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STUDIES ON THE TRANSCRIPTIONAL REGULATION AND DIFFERENTIAL SPLICING OF THE HUMAN PARATHYROID HORMONE (PTH)/ PTH-RELATED PEPTIDE (PTHRP) RECEPTOR GENE (PTHR).

By

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A thesis submitted to the faculty of graduate studies and research, McGill University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT.

Parathyroid hormone (PTH) and PTH-related peptide (PTHrP) stimulate signal transduction in target cells by binding to the same G protein-linked receptor (PTHR). The PTHR mediates PTH signaling in kidney and bone, and PTHrP binding to the receptor has been shown to be essential for normal endochondral bone formation in humans and mice.

Expression of the murine PTHR gene is controled by two promoters that are regulated differently. Whereas the activity of the upstream promoter (P1) is mainly restricted to the kidney, where it provides the bulk of the gene transcription, the downstream promoter (P2) is more widely active. We characterized the upstream regulatory region of the human PTHR gene and showed that its organization is very similar to that of the mouse PTHR gene. RNase protection experiments revealed, however, that the homologue of the mouse renal-specific promoter P1, is only weakly active. This observation led us to identify andcharacterize a third promoter (P3) that is responsible for the expression of more than 80% of human renal transcripts, but is apparently not active in mouse kidney. A study of the tissue distribution of the activity of P1, P2 and P3 showed that, while P2 and P3 are widely active, function of P1 is restricted to renal tissues. This study further revealed the existence of a shorter, differentially spliced variant of P2 derived transcripts. P1 and P3 were found to be developmentally regulated, as no activity was detected at mid-gestation. Hence, expression of the PTHR gene until this stage is driven solely by P2. Furthermore, the shorter P2-specific transcript

observed in adult not detected in fetal tissues, suggesting that differential splicing of PTHR mRNA is developmentally regulated as well.

The observation that activity of P1 and P3 is developmentally up-regulated, and the presence of P3 within a CpG island, prompted us to examine whether DNA methylation could play a role in regulating PTHR promoter function. Our results show that all three promoters are sensitive to methylation in vitro and that the methylation pattern of P1 is renal specific and is established well before the onset of PTHR gene expression. Our results suggest that while the transcriptional regulation of the PTHR gene expression during development might be similar in mouse and human, expression in the adult is likely to be controlled by different mechanisms.

We also addressed the presence of structural alterations of the 5' regulatory region in patients with pseudohypoparathyroidism type Ib. Genomic southern blot analysis, as well as sequencing of the three upstream untranslated exons did not reveal any deletion or point mutation that could account for the kidney specific loss of PTH response observed in these patients.

RÉSUMÉ.

L'hormone parathyroidienne (PTH) et le peptide relatif à l'hormone parathyroidienne (PTHrP) activent les signaux secondaires dans les cellules cibles en se fixant au même récepteur (PTHR). Ce dernier, lié à la protéine G, est responsable de l'action de PTH dans les reins et dans les os, et est essentiel pour un développement normal des os chez l'homme et chez la souris.

L'expression du récepteur chez la souris est sous le controle de deux promoteurs qui sont régulés de façon différente. Le promoteur en amont (P1) est principalement actif dans les reins où il est responsable de la vaste majorité de l'expression du gène. Le promoteur placé en aval (P2), est actif dans un grand nombre de tissus. Nous avons isolé la région régulatrice du gène humain codant pour le PTHR. L'organisation de cette région chez l'homme est très similaire à celle de la souris. Des experiences de protection à la RNase ont demontré que l'homologue humain du promoteur P1 est faiblement actif. Cette observation nous a ammenée à caractériser un troisième promoteur (P3) qui dirige plus de 80% de la transcription du gène dans les tissus rénaux humains, et qui est apparement inactif chez la souris. L'étude, dans différents tissus, de l'activité des trois promoteurs a montré que P1 est actif uniquement dans les reins, alors que P2 et P3 sont actifs dans de nombreux tissus. Cette étude a de plus mise en évidence l'éxistence d'un transcrit plus court, résultant de l'épissage interne des transcrits provenant de P2. L'absence, à 18 semaines de gestation, de transcrits provenant de P1 et P3 suggére que ces deux promoteurs sont régulés différement au cours du développement. Il semble donc que l'expression du récepteur jusqu'à ce stade est sous le control exclusif de P2. De plus,

les transcrits plus courts dérivant de P2 ne sont pas présents chez le fœtus, ce qui suggère que cet épissage est aussi régulé au cours du développement.

Le fait que P1 et P3 sont actifs chez l'adulte et non pas chez le fœtus, et la présence de P3 dans un îlot CpG, nous ont poussés à examiner la possibilité que la transcription du gène PTHR soit régulée par des phénomènes de méthylation de l'ADN. Nos résultats montrent que les trois promoteurs sont sensibles à une méthylation in vitro, et que le mode de méthylation de P1 est specifique aux tissus rénaux et est établi plusieurs semaines avant l'établissement de l'activité de ce promoteur.

Nous avons aussi analysé la possibilité qu'une délétion, ou une mutation ponctuelle de la region promotrice, puissent être responsible de l'apparition du syndrome de pseudo-hypothyroidie de type Ib. Aucune deletion ou mutation ponctuelle n'ont pu être détectée dans l'ADN génomique provenant de patients souffrant de ce syndrome.

FOREWORD

The following excerpt is taken from the guidelines concerning thesis preparation, faculty of graduate studies and research, McGill University, and applies to this thesis.

Candidates have the option of including, as part of the thesis, the text of a paper(s) submitted or to be submitted for publication, or the clearly duplicated text of a published paper(s). These texts must be bound as an integral part of the thesis.

If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the " guidelines for thesis preparation". The thesis must include: A table of contents, an abstract in English an French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscritps co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these

cases, it is in the candidate's interest to make perfectly clear the responsabilities of all the authors of the co-authored papers. Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis.

PREFACE.

The work described in chapters 2 and 3 of this thesis has been published in the following journals.

Chapter 2: Bettoun J.D., M. Minagawa, M.Y. Kwan, H.S. Lee, T. Yasuda, G.N. Hendy, D. Goltzman, and J.H. White 1997. Cloning and characterization of the promoter regions of the human parathyroid hormone (PTH)/PTH-related peptide receptor gene: Analysis of deoxyribonucleic acid from normal subjects and patients with pseudohypoparathyroidism type 1b. J. Clin. Endocrinol. & Metab., **82**: 1031-1040.

Chapter 3: Bettoun J.D., M. Minagawa, G.N. Hendy, L.C. Alpert, C.G. Goodyear, D. Goltzman, and J.H. White 1997 accepted for publication in the Journal of Clinical Investigation with minor revisions. A third manuscript describing studies on methylation (fig 3.7-3.9) is in preparation.

The work presented in chapter 2 and 3 is my own except for the contribution of my co-authors as follow:

In chapter 2:

- Dr Minagawa performed the experiments in figure 5.
- Dr Yasuda provided table 1.
- M.Y.Kwan helped in sequencing.
- H.S. Lee helped for RNase protection.

In chapter 3:

- Dr Minagawa did the in-vitro methylation experiments and the hybridization of the genomic southern of DNA from 18 weeks fetal kidney.
- Dr Goodyear kindly provided human fetal sample tissues.
- Dr Alpert kindly provided adult human tissues.
- Dr Hendy allowed us the use of his address book.

ACKNOWLEDGMENT.

I am indebted to my supervisors, Dr John H. White and Dr David Goltzman, for their scientific counseling, and everlasting patience and support during my Ph.D. Making me realize that there is a difference between being capable of understanding a scientific publication and being a scientist is probably the least of their achievements.

Considering the reported sedative effect of this thesis, this acknowledgment section should really have begun by thanking my wife, Jack Lavan and Lina Muhtadie, who not knowing the difference between PTHrP and PST, spent long hours trying to make unnoticeable the paucity of my English writing skill.

For its resourcefulness and his endless knowledge, both of which he shares happily, I thank Geoffrey N. Hendy.

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To my grandmother, Aïsha Hazan.

To my Parents, Esther and Avraham Bettoun.

To my wife Bracha, and for my children

Shalom Meshulam Zushia and Malka Aïsha.

To my Mother and father in law.

TABLE OF ILLUSTRATION

LIST OF ABBREVIATIONS.

Bp : base pair.

cAMP: cyclic adenosine monophosphate.

Dex. Dexamethasone.

Di (OH): di-hydroxy.

FA: fetal age.

Inr: initiator.

IP3: inositol triphosphate.

MeC-BP: methyl cytosine binding protein.

Na+: Sodium cation.

PHP: pseudohypoparathyroidism.

PIC: preinitiation complex.

PKA: protein kinase A

PKC: protein kinase C

PLC: phospholipase C

PTH: parathyroid hormone.

PTHR: PTH/ PTHrP receptor.

PTHrP: parathyroid hormone related peptide.

RNase: ribonuclease.

RT-PCR: reverse transcription-polymerase chain reaction.

SDS: sodium dodecyl sulfate.

TAF: TATA binding protein-associated factor.

TBP: TATA binding protein.

Wt1: Wilm's tumor 1.

TABLE OF CONTENTS.

OBJECTIVES AND METHODS.

The research described in this thesis aimed at characterizing the upstream regulatory region of the human parathyroid hormone (PTH)/ PTH-related peptide (PTHrP) receptor gene (PTHR).

Studies in mice showed that this gene is under the control of two promoters. While the downstream promoter (P2) is ubiquitously active, expression from the upstream promoter (P1) is mainly restricted to kidney.

The original hypothesis was that a similar situation was likely to be found in human, which could provide the molecular basis for a number of human pathologies.

Using molecular techniques, we show that while the transcriptional mechanisms regulating PTHR expression during embryonic development are probably similar in the two species, those controlling expression in adult tissues are different.

CHAPTER 1:

INTRODUCTION

SECTION I:

CALCIUM HOMEOSTASIS.

- CALCIUM LEVELS.
- ABSORPTION AND SECRETION: BALANCING CALCIUM CONCENTRATION.
- HORMONAL REGULATION OF CALCIUM HOMEOSTASIS.
- PTHrP.

Calcium is acutely required in the regulation of a wide array of biological processes ranging from synaptic transmission to blood clotting. The important role of calcium in biological processes can be better understood when considering the potential of this ion to interact in a well defined way with calcium binding sites within proteins and to modulate their behavior. Upon binding, calcium is capable of eliciting conformational changes or constraints that will be translated by proteins into changes in their biochemical properties. Though the most obvious modifications are concerned with the acquisition or loss of enzymatic catalytic activity, other parameters can also be modified. For example,upon calcium binding, Thermolysin, a secreted thermostable endopeptidase isolated from *Baccilus thermoproteolyticus*, undergoes, a conformational change that enables it to sustain elevated temperatures. Likewise, the capacity of trypsin to avoid autolysis is mainly dictated by the presence of a calcium ion at its calcium binding sites. Calcium interacts essentially with oxygen atoms of carboxyl residues, and to a lesser extent, with those of peptide-carbonyl or hydroxyl groups. The coordination number of calcium as well as the nature and origin of the donor and the binding mode will dictate the length of a bond. The precise complement of donor groups at a binding site, along with their relative topographies, is probably the main factor in regulating the affinity and the specificity of a protein for calcium. Moreover, the biophysical properties of calcium dictate the formation and solubility of its phosphate salts. Hence, although both calcium and magnesium form a soluble salt with organic phosphate, the smaller radius of magnesium for a given coordination does not allow it to form a structure similar to the most insoluble of the calcium-phosphate salt, hydroxyapatite. The kinetics regulating the formation of these salts will in turn play an important role in both calcium and phosphate homeostasis, as will be discussed later (Martin RB. 84).

Calcium affinity is usually in the millimolar range for extracellular proteins and in the micromolar range for those inside the cell. This distribution reflects closely the relative distributions of calcium concentration between the inside and the outside of a

cell. The correspondence between the affinity for calcium and the surrounding concentration of this ion allows for a prompt response of calcium-regulated proteins to variations in calcium concentration.

A) Calcium levels in the organism.

Calcium is present in the body as ions, bound to proteins, complexed to anions or deposited as insoluble crystals in bone or tooth. Calcium homeostasis is primarily concerned with maintaining plasma calcium concentrations within the physiological range.

Calcium is present in the plasma free, complexed to organic or inorganic ions or bound to proteins. The relative distribution of plasma calcium between these three groups varies in function of the metabolic state. Under normal conditions, 50% of the plasma calcium is in the form of free ions, 5-10% is complexed and 40-45% protein-bound. Most of the proteins that bind calcium (such as albumin and globulins) are far from their binding saturation limits, even at high calcium concentrations. It ensues that proteins will bind a given proportion of ionized calcium rather than a fixed amount. In a normal individual, the total serum concentration varies between 2.1 to 2.6 mM, in which the ionized calcium will contribute from 1 to 1.3 mM. Any condition resulting in a disturbance of fluid balance would result in changes in the calcium distribution between its different plasma constituents (Nordin BEC. 1990).

Calcium represents the fifth most abundant element in the body after hydrogen, oxygen, carbon and nitrogen. About 99% of this calcium is contained in bone (750 to 1000mM). The large pool of miscible calcium contained in this tissue contributes largely to daily maintenance of the normal serum ionized calcium concentration. This 'buffer' calcium allows the daily dietary intake to double, or the daily renal excretion to be multiplied by four, without significantly altering the serum calcium concentration. This

apparent lack of effect of the daily intake or output of calcium is largely due to the fact that exchange of calcium between blood and bone occurs in the molar range, whereas absorption or excretion occur in the millimolar range. Hence by varying slightly the balance of exchange between bone and blood, a healthy individual will be able to cope with normal daily variations in calcium balance (Nordin BEC. 1990).

Mild hypo- or hypercalcemia (between 1.75 to 3.0 mM of total serum calcium) are usually asymptomatic. More pronounced hypocalcemia can result in severe neuro-muscular and organic defects that can lead to death following cardiac arrest or seizures. Likewise, hypercalcemia gives rise to a number of severe conditions that can result, in extreme cases, in coma and death (Neer RM. 82). Both hypo-and hypercalcemia are generally associated with a failure of the mechanisms normally responsible for tightly maintaining calcium concentration within its physiological values. Such a failure can be due to various clinical conditions that directly affect one of the components of the calcium regulatory mechanisms, or that disturb calcium homeostasis beyond the range of action of the regulatory mechanisms.

Thus, calcium homeostasis will be compromised in any organic or genetic condition affecting the functioning of the organs involved in calcium trafficking, and/or those responsible for regulating exchanges.

B) Absorption and secretion: balancing calcium concentration.

Extracellular fluid calcium concentration is the result of the difference between absorptive and secretive fluxes that take place in the intestine, kidneys, bones and teeth, as well as transfer of calcium to the fetus or to the milk in the case of a pregnant or lactating woman. The minor component, resulting from inevitable loss (dead cells, bleeding, nails, etc.), will not be considered here. As might be expected, hormones regulating calcium homeostasis will act at all the sites of calcium trafficking and utilization.

1. Calcium absorption in the intestine.

The net absorption of calcium by the intestine is the balance between the dietary intake and the amount lost in the feces. The loss of calcium is the sum of calcium that has not been absorbed and that, which has been secreted, in the digestive fluids. Since the latter does not depend on intake, the net calcium absorption can be negative at low calcium intake (<4.5millimoles).

The existence of both an active saturable and passive components of calcium transport results in a bi-phasic mechanism of absorption. The percentage of calcium absorption increases from negative values at intake levels lower than 4.5 mmoles, to 30% at 14mmoles. At this amount, the active transport component is mostly saturated and, as intake increases, represents a decreasing fraction of the calcium absorption. Above values of 14 mmoles, the fraction of calcium absorbed declines slowly toward the value of the passive component, until only 5 to 10% of the ingested calcium is then reabsorbed (Nordin BEC. 90).

Calcium is absorbed along the entire gastro-intestinal tract. The contribution of each segment to absorption has been determined using radioactive calcium and depends on both the absorptive activity of the cell within a segment and on the time spent in a segment. While the absorptive activity is the highest in the duodenum and declines progressively thereafter, the highest level of absorption takes place in the ileum where 67% of the calcium is absorbed. The contributions of other segments to absorption are: 17% in the jejunum, 8% in the colon, 7% in the duodenum and 2% in the stomach.

In each segment, absorption is the result of the sum of absorptive fluxes from the mucosa to the serosa, and secretive fluxes in the inverse direction. While the absorptive fluxes are subject to hormonal regulation, secretion appears to be constant.

The absorptive fluxes can be described by an equation that considers both the active saturable and passive components.

$$J_{m\text{-}s} = (J_{max} * Ca / Ca + Kt) + D * Ca$$

- Jmax is the maximum absorptive activity of the active component, which is directly influenced by the level of vitamin D.
- D is the diffusion coefficient of the non-saturable passive component.
- Kt is the absorption constant of the active component and is similar in all the segments of the gastro-intestinal tract, with a value of 1-2mM.

The net calcium absorption will therefore depend on the maximum velocity of the active transport, the value of the diffusion coefficient and the calcium concentration in the lumen of the intestine. This latter parameter will in turn reflect both the calcium content of the diet and the acidity at the site of absorption, since a basic environment activates the formation of insoluble calcium complexes with phosphate ions, free fatty acids and other organic compounds. The permeability coefficient, D, reflects the diffusion of calcium through paracellular ways. The Jmax is a composite of several transport mechanisms through the cell membranes. Some data support the idea that Jmax is predominantly a reflection of transport mechanisms from the lumen to the inside of the cell. As stated above, vitamin D acts mainly by increasing this component of the calcium transport. Assuming an average lumen and blood calcium concentration of 1-1.5mM and a cellular concentration of 1-0.1μM, the transport of calcium within the cell involves a diffusion down a steep electrochemical gradient, and an up-hill energy-consuming extrusion of this calcium out of the cell and into the blood. The physico-chemical characteristics of the phospholipid bilayer hinder the passage of large cations like calcium, and despite the electrochemical gradient, calcium entry within the cell needs to be facilitated. The nature of the transport mechanisms for calcium ions at brush border membranes is not really known. Both calcium channels and a chloride co-transporter have been postulated, however the only consensus about apical calcium entry is that very few studies have concentrated on the subject (Friedman PA. and FA. Gesek; 95 Bronner F. 92).

Extrusion of calcium through the basolateral membrane occurs against a non-favorable gradient, and as expected, requires energy. Two transporters have been shown to participate in calcium extrusion: a high affinity Ca^{2+}ATPase pump, and an electrogenic Na^{+}/Ca^{2+} antiporter, both of which are located at the basolateral membrane of epithelial cells. Of these two transporters, the Ca^{2+}ATPase displays an increased activity along the different segments of the gastro-intestinal tract, whereas that of the sodium exchanger is constant. Electrochemical data suggest that the higher absorptive capacity of the duodenum is predominantly due to the presence of a highly active Ca^{2+}ATPase.

2. Renal handling of calcium.

Calcium ions and ionic complexes are freely filtered by the glomeruli. Of the approximately 260 mmoles of calcium filtered daily, 1-2% (2.5-5 mmoles) are excreted in the urine.

The relative contributions of the different segments of the nephron to calcium reabsorption varies between different species. Between 40 to 60% of the filtered calcium is reabsorbed in the proximal tubule, 20% in the thick ascending limb of the Henle's loop, 10% in the distal convoluted tubule, and a further 5% before the final urine.

The nature and mechanisms of calcium absorption also vary along the nephron. As for the transport mechanisms in the gastro-intestinal tract, calcium transport occurs along a thermodynamically favorable gradient to the inside of the cell, and against a similar gradient from the inside of the cells to the peritubular fluid. The flux of calcium from the lumen into the cell is mediated by a passive facilitated mechanism. Absorption of water, calcium and sodium in the proximal convoluted tubule depends on the presence of sodium in the peritubular fluid and on calcium within the cells, stressing the role played by the basolateral Na^{+}/Ca^{2+} exchanger in this segment (Gmaj P. and H. Murer

84, Friedman PA. and FA. Gesek 95). This electrogenic antiporter, like the one in the intestinal epithelium, exchanges 3 Na^+ for one calcium.

The coordinated absorption of calcium and sodium is no longer found in the straight part of the proximal tubule, where calcium is reabsorbed in excess of sodium. The thin descending limb is relatively impermeable to calcium. In contrast, cells in the thick ascending portion of Henle's loop display a high absorptive activity, albeit with poorly defined transport mechanisms (Friedman PA. and FA. Gesek 95). The abolition of calcium absorption by drugs that inhibit chloride transport suggests that in this segment, transport could be derived from the electrical gradient created by the active absorption of chloride. Heterogeneity also exists between the cortical and medullary parts of this segment with respect to calcium permeability; the cortical part being more permeable to calcium.

As for the straight part of the proximal tubule, the rate of calcium reabsorption in the distal tubule is higher than that of sodium and is independent of its reabsorption or of electrical potentials. The rate of reabsorption there appears proportional to the calcium load, with transport mainly occuring through the transcellular pathways (Friedman PA. and FA. Gesek 95). The convoluted part of the distal tubule is furthermore the primary site for regulation of calcium excretion by the parathyroid hormone.

The exact site of reabsorption of 5% of the calcium between the end of the distal tubule and the urine is more difficult to localize precisely, but is believed to occur in the early portion of the connecting tubule (granular segment of the cortical collecting duct). Calcium reabsorption in this segment depends totally on the presence of PTH.

The mechanisms by which calcium is reabsorbed are diverse. The existences of various electrical or chemical gradients along the nephron provide numerous paracellular routes for calcium to translocate from the lumen to the peritubular space. The permeability for calcium varies not only in different segments but also for the same

segment in different species. Any condition affecting luminal osmolality or the transluminal electrical gradient will certainly affect the passive diffusion of calcium.

Transcellular pathways for calcium absorption comprise the three steps of apical entry, cellular translocation and basolateral extrusion described for intestinal transcellular calcium translocation. Similar biochemical processes are thought to mediate calcium movement in the intestine and kidney; however it has not been established whether the same proteins mediate these transport mechanisms in the two organs. For example, although in certain species the same type of CaBP-D is found in both systems, other species seem to display a differential pattern of expression with respect to the different types of CaBP-Ds (Johnson JA. and R. Kumar 94 and see later for the role of this protein).

3. Calcium deposition in bones.

Stated naively, the main function of bones is to sustain the body structures. Bones also serve as a calcium reservoir as they contain about 99% of bodily calcium. By processes of exchange with blood, bones are capable of buffering the daily variations of calcium balance. Rapid calcium exchanges occur mainly at the trabeculae and result from changes in phase between ionized and insoluble calcium phosphate salts. The formation and solubility of these salts depends tightly on the acidity and phosphate concentration of the environments as well as on the extracellular matrix environment (Martin RB. 84, Wuthier RE. 84, Bronner F. 92). The contribution of bones in actively maintaining a normal serum calcium concentration appears to be more important in situations of active metabolic demand like pregnancy and lactation. The onset of fetal bone calcification during the third trimester of pregnancy has been correlated with an increase in intestinal absorption and a rise in the levels of markers indicative of bone resorption such as tartrate-resistant alkaline phosphatase or hydroxyprolin. Markers of bone formation, on the other hand, do not appear to vary significantly. The increased intestinal calcium

absorption and bone resorption might result from the concomitant rise of PTHrP and 1,25 dihydroxy-vitamin D levels throughout pregnancy (Kovacs CA. and HM. Kronenberg 97).

Lactation is associated with profound effects on calcium metabolism in general, and on bones in particular. Studies in rat showed that up to 35% of bone mass could be lost during lactation. Although bone loss is not as pronounced in humans, lactation periods of 2 to 6 months can result in bone mineral loss ranging from 3 to 8% (Kovacs CA. and HM. Kronenberg 97).

A number of endocrine conditions that affect the mechanisms of calcium absorption or excretion or their hormonal regulations also have an effect on bones. It is, however, beyond the goal of this introduction to review all the mineral and skeletal effects that these endocrine conditions may have.

C) Hormonal regulation of calcium homeostasis.

Two components should be envisaged when considering calcium homeostasis. The first is related to the organs controlling calcium movements to and form the blood, i.e. the intestine, kidney and bones. The second component is concerned with regulating these movements in relation to the blood calcium levels, and involves the different calciotropic hormones, PTH, 1,25 dihydroxy-vitamin D, calcitonin, PTH related peptide (PTHrP) and calcium itself. A set point can be defined in each compartment that is the serum calcium concentration that will affect variations in the behavior of this compartment *vis-a-vis* calcium handling. For example, the kidney will linearly increase calcium excretion when the plasma calcium concentration rises above 1mM. Likewise, the bi-directional flux of calcium between bone and blood is kept at about 1000 mg per day under conditions where the plasma calcium concentration is at 1mM. If the plasma concentration were to decrease consistently, a new value for the total amount of calcium

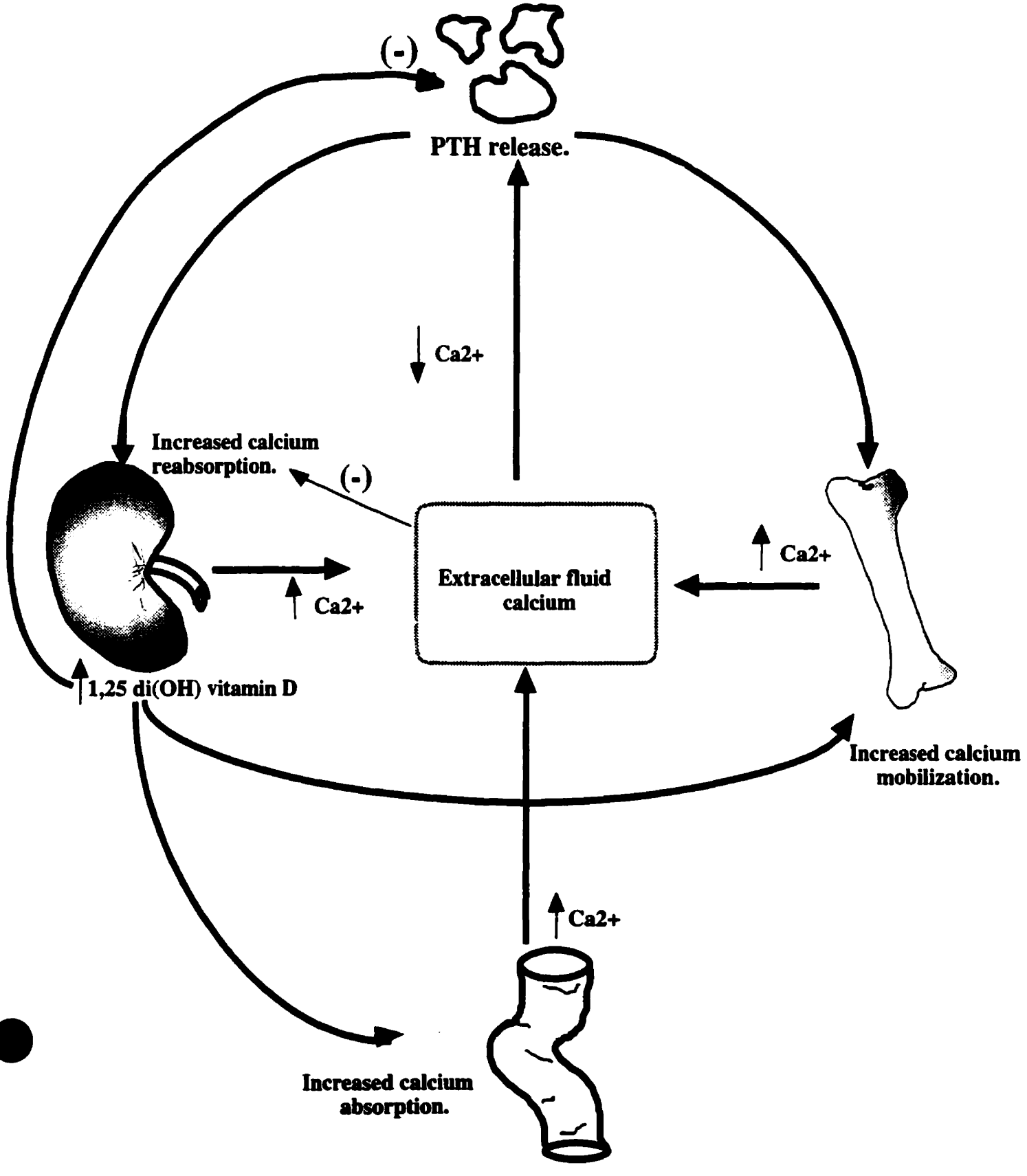
exchanged per day would allow the maintenance of calcium concentration at its safe value, by allocating less calcium to the resorption and formation processes in bones. It has been shown that in the absence of both PTH and Vitamin D the kidneys allow calcium excretion at lower serum concentrations, which demonstrates that the set-point is under hormonal regulation (Hebert SC. et al 97).

The capacity of PTH, calcitonin and Vitamin D to affect the ability of the kidneys to handle calcium filtration is dictated by the circulating levels of these two hormones, which itself depends on the serum calcium concentration. The parathyroid glands also have a set point under or above which PTH secretion is stimulated or inhibited. The set point for the parathyroid gland is defined as the concentration of calcium under which PTH secretion is half-suppressed; as for the renal set point, it varies under pathological conditions affecting the function of the parathyroid glands (Goltzman D. and GN.Hendy 90, Brown EM. 95). The parathyroid glands thus have the role of discriminating between normal and abnormal circulating calcium values, and of accordingly adjusting PTH secretion. Starting with a variation in the serum calcium concentration, figure 1.I.1 depicts the regulatory processes that take place to adjust plasma calcium concentrations.

The mechanisms by which the parathyroid glands are capable of detecting variations of the plasma ionized calcium concentration have become clearer in recent years with the characterization of the so-called "calcium-sensing" receptor (Brown EM et al 93). This receptor, coupled to G proteins, can activate a number of intracellular second messenger pathways upon calcium binding. Activation or inactivation of the receptor follows variations in calcium concentration in the millimolar range. The resultant variations in intracellular calcium affect PTH secretion. The calcium receptor is therefore a good candidate in establishing the calcium set point in the parathyroid glands. Its presence has been demonstrated on the surface of parathyroid glands, as well as in a number of other tissues (Brown EM. 95). Mutations affecting the calcium receptor have been associated with various forms of calcemic disorders. Individuals carrying an

Figure 1.1: Schematic representation of the endocrine mechanisms of calcium homeostasis. A decreased extra-cellular fluid calcium concentration causes PTH secretion by the parathyroid glands. PTH acts on the kidneys to increase calcium reabsorption, phosphate secretion, and 1a-hydroxylase activity. It also stimulates calcium mobilization from bones. 1,25 di (OH) vitamin D stimulates Calcium absorption by the intestine, potentiates PTH action on bone, and inhibits PTH gene transcription. These combined actions restore ECF calcium to normal values. Note that calcium can affect its own reabsorption by acting on the kidney. See chapter 1.I.C for more details.

Schematic representation of calcium homeostasis.



inactivating mutation in one allele display familial hypocalciuric hypercalcemia. This syndrome is characterized by a mild hypercalcemia accompanied by lowered levels of calcium excretion. As PTH levels are mostly normal in these patients, the renal calcium retention can not be associated with PTH over-expression. The presence of mutations in both alleles has been shown to cause neonatal severe hyperparathyroidism. Patients harboring this double mutation develop a severe hypercalcemia within hours following birth, demonstrating the acute role played by the calcium sensing receptor in regulating calcium concentration. The concurrence of PTH hypersecretion and hypercalcemia can be explained by a failure to suppress PTH secretion at the normal circulating calcium concentration, or in other terms, by a shift in the parathyroid glands set-point toward higher values (Brown EM. et al 95).

The calcium receptor is also present in the nephron (Riccardi D. et al 95), where its activation by an elevated level of calcium in the ultrafiltrate inhibits sodium and chloride reabsorption in the thick ascending limb and antagonizes the action of anti diuretic hormone in the collecting duct. This results in a decrease in the generation of the positive voltage in the lumen that drives calcium absorption in the thick ascending limb, and in impaired water reabsorption. Thus the calcium receptor might be an important component in the establishment of the renal set-point for calcium excretion ensuring the elimination of excess calcium loads without necessarily requiring changes in circulating PTH levels (Hebert SC. et al 97).

1. Effect of the parathyroid hormone.

Parathyroid hormone is produced exclusively in the parathyroid glands. The active hormone is released in the circulation following cleavage of a pre-pro-hormone to a pro-hormone upon translocation to the endoplasmic reticulum. Further maturation of PTH involves cleavage of the pro-hormone, to yield the active form in the trans-Golgi apparatus. The hormone is then stored in secretory granules. In the presence of the

appropriate extracellular stimulus, i.e. hypocalcemia, the parathyroid glands secrete the biologically active form of PTH consisting of 84 amino acids. In the presence of high plasma calcium content, an increase in the intracellular metabolism of PTH is observed. Under such circumstances, biologically inactive mid-molecule and carboxy- terminal fragments are secreted in the blood. In a situation of sustained hypocalcemia, the rapid depletion of preformed stocks of the hormone is prevented by transcriptional and post-transcriptional activation of PTH expression. The importance of these second-line regulatory mechanisms is emphasized by the development of secondary hyperparathyroidism due to glandular hyperplasia in patients with chronic renal failure. A loss of responsiveness to 1,25 dihydroxy-vitamin D in PTH-producing cells is believed to cause the secondary hyperparathyroidism in these patients. Bolus injection of 1.25 dihydroxy-vitamin D has been shown to suppress the condition (Kurokawa K. 94). Apart from the hyperplasia associated with CRF, sustained hypocalcemia will also lead to increased PTH secretion by mechanisms involving cell proliferation in the parathyroid gland (Goltzman D. and Hendy G.N. 90).

Sequential carboxy deletion of PTH showed that the peptide comprising amino-acids 1-34 retains full biological activity. Studies of the relative activities of PTH molecules deleted or substituted at their NH2- or COOH- termini have led to the characterization of the amino acids necessary for activity, allowing the definition of a number of partial agonists and antagonists in different species. Thus, the first three amino acids are obligatory for PTH action, while removal of residues before amino acid 34, results in a progressive loss of PTH activity (Goltzman D. and Hendy G.N. 90).

The principal secretatogue of PTH is calcium, although other cations, anions or peptides have been shown capable of eliciting PTH secretion. Upon release, PTH acts in an endocrine fashion to regulate circulating ionized calcium concentration. The strikingly short half-life of PTH in the circulation, less than three minutes, further emphasizing the acute nature of PTH action (Fitzpatrick LA. and Bilezikian JP. 96).

The two conventional PTH target organs are bone and kidney, where the hormone binds a G-protein coupled receptor at the surface of target cells and affects calcium transport or utilization. The structure, function and localization of the receptor will be addressed extensively in the next section.

The means by which PTH affects calcium handling is a direct consequence of how physiological properties of target cells are altered upon PTH binding. The binding of PTH is relayed intracellularly by different second messenger systems, and translated into activation or inhibition of cellular processes. A comprehensive understanding of how PTH affects cells involved in calcium homeostasis is limited by the lack of detailed knowledge of the cellular mechanisms pertaining to calcium homeostatic processes.

i) Renal action of PTH: The overall effect of parathyroid hormone on the kidney is to reduce calcium and increase phosphate excretion. PTH-mediated increase in calcium absorption is the result of its action on the transcellular calcium transport capacity of three of the distal tubular segments of the nephron: the thick ascending limb, the distal convoluted tubule and the connecting tubule. In the proximal tubule, PTH inhibits phosphate reabsorption and Na+/ H+ exchange at the brush border (Muff R. et al 92)
An increase in the apical calcium entry, transcellular transport or extrusion at the basolateral membrane could account for higher level of calcium reabsorption. The appearance, upon PTH treatment, of dihydropyridine-sensitive calcium channels at the apical membrane of cells in the cortical part of the thick ascending limb of mouse nephrons has been reported. The activity of the Na^{+}/Ca^{2+} antiporter at the basolateral membrane is also enhanced in the presence of PTH. In most instances however, the molecular nature of the cellular components affected by PTH remain purely hypothetical or subject to contradictory results (Bourdeau JE. 93, Friedman PA. and FA. Gesek 95).

Most of the electrochemical observations made under PTH treatment can be reproduced with an activator of adenylyl cyclase or with diBut-cAMP, suggesting that PTH action is mediated by activation of PKA pathways. A role for PKC was also

suggested by the demonstration that PTH application could trigger the appearance of calcium transients in proximal tubule cells, and a more sustained intracellular calcium elevation in cells from the distal collecting duct. Although their significance in the regulation of calcium homeostasis is not known, the molecular bases for these phenomena became clearer with the demonstration that the cloned PTH/PTHrP receptor is capable of activating several intracellular pathways (see the chapter on the PTH/PTHrP receptor).

A further effect of PTH in the kidney relevant to calcium homeostasis is the stimulation of 1-alpha-hydroxylase activity in cells from the proximal tubule, which leads to an increased circulating level of 1,25 dihydroxy-vitamin D (see below).

ii) Skeletal effects of PTH: PTH exerts a catabolic effect in bones by stimulating the resorption of the calcified bone matrix and therefore the release of calcium and phosphate in the circulation. The osteolytic activity of PTH in bones is mediated by a proliferation and activation of osteoclasts. The effect of PTH on bone is bi-phasic, comprising an initial rapid effect, which leads to the liberation of calcium in the blood within minutes, and a delayed response that involves more complex changes (Goltzman D. and GN. Hendy 90, Fitzpatrick LA. and Bilezikian JP. 96). The initial phase is apparently derived from the activation of preexisting osteoclasts or of other cell types, and results in an enhanced mobilization of labile calcium. The persistence (over 24 hours) of high levels of circulating PTH will induce the appearance of more osteoclasts, as well as ultra structural and biochemical changes leading to an increase of resorptive activity (Bronner F. 92, Fitzpatrick LA. and Bilezikian JP. 96). Bone resorption by osteoclasts first involves dissolution of bone mineral, and subsequently a degradation of the organic matrix. The sole documented agent capable of dissolving hydroxyapatite is the proton, and acidification of the space between the bone matrix and the osteoclast's ruffled membrane is primarily responsible for crystal solubilization. Following acidification of the lacuna,

proteinases degrade the bone matrix. The exact nature of the enzymes in this process and their origin is controversial, but both collagenase and secreted lysosomal cysteine proteinases have been proposed to play a role in this process (Vaananen K. 96).

