrogen affects the growth plate in part by regulating PTHrP gene expression.^{137,138} Also, the hair cycle is significantly modulated by both androgens and estrogens and these steroids may influence the hair cycle-dependent expression of PTHrP.¹³⁹

IX. SUMMARY

Despite the efforts of multiple laboratories, there is still much to understand about how PTHrP gene expression is regulated, both in cancer and under normal physiological conditions. Several very basic questions remain to be addressed. These include the following (listed in no particular order): Is the gene controlled by enhancer and insulator sequences and if so, where are they located? What is the role of chromatin structure in the control of PTHrP gene expression? Why is PTHrP expressed at very low levels in the normal tissues of the adult? What *cis*-acting sequences activate PTHrP gene expression in the cells of developing organs? What is the level of PTHrP gene expression in tumor cells that have metastasized to bone as compared to other sites in the body? Do changes in the stability of PTHrP mRNA isoforms affect PTHrP gene expression in tumors or developing organs?

These questions and others will soon be answered using a number of technologies. Quantitative RT-PCR can be used to quantify gene expression in tumors and normal tissue and can also be used to investigate PTHrP mRNA stability. Chromatin immunoprecipitation-PCR will enable the identification of enhancer and insulator sequences and will assess the global chromatin structure of the PTHrP gene and its role in gene expression. Transgenic technology will be employed to understand PTHrP gene expression in developing organs. Also, the identification of guanosine nucleotides as specific inhibitors of PTHrP gene transcription in both breast (P3) and lung cancer (P1 and P3) cell lines¹⁴⁰ will provide a powerful tool to enhance studies of PTHrP gene regulation.

In conclusion, PTHrP is expressed by the majority of epithelium-derived cancers and a subset of hematological malignancies, whereas in normal tissues, PTHrP expression is at or below

the limit of detection. This is likely due in part to the activation of growth factor production, growth factor receptors, or second messenger pathways associated with transformation events that give rise to these cancers which, in turn, activate the PTHrP-P3 promoter. Dysregulation of hormonal influences, such as decreased production of active vitamin D or mutations in steroid hormone receptors, could augment these growth factor pathway-induced activations of the PTHrP promoter. Finally, in some cases, either viral transcription factors or the mutation of tumor-suppressor genes probably contribute to a permissive environment for the activation of the PTHrP gene. However, it is unclear whether these changes in cellular control of gene expression would provide sufficient explanation for the very high levels of PTHrP produced in solid tumors that cause HHM. It will take a new generation of studies to ultimately answer the questions that have driven this field for the past 15 years.

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FIGURE 1.

Schematic of the PTHrP gene. (A) The entire PTHrP gene. Exons are indicated by boxes and intron sequences by lines. Potential splicing combinations are indicated by dashed lines. Promoters are designated with arrows and the protein coding sequences are colored. The translation start site is designated as +1 and bases 5' are indicated with negative numbers with this as the origin, whereas bases 3' are designated as positive numbers. (B) 5' regulatory region of human PTHrP gene. This is drawn to scale with 720 bp represented as 1 inch. Exons are boxes and noncoding sequences are represented by a horizontal line. Transcription factor binding sites confirmed in transient transfection or EMSA assays are designated with vertical lines. The GC island is indicated with a stippled box, whereas methylated CpG residues are represented as lollipop structures. Arrows represent transcription start sites for each promoter. (C) Sequence of P3 core-promoter region. Key binding sites are boxed within the sequence. The TATA box is at the 3'-end of the sequence.

FIGURE 2.

The Ras-MapK pathway. The figure illustrates the key signaling molecules involved in transduction of signals from the plasma membrane to the nucleus for the three arms of the Ras-MapK pathway. All of the pathways appear to converge on the Ets proteins that interact with the PTHrP-P3 core promoter. Abbreviations not defined in the text: RTK, receptor tyrosine kinase; ER endoplasmic reticulum; ASK1, apoptosis signal-regulating kinase; Cdc, cell division cycle; RAF, ras-associated factor; MEK, MapK ERK Kinase; MKK, Map kinase kinase; PI3K, phosphatidylinositol 3-kinase.

HTLV-1 TAX

TGF-beta

Ras-MapK

FIGURE 3.

Activation of the P3 promoter. The transcription factors and coactivators proposed to mediate (A) Tax gene activation; (B) TGF- β -induced activation; and (C) Ras-MapK induced activation of PTHrP transcription.