

Developmental Upregulation of Human Parathyroid Hormone (PTH)/PTH-related Peptide Receptor Gene Expression from Conserved and Human-specific Promoters

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Abstract

The parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor (PTHrP) functions in skeletal development and mediates an array of other physiological responses modulated by PTH and PTHrP. PTHrP gene transcription in mouse is controlled by two promoters: P1, which is highly and selectively active in kidney; and P2, which functions in a variety of tissues. P1 and P2 are conserved in human tissue; however, P1 activity in kidney is weak. We have now identified a third human promoter, P3, which is widely expressed and accounts for ~80% of renal PTHrP transcripts in the adult. No P3 activity was detected in mouse kidney, indicating that renal PTHrP gene expression is controlled by different signals in human and mouse. During development, only P2 is active at midgestation in many human tissues, including calvaria and long bone. This strongly suggests that factors regulating well conserved P2 control PTHrP gene expression during skeletal development. Our results indicate that human PTHrP gene transcription is upregulated late in development with the induction of both P1 and P3 promoter activities. In addition, P2-specific transcripts are differentially spliced in a number of human cell lines and adult tissues, but not in fetal tissues, giving rise to a shorter and less structured 5' UTR. Thus, our studies show that both human PTHrP gene transcription and mRNA splicing are developmentally regulated. Moreover, our data indicate that renal and nonrenal PTHrP gene expression are tightly coordinated in humans. (*J. Clin. Invest.* 1998. 102:958–967.) **Key words:** gene transcription • differential splicing • fetal development • calcium homeostasis • tissue-specific promoter

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Introduction

Signaling through the parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor (PTHrP) is essential for normal development and a wide array of physiological responses stimulated by PTH and PTHrP. PTH is released from the parathyroid glands in response to decreased extracellular fluid calcium levels, and binds to PTHrPs expressed in kidney and bone. Stimulation of renal and osseous PTHrPs increases extracellular fluid calcium concentrations by enhancing calcium reabsorption and mobilization and phosphate excretion (1, 2). PTHrP can mimic many of the effects of PTH when overexpressed by cancers. Physiologically, PTHrP acts in a paracrine/autocrine manner, in contrast to PTH. Whereas production of PTH is restricted to the parathyroids, PTHrP is widely expressed and functions to modulate cellular growth and differentiation (3–6). Like PTHrP, the PTHrP is widely expressed. In the rat, the PTHrP is expressed at its highest levels in kidney and at lower levels in a number of tissues, including bone, bladder, aorta, heart, liver, lung, and spleen (7, 8).

The essential role of the PTHrP in skeletal development has been highlighted by studies of Jansen-type metaphyseal chondrodysplasia, a rare form of dwarfism associated with hypercalcemia despite near normal circulating levels of PTH and PTHrP. Mutations in transmembrane domains of the PTHrP in these patients lead to constitutive activation of the receptor (9–11). Gene ablation studies in mice have shown that PTHrP-PTHrP signaling is essential for normal endochondral ossification (12–14). PTHrP and PTHrP null mice displayed similar phenotypes, with reduced proliferation and accelerated maturation of chondrocytes associated with accelerated ossification.

The PTHrP belongs to the vast family of G protein-coupled receptors containing seven transmembrane domains. Binding of ligand can stimulate production of intracellular cAMP and inositol 1,4,5-trisphosphate (15–17). The structure of the PTHrP gene is very similar to those of receptors for peptide hormones, including calcitonin, vasoactive intestinal peptide, glucagon, and growth hormone releasing peptide (18–23), providing evidence that these receptors have evolved from a common precursor.

Two promoters control PTHrP gene transcription in the mouse and rat, P1 and P2, which give rise to transcripts differing in their 5' untranslated regions (UTRs) but not their coding sequences (18, 24, 25). P1 is highly and selectively active in mouse kidney and accounts for at least 90% of renal PTHrP

1. *Abbreviations used in this paper:* 5' UTR, 5' untranslated region; PHP1b, pseudohypoparathyroidism type 1b; PTH, parathyroid hormone; PTHrP, PTH-related peptide; PTHrP, PTH/PTHrP receptor; SS, single stranded.

transcripts (25, 26). P1 activity in bone and cartilage is very weak or absent (25, unpublished results). Unlike P1, P2 is expressed at moderate levels in a number of tissues and controls the broad expression pattern of the PTHR. P2 is highly (G + C)-rich and has all the hallmarks of a housekeeping promoter. The expression patterns of P1- and P2-specific transcripts in mouse suggest that renal and nonrenal transcription of the PTHR gene are regulated largely by different mechanisms.

The exon-intron structures of the coding regions of the human and rodent PTHR genes are highly conserved (19). P1 and P2 are also conserved between mouse and human (27). However, studies with kidney RNA suggested that human P1 is not as active as its mouse counterpart. Here we have further characterized the human PTHR gene and have discovered a third promoter, P3, which is active in kidney, bone, and other tissues. P3 accounts for ~80% of detectable renal PTHR transcripts in human but is apparently inactive in mouse kidney. Elucidation of the normal regulation of human PTHR gene expression will provide insights into various disorders of calcium homeostasis and development. Our data show that both PTHR gene transcription and mRNA splicing are developmentally regulated and suggest that PTHR gene expression in adults is coordinated differently in human and mouse.

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 wk fetal age) 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 (FA) was determined by foot length (28). Total RNA was isolated from adult tissue or cultured cells by CsCl gradient centrifugation and from fetal tissues using Trizol (GIBCO BRL Life Technologies, Grand Island, NY) extraction.