The understanding of the mechanisms that regulate resorption versus formation have been limited by the lack of suitable cellular models for studying osteoclasts. It appears that PTH action is likely to be mediated by osteoblasts or stromal cells and to involve soluble factors. This concept is reinforced by the observation that PTH receptor is not expressed in mature osteoclasts. A number of cellular factors known to enhance osteoclastic activity, have been shown to be produced by osteoblasts in response to PTH. Among them, the more potent, as suggested by in vivo studies, are interleukin 1 (Il 1) and the macrophage colony-stimulating factor (M-CSF) (Roodman GD. 96).

2. 1,25 di-hydroxy vitamin D and calcium homeostasis.

Production of the active component of vitamin D requires its hydroxylation at both positions 1 and 25. Vitamin D is produced in the skin by a UV induced photochemical reaction, which breaks open the B ring of 7-dehydrocholesterol between carbons 9 and 10; it is also acquired through the diet. Hydroxylation at position 25 takes place in the liver and leads to the production of 25(OH) vitamin D. This compound is the most abundant metabolite of vitamin D in the blood, with circulating concentrations two or three orders of magnitude higher than that of 1,25 dihydroxy-vitamin D, and with a much longer half-life (Kanis JA. et al, 82). Further hydroxylation occurs in the kidney with the generation of 1,25 di (OH) or 24,25 di (OH) vitamin D, following hydroxylation by the mitochondrial enzymes 1α– and 24 hydroxylase, respectively. The calcitropic form of the hormone is 1,25 di-hydroxylated, although some activity is observed at high doses of 25 OH vitamin D.

The most documented action of 1,25 di (OH) vitamin D is the modulation of gene transcription upon binding to its nuclear receptor, the vitamin D receptor (VDR). This

receptor belongs to the superfamily of steroid nuclear receptors and modulates transcription upon binding, as a heterodimer with the 9-cis retinoic acid receptor (RXR). to so called Vitamin D response elements in the promoter region of target genes. The VDR, like other nuclear receptors, has a modular structure with distinct regions of the protein assuming discreet functions (Haussler MR. et al 98).

i) Modulation of intestinal epithelium calcium transport by 1,25 di (OH) vitamin D.

As for PTH, the stimulation of calcium translocation through the intestinal epithelium assumes the up-regulation of transport mechanisms that are only partially characterized.

Two modes of action have been described for 1,25 di (OH) vitamin D, a classical modulation of gene expression and a less characterized non-genomic action of the hormone. The use of 1,25 di (OH) vitamin D analogues have permitted the distinction between these two pathways.

Two proteins involved in transcellular calcium transport have been shown to be up regulated by 1,25 di (OH) vitamin D via activation of their genes transcription. The calcium binding proteins 9KD and 28KD ($CaBP\text{-}D_{9Kor28k}$) belong to the Troponin superfamily of high affinity calcium binding proteins. They are distributed in a ubiquitous manner, but their expression is dependent on 1,25 di (OH) vitamin D in only a restricted subset of tissues. 1,25 di (OH) vitamin D increases both the generation and the stability of transcripts encoding for the CaBP-9k (Johnson JA. and R. Kumar 94). The resulting increase in the intracellular concentration of the protein is thought to account for the 70-fold faster rate of calcium cellular translocation between the apical and basolateral membranes, that is found in 1,25 di (OH) vitamin D repleted animals (Bronner F. 92). Alternative roles for this protein have been proposed such as increasing the activity of the Ca-ATPase at the basolateral membrane of intestinal cells, and therefore favoring the

extrusion of calcium, or, acting as a buffering system at the basolateral membrane, which maintains the electrochemical gradient across the membrane even with higher intracellular calcium concentrations. Application of 1,25 di (OH) vitamin D and calcium or phosphate dietary depletion stimulates the transcription of the gene encoding this calcium transporter, which in turn stimulates calcium extrusion from the epithelial cells.

Non genomic effects of 1,25 di (OH) vitamin D-stimulated calcium transport are concerned with the transfer of the ion from the lumen to the cell via the apical membrane. This effect, known as transcaltachia, is characterized by a very rapid increase of calcium transport (within minutes) from the lumen upon local 1,25 di (OH) vitamin D perfusion. It is not hampered by the use of protein synthesis inhibitors but is blocked by 1β,25 di (OH) vitamin D (Kumar R. 95). The mechanisms underlying this phenomenon are uncertain and might involve changes in membrane fluidity.

It thus appears that 1,25 dihydroxy-vitamin D acts at the three cellular levels of entry, translocation, and extrusion, by affecting both genomic and non-genomic mechanisms. The relative importance of these two types of action for the transport of calcium is not clear. Some insights have been gained from patients with hereditary hypocalcemic vitamin D resistant rickets in which the vitamin D receptor is not functional (Haussler MR. 97). In these patients the rate of intestinal calcium absorption is low despite very high levels of circulating 1,25 di (OH) vitamin D, suggesting a marginal role for transcaltachia in vivo. On the other hand, activation of VDR by 1,25 di (OH) vitamin D analogues fails to increase the rate of absorption despite an up-regulation of the CaBP-D9k (Johnson JA. and R. Kumar 94), demonstrating that the activation of gene transcription by the hormone is not sufficient to mediate the physiological action of vitamin D.

ii) 1,25 di (OH) vitamin D and renal calcium absorption. On the basis of the observations made on intestinal calcium transport regulation by 1,25 di (OH) vitamin D, it is tempting to use the same cellular model to explain the enhanced calcium

translocation through late segments of the nephron in the presence of the hormone. The temptation becomes almost irresistible when considering that all the components of the 1,25 di (OH) vitamin D inducible machinery found in the intestine are also found in the appropriate segments of the kidney (Kumar R. 95). While some investigators support the notion of a similar model in the intestine and kidney (Johnson JA. and R. Kumar 94. Bronner F. 92), others are more cautious in their conclusions, in the view that there exist species differences in 1,25 di (OH) vitamin D regulation of renal calcium transport that cannot be accounted for by different responses to the hormone. Hence, whereas both calcium uptake and intracellular content are increased by the hormone in rabbit connecting tubule cells, mouse distal convoluted tubule cells do not exhibit such a response but seem rather to make use of the hormone to shorten the cell response to PTH. This qualitative improvement of PTH performance does not seem to require transcriptional activation, since it can be elicited in the relatively short time of two hours (Friedman PA. and FA. Gesek 95).

ii) 1,25 dihydroxy-vitamin D and bones. 1,25 di (OH) vitamin D is a potent activator of bone resorption. Its primary action is to stimulate osteoclastic osteolysis. Not surprisingly, the exact mechanisms underlying hormone activity are not well understood. As for the PTH/PTHrP receptor, mature osteoclasts do not express VDR, and like PTH, 1,25 di (OH) vitamin D is likely to act through the osteoblasts cells to modulate the secretion of factors that could in turn regulate osteoclastic activity. The presence of VDR in cells committed to become osteoclasts and the documented influence of both PTH and 1,25 di (OH) vitamin D in the generation of immature osteoclasts explain how these two hormones may influence osteoclast formation (Roodman GD. 96). One possible mode of action for 1,25 di (OH) vitamin D is to modulate the production of bone specific matrix component by osteoblastic cells. Both osteocalcin and osteopontin gene expression are up regulated by the hormone, whereas that of type I collagen is down regulated (Haussler MR. et al 97). Variations in the matrix composition are very likely to affect the stability

of the calcified tissue. Osteocalcin, for example, is known to inhibit hydroxyapatite precipitation, an increased production of which would result in a more labile bone matrix (Whutier RE. 84). A simultaneous effect of the hormone on bone formation can also be expected, as osteoblast differentiation is influenced by matrix composition (Stein G.S. and J.B. Lian 93).

3. Calcitonin and calcium homeostasis.

Calcitonin was originally characterized based on the observation that a faster and greater fall in plasma calcium is observed following thyroparathyroidectomy than following calcium perfusion of the parathyroid gland. Calcitonin was subsequently shown to be produced in the parafollicular cells of the thyroid gland in a number of species (Barlet J-P. 82). Since then, calcitonin production has been demonstrated in a number of other tissues. Calcitonin is a secreted peptide hormone. Its final size of 32 amino acids in humans results from the cleavage of both the NH2- and COOH- termini of a larger precursor. The physiology, function, and the relative importance of calcitonin in humans is controversial. Thus neither calcitonin over-production by medullary thyroid cancer, nor its absence, seems to affect calcium metabolism significantly (Becker LK. 90). Calcitonin has, however, been extensively used for the treatment of Paget's disease and for short term therapy of hypercalcemia (Becker LK. 90).

The wide distribution of the calcitonin receptor in kidney, intestine and osteoclasts would be consistent with a role of calcitonin in calcium homeostasis. The only undisputed effect of calcitonin, however, is to down regulate the expression of its receptor in osteoclasts. This effect probably accounts for the loss of responsiveness to calcitonin over time, in hypercalcemic patients treated with this hormone (Roodman GD. 96).

D) PTHrP and calcium homeostasis.

One of the main endeavors of the world of bone and mineral research in the eighties was the characterization of the factor responsible for the appearance of humoral hypercalcemia of malignancy (HHM). This syndrome, which is associated with a number of tumors, has a high incidence and leads to severe complications associated with hypercalcemia (Orloff JJ. et al 94). The factor responsible for HHM has been characterized in 1987 and has been shown to display a high homology to PTH in its amino terminal residues. Several different cDNAs have been isolated that are derived from differential promoter usage or splicing. The longer open reading frame (ORF) for the peptide is found in humans and encodes a 173 amino acid peptide. Other shorter transcripts encoding peptides of 139 to 141 amino acids have been characterized in chicken, mouse, rat and human. The human gene is composed of eight exons, four of which encode for the three 5' untranslated regions (Exons 1a, 1b, 1c and 2 in fig 1.I.2), and the remaining four the signal sequence (exon 3), the core of the peptide up to residue 139 (exon 4), and the two alternative carboxy-terminal extensions of the protein (exons 5 and 6). Transcription of the human gene proceeds from three different promoters, two containing TATA-like initiators of transcription (upstream of exons 1a and 2) and one being a (G+C) rich promoter (upstream of exon 1c). (Fig. 1.2).

PTHrP undergoes extensive post-translational processing that results in the generation and secretion of several potentially biologically active peptides. The primary amino acid sequence shows the presence of multiple potential endo-proteolytic sites that delineate functionally distinct domains. Diverse physiological activities have been ascribed to these domains. Hence PTHrP is known to have endocrine, paracrine, autocrine, and intracrine modes of action. Although it is not our purpose to review the numerous activities of PTHrP associated with normo- or pathophysiology, we will nonetheless summarize the different activities that might be relevant to the understanding of the function of the PTH/PTHrP receptor.

Residues 1-34 of PTHrP have been shown to be chemically and structurally homologous to PTH 1-34 and to be capable of eliciting all the actions of this hormone. This portion of the molecule is responsible for the appearance of HHM, as tumors secrete large amounts of PTHrP in the serum, which acts on kidney and bone to stimulate calcium reabsorption and release. PTHrP 1-34 has also been shown to be a potent relaxant of smooth muscle cells in a number of organs. Release of PTHrP upon cellular distension results in cellular relaxation. In the uterus, liberation of PTHrP diminishes the spontaneous contractility of the muscle. An increase in the urine volume in the bladder leads to PTHrP secretion and to a relaxation of the bladder wall.

The amino-terminal region of the peptide has been associated with organogenesis. In breast, skin and bones, alteration of the local production of PTHrP leads to defects in organ formation. Genetic ablation of the PTHrP gene in mice resulted in accelerated chondrocyte differentiation, and therefore of ossification, which leads to the development of osteochondrodysplasia (Karaplis A et al 94). Consistent with an accelerated differentiation in mice lacking the peptide, overexpression of PTHrP in bone leads to a delayed differentiation resulting in smaller transgenic animals compared to their normal littermates, with a disproportionate fore-shortening of the fore limbs and tail (Weir EC. et al 96). Targeted overexpression of PTHrP to myoepithelial cells of the mammary gland of transgenic mice produces a mild form of mammary hypoplasia due to defective branching morphogenesis. Likewise, in skin, PTHrP might be involved in regulating the growth and differentiation of keratinocytes. Conflicting results were obtained depending on whether the ligand was added to the culture medium, in which case PTHrP had a slight mitogenic effect, or whether the endogenous production was inhibited by the use of antisense technology, which resulted in a decrease in the expression of maturation markers of the keratinocytes (Henderson J. et al 91). The generation of transgenic mice in which PTHrP overexpression was targeted to the keratinocytes showed that excess production of PTHrP

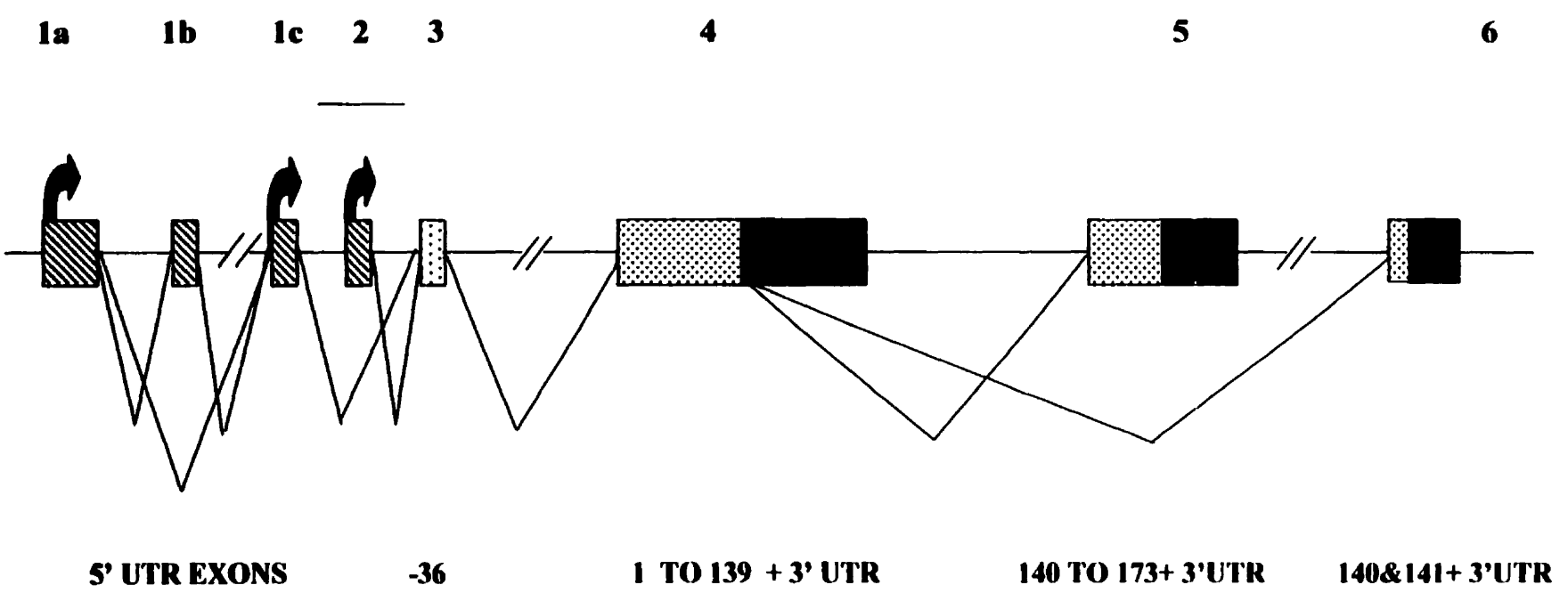


Fig 1.2. Schematic representation of the human PTHrP gene. The three promoters are represented by arrows. The multiple potential splicing are represented by a bent lines connecting the exons. The bar above exon 2 represents a CpG island. Note also that each exon encoding for the three possible carboxy-terminal parts of the molecule has a particular 3' UTR region.

is associated with a delay or failure in hair follicle development (Wysolmerski JJ. et al 94).

The growth regulatory actions of PTHrP might play an important role in the generation and development of malignancy. Whether the up-regulation of PTHrP observed in a number of tumors is coincidental or whether PTHrP actually plays a role in the apparition, development and progression of the tumors, is not known.

The presence of multiple splicing forms and the secretion of different processing products all provide the potential for a great diversity of actions as an autocrine, paracrine or endocrine factor. As we shall see in the next chapter, PTHrP production usually occurs in the close vicinity of its membrane receptor, the activation of which can elicit a variety of cellular responses. It is however important to note that an increasing amount of evidence suggests the existence of one or several additional membrane receptors and that, as will be mentioned later, PTHrP can act in an intracrine manner by being targeted to the nucleus.

There are several arguable reasons against the inclusion of a description on PTHrP in a chapter about calcium homeostasis. Under normal physiological conditions, PTHrP is not present in the plasma. Most of the instances where circulating concentrations of PTHrP are detectable are associated with deregulation of or changes in calcium homeostasis. Also, as opposed to PTH, PTHrP is expressed almost ubiquitously and acts in a paracrine, autocrine or intracrine manner. Finally, there does not seem to be any acute correlation between plasma calcium concentration and PTHrP production. On the other hand, there is no direct evidence that PTHrP is dispensable as far as calcium homeostasis is concerned. As we shall discuss in the chapter dealing with the PTH/PTHrP receptor, mice lacking PTHrP die in the immediate perinatal period (or after two month for genetically rescued mice) yielding no information about the effects of a lack of PTHrP on calcium homeostasis. Finally most of the recognized PTHrP activity

seems to be mediated by the seven transmembrane-spanning, G-protein linked receptor that can be liganded and activated by both PTH and PTHrP.

INTRODUCTION,

SECTION II:

THE PTH/PTHRP RECEPTOR.

- STRUCTURE.
- SIGNALING PATHWAYS.
- STRUCTURE-FUNCTION RELATIONSHIP.
- THE PTHR GENE.
- DISTRIBUTION OF THE RECEPTOR.
- REGULATION OF THE PTH/PTHRP RECEPTOR ACTIVITY AND EXPRESSION.
- ROLE OF PTHR SIGNALLING DURING DEVELOPMENT AND IN THE ADULT.
- OTHER RECEPTOR FOR PTH AND PTHRP ?

A) STRUCTURE OF THE PTH/PTHRP RECEPTOR.

A cDNA for the PTH/PTHrP receptor (PTHR) was originally isolated by expression cloning from an Opossum kidney cell cDNA library (Juppner H. et al 91). Analysis of the primary amino acid sequence derived from the open reading frame, showed no similarities with the previously reported G-protein linked receptors, except for the Calcitonin receptor (CR), whose cloning was described in the same issue of Science (Lin HY. et al 91). The hydrophobicity plot of the predicted amino acid sequence revealed the presence of ten hydrophobic regions, seven of which were assumed to span the membrane. Of the remaining three hydrophobic regions, the first, located immediately after the ATG codon, was assigned to the signal peptide. The presence of four putative N-glycosylation sites in a position carboxyl- to the second hydrophobic region precluded it being chosen as the first transmembrane domain, as was unlikely that the four glycosylation sites would be cytosolic. The rest of the transmembrane domains were speculatively attributed in order to match the putative secondary structure of the CR (which displayed only eight hydrophobic regions including the signal sequence). PTHR and CR, as well as the Secretin receptor (SR) characterized at the same period, defined a novel subfamily of seven transmembrane-spanning domains G-protein-coupled receptors. Subsequently, a number of peptide receptors were shown to be homologous to various extents to PTHR and to belong to the same subfamily. PTHR is related structurally to the receptors for, Secretin (SR), vasoactive intestinal peptide (VIPR1 and VIPR2), gonadotropin releasing factor (GRFR), pituitary adenylyl cyclase-activating peptide

(PACAPR), and to a lesser degree to the receptors for gastric inhibitory peptide (GIPR), glucagon and the glucagon like peptide 1 (GLP1R). Other receptors, like those for the corticotropin releasing factor (CRF); insect diuretic hormone (DHR) and calcitonin are more structurally distant. A partial genomic clone isolated from *C. elegans* also showed some similarity with PTHR. As we shall discuss later, the subsequent characterization of genes encoding these receptors also revealed related gene structures.

Apart from sequence homology, members of the so-called PTHR/CR subfamily of receptors present a number of structural similarities. The extracellular amino terminal extension contains multiple asparagine linked glycosylation sites and is of intermediate length between those of the adrenergic and muscarinic receptors and those of the glycoprotein hormones receptors such as TSH, LH and FSH. There is also a conserved pattern of cysteine residue distribution in the extracellular domain and in the first and second extracellular loops.

B) THE PTH/PTHrP RECEPTOR CAN ACTIVATE MULTIPLE INTRACELLULAR SIGNALING PATHWAYS.

Upon binding by one of either of its cognate ligands, the PTH/PTHrP receptor contacts and activates the intracellular signal transduction machinery. As indicated by their generic name, G protein-linked receptors function by contacting the inactive heterotrimeric G protein complex. This complex, composed of a α subunit and of a $\beta\gamma$ heterodimer, dissociates and each component can then activate the production of second messengers.

Early studies in which the cDNA for the receptor was expressed in COS7 cells showed that both PTH (1-34) and PTHrP (1-36) bind to the receptor with the same affinity (kd=4nM). The two ligands stimulated cAMP accumulation to the same extent

(Juppner H. et al 91, Abou-Samra A-B et al 92). The original report of the receptor cloning and subsequent studies demonstrated that PTHR could activate all of the characterized intracellular response to PTH. COS7 cells or LLC-PK1, transiently or stably transfected, respectively, with either the rat or opossum receptor, showed that the receptor is by itself capable of mediating a number of cellular responses to PTH following the activation of distinct transduction pathways (Juppner H. et al 91, Abou-Samra A-B 92).

The PTH/PTHrP receptor is capable of stimulating the intracellular accumulation of cyclic adenosine monophosphate (cAMP), as well as that of inositol triphosphate (IP3) and calcium.

cAMP accumulation is the result of receptor contact with the Gs class of G proteins. Upon dissociation of the heterotrimer, the Gsα subunit contacts membrane bound adenylate cyclase, which catalyzes the production of cAMP. The extent and the nature of the cellular response depends on the isotypes of adenylate cyclase, cAMP phosphodiesterase and PKA regulatory subunit, which are present within the cell (Houslay MD. and G. Milligan 97).

The coupling of the receptor to the Gq class of G proteins results in the activation of phospholipase C (PLC). This enzyme catalyzes the hydrolysis of phosphatidyl inositol diphosphate (PIP2) into diacyl-glycerol (DAG) and inositol triphosphate (IP3). Accumulation of DAG leads to the activation of protein kinase C (PKC), following its translocation to the cell membrane (Newton AC. 97).

In a number of cell lines, activation of the receptor also results in an elevation of cytosolic calcium concentrations. This increase can be due either to extracellular calcium entry via surface channels or to the liberation of calcium from intracellular stores. While the latter pathway involves IP3, the former can be due to the activation of PKA or PKC, or to the coupling of the receptor to other G protein classes. The mechanisms by which

PTH, or PTHrP, induces increases in cytosolic calcium appear to vary among different cell types (Massry SG. and Smogorzewski M. 95).

The capacity of the receptor to activate multiple signaling pathways is not the same in every cellular system. Hence, PTH or PTHrP can readily elicit cAMP, IP3 and calcium increase in some cell types (Abou-Samra A-B et al 92, Bringhurst FR. et al 93), while primary or clonal vascular smooth muscle cells (VSMC) only display cAMP accumulation. This selective activation of adenylate cyclase was attributed to regulatory mechanisms at the G protein level since a slight overexpression of $G_{q, 11}$ or $_{14}$ could restore PTH induced PLC activation (Maeda S. et al 96). Unlike VSMC, treatment of stably transfected porcine renal LLC-PK1 cells with PTH will invariably result in activation of adenylate cyclase, irrespective of the number of receptors expressed at the cell surface. PLC activation, on the other hand, will only be observed at higher levels of receptor expression (Guo J et al 95). The differential activation of the two signaling pathways is of particular interest, as it raises the possibility that a tissue or developmental specific up-regulation of the number of receptors at the cell surface can influence the production of the two potential second messengers.

The generation of different second messengers can elicit a myriad of cellular responses sometime opposite in nature depending on the cell line. Thus, while application of PTHrP is associated with an inhibition of DNA synthesis in VSMC (Maeda S. et al endo 96), its action in keratinocytes is associated with a slight increase of proliferation (Henderson JE.et al 92).

It has been demonstrated that the multiple biological responses to PTH or PTHrP within a cell can rely on distinct activation pathways. For example, in stably transfected LLC-PK1 cells, inhibition of DNA synthesis by PTH requires the simultaneous activation of PKA and PKC. In the same cell line, stimulation of sodium-dependent phosphate uptake does not seem to depend on PKA activation or calcium release (Bringhurst FR. et al 93, Iida-klein A. et al 97). In the case of the inhibition of the apical Na+/H+ exchanger

NHE-3 isoform in OK cells by PTH or PTHrP, both PKA and PKC are activated, but each pathway seems to act independently from the other (Azarani A. et al 95a). Interestingly, in the osteoblast like cell line UMR-106, PTH or PTHrP could activate the Na+/H+ exchanger NHE-1 isoform only via the PKA pathway (Azarani A.et al 95b). The action of PTH on the Na+/H+ channel became interesting in light of the report that PTH can cause a specific increase in the extracellular acidification rate. This newly described activity of PTH, which can be found in SaOS 2 cells but not in UMR-106, depends exclusively on the activation of the PKC pathway. The use of a Na+/H+ exchanger inhibitor, showed that these transporters do not mediate this phenomenon (Barrett MG. et al 97).

As detailed above, the ability of the receptor to stimulate the production of a specific second messenger depends on which Gα subunit the receptor will contact. The cellular specificity of the physiologic response to PTH or PTHrP binding is puzzling, given the high potential for diversity, which is present downstream of the receptor in many cell types. One possible explanation is the presence of cell- or stage-specific regulatory proteins that would modify the receptor capability to interact with a given class of effector. Other mechanisms could include the kinetics of deactivation of different Gα subunits, the stoichiometry of different classes of subunits, compartmentation of signaling proteins, and covalent modification. For a more detailed review of G protein-mediated transduction pathways see (Neer EJ. 95).

C) Structural determinants of the dual binding and signaling capacity of the PTH/PTHrP receptor.

The PTH/PTHrP receptor is capable of activating both the cAMP and IP3/Ca^{2+} pathways. The structural bases for both the dual ligand binding and the dual signaling capacity of the receptor have been the subject of extensive studies.

1. Role of extracellular residues. The first analyses of the structure-function relationship *per se* took advantage of discrepancies in the binding affinity of various PTH analogues or derivatives to receptors from different species. Although the rat and human receptors, which are 91% identical, bind PTH (1-34) with the same affinity, the human receptor has a 50-fold higher affinity for PTH (7-34) than the rat (Shipani E. et al 93). Chimeric constructs in which different portions of the receptor were swapped between the two species revealed that the amino-terminal extracellular domain bound the carboxy-terminus part of PTH (Juppner H. et al 94). A finer analysis in which discreet domains of the receptor were deleted showed that the proximal part of the extracellular extension (residues 31-47) and the third extracellular loop (residues 431-440) are critical for bovine PTH (1-34) binding (Lee C. et al 94). In these two studies, the mid-region of the extracellular extension was shown to be dispensable for receptor activity, and was subsequently replaced by a tag that allowed the immuno-characterization of the receptor in transfected cells.

The role of the third extracellular loop was confirmed in homologue-scanning experiments in which different segments of the rat PTHR were replaced by the corresponding segments of SR. Systematic amino acid substitution within this region singled out the tryptophan residue at position 437 and the glycine residue at position 440 as important for binding. Substitution of either of these two residues by neutral amino acids resulted in a significant loss of PTH (1-34) binding. This loss of affinity was accompanied by a decrease in cAMP accumulation upon treatment with agonist. An arginine for glycine substitution at position 440 resulted in a decrease in cAMP accumulation that was not correlated with a decrease in the binding to the receptor. It is

noteworthy that these mutants could bind PTH (3-34) with affinities similar to that of the wild type receptor, which suggests that the first two amino acids of PTH are not required for binding to the receptor (Lee C. et al 95). One of the mutant receptors, in which the carboxy-terminal part of the extracellular domain extension was exchanged for that of secretin receptor, displayed a dramatic loss of affinity with bPTH (1-34). Although this mutant was not characterized further, it was shown subsequently that this region is part of the agonist-binding domain of the receptor (Zhou AT. et al 97). In this report, Zhou et al, using photo-affinity cross-linking techniques, showed very elegantly that residues 173-189 could be covalently linked to a photo-reactive ligand. This set of experiments better defined the regions of the receptor important for ligand binding. The data they generated are however not exhaustive as they are restricted to areas of the receptor dispensable for expression of the receptor and its integration into the cell membrane. For example, Lee et al failed to recognize residues 173-189 as important for ligand binding since the receptor in which this region was deleted was not expressed at the cell surface (compare Lee C. et al 94 and Zhou AT. et al 97).

2. The intracellular loops and terminal extension. Studies on the intracellular domains of the receptor mainly focused on trying to understand the molecular determinants that enable the receptor to activate a dual-signaling pathway. Two studies, giving apparently contradictory results, emphasized the role played by the cytoplasmic carboxy-terminal extension in activating intracellular signaling pathways. Huang *et al* found that this region of the receptor was dispensable for both adenylate cyclase and PLC activation (Huang Z. et al 95a). Using a similar experimental approach, Iida-Klein *et al* showed that deletion of the cytoplasmic tail selectively activated cAMP accumulation without

affecting IP3 levels (Iida-klein A. et al 95). The discrepancy between these two results is probably due to the difference in the techniques used to achieve a uniform number of receptors at the cell surface for each mutant. While the former group diluted COS7 cells transfected with the wild type receptor with untransfected cells, the latter adjusted the amount of DNA transfected in order to achieve a similar density of receptor per cell for each construction. Alternatively, the differences could have arisen from the use of opossum versus rat receptor. The carboxy-terminal region also contains two amino acid segments important for the regulation of ligand induced internalization of the receptor. Deletion of the three amino acid residues located at the 7th transmembrane domain interface, enhanced internalization of the receptor, whereas a fragment of 19 residues located between positions 475 and 494 markedly reduced it (Huang Z.et al 94). Finally, the cytoplasmic terminus of the receptor has been shown to be the subject of basal and ligand-induced phosphorylation. Several serine residues appear to be phosphorylated by different kinases such as the protein kinases A and C (PKA, PKC) or the beta-adrenergic receptor kinase 1, βARK1 (Blind E. et al 96).

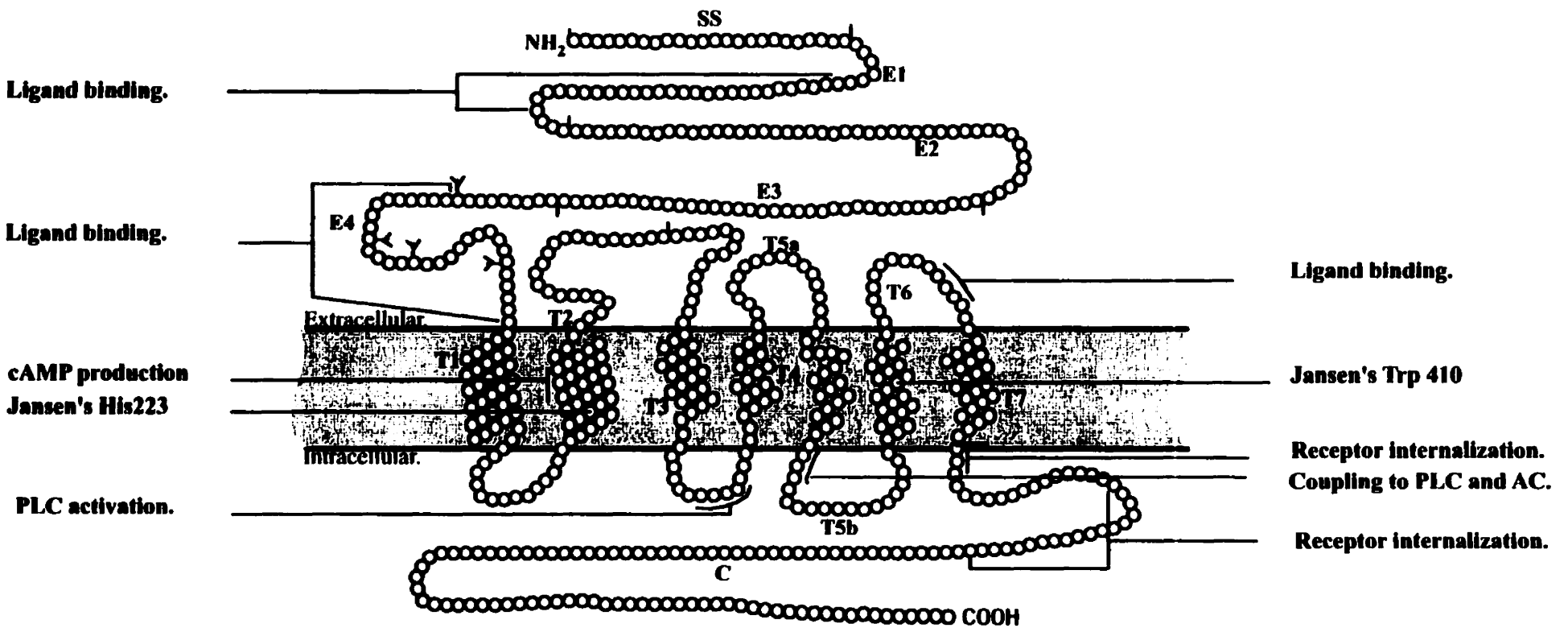
Coupling of the receptor with a particular G protein constitutes the main determinant for activation of a specific pathway (Neer E.J. 95). The ability of the PTH/PTHrP receptor to interact with different classes of G protein heterotrimers is mainly determined by residues contained within the three intracellular loops. A number of mutagenesis studies, as well as some naturally occuring point mutations, unraveled the molecular bases of the multiple activating capacity of the PTH/PTHrP receptor. Two amino acids, His 223 and Trp 410, were found mutated in patients with Jansen's metaphyseal chondrodysplasia. Both mutant receptor displayed a constitutive activation of the cAMP pathway. However,

while substitution of the histidine at position 223 by either an arginine or a lysine residue resulted in a failure to activate IP3 production upon PTH binding, substitution of the threonine at position 410 by any amino acid preserved the PTH induced IP3 response (Shipani E. et al 96). The absence of a particular requirement for the side chain of the residue at position 410 suggests that a threonine is required at that position to maintain the receptor in a repressed/inducible state (Shipani E. et al 97a). The different behaviors of those two mutants *vis-a-vis* IP3 accumulation further emphasize the complexity of the mechanisms controlling the dual signaling capacity of the receptor.

Random clustered mutations in the second intracellular loop demonstrated that four amino acids at positions 317-320, and in particular a lysine residue at position 319, were important for specifically activating phospholipase C (PLC). A mutant receptor in which these four amino acids were replaced could not elicit IP3 accumulation, whereas cAMP accumulation was unaffected (Iida-Klein A. et al 97). The amino terminal portion of the third intracellular loop is likewise involved in receptor coupling with cellular transduction pathways. Tandem alanine scanning mutagenesis of this region resulted in a sensible reduction of cAMP and IP3 accumulation in transfected COS7 cells. Single substitution of residues 377-379 failed, however, to affect cAMP response to PTH binding, while IP3 accumulation was markedly reduced (Huang Z. et al 96). Replacement of residues 381 and 382 resulted in a loss of adenylate cyclase activation, as well as of PLC activation in the case of amino acid 382. A detailed analysis of several characteristics of the response suggested that these mutations could lead to a decreased capacity of the liganted receptor to contact the G protein and/or an impaired ability to activate it once contacted (Huang Z. et al 96).

Figure 1.3. Schematic representation of the rat PTH/PTHrP receptor. The exon boundaries, as found in the gene, are indicated by small bars. Regions or individual residues that have been found important for either binding or intracellular pathways activation are indicated on the side. Refer to the text (chapter 1.II.B, C and D) for more details.

Structure and function of the PTH/PTHrP receptor.



Y Glycosyl groups.

3. Role of the transmembrane residues. Activation of seven transmembrane G protein-linked receptors by their cognate ligands is thought to induce a conformational change that separates the transmembrane domains, and positions intracellular residues in a proper conformation (Bourn HR. 97). The role of residues in the transmembrane domain was addressed by systematically mutating residues that are highly conserved between members of the PTHR subfamily of G protein coupled receptors. This study underlined the importance of an arginine and two serine residues located within the second transmembrane domain, at position 230, 226 and 233 respectively. The replacement of these amino acids by an alanine resulted in a loss of adenylate cyclase activation, suggesting that these residues may participate in maintaining the receptor in a proper conformation (Turner PR. et al 96a). The same group showed that exchanging the second transmembrane domain of the secretin receptor for that of the PTH/PTHrP receptor allows this receptor to bind PTH, and *vice-versa*. Furthermore, both chimeric receptors could activate intracellular signaling upon binding of the agonist specific for the second transmembrane domain. Although binding and intracellular pathway activation were weak, this result revealed that residues within the second transmembrane domain, particularly an isoleucine at position 234, could discriminate between ligands, and in turn transduce an intracellular signal upon binding of the appropriate ligand (Turner PR. et al 96b). Figure 2 depicts the different amino acid that have been found to play a role in either binding of the ligand or second messenger pathway activation.

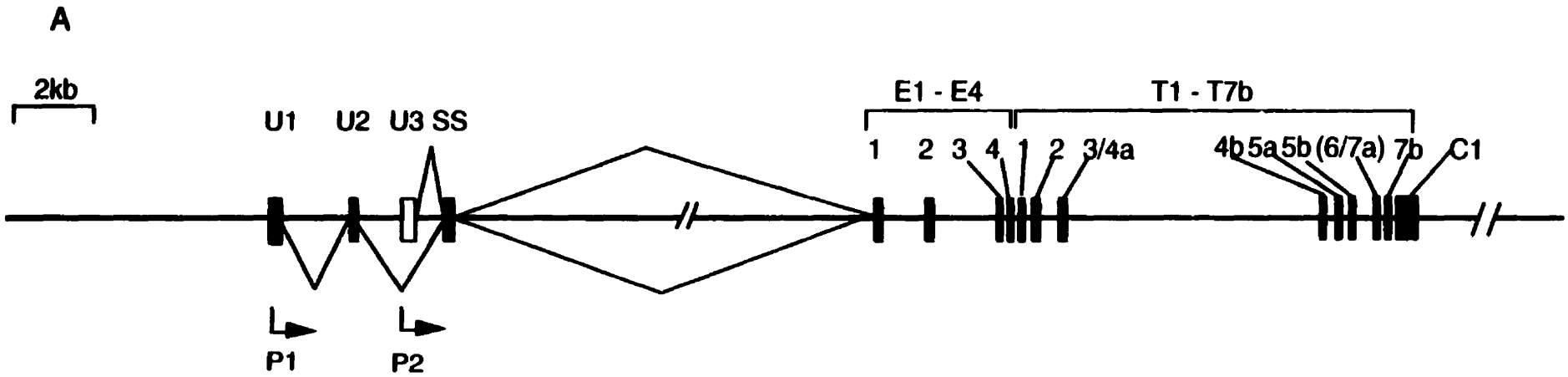
D) ORGANIZATION OF THE PTH/PTHrP RECEPTOR GENE.

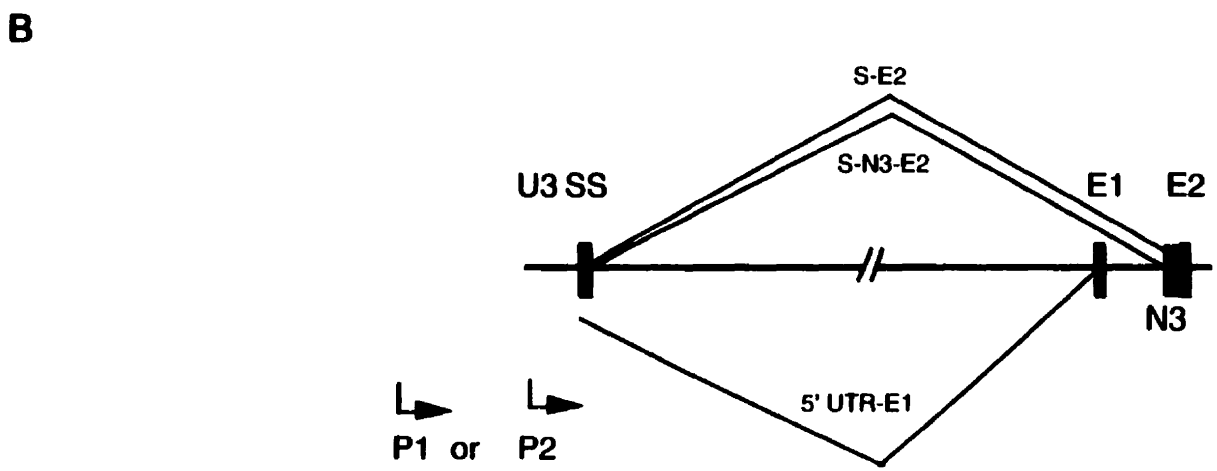
The gene encoding the PTH/PTHrP receptor has been characterized in rat, mouse, human and *Xenopus Laevis*. (McCuaig K. et al 94, Kong X-F. et al 94, Bergwitz C. et al 98). Its structure is highly similar in all mammalian species and it is located on chromosome 3, 8 and 9, in human, rat, and mouse respectively (Pausova Z. et al 94). The coding region of the cDNA is comprised of fourteen exons (figure 3). Both the signal sequence and the cytoplasmic tail are encoded by a single exon, whereas four exons encode the extracellular domain (E1-E4). The transmembrane domains and the extra- and intra- cellular loops are encoded by nine exons (T1, T2, T3/4a, T4b, T5a, T5b, T6/7a, T7b and C). A very large intron (>11kb) is invariably present in all three species between the signal sequence and exon E1.

Two reports describe differential splicing at, or near, the beginning of the coding region. In one instance, two shorter isoforms were amplified by RT-PCR. Both splice variants lacked E1, and the 3'end of the signal sequence was spliced directly to E2 (S-E2), or to sequence located 34bp upstream of E2 (S-N3-E2). This latter isoform could still drive the expression of a functional receptor which is very poorly translocated at the cell surface (Jobert AS. et al 96). Another report describes the splicing of an upstream untranslated exon to E1, which eliminates the signal sequence. The protein resulting from the translation of this isoform appeared to be mostly confined to the cytosol (Joun H. et al 97). Considering the extreme sensitivity of PCR, an RNase protection experiment would have been useful in determining to what degree these splice variants represent a significant fraction of the PTH/PTHrP receptor transcripts.

Figure 1.4: Schematic representation of the rodent PTHR gene. A: The three upstream untranslated and 11 coding exons are represented by boxes. The splicing of introns is represented by bent lines. Arrows indicate the posistion of the two promoters. B: representation of the differential splicing of the gene within the major intron. Note that N3 was originally described as part of the intron.

Genomic organisation of the rodent PTHR gene.





Although their respective mRNAs were shown to be polyadenylylated, no classical polyadenylylation signal AAUAAA was found in the human, mouse, rat or *Xenopus laevis* genes. Polyadenylylation of the transcript must therefore occur through an unrecognized signal (McCuaig K. et al 94, Kong X-F. et al 94, Bergwitz C. et al 98).

Transcription of the PTHR gene in mouse and rat proceeds from two different promoters (McCuaig K. et al 94, McCuaig K. et al 95, joun endo 97). Transcription from the first promoter, P1, is initiated at multiple start sites spanning more than 100 bp. Expression from P1 appears to be largely restricted to the kidney, where, in mouse, it accounts for more than 90% of PTHR transcripts (McCuaig K. et al 95, Amizuka N. et al 97). The 5' untranslated region of transcripts derived from P1 results from the splicing of two upstream exons, U1 and U2, either to the signal sequence exon (McCuaig K. et al 95) or directly to the first exon, E1, encoding the extracellular domain (Joun H. et al 97). A cDNA in which U1 is spliced directly to the signal sequence has also been described in pig (Smith DP. et al 96).

P2, the second promoter, is located in a (G+C)-rich region approximately 3.0 kb downstream of P1, and drives the expression of transcripts bearing an upstream exon (U3) spliced to the signal sequence. Unlike P1, P2 is active in all tissues examined. Table 1 summarizes the respective characteristic of P1 and P2.

The cloning of the genomic region encoding for mouse PTHR, VIPR1, CR and GRFR showed that these four genes share a very similar organization. The intron-exon boundaries in the primary amino acid sequences are similar in all of them, excluding an additional exon, E2, in the amino terminus of the PTHR gene and of a single exon encoding T5b T6 and T7a in the CT gene (Albrandt K. et al 95, McCuaig K. et al 94).

Although early reports emphasized the similar organization of genes encoding for some members of the PTHR receptor subfamily, the subsequent characterization of genes encoding for other members of this family, which have a different organization, was barely noticed. For example, the gene encoding for the Glucagon-like peptide receptor is encoded by 7 exons spanning more than 40kb (Lankat-Buttgereit B. and Goke B. 97). Likewise, the gene encoding for the gonadotropin-hormone-releasing factor is only composed of three exons (Fan NC. et al 95).

The recent characterization of cDNAs for the receptor from *Xenopus laevis* kidney, showed that this species has two non-allelic genes encoding for 95% identical receptors (Bergwitz C. et al 98). Interestingly, the two X.laevis genes did not have the equivalent of exon E2,confirming that this exon is dispensable for PTHR function (see the chapter on structure-function relationship).

	INITIATION OF TRANSCRIPTION	G+C CONTENT AND PROMOTER CONTENT	TISSUE SPECIFICITY	STRUCTURE OF THE 5'UTR
P1	• Multiple start-sites. • No recognizable initiator elements.	• ~ 50%. • Few consensus sites for general transcription factors	• Accounts for 90% of the expression in the kidney, mostly silent elsewhere	• Two UTR exons (U1&U2) spliced to the signal sequence.
P2	• Single start-site. • No recognizable initiator elements.	• ~85%. • Numerous sites for Sp1 and Ets transcription factors.	• Ubiquitously active.	• A single exon spliced to the signal sequence or to E1.

Table 1: Characteristic of the two promoters for the mouse PTHR gene.
From McCuaig K. et al 94, McCuaig K. et al 95, Joun H. et al 97.

E) Tissue distribution of PTH/PTHrP receptor mRNA.

The cDNA for the receptor was used as a probe in a thorough examination of receptor mRNA expression in various tissues or cell lines during different stages of development, and under different culture conditions.

The localization of receptor mRNA in classical PTH target organs has revealed features which shed new light on the role of PTH-PTHR axis in regulating calcium homeostasis. Detailed analysis of the cellular distribution of transcripts for the receptor has been performed in few tissues. An immunohistochemical study performed in rat kidney, detected the presence of the PTHR protein in both the basolateral and luminal membranes and in luminal sub-membranar vesicles of cortical and medullary epithelial cells. A weak immunoreactivity was present in the glomeruli, and at the surface of glomerular podocytes. Unexpectedly, a strong signal was present in the thin wall of cortical capillary endothelial cells. A detailed in-situ hybridization study of the 5' UTRs expressed in different renal cellular subtypes demonstrated a cellular specificity of promoter utilization. While transcripts derived from the upstream promoter P1 could be detected in all cell types in which the protein could be detected, those originating from P2 were barely detectable in the glomeruli, and were absent from vascular endothelial cells. Hence, in vascular endothelial cells, expression of the PTH/PTHrP receptor gene is driven solely from the kidney-specific P1 promoter. Both protein and transcripts were detected in the cortical and medullary collecting ducts. There, again, only P1 specific transcripts could be seen (Amizuka N. et al 97). This result contrasts with another report

in which no transcripts encoding for the receptor could be amplified by RT-PCR using RNA samples derived from the medullary collecting ducts (Riccardi D. et al 96). Failure to detect P1-specific transcripts in otherwise highly vascularized tissues suggests that some signal originating from kidney epithelial cells triggers the onset of this promoter in the adjacent vascular endothelial cells (McCuaig K. et al 95, Amizuka N. et al 97).

Localization of transcripts for the PTH/PTHrP receptor in rat endochondral bone showed that expression of the gene is spatially and temporally regulated. Transcripts for the receptor were detected in adult rats growth plate by in situ hybridization. There, a signal could be seen exclusively in maturing chondrocytes, as estimated by the overall absence of overlap between regions of the growth plate expressing type X collagen and PTHR. Osteoblasts at the surface of trabecular bone also displayed a high level of expression (Lee K. et al 92). The temporal and cellular pattern of expression during fetal bone development was established by the simultaneous detection of several markers for bone differentiation. The formation of endochondral bone during fetal development proceeds in a very orderly manner. The onset of receptor expression coincides with the early stage of mesenchymal condensation. Although very weakly expressed at this stage, a high level of expression subsequently develops in early chondrocytes and in the perichondrium. Further differentiation involves expression of the receptor in the zone of maturing chondrocytes and in cells of the perichondrium immediately adjacent to the maturation zone. As the bone grows, expression of the receptor extends along the whole perichondrial surface, with the exception of the zone where premature chondrocytes are situated , it is also found in the osteoblast within the primary ossification centers (Lee K. et al 95). This sequence of expression of the receptor in bone is relevant to the

understanding of the defects present in mice in which the PTH/PTHrP receptor has been ablated (see later). Consistent with the wide expression pattern of PTHrP during fetal development, other tissues express the receptor in the fetus. PTH/PTHrP receptor is generally expressed in mesenchymal cells lining epithelial cells, where PTHrP is expressed (Wysolomerski JJ. and Stewart AF. 98). Furthermore, there seems to be an inverse correlation between the intensity of PTHrP expression and that of its receptor. The role of the PTHrP/PTHR signaling axis during development is unclear as no structural or functional defects could be observed in any organs other than bone. Transcripts for the receptor were present in the dermis, tooth buds, sub-ephitelial layers of the intestinal wall and lung, heart, kidney, choroid plexus, and a number of other skeletal and extra-skeletal tissues in the head (Lee K. et al 95).

Distribution of PTHR transcripts in adult rats was mainly addressed by northern blot analysis. PTHR transcripts were detected in all the organs examined with the exception of prostate, pituitary, and thyroid gland (Urena P. et al 93). Interestingly, in kidney and liver, a 5kb and a 2.8-kb transcript can be detected in addition to the major 2.3-2.5-kb band. RNA from testes and skin shows additional smaller transcripts of 2.1kb and 1.4kb, respectively. The smaller transcript appears to be the major PTHR mRNA species in testes. A probe spanning the first 107 amino acids of the receptor failed to detect this transcript in the testes, and the corresponding band in the kidney. This suggests that some transcripts may be missing part of or the integrality of the sequence encoding for the extracellular domain. Alternatively, this band could represent closely related transcripts that would be recognized by the PTHR cDNA. Analysis by the same technique of mRNA distribution in human tissues showed minor differences as compared

to the rat. Transcripts were detected in prostate and larger transcripts, rather than smaller, were observed in testes and ovary (Usdin TB. et al 95).

Few reports are available on the cellular localization of the receptor in non-classical target organs in adults. The presence of the receptor has been demonstrated in epithelial cells along the gastro-intestinal tract, and the onset of its expression seems to precede that of PTHrP (Li H. et al 95). Transcripts for the receptor were co-localized with those for PTHrP in the stromal fraction of the spleen and a down regulation of the level of transcripts accompanied lipoplysacharide (LPS) treatment. The level of expression of the PTH/PTHrP receptor and of PTHrP appeared inversely correlated as the latter was up regulated under these circumstances (Funk JL. et al 95).

F) Regulation of the PTH/PTHrP receptor activity and expression.

Like most G-protein linked receptors, PTH/PTHrP receptor is subject to homologous or heterologous desensitization or down-regulation (Lohse MJ. 93). These terms generally refer to a reduction of the second messenger response to agonist binding, following a pre-incubation with either the agonist itself (homologous) or another agent (heterologous). Desensitization, or down-regulation, of the receptor can be the result of different events. Both a decrease in number or affinity of cell-surface receptors (down-regulation) or an uncoupling of the receptor from the G protein (desensitization) would result in a smaller apparent response of the cell to the agonist. This decrease can result from transcriptional, post-transcriptional or post-translational changes in response to a factor. All these modifications might or might not happen simultaneously in a cell type,

and might affect different aspects of the intracellular response to receptor activation (Segre GV. 96).

Numerous reports describe the regulation of receptor activity by different agents in different cell lines. We will here essentially focus on studies performed in osteoblast or kidney cell lines as they are of most relevance to calcium homeostasis. It should be noted, however, that homologous desensitization/down-regulation of the receptor is probably a widespread mechanism, as the level of expression of transcripts for PTHrP and for the PTH/PTHrP receptor seem inversely related in numerous organs (see the chapter on the distribution of the receptor).

Incubation of rat osteoblastic UMR-106 cells with either PTH or PTHrP for periods ranging from 8 to 48 hours, resulted in a decreased PTH-induced cAMP accumulation to 20% of that in untreated cells (Gonzalez EA. et al 96, Okano K. et al 94). Both ligand binding and stimulation of cAMP production were affected. The desensitization of the receptor *per se* was accompanied by a pronounced down-regulation of the steady state level of receptor transcripts (20% of control level after 24 hours). The down regulation of the mRNA steady state, as well as the decrease of PTH induced cAMP accumulation could be elicited by activators of the PKA pathway but not by those of PKC. In contrast, desensitization of the calcium response to PTH, which occurs within minutes following PTH treatment in the same cell type, required activation of the PKC pathway, and probably of other cellular mechanisms (Lee KL. and Stern P. 94). Studies in other osteoblast-like cell lines showed variations in the mechanisms of homologous desensitization of the receptor. In SaOS-2 cells, Fukayama *et al* showed that the mode of desensitization to PTH is achieved through PKA-dependent and independent pathways,

whereas the down regulation of the receptor was mainly independent of the PKA. Short PTH exposure (4-6 hours) resulted in a 75% desensitization of cAMP responsiveness and a 50% down-regulation of PTH binding in these cells. PTH also decreased the PTH/PTHrP receptor mRNA steady state in a manner that did not seem to involve transcript destabilization or require new protein synthesis (Fukayama S. et al 94). It is noteworthy that the extent of mRNA down-regulation and its time-course were not as acute nor rapid as that observed in other systems (see for example Okano et al 94 and Law F. et al 94). A slightly different scenario was reported in the rat osteosarcoma cell line ROS 17/2.8, where a homologous desensitization by PTH of the cAMP response and binding to PTH was also described. In this system, preincubation of the cell with PTH had no effect on the steady state level of receptor mRNA (Urena P. et al 94). Similar experiments performed in Opossum kidney (OK) epithelial cells, showed that both the desensitization and down-regulation of the receptor occur upon preincubation of the cells with an agonist. Similar to ROS17/2.8 cells, the down-regulation was not associated with changes in the steady state of the mRNA, but involved a removal of the receptor from the cell surface (at 24 hours), which was preceded by a loss of affinity of PTH for its receptor (at 4 hours) (Abou-Samra A-B. et al 94).

Although studies on receptor desensitization have mainly focused on the homologous action of receptor agonists, other factors are capable of affecting its desensitization. Among them is TGFβ, which is capable of inhibiting the differentiation process of cells of the osteoblastic lineage. A striking example of the effect of this growth factor on osteoblast-like cell differentiation was given by a report in which a 48 hour pulse of TGFβ on MC3T3 cells, following their plating, could irreversibly inhibit

differentiation of those cells into osteoblasts. The same treatment applied later, at a time when the differentiation program was already initiated, had only mild or negligible effects (McCauley LK. et al 95). Accordingly, TGFβ was shown to decrease both PTH induced cAMP production and the PTH/PTHrP receptor mRNA steady state level following a 6 hours treatment in primary osteoblast culture. This treatment had no effect on the binding of PTHrP. The action of TGFβ was suppressed by co-incubation with cycloheximide, but not by actinomycin (Jongen JWJM. et al 95). In opossum renal epithelial OK cells, a down-regulation of the receptor was observed that was sensitive to the use of an inhibitor of transcription (law F. 94), which suggests that different mechanisms can mediate similar actions of TGFβ in different cell lines. The effect of TGFβ appeared to differ in transformed cell lines such as ROS17/2.8, where it up-regulated the mRNA level of the receptor (Seitz PK. et al 92).

Other heterologous factors have been shown to have a differential action depending on the cell line. Angiotensin decreased all aspects of PTH/PTHrP signaling in primary aortic vascular smooth muscle cells but had no effect in UMR-106 cells (Okano K. et al 94).

Steroid hormones also display cellular specificity of action in the regulation of PTH/PTHrP receptor activity. For example, treatment of ROS 17/2.8 cells with the glucocorticoid receptor agonist dexamethasone (Dex) up-regulates the number of PTH binding sites, PTH-induced cAMP production, and the steady state level of receptor mRNA. None of these effects were observed in OK cells, where Dex could only potentiate the homologous down-regulation brought about by PTH (Urena P. et al 94). Conversely, 1-25 dihydroxy vitamin D3 decreased receptor mRNA in ROS17/2.8 (Xie

LY. et al 96) UMR-106 and MC3T3 cells (Gonzalez EA. et al 96, Amizuka N et al 98). Regulation of expression and activity of the receptor by steroid hormones has also been addressed in-vivo, in ovariectomized rat. A two hours treatment with estradiol (E_2), had a dramatic effect on the level of PTH/PTHrP receptor transcripts, with a greater than 80% decrease of the steady state mRNA level. This marked down-regulation was no longer observed after 24 hours of treatment, at which time the level of expression was back to 50% of the control level. Not surprisingly, the drastic decrease in the receptor's mRNA steady state level paralleled a marked increase in the transcript level for PTHrP as well as an impressive potentiation of PTHrP-mediated inhibition of uterus spontaneous contractions. Moreover, E_2's effect of on PTHrP transcripts was obliterated following a pretreatment with Dex (Paspaliaris V. et al 95).

A possible explanation to the differential responses to compounds by different cell lines assumes a role for the extracellular matrix (ECM) in modulating the effect of those compounds on PTH/PTHrP receptor expression. Although the effect of different ECM component on homologous and heterologous desensitization has not been addressed in a systematical manner, several reports suggested that the extracellular matrix composition could affect expression of the receptor and its signaling capacities (McCauley LK. et al 96, Hausmann S. et al 95). In that perspective, it is noteworthy that the ECM is suspected to affect both G-protein signaling and gene expression (Lafrenie RM. and Yamada KM. 96).

G) IMPORTANCE OF PTH/PTHrP RECEPTOR IN EMBRYONIC DEVELOPMENT AND IN ADULT PHYSIOLOGY.

The broad expression pattern of PTHrP and of the receptor both in adult life and during embryonic development as well as the role of PTHrP in numerous physiological responses suggest an important role for the PTH/PTHrP receptor in cellular growth and differentiation. Insight into the role that the PTHrP-PTHR signaling axis plays during embryonic development came from studies in which the function of the receptor was altered.

A genetically engineered ablation of most of the PTH/PTHrP receptor in mouse led to skeletal abnormalities (Lanske B. et al 96). Mice homozygous for the PTH/PTHrP receptor deletion (PTHR-/-) died in-utero during mid to late stages of embryonic development, between 12.5 and 18.5 days post-coitum (dpc). Their appearance was reminiscent of mice lacking PTHrP (PTHrP-/-) (Karaplis A. et al 94), in that they displayed a domed skull, short limbs, a protruding tongue, and short snout and mandible. A histological examination of the bones also revealed defects similar to those observed in PTHrP -/- mice. Hyaline cartilage of the ribs was differentiated into hypertrophic chondrocytes, and foci of prematurely ossified cartilage were observed. Columns of chondrocytes in tibia had a shorter and irregular zone of proliferation, which suggested an accelerated chondrocyte differentiation as in PTHrP-/- mice. The similarity of defects observed between the two type of mutant (PTHrP-/- and PTHR-/-) provided genetic evidence that the developmental action of PTHrP in bone is mediated by the PTH/PTHrP receptor. PTHrP-/- and PTHR-/- mice were used to define a feedback mechanism that

regulates the production of PTHrP in relation to the rate of differentiation of chondrocytes. In this model, terminally differentiating pre-hypertrophic chondrocytes release the morphogenic protein Indian Hedgehog (Ihh) in the surrounding milieu. This factor feeds back to the peri-articular perichondrium of the bone where it stimulates the production of PTHrP. PTHrP, in turn, represses chondrocyte differentiation by activating the receptor expressed at the surface of pre-hypertrophic chondrocytes. The loop is interrupted by a diminution of Ihh production, as the number of chondrocytes producing Ihh diminishes (Vortkamp A. et al 96, Kronenberg HM. et al 97).

The absence of any apparent phenotype in other tissues than bone is somewhat puzzling, given the broad distribution of transcripts for both the ligand and the receptor during embryonic development. This reflects the possibility that the PTHrP-PTHR signaling pathway is not active, or not essential, in any embryonic tissue but bone, until after birth. Alternatively, there might be compensatory mechanisms, which would correct for the lack of PTHR activity. It is noteworthy that prior to the appearance of skeletal defects, PTHR-/- mice were smaller than their littermates, with a proportional diminution of all the organs. This phenomenon was not observed in mice lacking PTHrP and so far remains unexplained. A subsequent report on the trans-placental calcium gradient in these two mutants underlined the fact that, whereas this gradient is usually observed in PTHR-/- mice, it was abolished in the PTHrP-/- mutant (see later and Kovacs CS. et al 97).

The death of embryos during gestation precluded any analysis of the importance of receptor signaling in post-natal development and adult physiology. A series of genetic experiments indirectly gave an idea of the importance of the PTHrP-PTHR signaling pathway in young mice. Expression of the mutant form of the human receptor associated

with patients with Jansen metaphyseal chondrodysplasia (see below and section on the structure-function relationships), was targeted to prehypertrophic chondrocytes in mice (TgA mice)(Shipani E. et al 97b). The expression of a constitutively active receptor in bones differentiating by endochondral formation led to slower maturation of proliferative chondrocytes and a consequently prolonged presence of hypertrophic chondrocytes. This phenotype was also observed in mice in which PTHrP over-expression was targeted to the same cell type (Weir EC. et al 97). Furthermore, mice presented a delayed vascular invasion of forming bone and an aberrant site of vascular entry. These abnormalities disappeared after two weeks, at which stage the mice looked histologically similar to wild-type animals. TgA mice were bred with PTHrP -/- mutants to target the expression of the constitutively active receptor in mice deleted for PTHrP. The resulting mice appeared similar to the TgA mice and survived up to two months after birth. However, growth plate closure and tooth eruption did not occur. This result confirmed that the post-delivery mortality in PTHrP-/- mice was due to skeletal defects and suggested an important role for the PTHrP/PTHR signaling pathway in adolescent or adult life. Furthermore, it highlighted the relative little importance of the PLC activating pathway in PTHR regulated endochondral bone formation.

The importance of signaling through the PTH/PTHrP receptor in bone development was further demonstrated by the finding that Jansen metaphyseal chondrodysplasia (JMC) is due to a mutation in the PTHR. Patients presenting this rare autosomal dominant form of short-limbed dwarfism carried a point mutation that causes the receptor to constitutively activate adenylyl cyclase (Shipani E. et al 95). The cellular bases for the skeletal abnormalities observed in these patients were unknown until the

generation of mice carrying the human mutant receptor under the control of a promoter that targeted its expression to prehypertrophic chondrocytes (TgA mice. See above). Mice had a slower rate of chondrocyte differentiation and maturation, resulting in gross bone abnormalities in the post-natal period (see above). Despite histologically normal parathyroid glands and normal or depressed levels of PTH or PTHrP, patients with JMC display severe hypercalcemia and hypophosphatemia, elevated urinary cAMP excretion, and normal and high levels of 25(OH)- and 1-25di(OH) vitamin D, respectively. This type of blood chemistry is reminiscent of that observed in cases of primary hyperparathyroidism. This suggests that renal PTHR signaling is as affected as that of bones (Kruse K. and Schutz C. 93). A patient with JMC was also reported to be unable to breast feed, and she and her daughter (also affected) had dry, scaly skin (Shipani E. et al 96). These findings were interesting, as PTHrP is expressed in mammary glands and keratinocytes and its over-expression there results in growth and development abnormalities (Wysolomerski JJ. and Stewart AF. 98). The absence of abnormalities in other organs could be interpreted as an indication that receptor signaling or cAMP responses to PTHrP are not essential in tissues other than bone and kidney (Shipani E. et al 96).

Pseudohypoparathyroidism (PHP) is another clinical syndrome associated with tissue specific alteration of PTH/PTHrP receptor activity. PHP is an apparent hypoparathyroidism in which the observed hypocalcemia and hyperphosphatemia occur in the presence of high circulating levels of PTH. PHP is therefore a generic term for a number of clinical conditions that describe end organ (i.e. kidney) resistance to PTH. With the development of biochemical and molecular diagnostics, PHP was further

subdivided into type I and II, with type I comprising the subtypes a, b and c. Briefly, PHP Ia and Ic are multiple hormone resistance syndromes caused by defects either in Gsα activity (Ia), or at a site downstream of Gsα (Ic). These patients usually show skeletal abnormalities referred to as Albright's Hereditary Osteodystrophy (AHO). Patients with type Ib do not have multiple hormone resistance. Finally, PHP II patients are characterized by the maintenance of an increased urinary cAMP excretion upon injection of PTH, which is the hallmark of the functional response of the kidney to PTH (Silve C. 95).

Based on the absence of AHO and on an apparent increase in bone resorption, consistent with a normal bone response to high levels of circulating PTH, it had been suggested that PTH resistance in patients with PHP Ib could be due to tissue-specific inactivation of the PTH/PTHrP receptor (Kruse K. et al 89, Murray TM. et al 93). This notion was further reinforced when two independent groups reported the absence of biologically significant mutations in the exons encoding for the PTH/PTHrP receptor (Shipani E. et al 95b, Fukomoto S. et al 95). A study that used a primary cultures of skin fibroblast isolated from PHP 1b patients, demonstrated heterogeneity of the cAMP response to PTH (Silve C. et al 86, Silve C. et al 90) Hence, patients affected with PHP1b could be classified into three subgroups. In one subgroup (a), fibroblasts did not show any cAMP accumulation in response to PTH; the response could, however, be restored following treatment with Dex. In another group (b), an acutely reduced, but not abrogated, cAMP response was not enhanced by treatment with Dex. Finally, some fibroblasts were responsive to PTH to the same extent as control fibroblasts, and like the controls, the response could not be enhanced by Dex (group c). The receptor cloning

allowed a reevaluation of these data at the RNA level, and a good correlation was found between the level of mRNA and response to PTH (Suarez F. et al 95). Furthermore, Dex was also found to affect the mRNA levels. These findings shed new light on the quest for the genetic defect responsible for PHP 1b. First and foremost, it demonstrated that, in some patients (groups c and b), the receptor is expressed and capable of responding to PTH, emphasizing that PHP 1b is not necessarily due to a non functional receptor. This result is in agreement with an independent study in which skin fibroblasts, derived from patients with PHP 1b or PHP 1a, had cAMP responses to PTH indistinguishable from that of normal individuals (Gupta A. et al 91). Second, in patients whose fibroblasts showed little or no response to PTH, the levels of transcripts expression were significantly lowered or almost nil (group b and a respectively). Finally, dexamethasone treatment could restore expression of the receptor with a concomitant up-regulation of the PTH/PTHrP receptor transcript level. While the first point emphasized the idea that, in patients with PHP 1b, the receptor can be expressed and functional in non-renal tissue, it became evident that PHP 1b could be related to a tissue specific defect in the PTHR gene transcriptional regulation or of transcript stability. This concept is consistent with reports that Dex can regulate receptor mRNA levels in certain cell lines or tissues, but not in others (Urena P. et al 94, Papspaliaris et al 95). The length of fibroblast treatment used in these studies (one week), precludes any conclusion to be drawn as to whether the up-regulation of the transcript levels was a direct consequence of the transcriptional up-regulation of the PTHR gene expression in fibroblasts, or whether it reflected more complex compensatory mechanisms (Suarez F. et al 95, Silve C. et al 90).

H) Other receptors for PTH or PTHrP ?

As already mentioned, the scope of defects observed in mice lacking PTHrP or PTH/PTHrP receptor is relatively restricted with respect to the broad range of physiological processes in which these two proteins have been implicated. The death of PTHrP-/- rescued animals (TgA x PTHrP -/-) after two months suggests that expression of PTHrP is essential for post-natal development. PTHrP could be necessary during embryonic development for the proper establishment of physiological systems that are pertinent to post-natal physiology. Alternatively, the presence of PTHrP might be necessary for the organism to cope with the new challenges encountered during post-embryonic development. Whether the death at two months resulted from the lack of activation of the cAMP or PLC pathways can not be inferred from the report by Shipani *et al.* as the expression of the constitutively active receptor was restricted to pre-hypertrophic chondrocytes. Further information on the relative importance of the two pathways will await the generation of mice in which expression of the constitutive receptor will be driven by a ubiquitously active promoter.

It is also possible that the action of PTHrP in organs other than bone, could be mediated by activation of cellular pathways which do not require the PTH/PTHrP receptor. In this respect, the report by Kovacs and coworkers (Kovacs CS. et al 97) that PTHrP is involved in the establishment of the fetal-maternal trans-placental calcium gradient through binding to a receptor distinct from the PTH/PTHrP receptor is noteworthy. The characterization of such a receptor would clarify the paradoxical situation of having two distinct ligands for a single type of receptor.

Data obtained in the emerging field of the PTHrP intracrine action provide further evidence that PTH/PTHrP receptor is not necessarily required for PTHrP activity. Following the original finding Henderson *et al,* that the 20 residues located between

positions 87 to 107 in the mid-region of PTHrP constitute a functional nuclear localization signal (Henderson JE. et al 95), a recent report showed that vascular smooth muscle cells stably transfected with PTHrP expression vectors display a higher proliferation rate than control (Massfelder T. et al 97). This effect was attributed to an intracrine action, as exogenous application of PTHrP peptide caused an opposite response, and as it required the presence of the nuclear localization signal characterized by Henderson et al. It thus appears, that in some instances, the PTH/PTHrP receptor is dispensable to the action of PTHrP. The characterization of a PTH specific receptor (PTHR2), whose expression is restricted to the pancreas and the central nervous system, tends to prove that this might also be the case for PTH (Usdin TB. et al 95). Although this receptor has proved useful in delineating the residues important for binding specificity in both PTH, PTHrP and the receptor (Gardella TJ. et al 96, Clark JA. et al 98), its restricted expression in non-classical PTH target tissues indicates that it is unlikely to play a critical role in calcium homeostasis, or in the genesis of organs implicated in calcium homeostasis.

INTRODUCTION

SECTION III:

TRANSCRIPTIONAL REGULATION OF CLASS II GENES.

- INITIATION OF THE TRANSCRIPTION: FORMATION OF THE PIC.
- TBP-ASSOCIATED FACTORS TAFs.
- THE MEDIATOR AND THE HOLOENZYME.
- DNA STRUCTURE AND TRANSCRIPTIONAL REGULATIONS.
- DNA METHYLATION AND GENE TRANSCRIPTION.
- TRANSCRIPTIONAL REGULATION OF THE PTHR GENE EXPRESSION.

The transfer of information from DNA to RNA is achieved through the process of transcription. In eukaryotic cells, mRNA molecules contain the information pertinent to the amino acid composition of the proteins which they encode (coding sequence) as well as that necessary for the post-transcriptional control of their expression.

Of the three possible steps at which control of gene expression can occur, transcriptional, post-transcriptional and translational, the mechanisms regulating transcription are considered the most important and appear very sophisticated.

Transcriptional control of gene expression can take place at all the levels of the transcription of a gene, from the initiation to the termination. Proper gene expression and therefore cellular functioning will depend in turn on the accurate initiation, elongation and termination of transcription.

The main determinants of the spatial and temporal regulation of transcription in both prokaryotes and eukaryotes are considered by many to be those regulating initiation. This level of control has therefore been the subject of extensive research that led to the elucidation of some of the mechanisms by which expression of a gene could be specifically activated or repressed in relation to the cell developmental or metabolic status.

A) Initiation of transcription: formation of the PIC.

During the initial steps of transcription, the basic transcriptional machinery in eukaryotes is mainly concerned with determining the site from where transcription should proceed, which means, to interact properly with the core-promoter region. Core-promoter elements, as defined by R.G Roeder, are the "minimal DNA elements that are necessary and sufficient for accurate transcription initiation by RNA Pol. II in reconstituted cell-free systems."(Roeder RG. 96). Most core-promoters for early identified class II genes

displayed a TATA box (TATa/tAa/t) located 20 to 30 bp upstream from the transcription start site, and an initiator region (inr, YYANt/aYY) near or at the transcription start site. Either one or both elements can be present, and can act either independently or synergistically. In-vitro initiation of transcription by RNA polymerase II at core-promoters requires the presence of additional cellular factors referred to as general initiation factors.

These factors are made of the association of several subunits and are capable, when added in a specific order, of promoting transcription of a naked DNA template from the TATA element-containing adenovirus major-late (AdML) promoter. The characterization of the ordered and stepwise pathway leading to **in-vitro** transcriptional initiation defined each factor's roles in this process (Roeder RG. 96). The first step of this ordered assembly of the preinitiation complex (PIC) consists in the binding of the TATA-binding protein (TBP) to the minor groove of the TATA element. TBP can, in many instances, substitute for TFIID, which was the originally characterized protein complex, and is one of the subunit of this general initiation factor (see later). TBP binding induces a bending of the DNA that brings sequences surrounding the TATA box in proximity with each other (Roeder RG. 96) and also smoothens the structural constraints associated with PIC assembly (Werner MH. and Burley SK. 97). Binding of TBP has been shown to be stabilized by subsequent binding of TFIIA to the complex. The presence of TFIIA is, however, only necessary when crude TFIID is used or when there is no strong TATA element (see below).

Further to the formation of this initial complex, TFIIB binds DNA at a site distinct from TFIIA and directs the subsequent recruitment of a complex made of the RNA Pol.II and TFIIF. The mode of interaction between TFIIB and RNA Pol II direct the exact positioning of the latter at the start site. The role of TFIIF seems to prevent interaction of the polymerase with non- specific DNA sequences. The next initiation factor to bind the intermediary PIC is TFIIE that does so by contacting RNA Pol II and

possibly TFIIF and TBP. Binding of the last general initiation factors, TFIIH, completes the formation of the PIC and provides for an helicase and a kinase activity that are necessary for elongation to proceed. Phosphorylation of the carboxy-terminal domain of the largest of the 12 subunits of the RNA Pol.II is thought to affect a conformational change that frees the polymerase from the PIC. The helicase activity of TFIIH requires ATP hydrolysis and promotes the melting of a 10 bp region in front of the PIC which facilitates elongation. TFIIE has been shown to play a role in these two phenomena as it can promote TFIIH kinase activity and has a DNA-unwinding capacity on its own.

Once elongation proceeds, the different components of the pre-initiation complex remain associated to the core-promoter (as for TBP or TFIID), the polymerase (TFIIF), or are sequentially released (first TFIIE and B than TFIIH). At the termination of transcription, a CTD-specific phosphatase dephosphorylates the polymerase rendering it ready for further association with intermediate PIC at the promoter. It is noteworthy that maintenance of TBP or TFIID at the core-promoter is probably what commits a particular gene for transcription; this mechanisms does not, however, seem to be universal (Roeder RG. 96).

As emphasized before, the original studies that demonstrated an ordered, stepwise assembly of the initiation complex were performed on the AdML promoter which contains both a strong TATA sequence and an initiator. On this type of promoter, TBP could substitute for the larger TFIID complex and the presence of TFIIA was helpful but dispensable. In the absence of such a strong TATA element, it appears that the presence of both TFIID and TFIIA is required. It became evident that in-vitro TBP could not support activation of the transcription by transcription factors, whereas TFIID could. Improvement of biochemical methods led to the demonstration that TFIID consists of the association of TBP and other factors (the TBP associated factors = TAFs). The characterization of TAF allowed for, not only a definition of some of the molecular bases

of core-promoter recognition by the PIC, but also for a characterization of important mechanisms of activated transcription initiation.

B) The roles of TAFs in regulating transcription.

Since their characterization as TFIID cofactors, several important functions have been ascribed to the TAFs. These factors have been shown to play a role in transcriptional activator recruitment of TFIID, core-promoter recognition, and conformational changes in the PIC that affect the topology of the promoter. Furthermore, distinct enzymatic activities have been demonstrated for some of the TAFs that are meaningful for activating transcription. Several cDNAs encoding different TAFs have been cloned in yeast, Drosophila and human, and some of them appear to be present in all three species (Tansey WP. and Herr W. 97).

As previously mentioned, the presence of TFIID and TFIIA is critical to activate transcription from a TATA-less promoter in-vitro. The current understanding of the respective roles of TBP and TAFs is that TBP will bind the TATA box, while one or several TAFs, as well as other cellular factors, will be utilizing the initiator element, or its equivalent, to enhance PIC assembly (Roeder RG. 96, Smale ST. 97). In the absence of a recognizable TATA element, TBP is still required but its TATA binding activity is not. There seems to be a bi-functional relationship between core-promoter structure and TAFs inasmuch as the presence of particular sequence in the core-promoter will determine which TAFs, as part of a TFIID complex, will be used to modulate the initiation of transcription, and the presence of certain TAFs (their expression) will permit the transcription of certain types of promoters (Verrijzer CP. and Tjian R. 96).

The recognition that TFIID and TFIIA are necessary for transcriptional activators to stimulate transcription in-vitro led to the hypothesis that TAFs could play a role in transcriptional activation. This hypothesis was reinforced by the demonstration that

transcriptional activators and TAFs can interact with each other. These interactions showed that formation of the PIC is one of the possible targets for activators activity. Two possible mechanisms by which interaction between TFIID and activator proteins could enhance transcription have been proposed. Transcription factors present on the DNA upstream, or downstream, of the core-promoter could assist TFIID recruitment to the core-promoter by specifically interacting with TAFs, thereby enhancing the rate of transcription initiation. Another, though not exclusive, hypothesis proposed that contact between activators and promoter-bound TFIID could affect the topology of the promoter by wrapping TFIID around the DNA. This topological effect would in turn facilitate subsequent initiation steps (Tansey WP. And Herr W. 97, Roeder RG. 96). The demonstration that particular transactivation domains specifically recognize certain TAFs, and the fact that the presence of particular TAFs can, in turn, dictate the formation or stabilization of the PIC at some core-promoters but not at others, suggest that TAFs may be important in modulating the differential effects of a given activator at different promoters. The possibility that different transcriptional activators recruit different TAFs could also provide the bases for the synergism of activation observed when multiple factors bind to a same promoter.

Although initial studies suggested a stepwise assembly of PIC, recent evidence showed that different types of partially pre-assembled complexes might exist in the cells. This notion of 'holoenzyme' was originally expounded concerning the characterization of the "mediator" activity in yeast (see below). Whether PIC formation proceeds by the gradual assembly of factors to an intermediate PIC, or whether it is the result of the attachment of giant protein complex to a TFIID-bound core-promoter is not clear. In either case, the protein-DNA and protein-protein interactions defined by biochemical studies are probably still relevant in the context of the holoenzyme model of PIC formation.

Complementing the data obtained by fractionation studies on in-vitro PIC assembly, genetic studies in yeast have unveiled apparent differences in the mechanisms of transcriptional regulation, and have allowed the characterization of a further level of transcriptional control in higher eukaryotes.

C) The mediator and the holoenzyme.

The name mediator was first applied to describe the activity of a component of yeast extract capable of suppressing the squelching effect observed between different transcriptional activators in an in-vitro transcription assay. The biochemical purification of this activity showed that it was made of a complex of more than 20 polypeptides that co-purified with RNA pol II. Some of them were subsequently identified as being members of the SRB complementation group. SRB proteins (which stands for suppressor of RNA pol B) were originally isolated in yeast as mutants capable of suppressing the phenotype associated with the expression of a RNA pol B truncated in its CTD (pol B is the yeast equivalent of pol II). The mediator's three main activities are to support basal and activated transcription and to stimulate TFIIH mediated phosphorylation of the CTD (Bjorkland S. and Kim Y-J. 96).

The characterization of the mediator component in yeast can explain the fact that TAFs play an apparently minor role in yeast. The contacts that occur between activators and the holoenzyme-bound mediator provide an alternate pathway by which the holoenzyme can be recruited to the promoter (Bjorkland S. and Kim Y-J. 96, Greenblatt J. 97). As far as higher organisms are concerned, the mediator provides an additional level of regulation of gene expression. Hence, activation of the transcription could result from an increased recruitment of TFIID and A at the core-promoter or that of the RNA pol II holoenzyme at a TFIID primed core-promoter.

The association of the mediator with the CTD, and the fact that it can stimulate its phosphorylation, suggests that, further to PIC assembly, the mediator could also have an effect on the elongation of the RNA and the recycling of the transcriptional machinery. The regulation of the elongation step seems primarly concerned with the prevention or the suppression of transcriptional arrest. Studies in yeast suggest that pausing suppression could also depend on the presence of promoter-bound transcriptional activators. Hence, in the presence of the appropriate inducer, the polymerase was mostly detected toward the 3' end of the transcribed gene, while it is normally (in the absence of the inducer) mostly located at the 5' extremity (Greenblatt J. 97).

D) Modifications of the DNA structure and transcriptional activation.

As mentioned earlier, the initiation of the PIC assembly by TBP or TFIID binding has been associated, in some instances, with structural alterations of the DNA template. As in-vivo DNA is found embedded in nucleosomes, it is likely that factors affecting the nucleosomes structure at the promoter will have an effect on transcription.

A number of TAFs have been shown to have an histone-like structure, and it has been suggested that, when attached to the promoter, these factors could substitute for histone and be embedded in higher chromatin structures during mitosis. After completion of mitosis, they would then to recommit the gene, on whose promoter they are bound, to transcription (Tansey WP. And Herr W. 97).

TAF 250 has an intrinsic histone acetylase activity (HAT) that is though to affect chromatin remodeling at the promoter. Acetylation of histone proteins at lysine residues result in the weakening of their affinity for DNA. Therefore the HAT of TAF 250 probably plays an important role in clearing the core-promoter region in preparation for the binding of general initiation factors. An alternative mechanism is that acetylation of histones could increase their affinity for nuclear factors that assist PIC assembly. Finally

acetylation could affect the activity of non-histone proteins required for activation of gene expression (Tansey WP. and Herr W. 97. Greenblatt J. 97. Roeder RG. 96. Pazin MJ. and Kadonaga JT. 97).

Consistent with an activation of transcription upon acetylation, certain transcriptional repressors act by targeting histone deacetylase to the promoter region of the gene they repress. In this scenario, the repressor binds to an intermediary protein, which in turn contact the deacetylase and targets it to the promoter (Pazin MJ. and Kadonaga JT. 97).

The distance between an activator binding site and the core promoter might necessitate long-range interactions between the activator and the transcriptional machinery. Looping of the DNA molecule is believed to be the major mechanism by which distal activators can affect transcription. The physical constraint applied to the intervening DNA, whether by protein binding, protein-induced bending, or by chemical modification, have been shown to influence the probability of bringing two distant protein complexes in proximity to each other (Rippe K. et al 95). In that respect, the characterization of transcriptional activators capable of affecting sharp bends in the DNA is particularly interesting. LEF-1, a pre-B and T lymphocyte specific DNA binding protein, can affect a sharp bend in the DNA upon binding to its recognition sequence in the TCRα promoter. This bend brings distant DNA sequences into close juxtaposition, which, in turn, allows the proteins bound at these sequences to interact with each other and to affect transcription. In a similar manner, HMG I affects DNA structure within the interferon β enhancer by reducing, rather than increasing, DNA bending thereby allowing NF-κb to bind to its cognate recognition site and to activate transcription (Werner MH. and Burley SK 97).

E) DNA methylation and gene transcription.

The demonstration in the late 70s that hypomethylation of genes correlated with transcriptional activity showed the importance of DNA methylation as a control mechanism for gene expression.

Cytosine residues, in the context of a 5'C-G 3' dinucleotide (referred to as CpG dinucleotide), can be methylated to form a 5-methyl cytosine. About 60-90% of the CPGs in the adult mammal genome appeared to be methylated, while most of them are demethylated at the blastula stage. The passage from a globally demethylated stage to a globally methylated one involves waves of de-novo methylation that are performed by DNA methyl-transferase at the implantation stage. The demonstration that mice lacking this enzyme do not survive past the mid-gestational embryonic stage emphasized the importance of DNA methylation for normal development (Szyf M. 96, Antequera F. and Bird A. 93, Razin A and Kafri T. 94). Thus, a number of promoters undergo demethylation at specific cytosine residue prior to the onset of their activity and abnormal demethylation has been associated with abnormal gene expression in cancer cells (Holt EH. et al 93). The use of DNA methylation inhibitors like 5-aza-cytidine provided a more direct demonstration that the relation between DNA methylation and gene expression is not fortuitous as treatment with this type of agent induces the expression of a number of genes. This relation is also supported by the fact that in-vitro methylation of genes prevents their expression when transfected into cells (Muizniek I. and Doerfler W. 94).

Three mechanisms by which DNA methylation could interfere with transcription have been proposed. Methylation of cytosine residues has been shown to influence the binding of some general transcriptional activators like NF-kB, AP-2, E2F, but not that of specific transcription factors. Conversely, the characterization of methyl-cytosine binding proteins that act as transcriptional repressors suggests that gene silencing by DNA methylation can be in some cases an active process. Finally, methylation could affect

chromatin structure by causing it to assemble into a transcriptionally inactive form, or by preventing the action of factors necessary to maintain it in a favorable transcriptional state (Razin A. and Kafri T. 94, Siegfried Z. and Cedar H. 97). Demethylation of promoter region seems therefore to be a means to trigger the expression of developmentally regulated genes by allowing the action of the transcriptional regulatory controls described earlier.

Some DNA regions are consistently unmethylated at all stages of development. They consist of (C+G) rich stretches of DNA that have a higher content of CpG dinucleotides than the rest of the genome, and are referred to as CpG islands. The relative paucity in CpG content of the genome (where the mean CpG to GpC ratio is about 0.2) is a direct reflection of some cellular enzymatic activities. Deamination of unmodified (non-methylated) cytosine yield uracil residues that are removed by the enzyme uracil-glycosidase. Methyl cytosine deamination on the other hand, results in the production of thymine, which converts CpG dinucleotide into TpGs (Antequera F. and Bird A. 93). Interestingly, CpG islands have mainly been found in promoter regions of housekeeping genes. These promoters usually lack TATA elements, and have numerous Sp1 recognition sequences. It is possible that PIC formation will result from the interaction of TFIID with promoter-bound Sp1.

F) Transcriptional regulation of the PTHR gene expression.

As described above (see section on the PTHR gene in chapter 2), two promoters drive the transcription of the gene in rodents. An upstream promoter, strongly and specifically active in kidney, directs transcription initiation from multiple start sites. A downstream promoter, more ubiquitously active, initiates transcription from a single start site. Although no information was available about the upstream regulatory region of the human gene, the cloning of a human kidney cDNA bearing a 5' UTR 70% identical to the

one derived from the upstream promoter in mouse suggests that the transcriptional regulation of the human receptor gene expression could be similar to that of rodent.

Several studies support the idea that the transcription of the PTHR gene could be deregulated in a number of pathological conditions:

- The selective down regulation of the receptor gene expression in kidney has been suggested in patients with pseudohypoparathyroidism type 1b (Suarez F. et al 95). Likewise, the down-regulation of the PTH/PTHrP receptor mRNA levels found in kidney and liver, in a rat model for chronic renal failure (CRF), suggests that changes in the transcription of the PTH/PTHrP receptor gene expression could account for the loss of PTH response, hypocalcemia and hyperphosphatemia associated with this condition (Urena P et al 94a, Tian J. et al 94)

- Consistent with its role in HHM, PTHrP has been found to be expressed and released in an unregulated manner in a number of malignant cell lines and its expression has been shown to play a role in tumor progression both in-vivo and in-vitro (Goltzman D. and Henderson JE. 97). Although the presence of high concentrations of PTHrP in medium derived from tumors or transformed cell lines is not a necessary indication that effect on cell proliferation requires the presence of the PTH/PTHrP receptor, a number of studies have reported the expression of the receptor in cancer cells or tumors. Moreover, it has been suggested that PTHR appearance in the course of tumor progression may be associated with the acquisition of a more aggressive phenotype (Carron JA. et al 97) or with the loss of proliferative capacity, which supports the acquisition of novel histological characteristics (Nakashima M. et al 95). Significantly, expression of PTHrP and of the receptor have been shown to be up-regulated in embryonic carcinoma and embryonic stem cells, following treatment with retinoic acid (RA)(Chan SDH. et al 90). The acquisition by these cells of a parietal endoderm phenotype was tightly associated with the apparition of the receptor (Van de Stople et al 93). In addition, PTHrP was shown fully capable of replacing dibutyryl cAMP as an inducer of parietal endoderm

differentiation of RA treated cells (Chan SDH. et al 90). These results suggest a role for the PTHrP/PTHR signaling pathway in RA induced differentiation and demonstrate that both partners are under similar transcriptional regulation. They further support the notion that ectopic expression of the receptor and of the ligand can affect the differentiation status of cells.

- The number of receptor molecules at the cell surface can determine the activation of the PLC/PKC limb of receptor signaling (Guo J. et al 95). Therefore, although the receptor has been found to be expressed almost ubiquitously, an up- or down-regulation of its expression could specifically activate or deactivate the PLC/PKC pathway. A similar selective activation of the PKA or PKC pathway could play an important role during fetal or post-embryonic development. Hence, whereas a clear role for the PKA pathway has been established during endochondral bone formation, very little is known about the contribution of the PLC/PKC pathway to fetal or post-embryonic development.

- Considering the effects of the absence of the PTH/PTHrP receptor on chondrocytes differentiation, and given the tightly regulated pattern of expression of the receptor in rat growth plate, it is likely that control of the transcription of the PTHR gene is critical for the proper embryonic development of bones. In this regard, the characterization of cis-acting elements and trans-acting factors that regulate expression of the receptor in bone will be of particular relevance to understand the mechanisms controlling bone development. The definition of local factors which can alter expression of the PTHR gene would be of interest in modulating bone turn-over.

In order to study the variations of the transcription of the PTH/PTHrP receptor gene associated with pathological and clinical conditions, we undertook to characterize the upstream regulatory region of the human gene. We show here that this region is very similar in human and mouse as the upstream untranslated exons found in mouse are also present in the human gene. However, the transcriptional mechanisms in the adult appear

different in the two species as we characterized an additional human promoter that is ubiquitously active and accounts for most of the gene expression in the kidney.

We also show that only one promoter is active during embryonic development. and that, in adult tissues, transcripts derived from this promoter are differently spliced. The characterization of the upstream regulatory region of the PTHR gene also permitted us to address whether any alteration at critical sites would be responsible for the appearance of type 1b pseudo-hypoparathyroidism. Analysis of PCR-amplified genomic DNA from patients with this syndrome did not reveal any obvious alteration of this region.

CHAPTER 2:

CLONING AND CHARACTERIZATION OF THE PROMOTER REGIONS OF THE HUMAN PARATHYROID HORMONE/PARATHYROID HORMONE-RELATED PEPTIDE RECEPTOR (PTHR) GENE: ANALYSIS OF DNA FROM NORMALS AND PATIENTS WITH PSEUDOHYPOPARATHYROIDISM TYPE 1B.

- INTRODUCTION.
- MATERIAL AND METHODS.
- RESULTS.
- DISCUSSION.

Expression of the parathyroid hormone (PTH)/parathyroid hormone related peptide (PTHrP) receptor (PTHR) in the mouse is controlled by at least two promoters. The downstream promoter (P2) is ubiquitously expressed, whereas expression of the upstream promoter (P1) is largely restricted to kidney. These observations may provide a genetic basis for a human PTH resistance syndrome, pseudohypoparathyroidism type 1b (PHP1b) in which renal, but not osseous, signaling by PTH is defective. We have therefore cloned and characterized the 5' end of the human PTHR gene, and found that its organization is very similar to that of the mouse. Transcription initiation sites of human P1 and P2 promoters are in similar but not identical positions to those of the mouse gene. The identification of a human P2 promoter is significant because no P2-specific human PTHR cDNAs have been isolated to date. Southern analysis of genomic DNA from 7 PHP1b patients did not reveal any rearrangements in proximal promoter regions or exons encoding 5' untranslated region sequences. No significant sequence differences were found in clones of normal and patient DNAs encompassing proximal promoter sequences, and untranslated region and signal sequence exons. Thus, in the seven PHP1b patients analyzed, no defects were identified which would influence initiation site selection, stability or splicing of renal PTHR transcripts. These data indicate that the genetic defect(s) in PHP1b in these patients lie in distal enhancer elements of the gene, in an essential transcriptional regulator, or in some as yet unidentified cofactor required for renal PTH signaling.

INTRODUCTION

Circulating concentrations of calcium ions are tightly maintained by the action of parathyroid hormone (PTH). PTH is released from the parathyroids following a decrease in the plasma calcium levels, and acts acutely in the kidney to stimulate calcium reabsorption and increase phosphate excretion (Kronenberg H. 93, Goltzman D. and Hendy GN 95). Parathyroid hormone-related peptide (PTHrP), originally characterized as the major mediator of hypercalcemia associated with a variety of malignancies, can mimic many of the effects of PTH when overexpressed by cancers. PTHrP is thought to act in a paracrine/autocrine manner under physiological conditions, in contrast to PTH. Moreover, whereas expression of PTH is restricted to the parathyroids, PTHrP is widely expressed, and functions to modulate normal cellular growth and differentiation (Suva LJ. et al 87, Ikeda et al 88, Stewart AF. 93, Strewler GD. and Nissenson RA. 93). The actions of PTH and PTHrP are relayed at the cellular level by binding to the same cognate receptor (PTHR), which can stimulate production of intracellular cyclic AMP and inositol 1,4,5-trisphosphate (Abou-Samra A-B et al 92, Juppner H. et al 91, Shipani et al 93).

The PTHR belongs to the vast family of G protein coupled receptors containing seven transmembrane domains. The organization of the PTHR gene shows that it is closely related to a subfamily of receptors for peptide hormones including calcitonin, vasoactive intestinal peptide, glucagon, and growth hormone releasing peptide (Lin S-C et al 93, McCuaig et al 94, Kong X-F et al 94, Maget B. et al 94, Zolnierowicz et al 94, Sreedharan et al 95). Expression of the mouse PTHR gene is controlled by a least two promoters, designated P1 and P2, which give rise to transcripts differing in their 5' untranslated regions but not their coding sequence (McCuaig et al 94, McCuaig et al 95)). In mice, expression from P1 is restricted mainly to kidney, giving rise to transcripts containing 5' untranslated regions composed of two exons, U1 and U2, spliced to the signal sequence exon (McCuaig et al 95). Expression of P2, on the other hand, is not

tissue specific, and is therefore likely to be largely responsible for the broad expression pattern of the PTHR. P2-specific transcripts contain a single untranslated region exon, U3, spliced to the signal sequence exon (McCuaig et al 94). Recently, a second PTH receptor (PTH2) has been characterized, with only 20% homology to the PTHR. PTH2 is expressed in the brain and to a lesser extent in pancreas, testes and placenta, but not in kidney (Usdin TB. et al 95). This receptor appears to bind PTH with much higher affinity than PTHrP (Gardella TJ. et al 96).

Several clinical syndromes characterized by end organ resistance to PTH have been described which are associated with hypocalcemia, high levels of endogenous circulating PTH, and the absence of a normal increase in urinary cAMP excretion following administration of exogenous PTH (Levine MA. et al 93). These have been collectively termed Pseudohypoparathyroidism (PHP). PHP type 1a (PHP1a) is associated phenotypically with a resistance to the action of several hormones in addition to PTH, and the presentation of Albright's hereditary osteodystrophy (AHO) (Levine MA. 93, Albright F. et al 42, levine MA. 83). The stigmata of PHP1a include obesity, short stature, round face, and brachydachtyly. PHP1a is often associated genotypically with one of a number of heterozygous mutations in the gene encoding Gsα, which disrupt its function. However, apparent cases of PHP1a have been characterized in which no mutations in Gsα have been found, suggesting that PHP1a can arise from more than one type of defect in PTH signalling (Phelan MC. et al 95, Shapira et al 96, Dixon PH. et al 96).

Pseudohypoparathyroidism type 1b (PHP1b) is not associated with a reduction in Gsα expression, or AHO. PHP1b patients show a defect in renal PTH signalling, but an apparently normal response to PTH in bone (Levine MA. 93), suggesting a defect in either renal PTHR function or expression (Suarez F. et al 95). Recent gene ablation experiments indicate that severe skeletal malformations are a consequence of disruption

of receptor signalling, indicating that mutations in the PTHR coding sequence are an unlikely site for the defect in PHP1b (Lanske B. et al 96). This has been confirmed by direct studies of genomic DNA from patients with PHP1b reporting the absence of such mutations (Shipani E. et al 95, Fukumoto S. et al 96). On the other hand, the existence of multiple promoters in the murine gene raised the possibility that, if such promoters are conserved in the human gene, mutations which disrupt tissue-specific expression of the PTH gene may be responsible for the syndrome. Two human PTHR cDNAs have been isolated from kidney libraries which contain 5'UTRs similar to those present in renal-specific transcripts in the mouse (Shneider H. et al 93, Adams AE. et al 95), raising the possibility that the mouse and human gene structures are conserved. Thus mutations in key elements controlling tissue-specific promoter activity, in one of the 5'UTR exons resulting in an abnormal splicing, stability, or translation of the PTHR mRNA could account for a kidney-specific defect in the PTHR action. To test this hypothesis, we have cloned and characterized the upstream region of the human PTHR gene and studied its expression in the human kidney. In addition, PTHR genomic sequences from normal individuals as well as from seven patients with PHP1b were cloned and analyzed for potential alterations affecting either expression or function of PTHR gene transcripts .

Materials and Methods

Diagnosis of patients with pseudohypoparathyroidism type 1b.

Patients with PHP1b included five Japanese and two Caucasians, all of which were sporadic cases. The diagnosis of PHP1b was established by the following criteria: hypocalcemia, hyperphosphatemia, elevated serum PTH levels (more than twice the upper limit of normal), normal serum magnesium levels, normal renal function, absence of AHO, and normal thyroid function. In addition, all patients showed minimal urinary cAMP elevation after injection of exogenous PTH (1-34). Details of characteristics of the patients are presented in Table I. Lymphoblastoid cell lines derived from PHP1b patients were established by Epstein Barr virus transformation (Katsuki T. and Hinuma Y. 75). All individuals in this study provided informed consent.

Generation of human PTHR gene 5'UTR probe.

The probe used for screening was generated using 4 overlapping primers (Fig. 1A). Primer sequences were derived from the 5' UTR of a human kidney cDNA clone of the PTHR (29), and are homologous to mouse exons U1 and U2. This strategy constructs a 148bp human PTHR 5'UTR probe using a two-step PCR strategy. Sequences of primers HPTHR1 (62mer) and HPTHR2 (63mer) overlap by 16bp. Primers HPTHR3 (33mer) and HPTHR4 (33mer), which overlap HPTHR1 and HPTHR2, respectively, extend the homology with human PTHR 5'UTR sequences to 148bp, and contain flanking EcoRI sites. The sequences of the primers are as follows: HPTHR1, GTGGCCAACTTGAGTCTGCTCTGCAGCTTTAGGCCCGACTTGGAAGGCCCATGG GCTGCAGA; HPTHR2, GTCCTGGACACTACCACTCTTCGGCTGTCTGGACCTCAGTTTCCTCATCTGCAG CCCATGGGC; HPTHR3, AATGAATTCGCCTCCCCGTGGCCAACTTGAGTC; HPTHR4, AATGAATTCAGTTGTGTGTCCTGGACACTACCA. PCR was performed as

follows. Five cycles (95°C, 1 min; 72 °C, 30sec; 50 °C, 1min) were performed with primers HPTHR1 and HPTHR2 using Vent polymerase (New England Biolabs, Beverly MA). One twentieth of the reaction was used for a second round of PCR with primers HPTHR3 and HPTHR4 for a further 25 cycles under the same conditions as above to yield a full length PCR product, which was digested with EcoRI and inserted in Bluescript SK+.

Screening of the human genomic DNA library.

One million independent clones of a λ DASH normal human genomic DNA library (Stratagene) were screened. Prehybridization and hybridization were performed for 2 hr. and overnight, respectively, at 42°C in 40% formamide, 6xSSPE, 1X Denhardt's, 10% dextran sulfate, 1% SDS, 25mM sodium phosphate (pH 7.5), and 10 μg/ml of denatured salmon sperm DNA. The hybridization was performed in the presence of 10^6 cpm/ml of probe labeled with ^{32}P by random priming. Positive clones were subjected to 3 rounds of plaque purification, and DNA was extracted using SDS/chloroform extraction of liquid cultures. Two positive clones were further characterized by Southern blot analysis using synthetic oligonucleotide probes hPTHR1 or hPTHR4 (Fig. 1A), or a 560bp XhoI-ApaI restriction fragment containing the mouse U3 exon. The two clones, designated A and B, contained inserts of 18 and 22 kb, respectively, which overlapped over a length of 18kb (Fig. 1B). The U1 region was subcloned as a 5.5kb BamHI fragment, whereas U2, U3, and SS exons were subcloned as a 3.6kb BamHI fragment.

Southern blot analysis of PHP1b patient genomic DNA.

DNA was extracted from whole blood and lymphoblastoid cell lines derived from PHP1b patients. Ten μg of genomic DNA were digested to completion with BamHI, KpnI, PstI, PvuII, and SacI restriction endonucleases under the conditions recommended by the supplier (Boehringer), electrophoresed on 0.8% agarose gels, and transferred to Hybond-N+ membrane (Amersham). Probes were labeled by random priming using a

Ready-To-Go DNA labeling kit (Pharmacia). Hybridization was carried out at 65°C or 68°C, depending on the probe used, for 18 hr in a solution containing 5 X SSPE, 5 X Denhardt's, 0.5% SDS and 20 μg of denatured salmon sperm DNA/ml. Filters were washed at room temperature with 2 X SSPE / 0.1% SDS twice for 10 min, at 65°C or 68°C with 1 X SSPE / 0.1% SDS for 15 min, and at 65°C or 68°C with 0.1 X SSPE / 0.1% SDS four times for 15 min. Autoradiography was performed at -70°C on Kodak XAR-5 film using intensifying screens.

PCR amplification of PHP1b patient genomic DNA.

PCR was performed using 10ng of DNA, 20 pmoles of each primers, 1x Promega Taq buffer, 200 μM of dNTPs, 1mM $MgCl_2$, 2 units of Taq DNA polymerase (Promega) in a total volume of 20μl. The primers for U1 were: forward, 5' ACAGAATCCTGGGCATCTGAAACACC 3'; reverse, 5' GAATTC GTCTGTCTGCCCATAGCAC 3'. Samples were cycled at 94°C, 1min; 50°C, 1min; 72°C, 30 sec for 5 cycles, and then 94°C, 30sec; 55°C, 1min; 72°C, 30sec for 30 cycles, followed by one cycle at 72° for 10 min. For U2 sequences, the forward and reverse primers were; 5' CAGAATTCTTGGGCTTGACAGATTTGC 3', and 5' ATACTGCAGAAACTGAGGCAGAGGGAC 3'. Samples were cycled at 94°C, 1min; 52°C, 1min; 72°C, 30sec for 5 cycles, and then 94°C, 30sec; 57°C, 1min; 72°C, 30sec for 30 cycles, followed by one cycle at 72°C for 10 min. To amplify sequences containing U3, nested PCR was performed using four primers. The first round of PCR was performed at 0.75mM $MgCl_2$, and samples were amplified at 95°C, 1min; 52 °C, 1min; 74 °C, 30sec for 5 cycles, and then at 95°C, 30sec; 57°C, 1min; 74 °C, 30sec for 35 cycles, followed by one cycle at 74°C for 10min, using the forward and reverse primers 5' AAGAATTCGCCTCTAGCGCAATGTCCC 3' and 5' CAATGGATCCGAGAC AGAGCAGCCTGCTGCTC 3', respectively. The second round of PCR was performed on one tenth of the first amplification product at a $MgCl_2$ concentration of 1mM under

cycling conditions identical to those described above, using forward and reverse primers 5' AAGAATTCTCTCGGCCTCTCCACACTC 3' and 5' CAATGGATCCGACTCCGGCCACTTCC 3', respectively. For the SS exon, the primers were; forward, 5' ACGGAATTCAGCCTGACGCAAGCTCTGCACC 3', and reverse 5' TTACGGATCCTGGATCAGAGGGGACTCTCAC 3'. Samples were cycled at 94°C, 1min; 54°C, 1min; 72°C, 30sec for 5 cycles, and 94°C, 30sec; 58°C, 1min; 72°C, 30sec for 30 cycles, followed by one cycle at 72°C for 10 min.

RNase protection analysis.

Probes for RNase protection were synthesized by *in vitro* transcription of DNA fragments using 3 units of T3 or T7 RNA polymerase, 0.5mM each of UTP, ATP, GTP, 10μM CTP, 5mM DDT, 1unit of RNAguard (Pharmacia), 50 μCi of ^{32}P-labeled CTP in 1x Promega transcription buffer at 37° for 1hr, and then treated with 100 units of FPLC pure DNase1 (Gibco-BRL) for 10 min at 37°C. The reaction was stopped by adding 80μl of 0.1% SDS, and the probe purified on a Sephadex G50 spin column. RNase protections were performed using 20 μg of total human kidney RNA or yeast tRNA (as a control). Samples were precipitated with 10^5 cpm of ^{32}P-CTP labeled probe, resuspended in 30ml of 80% deionized formamide, 40mM PIPES (pH6.4), 0.4M sodium acetate, 1mM EDTA and denatured for 5 min at 85°C and incubated overnight at 50°C. Digestion was performed for 1hr using 3 units of RNase 1 according to the manufacturer instructions, and products were run on a 6% polyacrylamide-urea gel. Autoradiography was performed for 48hr at -70°C with two intensifying screens. The integrity of the probe was checked by running 250 cpm in parallel on the gel.

Primer extension analysis. Ten pmol of primer were labeled for 1 hr at 37°C with ^{32}P-γ-ATP using T4 polynucleotide kinase. One tenth of this reaction was incubated with 10 μg of either human kidney total RNA, or yeast tRNA as a control, overnight at 55°C in 300mM KCl, 20mM Tris-HCl (pH 8.0), 2 mM EDTA in a final volume of 26μl. Samples

were then put on ice, and 4µl of 25mM Tris-HCl (pH 8.0), 60 mM $MgCl_2$, 10mM DTT, 5mM dNTPs, 2 units of RNAguard (Pharmacia), and 100 units of M-MLV reverse transcriptase (Gibco-BRL) were added. After 90 min at 43°C, the enzymes were inactivated for 10 min at 75°C, and the reaction was extracted once with phenol and ethanol precipitated. One quarter of the reaction was denatured for 2min at 80°C and run on a 6% denaturing polyacrylamide gel along with a sequencing reaction as molecular weight marker.

A) Results.

1. Cloning and Characterization of the Promoter Region of the Human PTHR Gene.

Human PTHR cDNA clones have been isolated containing 5'UTR sequences which correspond to mouse PTHR gene exons U1 and U2 (29). The human sequences were used to design oligonucleotides which were assembled by PCR to generate a 148 bp DNA fragment (Fig. 1A). This fragment was used to probe a human genomic DNA library, resulting in the isolation of two independent clones A and B, which are identical except for approximately 4 kb of additional sequence at the end of clone A (Fig. 1B). Sequences homologous to mouse UTR exons U1, U2 and U3, as well as the exon SS containing the signal sequence were found by Southern analysis (not shown) to be located on two contiguous BamHI fragments of 5.5 kb and 3.6 kb, respectively (Fig. 1B). The exonic structure of the 5' end of the PTHR gene is well conserved between mouse and human (Fig. 1B). Sequences corresponding to all three mouse untranslated regions region exons are present in the human gene, although the U1 and U2 exons in the human gene are more widely separated than their mouse counterparts.

2. Mapping the P1 and P2 Promoters of the Human PTHR Gene.

We have used primer extension and RNase protection analyses of human kidney RNA to map the sites of transcription initiation at the 5' ends of human exons U1 and U3. Several human P1 promoter transcription initiation sites were detected by RNase protection analysis (Figs. 2B and 3). Several sites were also found by primer extension which correspond closely to those detected by RNase protection (Fig. 2C). The human P1 promoter is similar to the mouse promoter (16) in that it is composed of several initiation sites. While the initiation sites are not identical in the mouse and human sequences, they are similarly distributed, being generally downstream of a conserved palindromic (A+T)-

Fig. 2.1. Cloning of the 5' end of the human PTHR gene. (A) Generation of a human PTHR cDNA probe corresponding to human U1 and U2 exons by PCR amplification using synthetic oligonucleotides. See Materials and Methods for details. (B) Characterization of two clones, A and B, isolated from a human genomic DNA library. A detailed map of two contiguous BamHI fragments containing sequences homologous to mouse exons U1, U2, U3 and SS is shown below. ApaI (A), BamHI (B), PvuII (Pv), PstI (P), KpnI (K), and SacI (S) sites are indicated. Also indicated are the positions of probes used for Southern analysis of genomic DNA of normals and PHP1b patients (see below). A schematic representation of the corresponding region of mouse genomic DNA is provided for comparison.

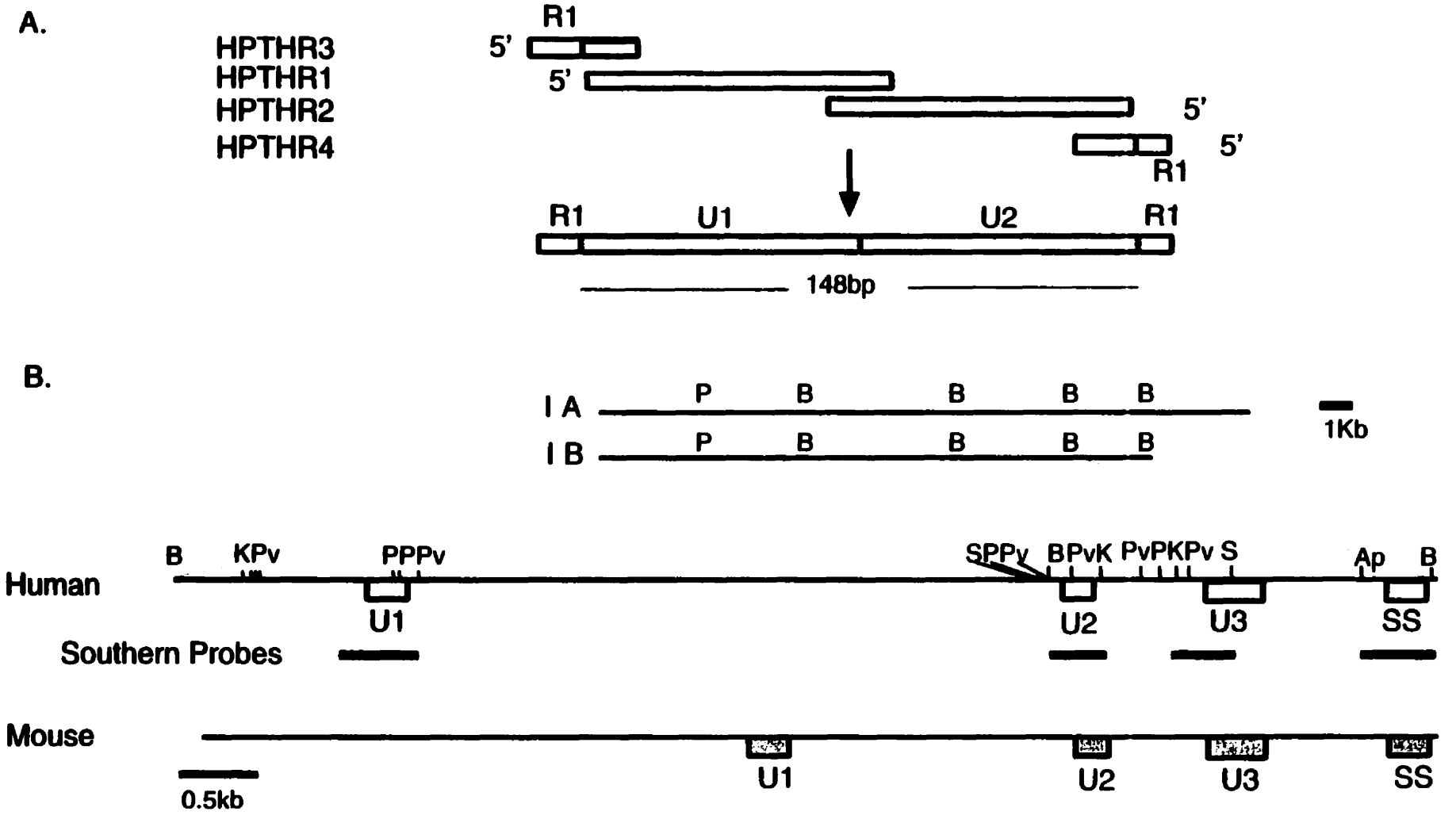
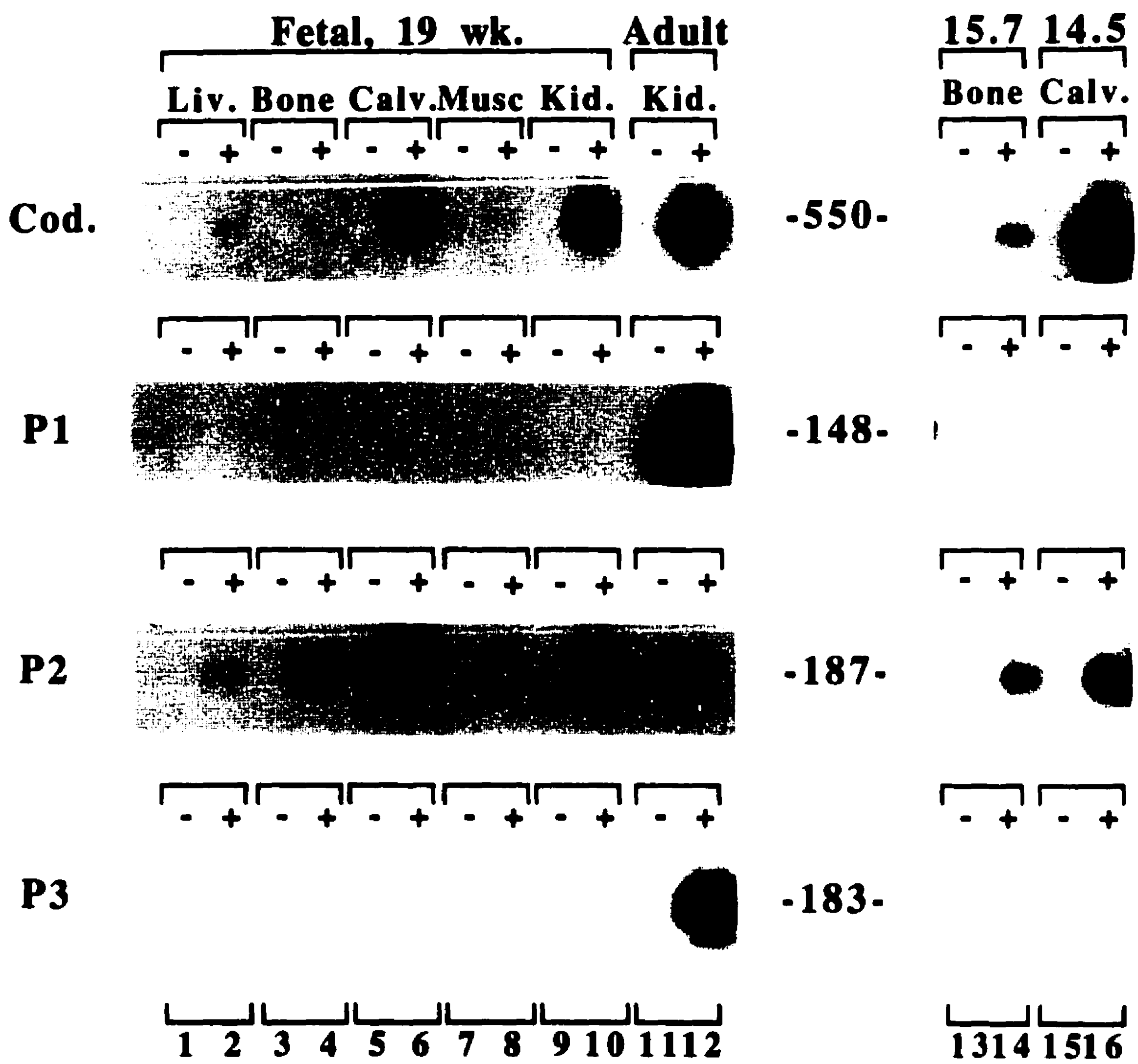

A.
HPTHR3
HPTHR1
HPTHR2
HPTHR4
R1
5'
5'
5'
5'
R1
R1
U1
U2
R1
148bp
B.
I A
I B
P
B
B
B
B
1Kb
Human
B
KPv
PPPv
SPPv
BPvK
PvPKPv
S
Ap
B
U1
U2
U3
SS
Southern Probes
Mouse
0.5kb
U1
U2
U3
SS

Fig.2.2. Mapping of the human P1 and P2 promoters. (A) Schematic representations of probes used for RNase protection analysis. (B) RNase protection analysis of human kidney RNA using P1- and P2-specific probes U1P (lanes 1 and 3), U3NS (lanes 2 and 4), and U3S (lanes 5 and 6). Human kidney RNA (kid.) or tRNA control were used as indicated. Fragments protected by U1P or U3S, are indicated by filled arrowheads, whereas that protected by U3NS is indicated by an open arrowhead. (C) Primer extension analysis of human kidney RNA using primers hU1PE and hU3P, complementary to U1 and U3 exons, respectively. U1 and U3-specific extension products (lanes 1 and 3) are indicated by filled and clear arrowheads, respectively. Transfer RNA controls are provided in lanes 2 and 4. Note that the DNA sequence used as a marker was taken from a longer exposure of the same gel. See Fig. 3 for positions of primers, and the results of promoter mapping studies.


Fetal, 19 wk.
Adult
15.7
14.5
Liv.
Bone
Calv.
Musc
Kid.
Kid.
Bone
Calv.
Cod.
-550-
P1
-148-
P2
-187-
P3
-183-
1 2 3 4 5 6 7 8 9 10 11 12
13 14 15 16

rich motif (Fig. 3A). It is noteworthy that the human P1 promoter contains a consensus AP-1 site which is lacking in the mouse promoter. (Fig. 4A).

Transcripts containing U3 sequences were detected by RNase protection analysis using two different probes (Fig. 2A). A probe complementary to 72b of U3 lying between NcoI and SacI sites protected a fragment of the expected size (Fig. 2B, lane 2). A probe complementary to sequences upstream of the SacI site in U3 protected a 95b fragment (Fig. 2B, lane 5; and Fig. 3), placing the initiation site for the human P2 promoter 15bp downstream from that of the mouse promoter (Fig. 4C). Consistent with this observation, a primer extension product obtained using the U3UP primer was detected which terminated at the same 3' nucleotide (Figs. 2C and 3). Thus, like their mouse counterparts, human P2 transcripts are initiated from a single site. The proximal P2 promoter regions are very similar, containing conserved Sp1 sites. Both promoters also contain consensus sequences recognized by the ets class of transactivators (Fig. 4C). The nucleotides around the mouse initiation site are not conserved in the human promoter, which may account for the difference in initiation sites. It is not surprising that the initiation sites of the P1 and P2 promoters are not conserved between mouse and human, given that neither promoter contains a TATA box to control start site selection.

The sequencing and promoter mapping studies are summarized in Figs 3 and 4. Sequence of 2.0kb of the U1 region, 2.4kb of contiguous sequence containing exons U2 and U3, and 400 bp containing SS is shown in Fig. 3. Human U1 and U2 exon share 74 and 73% homology, respectively, with the corresponding sequences in mouse (Figs. 4A and 4B). The human U3 exon is 92% homologous to the mouse U3 sequence, a degree of conservation which is remarkable considering that no human cDNAs containing U3 sequence have been identified to date.

Fig.2.3. Results of sequencing analysis of the 5' end of the human PTHR gene. Sequence of 2 kb of genomic DNA encompassing U1, 2.4 kb encompassing exons U2 and U3, and .4kb containing SS is presented. Positions of primers hU1PE, and U3UP used for primer extension analysis (Fig. 2.2) are indicated. Transcription initiation sites mapped by RNase protection (asterisks) and primer extension (arrowheads) are indicated. The 27 nucleotides of the human U3 exon downstream of the NcoI site (italics) are assigned based on homology with the mouse gene. The splice donor dinucleotide GT used in the mouse gene is conserved at the 3' end of the human U3 exon. The positions of the primers flanking U1, U2, U3, and SS used for PCR amplification of genomic DNA from PHP1b patients are double underlined.

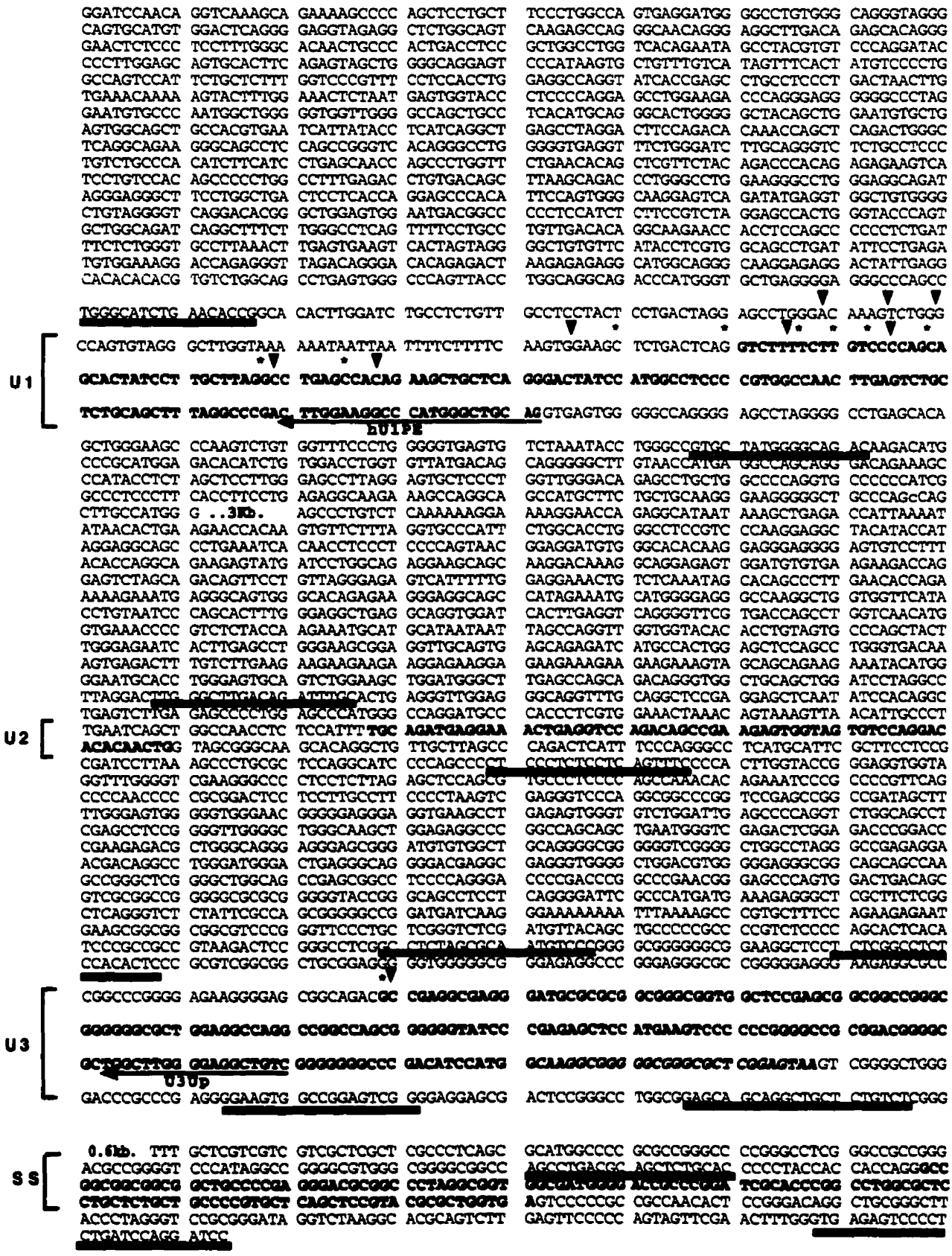
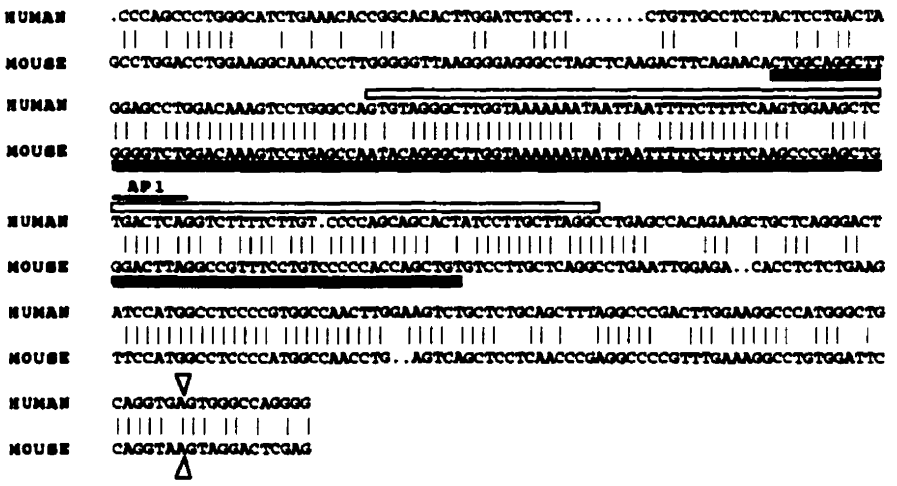

U1
hU1PE
U2
U3
U3Up
SS

Fig.2.4 Comparison of genomic sequence encompassing human and mouse U1 (A), U2 (B), and U3 (C) exons. Sequence comparisons were generated using the program Bestfit from the Genetic Computer Group (GCG) DNA Analysis Package. The range of transcription initiation sites mapped for the human and mouse P1 promoters are represented in A by clear and grey bars, respectively. The black arrowheads in B and C represent the beginning of U2 and the initiation sites of the P2 promoter, respectively. The 3' end of exons U1-U3 are represented by clear triangles.

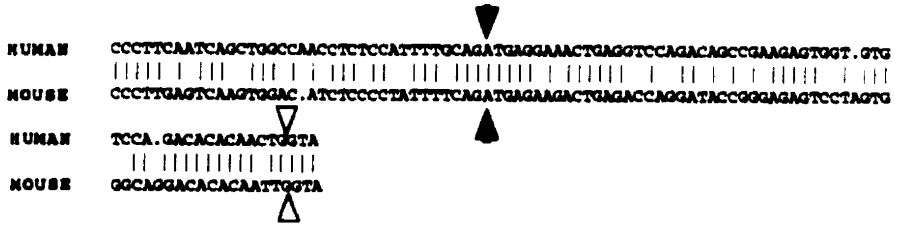
A.

Promoter P1, Exon U1



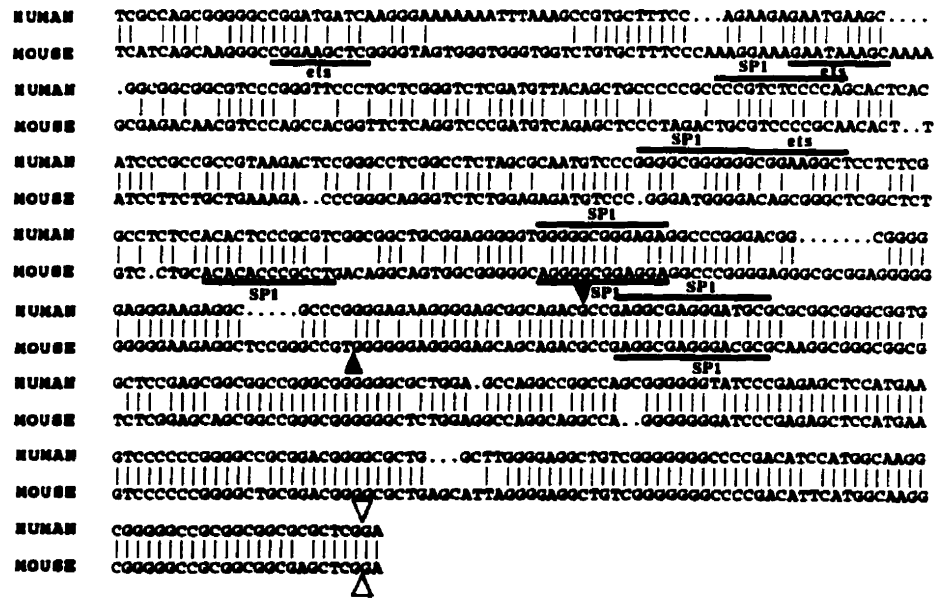
B.

Exon U2.



Promoter P2, Exon U3.

C.



CLINICAL AND LABORATORY CHARACTERISTICS OF PATIENTS WITH PSEUDOHYPERTHYROIDISM

Patient	Sex	AHO	Age	Ca (mg/dl)	P	PTH	1,25-(OH)$_2$D	TmP/GFR	Fold increase in urinary cAMP after PTH stimulation
P3	M	-	13	7.8	6.0	239 (20-65)	ND	6.0	6.2
P8	M	-	4	5.2	6.8	220 (20-65)	22	7.5	4.2
P9	F	-	9	6.2	5.7	870 (<550)	20	6.9	2.1
P13	F	-	6	4.0	6.6	288 (20-65)	ND	6.6	2.0
65	F	-	9	6.9	8.3	1150 (<550)	19	9.0	2.5
H2	M	-	13	7.4	5.6	780 (<270)	ND	6.7	2.5
H46	M	-	14	8.1	6.2	168 (20-65)	ND	6.0	5.1

Table 2.1:Albright's hereditary osteodystrophy (AHO) was determined to be negative (-) by X-ray and normal serum alkaline phosphatase. Serum concentrations of total calcium (Ca), phosphate (P), PTH, 1,25dihydrxyvitamin D [1,25-(OH)2D are indicated (ND, not determined)along with the ratio of tubular maximum of phosphate to the glomerular filtration rate (TmP/GFR). Values in parentheses represent the normal range for the index measure.

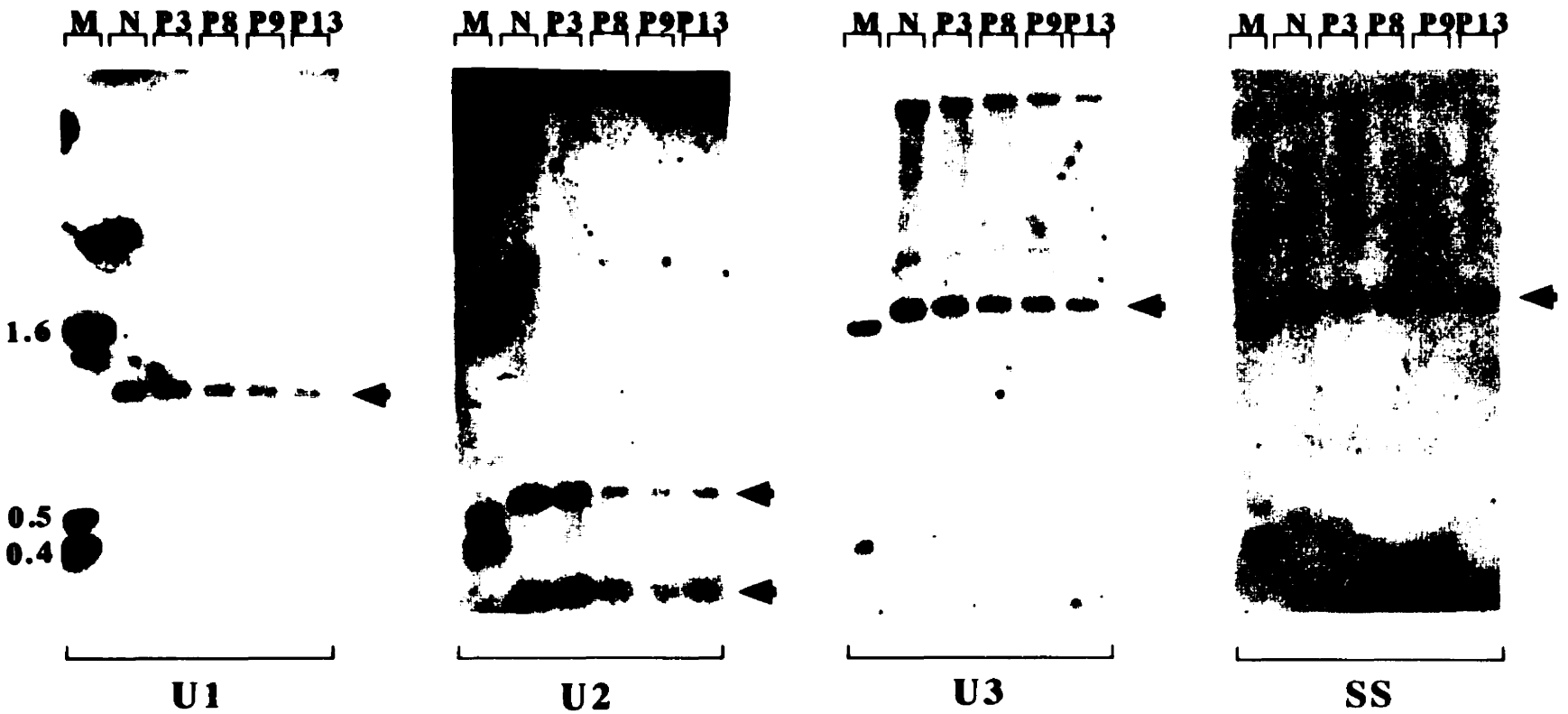
3. Southern blotting analysis of genomic DNA from PHP1b patients.

The structure of genomic DNA from a normal individual, and seven patients diagnosed with PHP1b was compared by Southern blotting analysis using four probes specific for the U1, U2, U3 and SS exons (Fig. 1B), which encompassed 7kb of genomic DNA. PvuII, PstI, SacI, or BamHI digests of normal DNA and DNA from four PHP1b patients which were analyzed with all four probes (Fig. 5; and Table I) generated restriction patterns predicted from the map of the normal PTHR gene (Fig. 1), and did not reveal any differences between the samples. Similarly, analysis of KpnI digests of DNA from all seven PHP1b patient samples with any of the four probes did not reveal any differences with normal DNA (Table I). These results suggest that there are no gross rearrangements of genomic DNA in the promoter region of the PTHR gene in the seven PHP1b patients tested.

4. PCR Amplification, Cloning and Sequencing of Exonic DNA from PHP1b patients.

A finer analysis of genomic DNA encompassing exons U1, U2, U3 and SS from all seven PHP1b patients and a normal control was performed by PCR amplification using the primers indicated in Fig. 3. In all cases, fragments were amplified from patient DNAs which appeared to be identical in length to the normal DNA control (not shown), and to the size predicted from the cloned normal gene. Fragments were subcloned into BlueScript SK+, and six recombinants of each cloned were sequenced. No changes in exonic DNA or spliced donor or acceptor sequences were found in any of the clones tested, strongly suggesting that the structure or splicing of the 5' UTRs of PTHR mRNAs is not affected in these PHP1b patients. These analyses also included 100bp and 80bp of P1 and P2 promoter sequences, respectively. Again, no differences were found in DNA sequences of PHP1b patients and those of normals (not shown). This suggests that the positions of transcriptional initiation of the two promoters is probably not affected in

Fig. 2.5. Analysis of genomic DNA from PHP1b patients by Southern blotting. (U1) Analysis of a PvuII digest of genomic DNA from a normal subject (N) and four (P3, P8, P9 and P13) of the seven PHP1b patients tested using a U1 specific probe (U1). The 1.6 kb fragment expected from restriction analysis of genomic DNA clones of normal DNA is indicated by the arrowhead. Molecular weight markers (M) of 1.6, 0.5 and 0.4 kb were used for each blot. (U2) Analysis of a PvuII digest of genomic DNA as in A using a U2-specific probe. The expected 0.5 kb and 0.35 kb bands are indicated by arrowheads. (U3) Analysis of a PvuII digest of genomic DNA as in A using a U3- specific probe. The expected 1.8 kb band is indicated by the arrowhead. (SS) Analysis of a PvuII digest of genomic DNA as in A using a SS exon-specific probe. The expected 1.8 kb band is indicated by the arrowhead.


M N P3 P8 P9 P13
1.6
0.5
0.4
U1
M N P3 P8 P9 P13
U2
M N P3 P8 P9 P13
U3
M N P3 P8 P9 P13
SS

PHP1b patients, given that promoter-proximal elements control initiation site selection. Taken together, the above results suggest that the structural integrity of the 5' untranslated regions PTHR mRNAs expressed from either P1 or P2 promoters is not affected in the PHP1b patients tested.

B) Discussion.

Our analysis of the upstream regulatory regions of the human PTHR gene showed that homologues of all three 5' UTR exons identified in the mouse are present in the human gene, with similarities varying from 92% for U3 to 73-74% for U1 and U2. The distance between U2 and U3 is conserved, whereas the distance between U1 and U2 is double that in the mouse gene. Transcripts originating from promoters P1 and P2 were detected by both RNase protection and primer extension techniques. Although the sequence and architecture of the two promoters are well conserved, the transcription initiation sites in the human promoters are not identical to those in the mouse. Little is known about the mechanisms controlling initiation site selection in TATA-less promoters, whether they be (G+C)-rich like P2, or non (G+C)-rich like P1. It will therefore be of interest to construct chimeric mouse/human sequences to determine which motifs and factors are controlling initiation from P1 and P2 promoters.

Both human and mouse P1 promoters contain initiation sites spread over at least 100 nucleotides. The striking common feature between the two promoters is a conserved 32bp palindromic (A+T)-rich sequence located in the initiation region. Interestingly, the proximal region of the human promoter contains a consensus AP-1 site which is not found in the mouse, suggesting that the two promoters may be differentially regulated. It is noteworthy that promoters such as P1 with multiple start sites have the potential to produce transcripts with 5' untranslated regions with differing capacities to generate stem-loop structures. Such structures have been proposed to control the efficiency of

translation of mRNAs (Yiu GK. et al 94). Our analyses of the 5' untranslated regions of different transcripts expressed from the human P1 promoter indicate that they can form stem-loop structures of differing stabilities, suggesting that they may be translated with varying efficiencies (data not shown).

The human and mouse P2 sequences are well conserved, and several Sp1 and ets factor binding sites are present in both species. Unlike the P1 promoters, the P2 promoters direct expression from single initiation sites which differ by 15bp in the mouse and human sequences. This shift may be due to the fact that nucleotides at or around the mouse start site are not conserved in the human sequence, or that unidentified factors controlling initiation site selection bind at different positions. The construction of promoter chimeras will be useful to distinguish between these two possibilities.

We have analyzed the proximal P1 and P2 promoter regions, and the 5' untranslated region and signal sequence exons of genomic DNA from PHP1b patients for defects which might lead to disruption of the tissue-specific expression of the PTHR gene. In contrast to PHP1a, where multiple endocrine systems may malfunction in addition to pathways controlled by PTH, functional hypoparathyroidism is the sole phenotype observed in PHP1b. Therefore, the locus of mutation in this latter disorder appears to be specific to the PTH signalling pathway. In addition, although PTH signalling in the kidney is defective, several reports have documented osteolytic lesions in PHP1b, consistent with the persistence of skeletal responsiveness to high circulating PTH concentrations (Murray TM. et al 93, Wemeau JL. et al 86). Consequently, the phenotype associated with PHP1b appears to emanate from a defect in PTH signalling restricted to the kidney. In view of the fact that we have identified multiple promoters controlling PTHR gene expression, the possibility arose that disruption of expression of a PTHR promoter, or of the function of a promoter-specific transcript could account for the phenotype of PHP1b.

	BamHI				KpnI				PstI				PvuII				SacI			
	U1 4.6	U2 3.0	U3 3.0	SS 3.0	U1 4.0	U2 4.0	U3 3.6	SS 3.6	U1 0.8	U2 3.3	U3 3.3	SS 1.1	U1 1.1	U2 3.3	U3 1.8	SS 1.8	U1 2.6	U2 0.5	U3 1.0	SS 2.3
P3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
P8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
P9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
P13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
65					+	+	+	+												
H2					+	+	+	+												
H46					+	+	+	+												

Table 2.2. Summary of analysis of genomic DNA of PHP1b patients by Southern blotting. Genomic DNA from seven PHP1b patients and one normal subject was digested with the enzymes indicated and probed with DNA fragments specific for U1, U2, U3 or SS exons (see Fig. 1 for positions of probes). The size of the expected DNA fragment is indicated. The symbol "+" indicates the expected fragment was seen. A band corresponding to the 0.5 kb SacI fragment containing U2 was not seen because it had migrated off of the gel.

Southern blotting analysis of the structures of the regulatory regions of the PTHR gene in the seven patients tested has excluded the presence of gross rearrangements or deletions which would disrupt promoter function. Genomic DNA from PHP1b patients amplified by PCR was sequenced to look for more subtle alterations which might affect promoter initiation sites, splice junctions or transcript stability. No differences were observed in the patterns of restriction sites in these regions or in the sequences of crucial elements. Thus in all seven PHP1b patients tested, the phenotype cannot be accounted for by mutations affecting the initiation, splicing or stability of transcripts expressed from P1 or P2. Coupled with the lack of mutations in the PTHR coding region found by other groups, our results suggest that mutations causing PHP1b could reside either elsewhere in the PTHR gene promoter, or in the coding or regulatory sequences of another gene. Preliminary observations have indicated that mutations in two families giving rise to PHP1b phenotypes may reside outside of loci encoding the PTHR and the PTH2 receptor (Ding C-L. et al 96). However, it is important to note that mutations at more than one locus have been shown to account for the phenotype of PHP1a (Phelan MC. et al 95, Shapira et al 96, Dixon PH. et al 96). Therefore, we cannot rule out the possibility that disruption of tissue-specific initiation, splicing or stability of a PTHR transcript may account for PHP1b in some patients.

Candidate loci responsible for PHP1b other than the PTHR may include those encoding transcription factors, which would control kidney-specific transcription of the PTHR. There are precedents for deficiencies in such factors. Defects in patients with insulin resistance and non-insulin-dependent diabetes mellitus have been ascribed to markedly reduced levels of transcription factors required for normal expression of the insulin receptor gene (Brunetti A. et al 96). To be implicated in PHP1b, such a factor would be required to be not only specific to the kidney, but also to the PTH signalling pathway. Disruption of its expression would not perturb expression of other genes essential for normal kidney function. Alternatively, the defect may lie in a factor required

for PTH-stimulated generation of cAMP, but acting downstream of the receptor, or in a factor which antagonizes functional PTH-PTHR interaction. Tissue-specific factors which regulate G protein signalling have been identified (Berman DM. et al 96). However, to be implicated in PHP1b, such factors would have to be specific to PTH signalling and not be important for other pathways. Characterization of factors controlling expression of the PTHR or modulating signalling through the PTHR will therefore be essential to fully understand the molecular mechanism of PTH-PTHR signalling and its disruption under pathological conditions.

CHAPTER 3:

DEVELOPMENTALLY REGULATED PROMOTER FUNCTION AND DIFFERENTIAL SPLICING OF THE HUMAN PARATHYROID HORMONE (PTH)/PTH-RELATED PEPTIDE RECEPTOR GENE.

- A) INTRODUCTION.
- B) MATERIAL AND METHODS.
- C) RESULTS.
- D) DISCUSSION.

The parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor (PTHR) is essential for normal skeletal development, and a wide array of physiological responses modulated by PTH and PTHrP. In the mouse, renal PTHR gene expression is controlled largely by the P1 promoter. Expression in many other tissues including bone and cartilage is driven by P2, indicating that renal and non-renal PTHR gene transcription are regulated differently in the mouse. P1 and P2 are conserved in humans, however, P1 activity in kidney is weak. We have now identified a major promoter, P3, which is active in humans but not in mouse. P3 accounts for ~80% of renal PTHR transcripts in adults and is active is many other tissues. Thus, in adults, PTHR gene expression is coordinated fundamentally differently in humans and mice. A study of 17.5-19 week fetal age (FA) tissues including kidney, calvaria, and long bone showed that only P2 is active at mid-gestation. Moreover, only P2-specific transcripts were detected in long bone and calvaria at earlier stages, indicating that factors regulating the well conserved P2 promoter control PTHR gene expression during skeletal development. We also found that P2-specific transcripts are differentially spliced in a number of human cell lines and adult tissues, but not in fetal tissues. The differential splice, which uses a nonconsensus splice donor, shortens the 5' UTR and eliminates an upstream open reading frame present in full length transcripts. P2 and P3 lie within a CpG island, whereas the CpG content of P1 is similar to bulk DNA. Function of all three promoters is inhibited by methylation in vitro. In vivo, P3 is fully demethylated in both fetal and adult kidney. P1 is partially demethylated in kidney, in a pattern that is established as early as 11.75 weeks FA, providing an example of an non-CpG island promoter that is tissue-specifically demethylated several weeks prior to function. These experiments also indicate that the activities of both P1 and P3 require factors expressed late in development. Our results show that expression of the human PTHR is subjected to multiple developmental switches, and that gene transcription is coordinated differently in humans and in mice.

A) INTRODUCTION.

Signaling through the parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor (PTHR) is essential for normal development, and for a wide array of physiological responses stimulated by PTH and PTHrP. PTH acts to tightly maintain circulating concentrations of calcium ions. It is released from the parathyroid glands in response to a decrease in extracellular fluid calcium levels, and binds to the PTHR expressed in kidney and bone to stimulate calcium reabsorption and increase phosphate excretion (Goltzman D., and Hendy GN. 95, Kronenberg HM. 96). PTHrP can mimic many of the effects of PTH when overexpressed by cancers. Under physiological conditions, PTHrP is thought to act in a paracrine/autocrine manner, in contrast to PTH. Moreover, whereas expression of PTH is restricted to the parathyroids, PTHrP is widely expressed, and functions to modulate normal cellular growth and differentiation (Ikeda K. et al 88, Stewart AF. 93, Strewler GD. and Nissenson RA. 93, Suva LJ. et al 87). Like PTHrP, the PTHR is widely expressed. The highest levels of receptor mRNA in rat are found in kidney, with lower levels expressed in a number of other tissues including bone, bladder, aorta, heart, liver, lung and spleen (Pausova Z. et al 94, Urena P. et al 93).

The most striking examples of function of the PTHR have come from genetic analyses. Gene ablation experiments in mice have shown that PTHrP-PTHR signaling is essential for normal endochondral ossification during development (Amizuka N. et al 94, Karaplis AC. et al 94, Lanske B. et al 96). Both PTHrP and PTHR null mice displayed similar phenotypes, with reduced proliferation and accelerated maturation of chondrocytes associated with accelerated ossification. Further evidence for the essential role of the PTHR in control of skeletal formation has come from studies of patients with Jansen-type metaphyseal chondrodysplasia, a rare form of dwarfism associated with hypercalcemia in spite of near normal levels of PTH and PTHrP. Mutations in

transmembrane domains of the PTHR have been discovered that lead to constitutive activation of the receptor (Juppner H. 96, Shipani E. et al 95 and 96).

The PTHR belongs to the vast family of G protein-coupled receptors containing seven transmembrane domains. Binding of ligand to the PTHR can stimulate production of intracellular cyclic AMP and inositol 1,4,5-trisphosphate (Abou-Samra A-B. et al 92, Juppner H. et al 91, Shipani E. et al 93). The structure of the PTHR gene is very similar to those of a subfamily of receptors for peptide hormones including calcitonin, vasoactive intestinal peptide, glucagon, and growth hormone releasing peptide, providing evidence that these receptors have evolved from a common precursor (Kong X-F et al 94, Lin S-C et al 93, Maget B. et al 94, McCuaig K. et al 94, Sreedaharan SP. Et al 95,Zolnierowicz S. et al 94).

Expression of the PTHR gene in the mouse and rat is controlled by two promoters, designated P1 and P2, which give rise to transcripts differing in their 5' untranslated regions but not their coding sequences (Joun H. et al 97, McCuaig K. et al 94 and 95). In mice, P1 is highly and selectively active in kidney, generating transcripts containing 5' untranslated regions composed of two exons, U1 and U2, spliced to the signal sequence exon SS (23,24). P1 is TATA-less but not (G+C)-rich, and has a number of start sites spread out over more than 100 bp (McCuaig K. et al 94 and 95). P2-specific transcripts contain a single untranslated region exon, U3, spliced to SS (McCuaig K. et al 94). Unlike P1, P2 is expressed at moderate levels in a number of tissues, and is responsible for the broad expression pattern of the PTHR. P2 has all the hall-marks of a housekeeping promoter. It is highly (G+C)-rich, contains multiple Sp1 sites and has a single major start site.

The exon-intron structures of the coding regions of the human and rodent PTHR genes are highly conserved (Kong X-F. et al 93). Recently, we have cloned the 5' regulatory region of the human PTHR gene (Bettoun JD. et al 97), and shown that P1 and

P2 are also conserved between mouse and human. However, RNase protection studies with kidney RNA suggested that human P1 is not as active as its mouse counterpart. In the present study, we have further characterized the regulatory region of the human PTHR gene and have discovered a major promoter, P3, which accounts for ~80% of detectable renal PTHR transcripts in human, but is apparently inactive in mouse. Our data provide evidence that PTHR gene expression in adults is coordinated differently in humans and mice, but is similar during development. We also show that the tissue-specific P1 promoter, which is not part of a CpG island, is demethylated at mid-gestation, several weeks before the onset of its function.

B) MATERIALS AND METHODS.

Tissues and RNA extraction. Protocols for obtaining human and mouse tissues were approved by local ethics committees. Tissues from human fetuses (n=7, 11.75-19 weeks FA) were obtained at the time of therapeutic abortion. All tissues were flash frozen and stored immediately at -70°C. Long bone samples were taken from the femur and muscle samples from the thigh. Fetal age was determined by foot length (Munsick RA. 84). Total RNA was isolated from adult tissue or cultured cells by CsCl gradient centrifugation and from fetal tissues using Trizol (Gibco) extraction.

Ribonuclease (RNase) protection analysis. The probes for RNase protection analysis of U1- and U3-containing transcripts have been described previously (Bettoun J. et al 97, and see Fig. 1a). A U4 probe was created from a 1.8 kb KpnI-BamHI fragment containing the SS exon inserted in Bluescript SK+, which was digested with ApaI and in-vitro transcribed with T3 RNA polymerase (New England Biolabs) under standard conditions (see Fig. 1a). Probes (10^5 cpm), purified on Sephadex G50 spin columns, were precipitated with 20 µg of total RNA, resuspended in 30 µl of hybridization buffer (80% deionized formamide, 40 mmol/L piperazine-NN'-bis-2-ethanesulphonic acid (pH 6.4), 0.4 mol/L sodium acetate and 1 mmol/L ethylenediaminetetraacetate), denatured for 5 min at 85°C and incubated overnight at 50°C. Digestions were performed for 1 hr at 37°C with 4 U of RNase 1 (Promega) according to the manufacturers specifications. Products were ethanol precipitated and electrophoresed on a 6% polyacrylamide sequencing gel. Autoradiography was performed overnight at -70°C with two intensifying screens. The integrity of the probe was verified by running 250 cpm of undigested mix.

Primer extension analysis. Ten pmol of primer P3Up2 (5'CTATGGGACCCCGGCGTCC3'), which is complementary to sequences starting 61 bp upstream of the SS exon, were labeled for 1 hr at 37°C with ^{32}P-γ-ATP using T4 polynucleotide kinase. One tenth of this reaction was incubated with 10 μg of either adult human kidney total RNA, or yeast tRNA as a control, overnight at 55°C in 300 mM KCl, 20 mM Tris-HCl (pH 8.3), 2 mM EDTA in a final volume of 26μl. Samples were then put on ice, and 4μl of 25 mM Tris-HCl (pH 8.0), 60 mM $MgCl_2$, 10 mM DTT, 5 mM dNTPs, 2 U of RNAguard (Pharmacia), and 100 U of M-MLV reverse transcriptase (Gibco-BRL) were added. After 60 min at 43°C, the enzymes were inactivated for 10 min at 75°C, and the reaction was extracted once with phenol and ethanol precipitated. One quarter of the reaction was denatured for 2 min at 80°C and run on a 6% denaturing polyacrylamide gel along with a sequencing reaction as molecular weight markers.

Reverse transcription (RT)-PCR analysis and cDNA cloning. Three μg of total RNA were precipitated, resuspended in 6 μl of 1x DNase I digestion buffer (Promega) containing 0.5 U of DNase I, incubated for 15 min at 37 C and for 10 min at 75°C, and put immediately on ice. Two μl were immediately added to 18 μl of a RT mix containing 0.5 mmol/L of each dNTP, 5mmol/L of DTT, 1 μmol/L of 6mer random primers, 75 mmol/L of KCl, 3 mmol/L of $MgCl_2$ in 50 mmol/L of Tris-HCl (pH 8.3) and incubated for 1 hr at 37°C, followed by 75°C for 10 min. For detection of P1 specific transcripts by PCR, 2 μl of the RT reaction were subjected to a first round of 30 cycles of amplification consisting of 95°C for 30 sec, 56°C for 1 min, 72°C for 20 sec, using 5'AGGGAATTCAGGTCTTTTCTTGTCCCCAGC3' as the forward primer (primer 1) and 5'GACGTCATCTGCATCCACCAGCGC3' (primer 6) as the reverse primer. One μl of the first reaction was then subjected to 30 more cycles of amplification consisting of 95°C for 30 sec, 58°C for 1 min, and 72°C for 5 sec, using forward primer 5'AGTTGTGTGTCCTGGACACTACCA3' (primer 2) and reverse primer

5'GCCTCCCCGTGGCCAACTTGAGTC3' (primer 3). PTHR and β-actin coding sequences were detected by submitting 2 μl of the RT reaction to 24 cycles of amplification consisting of 95°C for 30 sec, 56°C for 1 min, 72°C for 25 sec. The forward primer for the PTHR was 5'CACCACTACTACTGGATTCTGGTG3', the reverse primer used was 5'GATTTCTTGATCTCGCTTGTACC3'. For β-actin, the forward primer was 5'GCTGTGCTATCCCTGTACGC3' and the reverse primer was 5'GCCATGGTGATGACCGGC3'. All the above described reactions were performed in a 50 μl volume in 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl and 10 mmol/L Tris-HCl (pH 9.0) using 2.5 U of Taq DNA polymerase (Pharmacia). P3-specific transcripts were detected using 28 cycles of PCR, consisting of 95°C for 30 sec, 58°C for 45 sec and 72°C for 15 sec, performed using 2 μl of RT reaction in a 50 μl volume in 0.8 mmol/L $MgCl_2$, 50 mmol/L KCl and 20 mmol/L Tris-HCl (pH 8.3) using 2.5 U of Taq DNA polymerase (Pharmacia), and 5'ACGGAATTCCAGCCTGACGCAGCTCTGCACC3' (primer 5), and primer 6 (see above) as forward and reverse primers, respectively. Amplification of P2-specific transcripts was performed by subjecting 1 μl of the RT reaction to a touch-down PCR under the following conditions, 45 sec denaturation at 95°C, 20 sec elongation at 72°C and 1 min annealing starting at 58°C, down 1°C per cycle to 51°C, under which conditions 25 cycles of amplification were performed. The reaction was in 20 μl volume in 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl and 10 mmol/L Tris-HCl (pH 9.0) and in 5% formamide using 5 U of Taq DNA polymerase (Pharmacia). The forward primer was 5'GCCCGACATCCTGCAAGGC3' (primer 4), and the reverse primer was primer 6 (see above). PCR reactions (2/5th to 1/10th) were run on a 2% agarose gel, transferred overnight to a nylon Hybond N+ membrane (Amersham) and the DNA was covalently attached by baking the membrane at 80°C under vacuum for 2 hrs. Membranes were prehybridized for 30 min in 6xNET (100 mM Tris-HCl pH 7.5, 0.9 M NaCl, 6 mM EDTA), 10x Denhardt's, 0.5% SDS, 10 mg/ml denatured salmon sperm DNA at 62°C and then hybridized in 6xNET, 5x Denhardt's, 0.5% SDS, 1 mg/ml

denatured salmon sperm DNA with 10^6 cpm of end-labeled oligonucleotides for 5 hr at 62°C. The oligonucleotides used for probing were: 5'CCCTAGGCGGTGGCGATGGGGACC3', for P2- and P3-containing sequences, 5'CTGCAGCTTTAGGCCCGACTTG3' for P1-specific sequence, and 5'TGGCCATGAAGACAATGTAGTGGACG3' for coding sequence. To clone P2-specific cDNA sequences amplified from HOS cell extracts, cDNA fragments amplified as described above were subjected to 30 more cycles of PCR under the following conditions: 45 sec denaturation at 95°C, 20 sec elongation at 72°C and 1 min annealing at 51°C. The amplified band was purified from a 2% agarose gel, inserted in the TA cloning vector pPCR2 (Invitrogen), and characterized by DNA sequencing.

Construction of PTHR promoter-luciferase reporter vectors. To construct the P1-luciferase reporter plasmid pXP2-P1, a 1.6 kb fragment of P1 extending from a BamHI site 1428 bp upstream of the most 5' transcription start site (+1) to a PstI site at position +174 (Bettoun J. et al 97) was inserted in BamHI-PstI-digested pSKb, a derivative of pBluescript SK+ (Stratagene) in which the XbaI site has been converted into a BglII site. The insert was excised from pSKb as a BamHI-BglII fragment and inserted in the polylinker of the promoterless luciferase plasmid pXP2 (Nordeen SK. 88). To construct P2-luciferase recombinant pXP2-P2, a 1.1 kb SacI fragment (containing P2 sequences -959 to +101), whose ends were rendered blunt with T4 polymerase, was inserted into the SpeI site of pSKb. The orientation of the insert was confirmed by restriction digestion and DNA sequencing, and was then excised as a BamHI-BglII fragment and subcloned into pXP2. To construct pXP2-P3, a 1.1 kb NcoI-AvrII fragment (-843 to +245) was rendered blunt with T4 polymerase and inserted into the SpeI site of SKb. The resulting BamHI-BglII fragment was excised and inserted in the polylinker of pXP2. A HindIII-BamHI fragment containing a truncated Herpes Simplex virus thymidine kinase (tk) promoter (-109 to +52) was inserted into the polylinker of pXP2 to create tk-luciferase,

and a 370 bp KpnI-HindIII fragment containing the SV40 early promoter was inserted in the polylinker of pXP1 (27) to create SV40-luciferase.

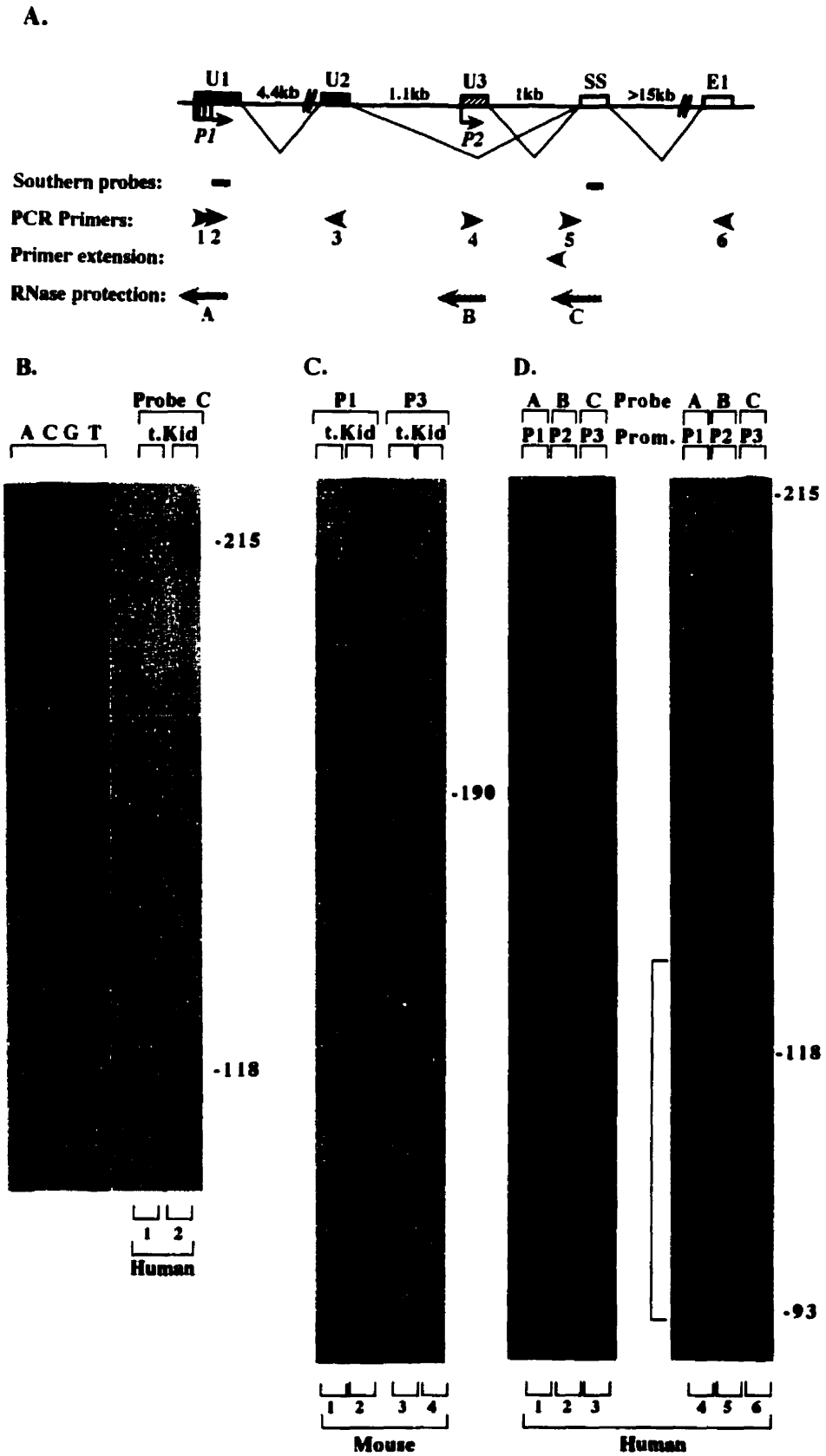
Methylation of reporter plasmids in vitro. Plasmids (5 μg) were treated with varying concentrations of either SssI or HpaII methylase (New England Biolabs) for 2 hr at 37°C, according to the manufacturer's instructions. Treated DNA was extracted twice with 1 volume of phenol/chloroform, and precipitated with ethanol prior to transient transfections.

Cell culture and transient transfections. SaOS-2 cells (ATCC) were propagated in McCoy 5A media + 10% fetal bovine serum (FBS). HOS cells (ATCC) were cultured in DMEM + 10% fetal calf serum. HK-2 cells (Ryan MJ. et al 94) were propagated in keratinocyte serum-free medium + 5 ng/ml EGF, and 50 μg/ml bovine pituitary extract (Gibco), and MCF-7 cells were propagated in Eagle's MEM + 10% FBS. Transient transfections were performed with COS-7 cells (50% confluent), cultured in DMEM + 10% FBS in 12-well plates. Cells were transfected with 1 μg of luciferase reporter plasmid, and 250 ng of β-galactosidase expression vector p610AZ (Kothary R. et al 89) using Lipofectin (Gibco), according to the manufacturer's instructions. Media were changed 12 hr later, and cells were harvested in 100 μl of Reporter Lysis Buffer (Promega) 48-72 hr after transfection. Luciferase assays were performed according to instructions (Promega), and β-galactosidase assays to normalize for transfection efficiency were performed as previously described (Tora L. et al 89).

Restriction digestion and Southern blotting of genomic DNA. DNA was extracted from peripheral lymphocytes as previously described (Treco D. 92). Ten μg of genomic DNA purified from either adult or 18 week FA kidneys was digested with either ApaI, ApaI-MspI, ApaI-HpaII, KpnI, or KpnI-TaiI overnight according to the manufacturer's

instructions (New England Biolabs). Digests were run on a 2% agarose gels and Southern blots were performed as described above. Genomic DNA in the region of P1 was detected by probing with a ^{32}P-labeled 1.2 kb ScaI-PstI fragment containing U1. P3 region sequences were detected using a ^{32}P-labeled 515 bp SmaI fragment extending from a naturally occurring SmaI site located 111 bp downstream of the 5' P3 start site upstream to a second site representing the 5' end of an exonuclease III digestion.

Fig. 3.1. Detection of a third human PTHR gene promoter. **(A)** Schematic representation of the 5 end of the human PTHR gene. Previously characterized 5' untranslated region exons U1, U2 and U3 are indicated along with the signal sequence exon (SS), and the first extracellular domain exon E1. Splicing patterns of transcripts expressed from P1 and P2 promoters are shown up to E1. The positions of probes used for Southern analysis, primers used for PCR (numbered 1 to 6) and primer extension, and probes A, B and C used for RNase protection assays of human RNA presented below are indicated. **(B)** RNase protection analysis of transfer RNA control (t.) and adult human total kidney RNA (Kid) samples using probe C which corresponds to human genomic sequences encompassing SS and sequences 5' to SS. The 118 b protected product expected from previously characterized splicing pattern as well as a 215 b product are indicated. **(C)** RNase protection experiments performed with tRNA (t.) control or mouse total adult kidney RNA (Kid) using a mouse P1-specific probe (lanes 1 and 2; ref 19), and a probe extending 1kb downstream from a BamHI site at the 3' end of U3 to an AvrII site 30 bp downstream of the 5' end of SS of the mouse gene (lanes 3 and 4). A major protected P1-specific fragment of 190 b is indicated. **(D)** RNase protection experiments of adult human total kidney RNA with human probes A, B, and C corresponding to P1, P2, and P3 promoter regions, respectively. Short (lanes 1-3) and longer exposures (lanes 4-6) of the same autoradiogram are presented. The 215 and 118 b fragments protected by the P3-specific probe C, and a 93 b product protected by the P2-specific probe B are indicated. Several bands protected by the P1-specific probe A are only seen in the longer exposure (lane 4, bracketed).


A.
U1
4.4kb
U2
1.1kb
U3
1kb
SS
>15kb
E1
P1
P2
Southern probes:
PCR Primers:
1 2
3
4
5
6
Primer extension:
RNase protection:
A
B
C
B.
Probe C
A C G T
t.Kid
-215
-118
1 2
Human
C.
P1
P3
t.Kid
t.Kid
-190
1 2
3 4
Mouse
D.
A B C
Probe
A B C
P1 P2 P3
Prom.
P1 P2 P3
-215
-118
-93
1 2 3
4 5 6
Human

C) RESULTS.

1. Identification of a major PTHR promoter, P3, active in human but not mouse kidney.

We have previously identified two promoters, P1 and P2 (Fig. 1A), controlling expression of human PTHR transcripts (Bettoun JD. et al 97). In this respect, the organization of the human gene is very similar to that of the mouse (McCuaig K. et al 94 and 95). Notably, however, expression of P1 in the human kidney is weak, whereas in the mouse P1 activity accounts for at least 90% of renal transcripts (Amizuka N. et al 97, McCuaig K. et al 95). Preliminary RNase protection experiments suggested that the combined activities of human P1 and P2 could not account for the levels of PTHR transcripts detected with coding sequence probes (data not shown). We therefore investigated whether there were other promoter sequences controlling PTHR gene expression in human kidney.

An RNase protection experiment performed with human kidney total RNA and a probe of human genomic sequence extending upstream of signal sequence exon SS (Fig. 1A; Probe C) protected a 118 b fragment corresponding to splicing of SS to the 5' untranslated region exons (5' UTRs) of transcripts expressed from P1 or P2 (Fig. 1B, lane 2). The probe also protected a larger fragment of 215 b, present at much higher levels. This is consistent with transcription from a promoter, hereafter called P3, giving rise to a fusion exon composed of a 5' UTR and the SS exon (see below). An identical result was obtained with total RNA from an independent kidney sample (data not shown). Significantly, no protected fragment was detected using a probe derived from mouse genomic sequences upstream of SS, under conditions where mouse P1-specific transcripts were detected at high levels (Fig 1C, lanes 2 and 4), indicating that similar transcripts are not expressed at significant levels in mouse kidney.

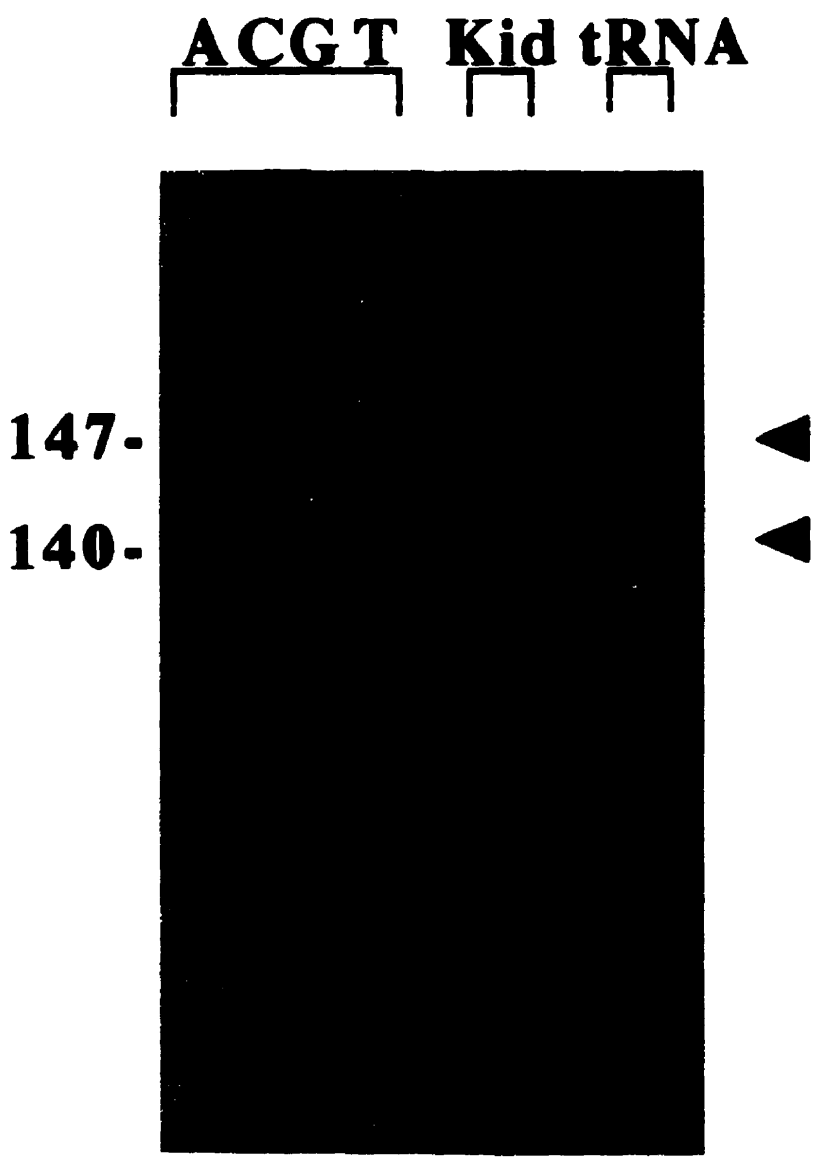
The relative intensities of the 215 and 118 b protection products in Fig. 1B suggested that P3 activity accounts for the majority of PTHR transcripts in the human kidney. These results were supported by a comparative analysis of promoter function using RNase protection probes A, B and C, which are specific to P1-, P2- and P3-specific transcripts, respectively. The results indicate that P3 is the major human renal promoter and that P2 activity accounts for most of the remaining transcripts detected (Fig. 1D, lanes 1-3). Indeed, under these conditions P1-specific transcripts were detected only after prolonged exposure of the autoradiogram (Fig. 1D, lane 4).

The initiation sites of P3 were mapped by primer extension analysis using a primer (Fig. 1A) whose 5' end lies 70 bp upstream of the 5' end of SS. Primer extension gave rise to two products of 140 and 147 b (Fig. 2, Kid). The location of those two start sites upstream of SS was confirmed by RNase protection experiments using a probe that extended 5' of those two sites (data not shown). Thus, transcription from P3 gives rise to a novel fusion exon combining a 5' exon of 210-217 bp in length (designated U4) and the previously identified SS exon. The ATG of the PTHR open reading frame lies downstream of a 253 b 5' UTR that contains an upstream open reading frame, suggesting that the transcript is subjected to translational regulation. These results are summarized in Fig. 3.

2. P2 and P3 promoters lie within a CpG island.

The sequence of human genomic DNA between exons U3 and SS was determined (Fig. 3B). The region encompassing P2 and P3 lies within a CpG island. In 1.3 kb of human sequence extending from 300 bp upstream of U3 to SS, CpG dinucleotides are found at a frequency of 11 per 100 bp, and the ratio of CpG/GpC is approximately 1 (Figs. 3A and B; Bettoun JD. et al 97). In contrast, a 1.1 kb region around human P1 contains only 11 CpG dinucleotides and a CpG/GpC ratio of 0.12 (Fig. 3A; and Bettoun JD. et al 97). Similar to other CpG island promoters (Antequera F. and bird A. 93, Lin Q

Fig. 3.2. Identification of P3 promoter initiation sites by primer extension analysis. Primer extension was performed with a primer whose 5' end lies 70 bp upstream of the 5' end of SS (see Fig. 1A). Two extension products of 140 and 147 b are detected using human kidney total RNA but not with a tRNA control. The sequencing ladder used as molecular weight markers is shown on the left.


ACGT
Kid
tRNA
147-
140-

et al 94, Wenger RH. et al 93), P3 contains no discernible TATA box, and is relatively (G+C)-rich (65%) between -250 and +100. The promoter contains a number of Sp1 sites, including one overlapping the initiation sites (Fig. 3B).

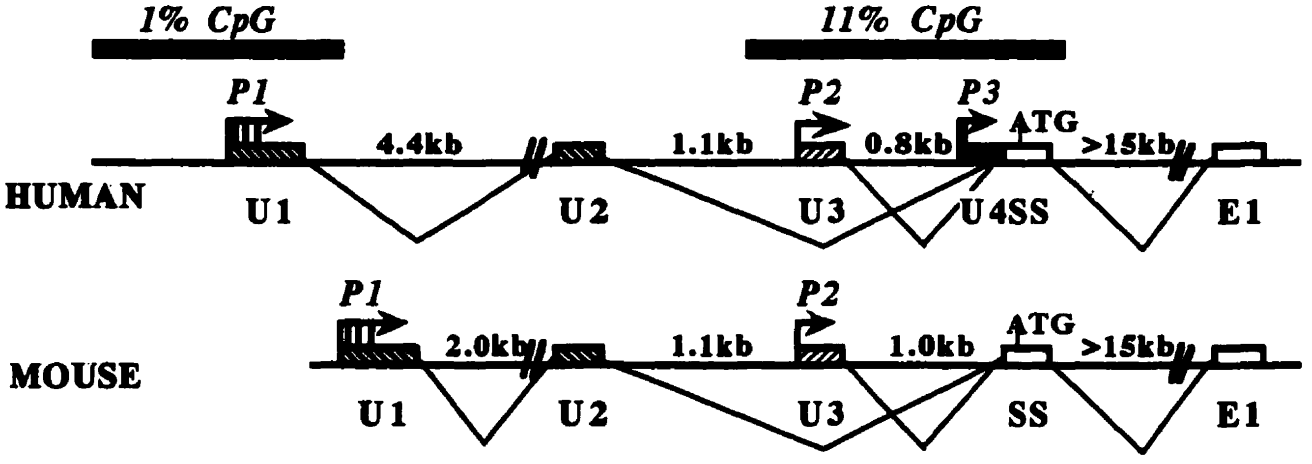
3. P3 is widely expressed in human tissues and cell lines.

In the mouse, activity of the major renal promoter P1 is largely restricted to kidney and is very weak or inactive in bone and cartilage (Amizuka N. et al 97, McCuaig K. et al 95 and unpublished results). It was therefore of interest to analyze the expression patterns of the three human promoters, and, in particular, to determine if P3 is expressed in tissues other than the kidney. Expression of PTHR coding sequence, and of P2- and P3-specific transcripts was examined by semiquantitative RT-PCR analysis using specific primers (see Fig. 1A). Low abundance P1-specific sequences were more highly amplified. In addition, β-actin sequences were amplified to control for differences in the relative total RNA concentrations.

As expected, the highest levels of expression of PTHR coding sequences were found in kidney (Fig. 4A, lane 14), with lower levels detected in spleen, and bowel (Fig. 4A, lanes 16 and 18). Low levels of expression were detected in human osteoblast-like osteosarcoma cell lines HOS (lane 6) and SaOS-2 (lane 10), and weak expression was seen in U2OS cells (lane 8). Transcripts were also detected in cultures of human skin fibroblasts (lane 12), the renal epithelial cell line HK-2 (lane 4), and at very low levels in the breast carcinoma cell line MCF-7 (lane 2). Actin sequences were amplified to similar degrees in all tissues and cell lines studied (Fig. 4, bottom), suggesting that the wide range of amplification of PTHR coding sequences from different tissues was due to variation in expression of receptor transcripts and not to variable RNA concentrations in reverse transcription reactions. This interpretation is supported by Northern analyses of PTHR transcripts showing a wide variation of expression levels in different rat and human tissues (Shipani E. et al 93, Urena P. 93 and data not shown).

Fig. 3.3. Sequence of the P3 promoter region. **(A)** Structures of the 5' ends of the regulatory regions of the mouse and human PTHR genes. The CpG content of sequences encompassing human promoter P1 and promoters P2 and P3 is indicated above. **(B)** Sequence of the human P3 promoter region. The start sites of the P3 promoter are indicated by arrowheads, with the upstream start site designated as +1. Sequences corresponding to consensus Sp1 sites are indicated. CpG dinucleotides are underlined. The sequence of the U4/SS exon is indicated in bold. The ATG start and TAG stop codons corresponding to the extremities of the open reading frame lying upstream of the PTHR coding sequence are boxed, as is the ATG (in italics) of the PTHR coding sequence.

A.



B.

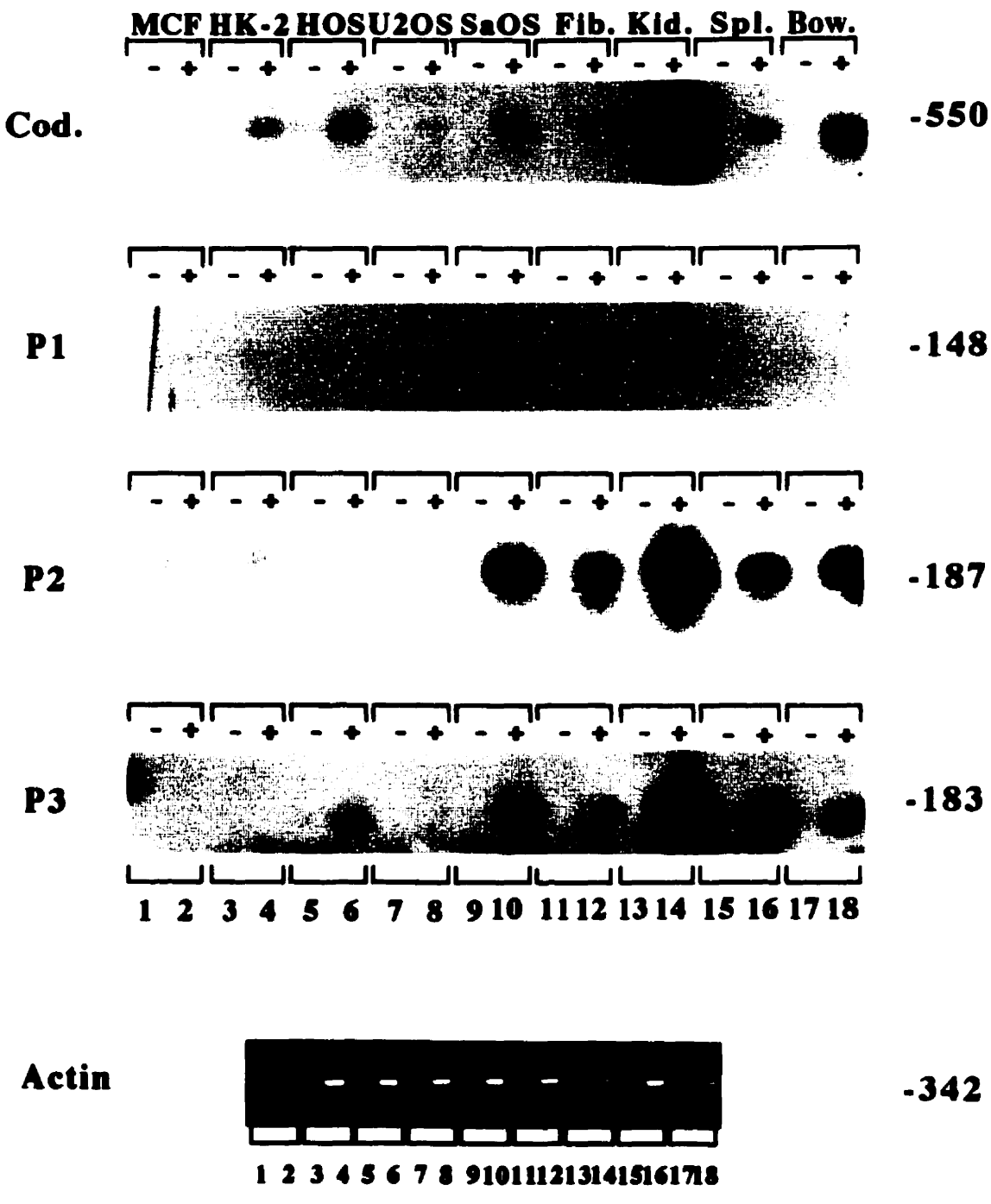
```
GTCGGCGGCG CTCCTCGGAA CCCGGGGGAG TCGCCAGCCC  CGCGCCGCTC GGCTCGGTGG CTTTTTTGGA AACTTGCAAA -632
TGTTTTCGTA GAGAGAAAAG GGGGAGGGAG GGAGCGAGGG  AGTGACCGAA ACGGAGCTTG GGGCCGCTGG AAGAACTGAG -552
GCCAAGGCCG GGGGAGCTAG AGACGGACTG ACAGACAGGC  AGACCGACAG AGCGTCGGGG CCGCTGCCCC GAGCCGCACA -472
GGCGCAAGGG GCTCTGGCAA GGATGGGGAA GGGGTGCGGG  AGGCGGCTGC CGAGGGTCTG GGATCTCAGG AGGCCGAACG -392
GCGGGGCTGG CGGCCGGAAC ACCTAAGGGC TCAGTGTGGC  TGCAAAGTTG AGATCGCACC CCCTAACTGC ACGCCCCGCG -312
CGGCTCAGAA CGCGCCCCCT GCCCGGCCCT GACTCCCTAC  CCCGAAAGTC GCGGAGCTAA AAATAACAGG TTCCTGCGCG -232
                    SP1
CCCCCCGCAG ACCGCGACCC CGACCCCTCC CCCGCCCCCT  CCCCCCACTG GGCGTGGGGC GAAGCCACAG CTCCCATTTC -152
                                  SP1       SP1     SP1
CCCAAAAGAA AAAAAAAGAA AGAAAGAAAG AAAGAAAGAA  AAGGCGGCGC GGGAGGGGGG CGGGGGGCGG GCCGGGGGCG -72
                                                                  SP1
GGGGGCCCGG CATATGGATG TGATTTCTTC GCTCCGAGGC  AGACGGGCCC GTCCGCAGCG GCTTGGCGCC CGCCGCCGCC +9
U4
CCCTCCGGCT CTTCTCCCCT CCCTCCTGTC CCTCCCTCCC  TCCCTCCTTT GCTCGTCGTC GTCGCTCGCT CGCCCTCAGC +89
GCATGGCCCC GCGCCGGGCC CCGGGCCTCG GGCCGCCGGG  ACGCCGGGGT CCCATAGGCC GGGGCGTGGG CGGGGCGGCC +169
                                U4 | SS
AGCCTGACGC AGCTCTGCAC CCCCTACCAC CACCAGGGCC  GGCGGCGGCG GCTGCCCCGA GGGACGCGGC CCTAGCCGGT +249
SS
GGCGATGGGG ACCGCCCGGA TCGCACCCGG CCTGGCGCTC  CTGCTCTGCT GCCCCGTGCT CAGCTCCGTA CGCGCTGGTG +329
AGTCCCCCGC
```

Consistent with results obtained in the mouse (McCuaig K. et al 95), P1 expression was detected in kidney (Fig. 4, lane 14), but not in any of the other tissues or cell lines studied, even after extended amplification. P2-specific transcripts were detected in all human tissues and cell lines expressing PTHR coding sequences, consistent with P2 activity in the mouse (McCuaig K. et al 95), although cDNA sequences amplified from HOS cell extracts were shorter than the predominant products amplified from other tissues and cell lines (Fig. 4, lane 6; and see below). Strikingly, P3-specific transcripts were detected in all PTHR positive tissues and cell lines except MCF-7, HK2, and U2OS (Fig. 4D, lanes 2, 4 and 8), demonstrating that P3 expression is not restricted to kidney. RT-PCR analysis was also performed on RNA from adult mouse tissues and cell lines using P3-specific primers complementary to mouse sequences and in all cases the results were negative (data not shown). Thus, although P3-specific transcripts are widely expressed in humans, we find no evidence for P3 activity in adult mice. Given that the major renal promoter in humans, P3, is widely active, and that predominant renal promoter in mouse, P1, is highly selective for kidney, our results indicate that PTHR gene expression is coordinated differently in the two organisms.

4. Expression of P2, but not P1 or P3, in fetal tissues.

Analysis of PTHR gene expression was extended to RNA derived from tissues of 19 week fetal age (FA) human fetus (Fig. 5). PTHR coding sequences were detected by RT-PCR in RNA from liver, long bone, calvaria, muscle and kidney (Fig. 5, lanes 1-10). No P1-specific expression was detected after extended amplification in any of the fetal tissues studied, including kidney, under conditions where P1-specific RNA was readily detected in an adult kidney sample (Fig. 5, lane 12). Consistent with its broad range of expression in adult tissues and cell lines, P2-specific transcripts were detected in all of the tissues tested. Strikingly, however, we did not detect any P3-specific transcripts in kidney or any of the other tissues, under conditions where an amplified product was detected

Fig. 3.4. Tissue-specific expression of PTHR transcripts in adult human tissues and cell lines. Promoter-specific expression was analyzed by RT-PCR using PCR primer combinations diagrammed in Fig. 1A. P1-specific sequence were amplified using primers 1 and 6, followed by amplification with primers 2 and 3. P2- and P3-specific sequences were amplified using primers 4 and 6, and 5 and 6, respectively. Coding region sequences (Cod.) were amplified using forward and reverse primers derived from sequences in exons T3/4a and C1, respectively, and detected using a probe derived from sequences in exon T6/7a (13). PCR conditions for coding region, P2, P3-specific and β-actin sequences were chosen so as to be in the linear range for amplification of reverse transcription products of kidney RNA (data not shown), whereas reactions performed with P1-specific primers were amplified beyond the linear range (see Materials and Methods for details of PCR conditions). Amplifications were performed on samples of total RNA from the breast carcinoma cell line MCF-7 (MCF), the renal epithelial cell line HK-2, osteoblast-like osteosarcoma lines HOS, U2OS, and SaOS-2 (SaOS), human skin fibroblasts (Fib.), and human kidney (Kid.), spleen (Spl.) and bowel (bow.) treated with reverse transcriptase (+), or untreated controls (-). The sizes of the amplified products are given to the right of each panel.


MCF HK-2 HOS U2OS SaOS Fib. Kid. Spl. Bow.
Cod.
-550
P1
-148
P2
-187
P3
-183
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Actin
-342
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

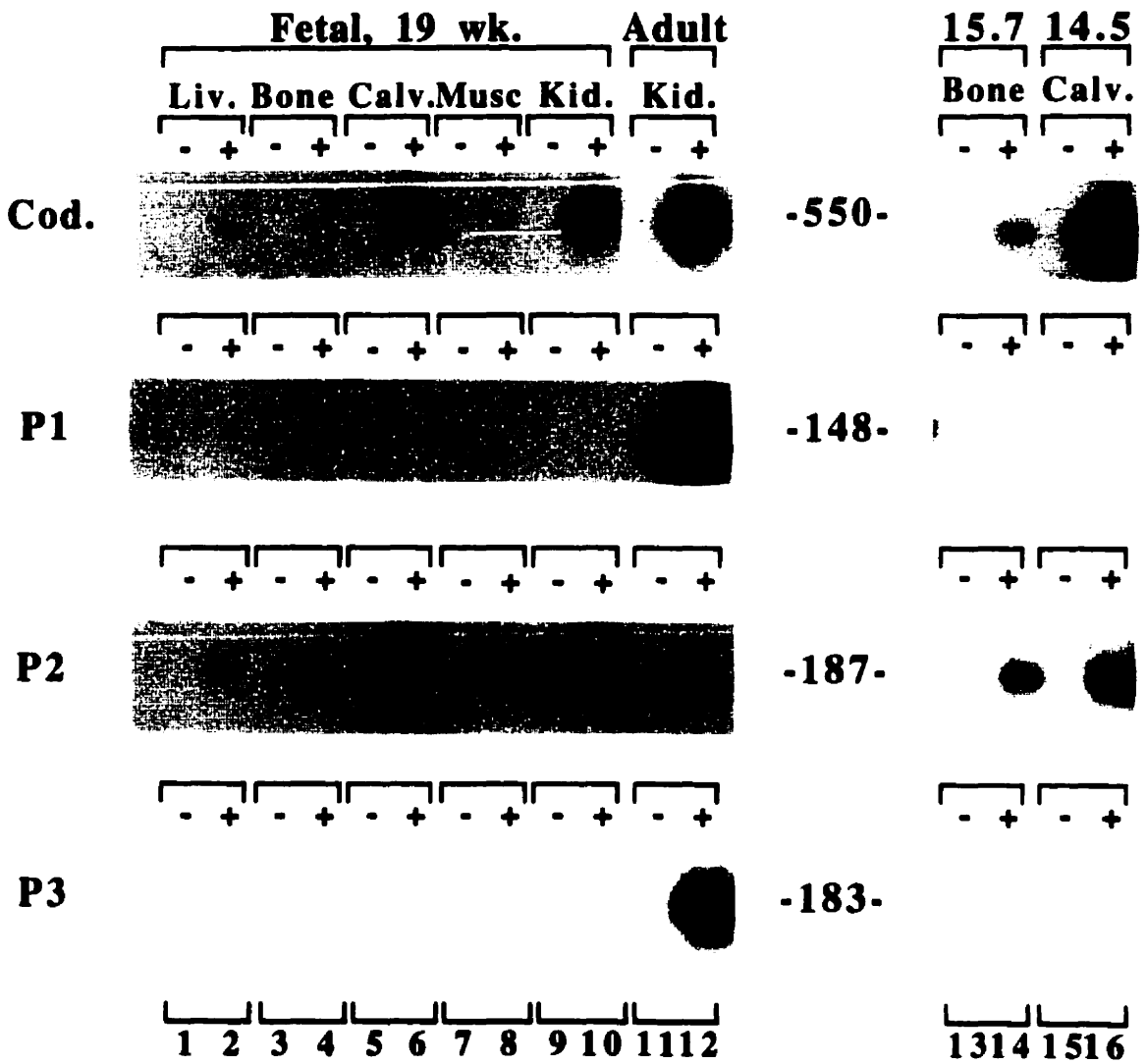
with adult kidney RNA (Fig. 5, lane 12). Essentially identical results to those presented in Figure 5 were obtained with tissues from a 17.5 week FA fetus (data not shown). Further analysis of PTHR expression in long bone at 15.7 weeks FA and calvaria at 14.5 weeks FA (Fig. 5, lanes 14 and 16) showed that only P2-specific PTHR transcripts were present. Thus, although both P2 and P3 are (G+C)-rich and lie within the same CpG island, they are clearly differentially regulated during development. Taken together, these results indicate that P2 is the major PTHR promoter active at mid-gestation, and, moreover, that the onset of expression of P1 and P3 in kidney occurs after 19 weeks of fetal age.

5. Differential splicing of P2-specific transcripts in adult, but not fetal tissues.

The nature of the shorter P2-specific RT-PCR product amplified from HOS cells (Fig. 4) was further invested by subcloning and sequence analysis. The shorter cDNA fragment arose from differential splicing between the U3 and SS exon using donor and acceptor sites distinct from those previously identified (Fig. 6). The sequence AGGCGG is present at both the donor and acceptor sites, preventing the unambiguous determination of the splice junction (Fig. 6, in bold). There is an AG dinucleotide in the splice acceptor region, however, no GT dinucleotide is present in the splice donor, suggesting that a nonconsensus sequence is used. It is noteworthy the this splicing event eliminates an upstream open reading frame present in U3/SS that may inhibit translation of the PTHR coding sequence.

The cell and tissue specificity of the alternative splice was analyzed by probing the P2-specific RT-PCR products examined in Fig. 4 with an oligonucleotide probe that encompasses the alternative splice junction (Fig. 6A), and detects both long and short cDNA fragments under the hybridization conditions used. The blot revealed the existence of truncated products amplified as expected from HOS cells (Fig. 6B, lane 6), and also from skin fibroblasts, kidney and spleen (Fig. 6B, lanes 12, 14 and 16), but not from MCF-7, HK-2, U2OS, SaOS-2, and bowel even after extended exposure (Fig. 6B, lanes 2,

Fig. 3.5. Expression of P2, but not P1 or P3 during the mid-gestational stage of fetal development. Expression of coding and promoter-specific PTHR sequences was analyzed by RT-PCR using the conditions described in Fig. 4, except that all products were amplified beyond the linear range (see Materials and Methods for details). Total RNA was from kidney (Kid.), liver (Liv.), muscle (Musc), calvaria (Calv) and long bone (Bone) of a 19 week FA fetus, as well as long bone and calvaria from samples of 15.7 and 14.5 week FA fetuses; long bone and muscle samples were taken from femur and thigh, respectively. An adult kidney RNA sample was used as a positive control. RNA samples treated with reverse transcriptase (+), or untreated controls (-) are shown.


Fetal, 19 wk.
Adult
15.7
14.5
Liv.
Bone
Calv.
Musc
Kid.
Kid.
Bone
Calv.
Cod.
P1
P2
P3
-550-
-148-
-187-
-183-
1 2 3 4 5 6 7 8 9 10 11 12
13 14 15 16

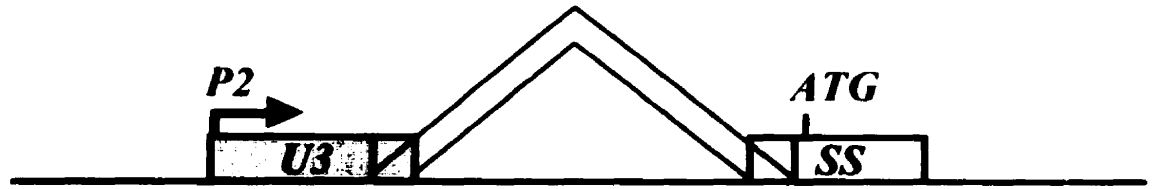
4, 8 and 18; and data not shown). No evidence for alternative splicing was observed in an RT-PCR analysis of 18 or 19 week FA tissue samples including kidney, bone and calvaria (Fig. 6C), and similar results were obtained with bone and calvarial samples from earlier fetal stages (data not shown). The observation that the alternative splice occurs in adult but not fetal kidney indicates that the event is developmentally regulated.

6. Inhibition of PTHR promoter activity by methylation.

Numerous studies have shown that expression of genes can be regulated by methylation of CpG dinucleotides in promoter regions (Antequera F. and Bird A. 93). Given that the activities of P1 and P3 are developmentally regulated, and that P2 and P3 lie within a CpG island, we were interested to determine if the activity of the three PTHR promoters could be controlled by methylation. The effect of methylation in vitro on the activity of P1, P2 and P3 was tested by transient transfection of promoter-luciferase recombinants (Fig. 7A) into COS-7 cells, as preliminary results had shown that all three promoters were active under these conditions (Fig. 7B). Luciferase genes controlled by the Herpes Simplex Virus tk promoter, and the SV40 early promoter were used as positive and negative controls, respectively (Muizinieks I. And Doerfler W. 94). Plasmids were treated with increasing concentrations of either SssI or HpaII methylase prior to transient transfection. The activities of all three PTHR promoters were inhibited by methylation to similar degrees, and were approximately as sensitive as the thymidine kinase promoter positive control (Fig. 7B). Under the same conditions, activity of the SV40 early promoter was not significantly affected by methylation with either methylase (Fig. 7B), indicating that the effects observed with the PTHR and tk promoters were specific to these particular promoter sequences.

Fig. 3.6. Differential splicing between exons U3 and SS gives rise to shortened P2-specific transcripts. **(A)** Schematic representation of the splicing patterns between U3 and SS. The sequences of the 3' end of U3 and all of SS are presented below. The previously characterized splice donor site at the 3' end of U3 and acceptor and donor sites at the extremities of SS are in italics. The ATG start and TAG stop codons corresponding to the extremities of the upstream open reading frame lying upstream of the PTHR coding sequence are boxed and the open reading frame is underlined. The ATG (in italics) of the PTHR coding sequence is also boxed. The putative splice donor and acceptor sites (arrowheads) were chosen based on the use of the consensus splice acceptor sequence. Note that splice donor an acceptor sites cannot be determined unambiguously because of the sequence AGGCGG (in bold) present in both exons. The sequence of the oligonucleotide probe used for Southern analysis that corresponds to the fusion between U3 and SS cause by the differential splice is presented at the bottom. **(B)** Southern analysis of the blot of P2-specific RT-PCR products presented in Fig. 4 probed with the oligonucleotide presented in A. Products corresponding to previously characterized splice sites (187 bp) and the differentially spliced product (129 bp) are indicated. **(C)** Southern analysis of P2-specific RT-PCR products amplified from fetal RNA probed with the oligonucleotide presented in A. Muscle (musc.), calvaria (calv.), bone and kidney (kid.) were from 18 week FA tissue, whereas liver (liv.) was from a 19 week FA sample.

A.



U3

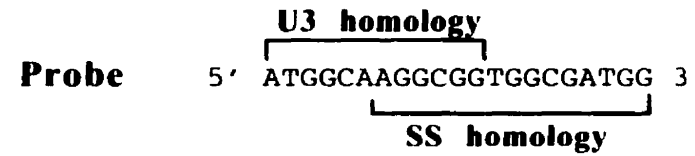
....GGGTATCCCGAGAGCTCCATGAAGTCCCCCCGGGGCCGCGGACGGGGCGCTGGCTTGGGGAGGCTGTCGGG

GGGGCCCGACATCCATGGCAAGGCGGGGGCGGGCGCTCGGA *GTAA*....

SS

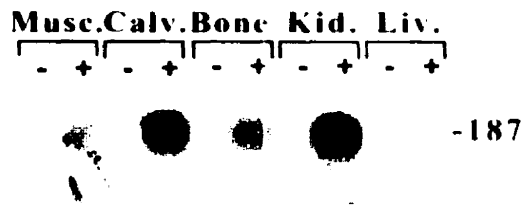
....*CCACCAG* GGCCGGCGGCGGCGGCTGCCCCGAGGGACGCGGCCCTAGGCGGTGGCGATGGGACCGCCCG

GATCGCACCCGGCCTGGCGCTCCTGCTCTGCTGCCCCGTGCTCAGCTCCGTACGCGCTG GT*GAGT*....



B.

C.



7. Methylation status of promoters P1 and P3 in vivo.

The results presented above indicate that all three PTHR promoters are susceptible to regulation by methylation in vitro. Given that P1 and P3 are inactive in human fetal kidney (Fig. 5), it was of interest to analyze the extent of methylation genomic DNA in fetal and adult kidney samples to determine if there is a correlation between methylation and promoter activity in vivo. Genomic DNA derived from adult peripheral lymphocytes, adult kidney, and 11.75 and 18 week FA kidney was digested with combinations of either ApaI and MspI or HpaII, or KpnI and TaiI. In control experiments, pretreatment of plasmid DNA with SssI methylase completely blocked digestion with TaiI (recognition sequence ACGT) under conditions where the activity of MspI was unaffected (data not shown), showing that TaiI can be used in the analysis of the methylation status of non (G+C)-rich promoter regions. Southern analysis of the P1 region of the PTHR gene was performed using with a 1.2 kb ScaI-PstI fragment that encompasses the P1 transcription start-sites (Fig. 8A). Comparison of ApaI, ApaI-MspI and ApaI-HpaII digests of genomic DNA derived from peripheral lymphocytes showed that both upstream and downstream HpaII sites are highly methylated (Fig. 8B, lanes 1-3). The same series of digests of genomic DNA from adult kidney revealed that the HpaII sites are 30-50% demethylated (Fig. 8B, lanes 6-10). Essentially identical results were obtained with preparations of genomic DNA from independent lymphocyte and kidney samples (data not shown). The pattern of demethylation of TaiI sites is more complex. The downstream TaiI site is demethylated to similar degrees in lymphocyte and kidney DNA (Fig. 8B lanes 4,5,9 and 10; 255 bp band). The upstream TaiI site is cleaved <10% in lymphocyte genomic DNA, whereas ~60% cleavage is observed in adult kidney DNA (Fig. 8B, lanes 9 and 10; compare 596 and 457 bp bands). These results indicate that in adults partial demethylation of the P1 promoter correlates with its activity in kidney. Interestingly, analysis of genomic DNA from both 11.75 and 18 week FA kidney samples revealed partial demethylation of HpaII and TaiI sites in the P1 promoter region (Fig.

Fig. 3.7. Effect of methylation in vitro on activity of PTHR promoters. **(A)** PTHR-luciferase reporter recombinants. A schematic representation of promoter-reporter cassettes containing sequences from PTHR promoters P1, P2, and P3, and truncated Herpes Simplex Virus thymidine kinase (TK), and SV40 early (SV) promoters is shown. **(B)** Activities of P1, P2 and P3 promoter-luciferase recombinants and promoterless luciferase vector pXP2 in transiently transfected COS-7 cells. Luciferase activities are presented as fold inductions relative to pXP2. **(C)** Effect of treatment in vitro with increasing concentrations of HpaII (left hand panel) and SssI methylases (right hand panel) on luciferase expression of transiently transfected promoter-reporter vectors. See Materials and Methods for details of methylase treatments.

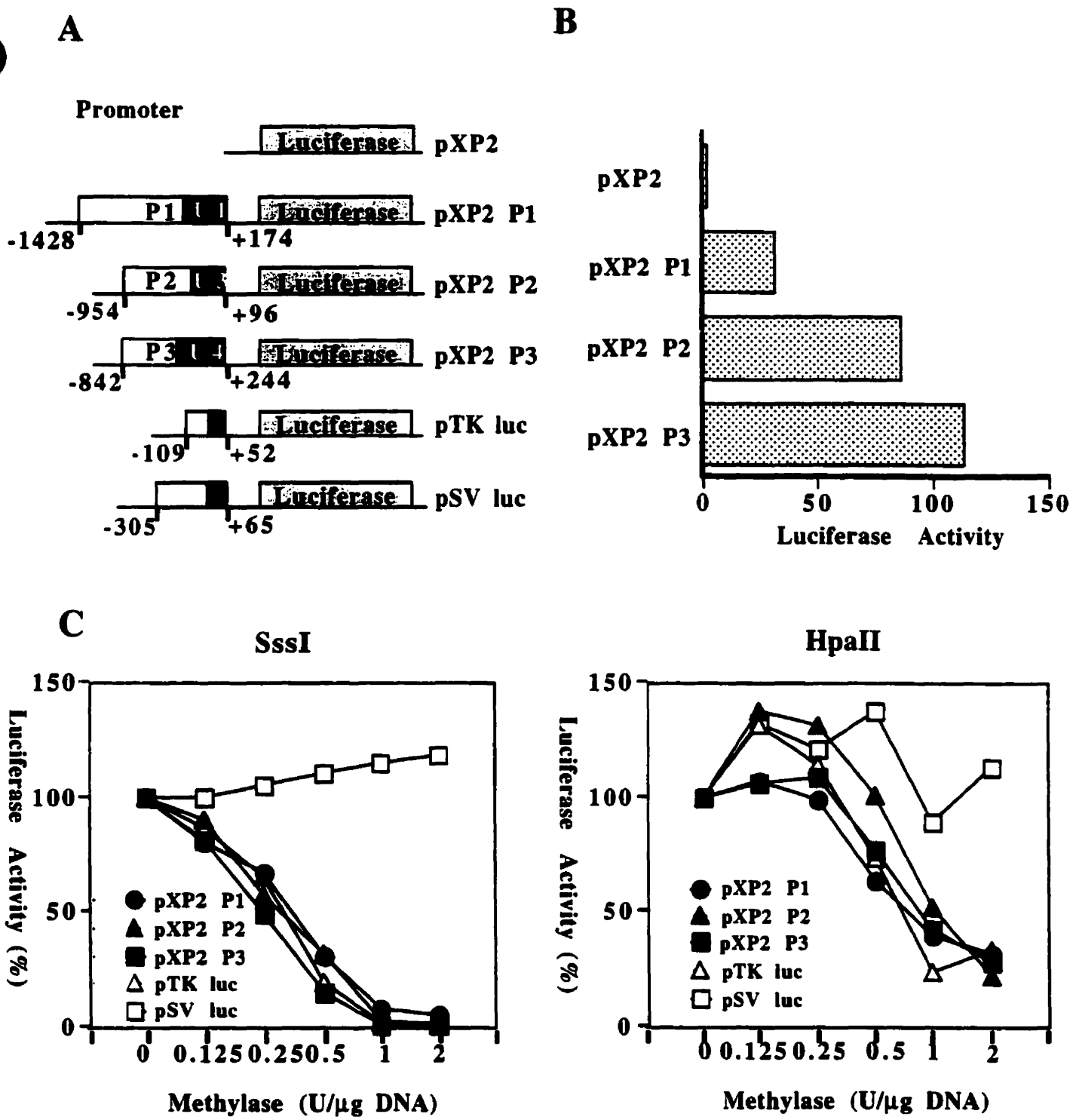
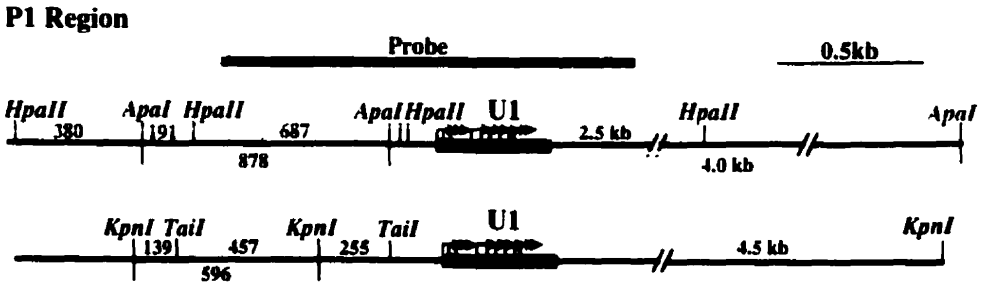
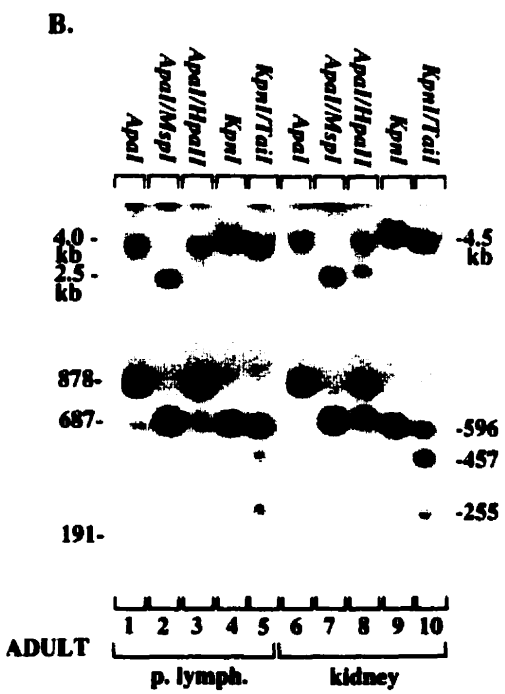

A
Promoter
Luciferase
pXP2
P1
pXP2 P1
-1428
+174
P2
pXP2 P2
-954
+96
P3
pXP2 P3
-842
+244
pTK luc
-109
+52
pSV luc
-305
+65
B
pXP2
pXP2 P1
pXP2 P2
pXP2 P3
0
50
100
150
Luciferase Activity
C
SssI
HpaII
Luciferase Activity (%)
150
100
50
0
pXP2 P1
pXP2 P2
pXP2 P3
pTK luc
pSV luc
0 0.125 0.25 0.5 1 2
Methylase (U/μg DNA)

Fig. 3.8. Analysis of the methylation status of P1 promoter region by genomic Southern blotting. **(A)** Restriction maps of region of the human P1 promoter detected by Southern analysis. **(B)** Genomic Southern analysis of the P1 region of the PTHR gene using DNA isolated from adult lymphocytes (lanes 1-5), and adult kidney (lanes 5-10). ApaI, ApaI-MspI, and ApaI-HpaII digests (lanes 1-3, and 6-8), and KpnI and KpnI-TaiI digests (lanes 4, 5, 9, 10) of genomic DNA are shown. **(C)** Genomic Southern analysis similar to that performed in B on genomic DNA from 11.75 and 18 week FA human kidney samples as indicated.

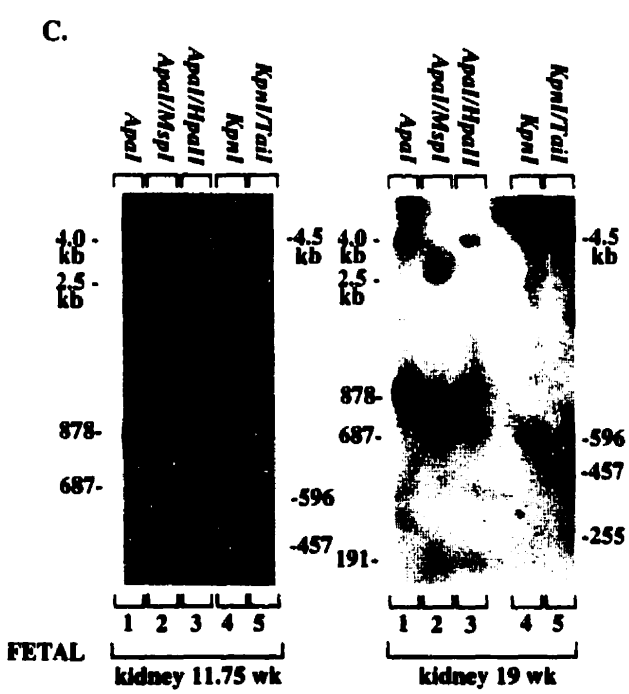
A.


P1 Region
Probe
0.5kb
HpaII
ApaI
HpaII
ApaI
HpaII
U1
HpaII
ApaI
380
191
687
878
2.5 kb
4.0 kb
KpnI
TaiI
KpnI
TaiI
U1
KpnI
139
457
255
596
4.5 kb

B.


ApaI
ApaI/MspI
ApaI/HpaII
KpnI
KpnI/TaiI
4.0 kb
2.5 kb
878
687
191
-4.5 kb
-596
-457
-255
1 2 3 4 5 6 7 8 9 10
ADULT
p. lymph.
kidney

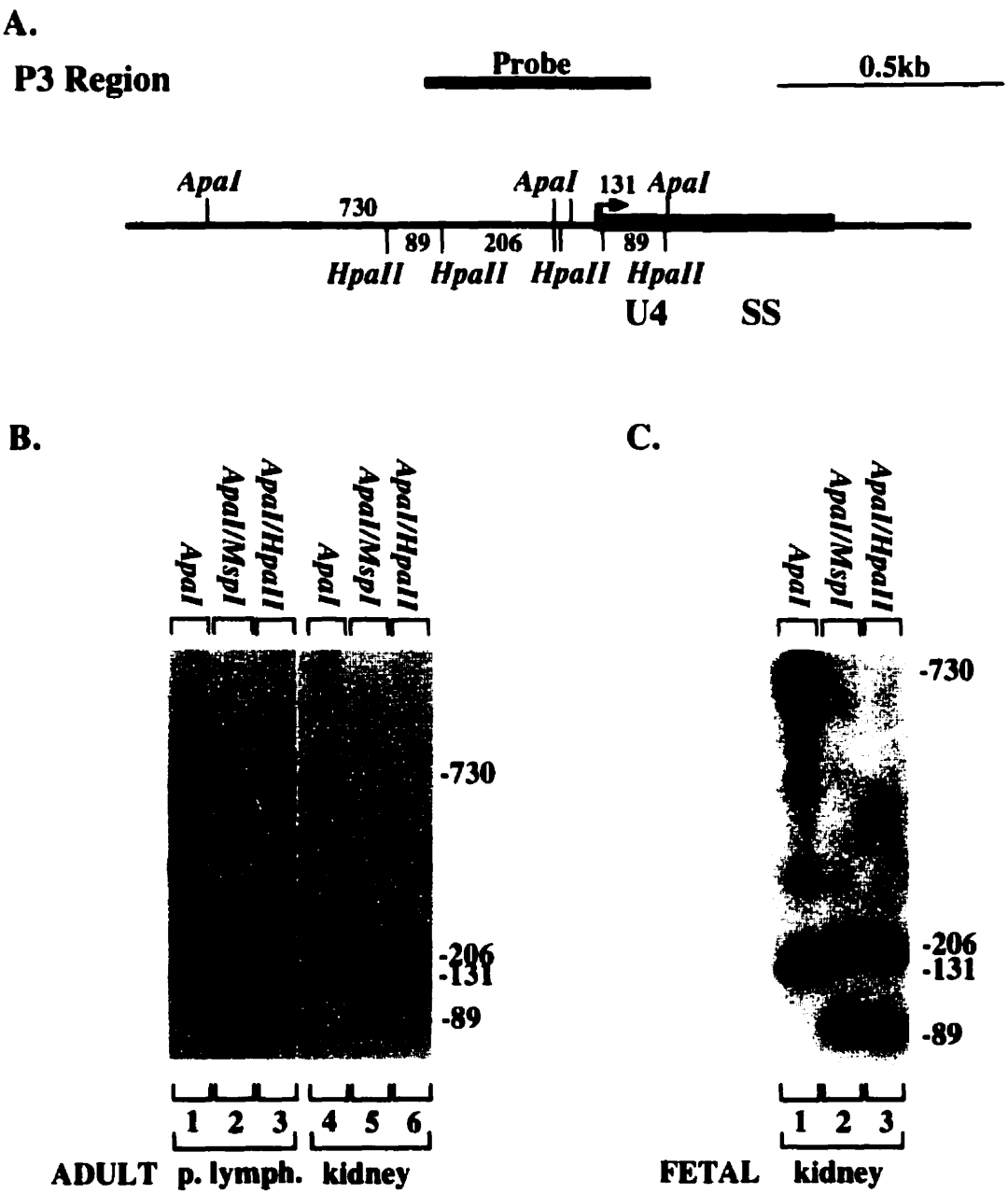
C.


ApaI
ApaI/MspI
ApaI/HpaII
KpnI
KpnI/TaiI
4.0 kb
2.5 kb
878
687
191
-4.5 kb
-596
-457
-255
1 2 3 4 5
FETAL
kidney 11.75 wk
1 2 3
kidney 19 wk

8C). Similar results were obtained with a 13 week FA kidney sample (data not shown). The only exception was the downstream HpaII site (generating a 2.5 kb band), which was only ~10% demethylated (Fig. 8C). Whether this reflects the lack of expression of the P1 promoter in fetal kidney, or is due to the fact that the fetal DNA was purified from whole kidney whereas the adult sample was taken from a section of cortex/medulla is not clear. In any case, the results indicate that partial demethylation of proximal P1 promoter sequences precedes the onset of P1 function by several weeks, providing a clear example of a non-CpG island tissue-specific promoter whose demethylation occurs long before induction of promoter activity.

A Southern blot of of genomic DNA from peripheral lymphocytes and adult kidney was probed with a 492 bp SmaI fragment encompassing the P3 transcription start sites (Fig. 9A). There are several MspI/HpaII sites in the promoter proximal region of P3, and autoradiography revealed that both the 730bp and 131bp ApaI fragments were cleaved to similar degrees with MspI and HpaII to generate detectable fragments of 209 and 89 bp (Fig. 9B). This indicates that the corresponding HpaII sites are unmethylated in both adult kidney and peripheral lymphocytes. Similar results were obtained with DNA isolated from 19 week FA kidney (Fig. 9C). The same Southern blot of fetal kidney DNA probed with a longer NcoI-AvrII fragment (-842 to +241) revealed that HpaII sites lying further upstream are also unmethylated (data not shown). Thus, the proximal P3 promoter region is unmethylated in kidney at mid-gestation, and in adult kidney and peripheral lymphocytes. These data show that demethylation of the P3 promoter in kidney occurs prior to the onset of its function after week 19 of fetal development. Moreover, the results of Figs. 8 and 9 indicate that induction of the activities of P1 and P3 requires factors that are expressed late in development.

Fig. 3.9. Analysis of the methylation status of P3 promoter region by genomic Southern blotting. **(A)** Restriction maps of the human P3 promoter region detected by Southern analysis. See Materials and Methods for details of probe constructions. **(B)** Genomic Southern analysis of ApaI, ApaI-MspI, and ApaI-HpaII digests of the P3 region of the PTHR gene using DNA isolated from peripheral lymphocytes (lanes 1-3) and adult human kidney (lanes 4-6). Fragments smaller than 89 bp ran off the bottom of the gel. **(C)** Genomic Southern analysis as in B of the P3 region of the PTHR gene using DNA isolated from an 18 week FA human kidney sample.


A.
P3 Region
Probe
0.5kb
ApaI
730
ApaI
131
ApaI
89
206
89
HpaII
HpaII
HpaII
HpaII
U4
SS
B.
ApaI
ApaI/MspI
ApaI/HpaII
ApaI
ApaI/MspI
ApaI/HpaII
-730
-206
-131
-89
1 2 3
4 5 6
ADULT p. lymph. kidney
C.
ApaI
ApaI/MspI
ApaI/HpaII
-730
-206
-131
-89
1 2 3
FETAL kidney

D) DISCUSSION.

1. Regulation of PTHR gene expression is coordinated differently in humans and in mouse. The results presented above show that the majority of PTHR gene transcription in human adult kidney is driven by the P3 promoter. P3-specific transcripts are also expressed in a number of other tissues and cell lines, suggesting that common regulatory signals control PTHR gene expression in renal and non renal tissues. Our data indicate that although human P1 is active specifically in adult kidney, it accounts for only a small proportion of renal transcripts (Fig 1; Bettoun JD. et al 97). In contrast, P1 activity in the adult mouse accounts for at least 90% of PTHR transcripts in kidney (Amizuka N. et al 97, McCuaig K. et al 95). Thus, renal PTHR gene transcription in the mouse is largely controlled by kidney-specific regulatory sequences of P1, whereas P2 drives PTHR gene transcription in most other tissues.

Recent immunohistochemical and *in situ* hybridization analyses of PTHR mRNA and protein expression in murine kidney have provided evidence for a cell-specific distribution of receptor protein and P1- and P2-specific mRNAs (Amizuka N. et al 97). These studies showed that the highest densities of PTHR protein and transcripts were found in peritubular endothelial cells and in vascular smooth muscle, and that PTHR mRNA expression in these cells was controlled exclusively by P1. Activity of P1 was also detected at moderate levels in tubular epithelial cells and in glomerular podocytes. P2 activity was more restricted, with moderate levels of transcripts being detected only in tubular epithelial cells (Amizuka N. et al 97). In this regard it is noteworthy that human P2-specific transcripts, but not those of P1 or P3, were detected in the renal epithelial cell line HK2 (Fig. 4). The relatively low levels of P1 activity detected in human kidney by RNase protection suggest that either P1 is widely active but at low levels, or that the expression pattern of P1-specific transcripts is much more restricted than that of mouse kidney. The observation that P1 sequences are ~50% demethylated in kidney would argue

for a relatively broad expression pattern in kidney if the promoter is demethylated only in cells in which it is active. It should be noted that human kidney samples used in this study, which are derived from cortex and medulla, may not be fully representative of the renal cell types in which the PTHR is expressed, and it cannot be excluded that P1 activity may be somewhat higher in specific cell types. However, taken together, our data suggest that the distribution and expression levels of the PTHR in kidney may well differ between mouse and human. It will therefore be important to determine the cellular distribution of PTHR transcripts and protein in human kidney.

Human and mouse P2 sequences are over 90% conserved (Bettoun JD. et al 97). Moreover, analyses of the tissue distribution of human (Fig. 4) and mouse P2-specific transcripts (McCuaig K. et al 95) suggest that they share the same broad patterns of activity. P2 is active in the mouse osteoblast-like line MC3T3, in secondary passage mouse osteoblasts, and in mouse bone and cartilage (McCuaig K. et al 95; and data not shown). P2 activity was also detected in a number of human osteoblast-like cell lines, and in human fetal bone and calvaria. However, PTHR gene expression in human adult bone is likely to be more complex than in the mouse as activity of P3 was detected in both HOS and SaOS-2 osteoblast-like lines.

2. P2 is the major human PTHR promoter during the mid-gestational stage of fetal development.

Although P3-specific transcripts were readily detectable by RT-PCR in adult human tissues and several cell lines, no P3-directed expression was observed in tissues from human fetuses ranging from 14.5 to 19 weeks FA, even after extensive amplification by PCR. Similarly, no evidence was found for activity of P1 in 17.5 or 19 week FA kidney (Fig. 5 and data not shown). In contrast, P2-specific transcripts were detected in several fetal tissues, including kidney, long bone and calvaria (Fig. 5, and data not shown). This indicates that PTHR gene expression in kidney is controlled exclusively by the highly

conserved P2 promoter at this stage, and that the onset of P1 and P3 expression occurs later in kidney development.

Onset of P1 and/or P3 activity could occur in preparation for, or in response to, the dramatic changes in kidney function that occur at parturition, when the fetus leaves an environment in which calcium homeostasis is controlled largely by the placenta. At this stage, it would be expected that PTHR expression would be upregulated to accommodate the increased function of the kidneys. It is not clear at this time whether expression of the PTHR is important for kidney development or maturation of kidney function ex utero. In gene ablation experiments, loss of receptor function did not result in any gross abnormalities in renal development in mice that survived to 18.5 days pc (Karaplis A. et al 94, Lanske B. et al 96). However, in rodents, kidney development continues after birth (Wintour EM. 97), and it has not been determined whether PTHR null mice show impaired nephron development.

While a role of the PTHR in kidney morphogenesis is uncertain, PTHR gene expression is clearly essential for normal skeletal development. PTHR and PTHrP null mice display similar defects in endochondral ossification, with reduced proliferation and accelerated ossification of chondrocytes (Karaplis A. et al 94, Lanske B. et al 96). Several results suggest that transcription of the PTHR gene during endochondral ossification is controlled by signals regulating P2 activity. RNase protection and *in situ* hybridization experiments in cell lines and tissue sections have shown that P2 is the major promoter controlling PTHR expression in both chondrocyte and osteoblast lineages in mice after birth (McCuaig K. et al 95; and data not shown). Results presented here indicate that P2 is the major PTHR gene promoter in long bone and calvaria from 14.5 to 19 weeks FA in human fetuses (Fig. 5). In humans, ossification is initiated at approximately 7 weeks FA in the femur, at 9 to 12 weeks FA in calvarial bone, and continues throughout development (Crelin ES. 81). Taken together, these results suggest that, in spite of the

differential regulation of PTHR gene expression in adult tissues in human and in mouse, the signals controlling PTHR gene expression during skeletal development are conserved.

3. Differential splicing of P2-specific transcripts.

We also found that P2-specific transcripts are differentially spliced between U3 and SS using donor and acceptor sites distinct from those previously characterized, giving rise to a shortened 5' UTR. RT-PCR analysis showed that both long and short UTRs are formed in kidney, spleen and skin fibroblasts, whereas only the short UTR was detected in HOS cells (Fig. 6). No evidence for alternative splicing was found in fetal tissues. The splice acceptor region contains the sequence UAGG, which corresponds to the consensus YAGG for AG acceptor sites. However, the corresponding splice donor sequence, GCGGGG, diverges considerably from the consensus GURAGU (Keivi J-P. and Lamond A.I. 96, Tarn W-Y. and Steitz JA. 97). While these differential splicing events may represent minor portions of P2-specific mRNAs in kidney, spleen and skin fibroblasts, they may be more efficiently translated because their shorter UTR lacks an upstream open reading frame present in full length transcripts (Fig. 6).

4. Differential control of PTHR promoter activities by methylation.

Sequence analysis has shown that promoters P2 and P3 are within a contiguous CpG island of at least 1.5 kb that extends from upstream of U3 to downstream of SS (Fig. 3). In contrast, the P1 promoter is not (G+C)-rich and contains relatively few CpG dinucleotides. Methylation in vitro by SssI or HpaII methylase of luciferase reporter plasmids containing P1, P2 or P3 regulatory sequences led to a dramatic inhibition of expression of transiently transfected reporter genes (Fig. 7), suggesting that all three PTHR promoters may be susceptible to regulation by methylation in vivo. Examination of genomic DNA by methylation-sensitive restriction digestion suggested that the P3 region of the gene is demethylated in 18 week FA and adult kidney (Fig. 9). This result is

not unexpected given that CpG island promoters are extensively demethylated during embryonic development (Antequera F. and Bird A.93, Monk M. 95). As P3 is not active at this stage of development, the data indicate that the onset of P3 activity requires the function of trans-acting factors expressed at a later stage of development.

Analysis of the methylation status of HpaII and TaiI sites in P1 in adult kidney and peripheral lymphocytes provided a correlation between P1 activity and partial demethylation of the promoter (Fig. 8). Moreover, this partial demethylation is established early in development, as a similar pattern of demethylation was seen in genomic DNA from fetal kidney samples of 11.75, 13 and 19 weeks FA (Fig. 9, and data not shown). This provides a clear example of a non CpG island promoter whose tissue-specific demethylation occurs several weeks before the induction of its activity. Similar to P3, the results also indicate that P1 function requires factors expressed late in development.

In summary, we have shown that expression of the PTHR gene in humans and in mice is regulated differently in the adult. The P3 promoter, which is apparently specific to humans, drives the majority of PTHR gene transcripts in kidney, and is active in a number of other tissues. P2 promoter activity is apparently well conserved between humans and mice, whereas P1 activity is much weaker in humans than in mice. The P2 promoter drives PTHR gene expression at mid-gestation, indicating that factors controlling P2 regulate PTHR gene expression during skeletal development. We also show that differential splicing occurs between U3 and SS in P2-specific transcripts in adult but not fetal tissues, giving rise to a truncated 5'UTR that lacks an upstream open reading frame. Our data also show that demethylation of the tissue-specific P1 promoter occurs several weeks before the onset of its function, and provide evidence that induction of P1 and P3 activities requires factors expressed late in development.

CHAPTER 4:

GENERAL DISCUSSION.

- SUMMARY OF EXPERIMENTAL RESULTS.
- TRANSCRIPTION OF THE PTHR GENE IS DRIVEN BY MULTIPLE PROMOTERS.
- PHP 1B.
- DIFFERENTIAL SPLICING OF P2-DERIVED 5' UTR.
- PTHR MRNAS CAN HAVE MULTIPLE 5' UTRS.
- CONCLUSION AND PROSPECTS.

A) Summary of the experimental results.

The data presented in chapters two and three describe the characterization of the upstream regulatory region of the human PTHR gene. This region has a similar organization in human and mice. Hence, segments highly homologous to the three mouse upstream-untranslated exons were found in the human gene and the distances between them were mainly the same. RNase protection assays performed on human total RNA demonstrated the presence of transcripts bearing UI or U3 sequences in the kidney. These experiments, and primer extension analysis, established that these transcripts originated from the human equivalent of the mouse P1 and P2 promoters. Start site selection was also similar. We showed that in human, like in mouse, P1 utilizes multiple start-sites extended over 100bp whereas a single start site was detected for P2.

Despite the architectural similarities between the mouse and human upstream regulatory regions, differences were noted:

- The precise location of the start-sites was not the same in the two species.
- The activity of the P1 promoter, relative to that of P2, was much weaker in human than in mouse.
-The distance between exon U1 and U2 in human is double that in mouse.

The weak activity of P1 in humans was surprising since expression from this promoter, in mouse kidney accounts for the vast majority of the PTHR gene transcription (McCuaig K. et al 95, Amizuka N.et al 97). Given the importance of the receptor expression in this organ, we performed control experiments to check whether the whole of the expression of the PTHR gene in kidney could be accounted for by P1 and P2-derived transcripts. These experiments led us to characterize a third promoter, P3, located 200 bp upstream of the signal sequence exon. This promoter is specific to the human gene and drives the majority of the PTHR gene expression in kidney.

Primer extension analysis detected two start sites distant of 7bp from each other. The sequencing of P3 revealed a high (G+C) content, and the presence of an hitherto unrecognized CpG island, that originated immediately upstream of U3, and extended to the end of the signal-sequence exon (fig. 3.3).

RT-PCR analysis of total RNA samples obtained from human adult and fetal tissues or from different human cell lines revealed in the adult that:

- P1 expression is restricted to the kidney.
- P2 and P3 are widely active.
- The human osteosarcoma cell line HOS expressed a transcript that is apparently shorter than that of other tissues or cell lines.

The same analysis performed on fetal sample showed that:

- P2 is the only active promoter.
- The so-called "HOS-specific" splice variant can not be detected.

The developmental up-regulation of P1 and P3, and the presence of a CpG island encompassing P2 and P3 suggested that DNA methylation could play a role in regulating PTHR gene transcription. Unexpectedly, in-vitro methylation experiments showed that all three promoters were sensitive to DNA methylation. Analysis of the methylation status of genomic DNA derived from different tissues revealed that P1 was partially and specifically methylated in kidney, and that this methylation pattern was established early during embryonic development.

Thus, the upstream regulatory region of the human PTHR gene can direct the expression of a least four types of transcripts, which differ in their 5' UTR, in a tissue and developmental specific manner.

B) Transcription of the PTHR gene is driven by multiple promoters.

The use of multiple promoters to drive transcription has been documented for a number of genes (Ayoubi TAY. and WJM. Van De Ven 96). The presence of several promoters controlling gene expression allows for a greater diversity of regulatory mechanisms. Many physiological scenarios, requiring differential promoter usage, can be envisaged and have been described, from cell and developmental specific control of gene expression, to drug or pathological control of promoter activity. Since core promoter regions are considered important for the precise positioning of the pre-initiation complex (see chapter 1 section III, Roeder RG. 96 and Smale ST. 97), it is noteworthy that P1, P2, and P3 display different characteristics in terms of start site selection. In light of the current view that links transcriptional control of gene expression to PIC formation, it is likely that the architectural differences in PTHR gene promoters play a major role in the regulation of their respective activities.

1- P1 is a developmentally regulated and tissue specific promoter.

Very little information is available concerning PIC assembly at multiple start-sites promoters. A study aiming at identifying common structural elements in promoters using multiple start sites proposed that a consensus sequence called MED-1 (for multiple start sites element downstream 1), typically located 25 to 30 bp downstream from the most 3' initiation site and about 100bp of the most 5', could direct initiation in this type of promoters (Ince TA. and KW. Scotto 95). Neither TATA nor Inr or MED-1 sequences could be found in P1 in mice or human, leaving unanswered the question of how the transcriptional machinery can recognize transcription start sites in P1.

Our finding that P1 activity is sensitive to methylation in-vitro and that its methylation status correlates with its tissue specificity, shows this promoter to be under the control of methylation sensitive transcriptional mechanisms. Our data suggest

however that DNA methylation of this region is more likely to determine its tissue specificity rather than its developmental specificity. Thus, the establishment of the methylation pattern in the adult, several weeks before the onset of activity, shows that demethylation is not sufficient to activate the promoter and that it requires additional factors that are not present or functional until after 19 weeks of fetal age.

The low abundance of P1 derived transcripts in human kidney, compared to that in mouse, is indicative of either a more restricted pattern of promoter activity in human or of an overall weaker activity. The presence of an enhancer which would be responsible for the very high level of expression from P1 in mouse kidney has been indirectly suggested by in-vitro transfection experiments performed in cells in which P1 is not endogenously active. Under these conditions, P1 failed to expressed a reporter gene placed under its control (Minegawa et al, manuscript in preparation). The existence of a distal enhancer is also supported by the report by Karperien et al that sequences located up to 4kb upstream of P1 are unable to direct high levels of expression of a reporter gene in transgenic mice (Karperien et al 97). This suggests that sequences located elsewhere in the PTHR gene can enhance P1 activity. It remain unclear whether a similar enhancer activity would also be responsible for the developmental up- regulation of P1 activity in human.

A number of transcription factors have been shown to be sequentially expressed during renal development and gene ablation experiments have demonstrated their importance in the organogenesis of the kidney.

Renal development in mammals proceeds from the differentiation of anterior intermediate mesoderm into a structure called the nephric duct. Following an elongation of the nephric duct, lateral cells differentiate into a structure called the mesonephron. These structures will serve excretory function during early embryonic development. The appearance of more sophisticated and permanent structures, called metanephric kidney, results from the induction of metanephric mesenchyme by an uro-genital tract extension,

called ureteric bud. Upon induction, mesenchymal cells aggregate, differentiate into epithelial cells, and undergo extensive morphogenic modification that leads to the formation of nephrons. Cells derived from the ureteric bud give rise to the intra-renal pelvis and calyces, to the ureter and possibly to the collecting tubules (Herzlinger D. 95, Stuart R.O. and SK. Nigam 95)

Pax2 is a member of a large family of transcription factors that are homologous with segmentation genes that act to subdivide the Drosophila embryo along the antero-posterior axis. The onset of expression of Pax-2 in the metanephric mesenchyme coincides with induction of this structure by the ureteric bud. As epithelial differentiation proceeds, Pax2 expression is repressed and is eventually restricted to the ductal epithelium. The transient nature of Pax2 expression in the early stages of the metanephric development makes unlikely a role for this factor in promoting P1 activity (Dressler G. 95).

Wilm's tumor suppressor gene (Wt1), originally characterized for its role in the appearance of this embryonic tumor, has also been shown to be expressed in a timely defined fashion during the urogenital-tract development. The level of expression of Wt1 was shown to be at a peak between 12 to 18 weeks of fetal age. In the mesonephron, Wt1 was expressed in the same structures as in the metanephros. In the metanephros, Wt1 expression was first detected at a low level in all the derivatives of the mesenchyme, and was later observed at a higher level in the lower cell layer of the anterior cleft of the S shaped bodies, which constitute the precursor of podocytes. Podocytes are the only post-natal renal cells to maintain Wt1 expression (Mundlos S. et al 93, Dressler GR 95). Wt1 can repress the transcription of a number of genes by binding to a recognition sequence that is common to Wt1 and to the transcription factor Egr1 (Drummond IA. et al 92, Kinane TB. et al 95, Harrington MA. et al 93). Incidentally, a number of sites resembling that for Egr binding (GCGGGGCG) are present between U2 and U3. Furthermore, the time course of Wt1 expression and its transcriptional repressor activity make it a good

candidate for down-regulating P1 and P3 activity during embryonic development. The description by Amizuka et al that P1 is active in mouse podocytes (Amizuka N. et al 97) does not support the idea that Wt1 could down-regulate PTHR expression during development. However, the characterization of a nuclear factor capable of modulating Wt1 activity (Johnstone RW. et al 96) suggests that the persistence of Wt1 expression in adult tissue does not preclude a role in down-regulating PTHR gene transcription during development. Furthermore, P1 activity in adult mouse podocytes does not necessarily imply that this promoter will also be active in human podocytes. It is therefore possible that the gradual down-regulation of Wt1 could contribute to the onset of P1.

Our observation that in-vitro methylation inhibits P1 activity in COS 7 cells could also be viewed as supporting the idea that P1 is actively repressed during development. As it is unlikely that P1 activity in these cells could result from the presence of a tissue-specific transcription factor, these data suggest that methylation prevents the binding of general transcription factors. Hence, we can hypothesize a model by which methylation of P1 in non-renal tissues would repress its function, by preventing the binding of general transcription factors, while, in the developing kidney, repression would be achieved by the presence of a repressor.

In both mouse and human, P1 has very few recognizable binding sites for general transcription factors. The start site region of the mouse promoter displays a glucocorticoid response element half site and a consensus sequence for the CCAAT box binding protein, but neither of these sites are present in the human gene at an equivalent position. Likewise an AP1 consensus sequence is present in the start site region of the human but not of the mouse promoter (McCuaig et al 95 and chapter 2).

The characterization of the mechanisms regulating P1 activity suffers from the lack of a suitable cell line, in which P1 would be endogenously active. The demonstration that a number of carcinoma cell lines expressing PTHrP also express the receptor (chapter 1, section II.E) hints that human renal carcinoma commonly expressing PTHrP (Weir EC.

et al, 88) might also support expression of the receptor. In such a case, the RT-PCR protocols we used, should be useful to address whether P1 is active in any of these cell lines.

2- P3 is a developmentally regulated ubiquitous promoter.

In view of our results, it is tempting to propose that P1 and P3 could be coordinately regulated. Although it is a GC rich promoter which drives transcription from a limited number of start-sites, P3, like P1, is silent during embryonic development and active in adult kidney. Functionally, P3 replaces P1, and is responsible for most of PTHR gene expression in the kidney. Finally, although P3 function is not limited to renal tissues, in as much as the specificity of P1 expression seems to be determined by tissue specific demethylation, P3, being contained in a CpG island, is compelled to be active ubiquitously.

Three different classes of promoters have been described based on CpG/GpC ratios (Edwards YH. 90). Promoters with absolute or limited tissue specificity generally have a CpG/GpC ratio comprised between 0.1 and 0.5. P1 falls into this category as it has a CpG/GpC ratio of 0.2 and is exclusively expressed in kidney. Promoters with ratios between 0.5 and 0.8 are expressed in one or a subset of tissues, or can be ubiquitously active. Above 0.8 most promoters are ubiquitously active or, conversely, are strictly tissue specific. In this latter category, the tissue and developmental specificity have been shown to be conferred by the presence of an enhancer. For example, the alpha-globin gene promoter is associated with a CpG island that is unmethylated in all tissues. The restricted expression of the gene in erythroid cells in a developmentally regulated manner depends on the activity of a tissue specific enhancer located 40kb upstream of the start site (Jarman AP. et al 91).

P2 has a CpG/GpC ratio of 1 and conforms to the characteristics of ubiquitously active promoters. It is demethylated and functions at moderate levels in all tissues tested.

On the other hand, the function of P3 differs from the previously characterized CpG island promoters. Silent during development, it is expressed at low levels in many organs and cell lines in the adult, but is highly active in the kidney. This behavior suggests a dual mechanism of activation by which, at some point after 19 weeks of fetal age, P3 becomes active in a number of tissues at basal levels, and is specifically enhanced in the adult kidney, giving rise to at least 80% of renal PTHR transcripts. The low activity of P3 in a broad range of tissue and cell lines suggests that widely expressed general transcription factors are responsible for low levels of expression, whereas high levels of activity in kidney are likely to depend on a renal specific enhancer activity.

It is possible that in vivo, the CpG island would only influence P2 expression, and that P3 would be controlled by different mechanisms. This hypothesis is unlikely for two reasons. First, P3 has all the known characteristics of a CpG island promoter(Antequera F. and A. Bird 93, Edwards YH. 90) in that it has a high (G+C) content, a high CpG to GpC ratio, and it contains several Sp1 binding sites and has been found unmethylated in expressing or non expressing tissues . Second, methylation-induced down-regulation P3 in COS7 cells is unlikely to be incidental, or due to methylation of sites outside the CpG island, since the promoter region used in the experiment was entirely contained within the CpG island and since, under the same conditions, the AdML promoter was not affected by methylation of the vector.

3- P2 is the housekeeping promoter of the PTHR gene.

Transcripts for the PTH/PTHrP receptors have been observed in numerous tissues during rat and mouse development (Lee K.et al 95, Karperien M. et al 94). In these species, the gene is under the control of two promoters, equivalent to the human P1 and P2. Since P1 activity is restricted to the kidney, P2 is probably responsible for PTHR gene expression during mouse development. The developmental defects associated with the loss of a functional receptor for PTH and PTHrP have shown the importance of the

transcriptional regulation of the PTHR gene expression in bone, where, as previously mentioned, the receptor is expressed in a spatially and temporally regulated manner. Our finding that P2 is the only promoter active in bone or in other tissues at mid-gestation in human showed that mice probably constitute a suitable model to study the mechanisms regulating PTHR transcription.

P2 shares a number of features with P3. Like P3, it has a high (G+C) content. It bears numerous Sp1 sites, and it is contained within the same CpG island. Unlike P3 however it initiates transcription from a single start site and does not seem to be expressed at elevated levels in any organs (table 4.2).

P2 structure is very similar in human and mouse, apart from a number of NF-IL6 response elements present in the mouse promoter but not in the human's (McCuaig 94 and chapter 3). Numerous binding sites for Sp1 and Ets transcription factors were also present in the proximal promoter region of both species (fig.2.2).

Ets and Sp1 have been shown required for retinoic acid-induced activation of PTHrP expression in the P19 mouse embryonal carcinoma cell line (Karperien M. et al 97). As RA also induced the expression of the receptor in this cell line (Eggenberger M. et al 98), it would be interesting to test whether the presence of Ets or Sp1 binding sites upstream of P2 are necessary to mediate the up-regulation of PTHR expression observed in the presence of retinoic acid.

Both Ets and Sp1 binding sites have also been implicated in transcriptional regulatory mechanisms connected to DNA methylation. Thus, functional Sp1 sites in the promoter of the mouse adenine phosphoribosyltransferase (aprt) gene are required to maintained the unmethylated state of the CpG island associated with this gene (Macleod D. et al 95). Methylation at an Ets site located downstream of the keratin 18 CpG island promoter has been shown to participate in the repression of the gene in non-expressing tissues (Umezawa A. et al 97). It would be interesting to address whether similar mechanisms apply to human PTHR gene regulation.

In relation with the function of PTHR during bone formation, it is noteworthy that two binding sites for the CBFa1 transcription factor are present upstream of P2. The potential importance of these sites became more clear with the simultaneous report by two different groups that genetic ablation of this transcription factor resulted in mice lacking bone. Furthermore, a mutation in this gene was shown to be responsible for the appearance of cleidocranial dysplasia (Rodan GA. 97).

Our results suggest that P2 is the only promoter active during mid development. The presence of binding sites for specific transcription factors upstream of the start site suggests, however, that this promoter might be actively regulated. Consistent with this idea is the report that no transcripts for the PTH/PTHrP receptor could be detected in embryonic tissue prior day 9.5 p.c. in mouse (Karperien M. et al 94).

C) PHP 1b.

The amplification of genomic DNA from PHP 1b patients did not revealed any deletion of promoter regions or any mutations at splice donor or acceptor sites, nor at transcription start sites.

Our finding that P3 is the major renal promoter sheds a new light on the understanding of the molecular defects associated with PHP 1b. The differential regulation of P3 in renal and non-renal tissues could account for the existing data on the gene expression in patients. More specifically, one can envisage how the loss of P3 function would result in a blunted renal response to PTH without abrogating the receptor expression in other organs. Alterations of P3 function are, however, unlikely to be the sole mechanisms responsible for the development of PHP 1b, since some patients presented a complete loss of PTHR gene transcriptional activity, suggesting that P2 function is also affected.

D) Differential splicing of the 5' UTR.

Although RNA blotting of rat or human RNA indicates that, in some tissues, transcripts of various length are present, few alternatively spliced transcripts have been described for PTHR, and none of them could account for the differences in length observed on northern blots (see chapter 1, section II). A transcript lacking the equivalent of exon U2, similar to that isolated from pig, still has to be characterized in other species. The PCR protocol we used to amplify P1-specific transcripts did not allow for the detection of such isomer, as the reverse primer used for the nested PCR was located in U2. The presence of transcripts derived from P1 that lack exon U2 can therefore not be ruled out. Likewise the use of a reverse primer located in exon E1 precluded the amplification of transcripts lacking this exon that have been described elsewhere (Jobert AS. et al 96). It is not clear whether any of these splice variants is present in human tissues.

The amplification of a shorter transcript in HOS cells led us to the characterization of a differentially spliced isomer resulting in the removal of the distal part of exon U3 and the proximal part of the signal sequence exon. The finding that this isomer is widely present in tissues demonstrated that it is not the result of a genomic deletion or differential splicing unique to HOS cell. The possibility that this splicing would result from the utilization of a cryptic site is not supported by the fact that it is not present at a detectable level in SaOS2 cells, in bowel, or more generally during development.

The fact that in clonal cell lines, both P2 and P3 are active suggests that, , these two promoters are likely to be active in the same subset of cells in-vivo. In the same line of reasoning, the presence of a single type of P2-derived transcript in HOS and could have indicated that SaOS2 cells display a more fetal phenotype. The expression of P3 in these cells suggests that the two transcripts might be segregated between different cell types in-vivo.

Splicing of pre-mRNA introns involves the recognition of sequences at the 5' donor, 3' acceptor and at the intronic branch site by snRNP. Two types of consensus sequences at the exon-intron boundary, GT-AG and AT-AC, have been described, each recognizable by a particular combination of snRNP (Tarn W-Y. and JA. Steitz 97, Kreivi J-P. and AI. Lamond 96). The presence of a GC dinucleotide in place of a GT, in the intron immediately after the exon, has been described in other genes and have been associated with a splicing reaction involving the same components as those required for the splicing of the conventional GT-AG pre-mRNA exon (Jackson IJ 91).

E) PTHR mRNAs can have multiple 5' UTRs.

Multiple promoter utilization and alternative splicing of transcripts derived from P2 can give rise to four different 5' UTRs (see 4.2). Assuming that the three promoters could all be active in a restricted subset of renal cells, the four types of transcripts are likely to be simultaneously present only in kidney. In other cells or organs the simultaneous activity of P2 and P3 and the shorter U3-SS splice variant can give rise to two or three different untranslated regions (depending on whether or not the two P2 derived transcripts are segregated in different cells).

In contrast to adult tissues, a single type of 5' UTR can be expected in embryo, since P2 is the only functional promoter and as the splice site variant found in adult tissues is not present.

Untranslated regions in eukaryotic mRNA are involved in a number of post-transcriptional regulatory processes. The three major aspects (or at least the most studied) of post-transcriptional regulation of gene expression, at the level of RNA, are concerned with mRNA translatability, stability and localization. Whereas the stability and sub-cellular localization of an RNA is mainly determined by its 3' UTR (Kozak M. 91,

Position		Content
Nt from cap site	Exon	
141	U1	CTATCCATGG
202	U1	AGGCCCATGG
216	U2	GTGCAGATGA
13	U3	CGAGGGATGC
102	U3	AGCTCCATGA
159	U3	ACATCCATGG
91	U4	CAGCGCATGG
Main ORF ATG	SS	GTGGCGATGG
Kozak consensus sequence		GCCPuCCATGG

Table 4.1. Position and context of ATGs located upstream of the main ORF in human PTHR transcripts. The position relative to the cap site or within the gene is shown in the left column. In the right column is given the nucleotide context in which the different upstream AUGs can be found. Underlined nucleotides represent a perfect match with the Kozak consensus sequence (given in the last row).

Wilhem JE. and RD. Vale 93), the efficiency of translation depends on its 5' UTR (Kozak M. 91, Kozak M. 92).

Of the four structural features of eukaryotic 5' UTR that play a role in affecting mRNA translation or recruitment by the translational machinery, three are potentially applicable to PTHR transcripts. The fourth one, which associates longer untranslated regions with a better translatability, is unlikely to be important as all four 5' UTRs have roughly the same length (~200 bp). Although the differentially spliced U3 isomer is 60bp shorter than its unspliced counterpart, these type of transcripts, resulting from the internal splicing of a longer form, are usually associated with a higher translation efficiency as we shall see later.

The three remaining structural features influencing translation efficiency are: a) The presence of upstream AUGs. b) The nucleotidic context around the AUGs. c) The presence of putative secondary structures and their thermo-dynamic stability.

All PTHR 5' UTRs display upstream AUG codons, several in the case of U1U2 and U3 bearing mRNAs and a single one for U4 (table 4.1). Except for one of them, none is found in a favorable Kozak consensus context (GCCA/GCCAUGG). In this context, nucleotides at position –3 and +4 have been shown to fit the consensus in 97% and 46% of the examined ORFs in animal cells. The only AUG that would be in a potentially good context (The first in U3, AGGGAUGC) is located within 13 nucleotides of the cap site, which is considered too close to the CAP site to interfere with translation initiation at the 'official' AUG is concerned (Kozak M. 92). It seems, therefore, that upstream AUGs are unlikely to affect the efficiency of translation, as most of them are located in a poor context. It is probable that upon encountering upstream ATGs, leaky scanning of the ribosome will allow accurate initiation of the translation (Kozak M. 92).

mRNA translatability and stability have been associated in many instances with secondary structures within the 5'UTR. The paradigm of postranslational regulation at secondary structures, that involves RNA translatability or stability, is associated with the

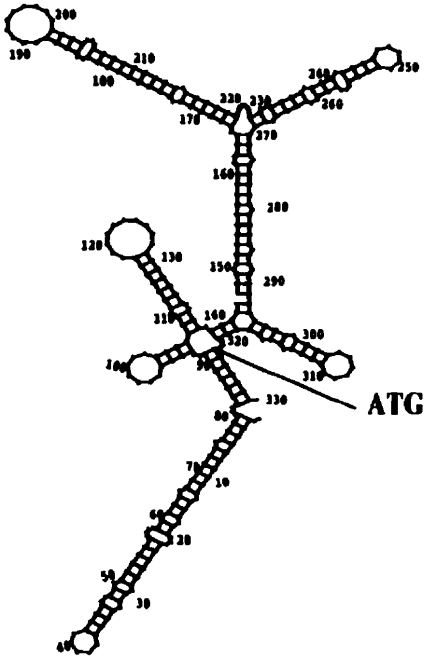
Figure 4.1: Secondary structures of P1- or P2-derived 5' UTRs. The top panel shows the secondary structures associated with transcripts derived from the distal (left) or the proximal (right) P1 start sites. Note the disappearance of three stem-loop structures and the decrease in thermodynamic stability. Also the ATG codon, buried in secondary structures in the longer transcript, is more accessible in the shorter.

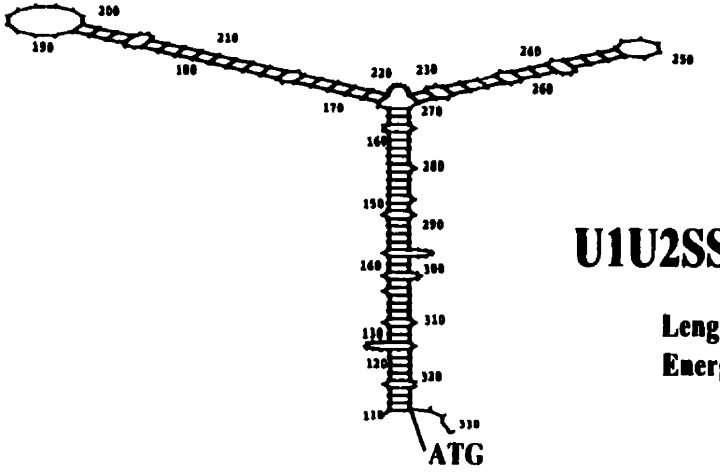
The bottom part of the figure depicts the secondary structures of the short (left) or long (right) P2-derived differentially spliced transcripts. Note the greater accessibility of the ATG and the decreased stability associated with the shorter transcript.

5'UTR SECONDARY STRUCTURES ASSOCIATED WITH P1 DIFFERENTIAL START SITES UTILIZATION OR WITH U3 DIFFERENTIAL SPLICING.

U1U2SS First start site.

Length: 332
Energy: -115.8



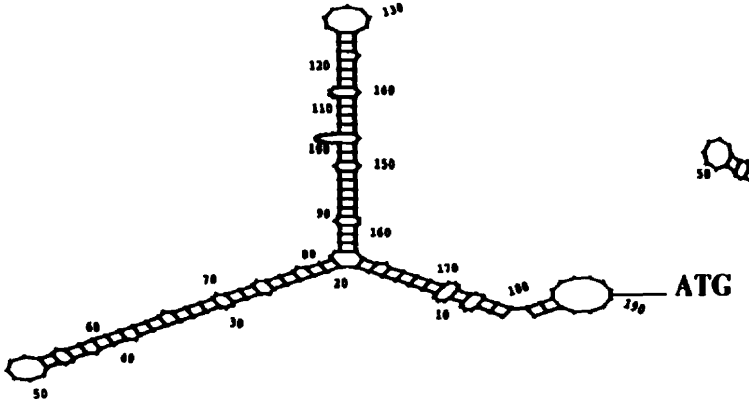


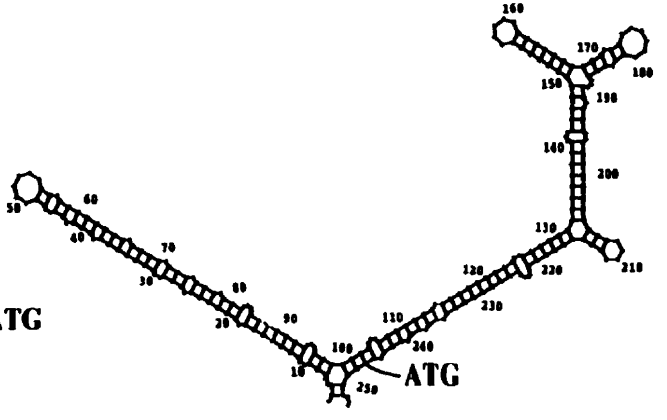
U1U2SS Last start sites

Length:223
Energy:-87.4

SHORT U3

Length: 196
Energy: -79.2





LONG U3

Length:254
Energy:-112.8

regulation of the expression of the proteins involved in iron utilization. A stem-loop structure in either the 5' or 3' untranslated regions of those mRNAs is recognized specifically by an RNA binding protein in the presence of iron and can affect the translation or the degradation of the transcripts to which it binds (Kinikis Z. et al 95). In other instances, the presence of a protein capable of binding the RNA does not even seem necessary. Thus, the introduction of an artificial stem-loop structure in the 5'UTR of transcripts encoding for the *Drosophila* Hsp70 protein can inhibit its translation (Hess MA. and R. Duncan 96). The extent of the inhibition depends on the stability of the structure, as assessed by the free energy value, corresponding to the energy required to melt the structure (Kozak M. 91). Interestingly, when submitted to a computerized folding, all the 5' UTRs display very stable structure with free energy values similar to that usually associated with viral genes or oncogenes (Patzel W. and G. Sczakiel 97).

The Insulin-like growth factor II (IGF II) gene constitutes a good example of how differential promoter utilization can give rise to differentially translatable transcripts. Of the two 5' UTR regions generated by alternate promoters usage, which have been described for IGF-II, only one was shown associated with the polysomal fraction in a rhabdo-myosarcoma cell line. This discovery was relevant in a number of pathologies in which an increase in the presence of the least translatable transcript was observed (Nielsen FC. et al 90).

Our results also show a potential for varying the degree of stability and the overall structure of the transcripts derived from P1 and P2 promoter. Hence, the decrease in the length of the 5' UTR of U1/U2 containing transcripts, resulting from the utilization of different start sites by P1, is associated with a concomitant decrease of the overall stability of the secondary structures (fig.4.1). It is noteworthy that the translatability of P1-derived transcripts is likely to be linked to the transcriptional state of the cells, as occupation of distal start sites by general transcription factors will likely result in an increased utilization of proximal start sites by the transcriptional machinery. Likewise, a

computerized analysis of putative secondary structures in the two U3 variants, shows that the shorter UTR derived from P2 has a less stable potential secondary structure than its longer counterpart. Significantly the shorter of the two UTRs lacks a long stem-loop structure at its terminus that, in the long variant, encompasses the translational start site (see fig 4.1).

Highly structured untranslated regions impede translation (Kozak M. 92). As mentioned previously the decrease in the overall secondary structures stability associated with the use of more proximal start sites, can result in more efficiently translated transcripts (Yiu GK. et al 94). Alternative splicing, in which part of a proximal untranslated exon or an entire exon is removed has been described for the rat insulin-like growth factor I and for the ornithine aminotransferase (OAT) gene. A study of the relative partitioning of the different 5' UTRs derived from these two genes showed that the short form is preferentially associated with the polysomal fraction. Significantly, association of the short OAT isoform with polysomes was cell specific whereas the longer transcript was poorly translated, irrespective of the cell types. The cellular specificity was shown to be due to a low level of expression of the general translation initiation factor ei4f (Foyt HR..et al 91, Fagan RJ. et al 91).

Translational regulatory mechanisms associated with U3 could explain the absence of abnormalities in any tissues except bone, in mice lacking the receptor. Hence, it is possible that translational mechanisms, rather than transcriptional, are the major mode of regulation of the receptor expression during development. In this hypothesis, the gene would be transcribed in many tissues, but the receptor would only be expressed in prehypertrophic chondrocytes.

This hypthesis supposes that the receptor is expressed from the same promoter during embryonic development in mice and human. Although no data are available concerning promoter usage during bone development in mouse, Dr Amizuka showed that P2 is the only detectable promoter activity in bone in adult mice (Amizuka N.

	INITIATION OF THE TRANSCRIPTION	G+C CONTENT AND PROMOTER CONTENT	TISSUE SPECIFICITY	ACTIVITY DURING DEVELOPMENT.	STRUCTURE OF THE 5'UTR
P1	• Multiple start-sites. • No recognizable initiator elements.	• ~ 50%. • Few consensus sites for general transcription factors. • CpG/GpC=0.2	• Renal exclusively. • Accounts for 1-2% of renal expression.	• Silent.	• Two UTR exons (U1&U2) spliced to the signal sequence.
P2	• Single start-site. • No recognizable initiator elements.	• ~85%. • Numerous sites for Sp1 and Ets transcription factors. • CpG/GpC=1.	• Ubiquitously active at low level. • Internal differential splicing.	• Active. • Single splice variant.	• A single exon spliced to the signal sequence.
P3	• Two start-sites. • No recognizable initiator elements.	• ~85%. • Numerous sites for Sp1. • CpG/GpC=1.	• Ubiquitously active at low level. • High activity in kidney. Accounts for 90% of the receptor expression	• Silent.	• A single exon spliced to the signal sequence.

Table 4.2. Summary of the structural features, distributions and activities of the three promoters controling the expression of the human PTHR gene.

unpublished results). As it is unlikely that P1 would be expressed in bone during development, one can reasonably assume that the same promoter direct expression of the PTH/PTHrP gene in mice and human at this time. The greater than 94% homology found between the murine and human U3 region might indicate that this exon carries the same functions in the two species.

The assumption that translational mechanisms regulate the appearance of the receptor during embryonic development also infers the existence of factors that would promote translation of U3-containing transcripts exclusively in prehypertrophic chondrocytes. Though the presence of such factors is at this stage purely speculative, the demonstration, by Fagan and co-workers, that the translational regulation of OAT expression is cell specific shows that it is not inconceivable.

To my knowledge, no studies specifically addressed post-transcriptional regulation of PTHR expression, although a number of data suggest however that they could play a role in regulating expression of the PTH/PTHrP receptor (see chapter 1, section II). Most significantly, binding of PTH agonists was not observed in rat aortic primary vascular smooth muscle cells despite the presence of transcripts for the receptors (Wu S. et al 93). A subsequent study that used clonal cells of similar origin, stably transfected with a cDNA for the receptor, showed a good expression of the receptor (Maeda S. et al 96). Since the cDNA used in this study, lacked the 5'UTR, it can be inferred that the endogenous 5'UTR is responsible for the lack of expression reported in the study by Wu et al. It is therefore possible that similarly the presence of P2 derived transcripts during embryonic development is not necessarily correlated to surface expression of the receptor. There are no reports of immuno-histochemical detection of the receptor in embryonic tissues, whereas the presence of the ligand has been documented at the protein level in both mouse and human (Van de Stople A. et al 93, Moniz C. et al 90).

F) Conclusion.

The developmental up-regulation of P1 and P3 and the appearance of the short P2 derived isomer may have important consequences for the receptor signaling. The demonstration, by Guo et al, that the density of cell surface receptors determines the ability of PTH or PTHrP to activate the PLC/PKC pathway, provides a clear example of how up-regulating PTHR gene transcription can influence cellular physiology. As we discussed previously (Chapter 1, section II F), many PTH-inducible cellular processes are exclusively dependant on PLC/PKC activation. If the results obtained by Guo et al in porcine LLCPK1 cells are confirmed in human, our finding that two of the three human PTHR gene promoters are developmentally up–regulated could be relevant in the appearance of PKC activated PTH-regulated biological processes.

Our results are also relevant to the understanding of the pathogenesis of PHP1b and CRF. While our early data showed that no gross deletions or mutations could account for the appearance of PHP1b in seven patients (chapter 2), the characterization of P3 as the major renal promoter shows that the transcriptional deregulation of renal PTHR expression associated with PHP1b is likely to affect P3. However, in the absence of more precise data on the sub-cellular promoter utilization, the presence of defects affecting P1 can not be ruled-out. An examination of promoter activity in fibroblast derived from PHP 1b patients (chapter 1 section II-G) will be useful in addressing the functionality of the three promoters in these patients.

A decrease in the steady state level of transcripts for PTHR have been observed in rat model for chronic renal failure (CRF) in various organs, including bone and kidney, suggesting that alteration of the PTHR expression could participate in the general deregulation of calcium homeostasis associated with the progression of CRF. Our finding that transcription of PTHR gene is mostly different in rodent and human raises questions about the use of rat as a model to study alteration of expression of PTHR in CRF. A more detailed study aiming at determining whether P2 and P3 behavior is coordinated under

different conditions, will be helpful in assessing to what extent data derived from rat can be applied to human.

If, as hypothesized by several, deregulation of the PTHrP/PTHR signaling axis would influence the cell phenotype, transcription of both PTHrP and PTHR gene can be predicted to play an important role in tumor progression. Our finding that PTHR gene expression is under the control of methylation sensitive mechanisms is particularly interesting in the light of the report that ectopic expression of PTHrP in renal carcinoma cell lines correlated with demethylation of this gene's promoter. The use of demethylating agents has been associated with either promotion or inhibition of neoplasia (Szif M. 96). Our data provide a possible mechanism by which demethylation-induced PTHrP expression could be paralleled by a transcriptional activation of the PTHR gene, thereby providing the neo-transformed cells with a functional intracrine or paracrine axis.

PTHR expression by prehypertrophic chondrocytes and by trabecular lining cells in rat long bone emphasized the precise mode of regulation of the gene during bone morphogenesis. Our finding that only P2 is active at a time of bone formation in human showed that this promoter is likely to be the one driving PTHR gene expression in this organ. Several transcription factors play an essential role during bone development. A more detailed analysis of the cis-acting sequences controlling P2 activity in chondrocytes will be informative about the transcriptional events involved in the process of bone formation.

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ORIGINAL CONTRIBUTION TO KNOWLEDGE.

1. Organization of the 5' regulatory region of the human PTHR gene is similar to that in mouse.
2. Transcription of the human PTHR gene proceeds from three different promoters, P1, P2, and P3. The transcription start sites has been characterized for all three promoters.
3. P1 activity is weak and is restricted to adult kidney.
4. P2 activity has been observed in all tissues and cell lines and at all developmental stages examined. Expression of the receptor up to week 19 of fetal age is under the exclusive control of P2.
5. A differentially spliced transcript, derived from P2 promoter, has been characterized. This type of transcript is present in most, but not all, adult tissues and cell lines but was not detectable up to week 19 of fetal age.
6. P3 is a CpG island promoter and is ubiquitously active in adult tissues and cell lines, and silent up to week 19 of fetal age. It is highly active in kidney where it is responsible for 80% of PTHR gene expression.
7. All three promoters are sensitive to in-vitro methylation.
8. Study of methylation patterns in-vivo shows that, while P3 is demethylated in both expressing and non-expressing tissues, P1 is partially demethylated in both adult and fetal kidney whereas it is completely methylated in circulating lymphocytes.