Ribonuclease (RNase) protection analysis. RNase protection probes for U1- and U3-containing transcripts have been described (reference 5; Fig. 1 A). A U4 probe was created from a 1.8-kb KpnI-BamHI fragment containing the single-stranded (SS) exon inserted in Bluescript SK+, which was digested with ApaI and in vitro transcribed with T3 RNA polymerase (New England Biolabs, Beverly, MA) under standard conditions (see Fig. 1 A). 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/liter piperazine-N,N'-bis-2-ethanesulphonic acid (pH 6.4), 0.4 mol/liter sodium acetate, and 1 mmol/liter ethylenediaminetetraacetate), denatured for 5 min at 85°C , and incubated overnight at 50°C . Digestions were performed for 1 h at 37°C with 4 U of RNase 1 (Promega Co., Madison, WI) according to manufacturer's specifications. Products were ethanol precipitated and electrophoresed on a 6% polyacrylamide sequencing gel. The integrity of the probe was verified by running 250 cpm of undigested mix.

Primer extension analysis. Ten picomoles of primer P3Up2 (5'-CTATGGACCCCGCGTCC-3'), which is complementary to sequences starting 61 bp upstream of the SS exon, was labeled for 1 h 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 26 μl . Samples were then put on ice, and 4 μl of 25 mM Tris-HCl (pH 8.0), 60 mM MgCl₂, 10 mM DTT, 5 mM dNTPs, 2 U of RNAGuard (Pharmacia, Uppsala, Sweden), and 100 U of M-MLV reverse transcriptase (GIBCO BRL) was added. After 60 min at 43°C , enzymes were inactivated for 10 min at 75°C , and the reaction was extracted

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 sequencing reactions as molecular weight markers.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis and cDNA cloning. Three micrograms of total RNA was precipitated, resuspended in 6 μl of $1\times$ 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 on ice. Two microliters was added to 18 μl of a RT mix containing 0.5 mmol/liter of each dNTP, 5 mmol/liter of DTT, 1 μmol /liter of 6mer random primers, 75 mmol/liter of KCl, 3 mmol/liter of MgCl₂ in 50 mmol/liter of Tris-HCl (pH 8.3), and incubated for 1 h at 37°C , followed by 75°C for 10 min. For detection of P1-specific transcripts by PCR, 2 μl of the RT reaction was subjected to a first round of 30 cycles of amplification (95°C for 30 s, 56°C for 1 min, 72°C for 20 s), using 5'-AGGGAATTCAGGTCCTTTCTTGTC-CCCAGC-3' as the forward primer (primer 1) and 5'-GACGT-CATCTGCATCCACCAGCGC-3' (primer 6) as the reverse primer. One microliter of the first reaction was then subjected to 30 more cycles of amplification (95°C for 30 s, 58°C for 1 min, and 72°C for 5 s), using forward primer 5'-AGTTGTGTGTCCTGGACACTACCA-3' (primer 2) and reverse primer 5'-GCCTCCCCGTGGCCAACT-TGAGTC-3' (primer 3). PTHR and β -actin coding sequences were detected by amplification of 2 μl of the RT reaction for 24 cycles (95°C for 30 s, 56°C for 1 min, and 72°C for 25 s). The forward primer for the PTHR was 5'-CACCCTACTACTGGATTCTGGTG-3', and the reverse primer used was 5'-GATTTCTTGATCTCGCTTG-TACC-3'. For β -actin, the forward primer was 5'-GCTGTCTATC-CCTGTACGC-3' and the reverse primer was 5'-GCCATGGTGAT-GACCGGC-3'. All of the above reactions were performed in 50 μl of 1.5 mmol/liter of MgCl₂, 50 mmol/liter of KCl, and 10 mmol/liter of Tris-HCl (pH 9.0) using 2.5 U of Taq DNA polymerase (Pharmacia). P3-specific transcripts were detected using 28 cycles of PCR (95°C for 30 s, 58°C for 45 s, and 72°C for 15 s), performed using 2 μl of RT reaction in 50 μl of 0.8 mmol/liter of MgCl₂, 50 mmol/liter of KCl, 20 mmol/liter of Tris-HCl (pH 8.3), and 2.5 U of Taq DNA polymerase, and 5'-ACGGAATTCAGCCTGACGCAGCTCTGCACC-3' (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 as follows: 45 s denaturation at 95°C , 20 s 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 of 1.5 mmol/liter of MgCl₂, 50 mmol/liter of KCl, and 10 mmol/liter of Tris-HCl (pH 9.0) and in 5% formamide and 5 U of Taq DNA polymerase. The forward primer was 5'-GCCCCGACATCCT-GCAAGGC-3' (primer 4), and the reverse primer was primer 6 (see above). PCR reactions (2/5 to 1/10) were run on a 2% agarose gel, transferred overnight to a nylon Hybond N+ membrane (Amersham, Arlington Heights, IL), and the DNA was covalently attached by baking the membrane at 80°C under vacuum for 2 h. Membranes were prehybridized for 30 min in $6\times$ NET (100 mM Tris-HCl [pH 7.5], 0.9 M NaCl, 6 mM EDTA), $10\times$ Denhardt's, 0.5% SDS, 10 mg/ml denatured salmon sperm DNA at 62°C , and then hybridized in $6\times$ NET, $5\times$ Denhardt's, 0.5% SDS, 1 mg/ml denatured salmon sperm DNA with 10^6 cpm of end-labeled oligonucleotides for 5 h at 62°C . Oligonucleotides used for probing were: 5'-CCCTAGGCGGTGGC-GATGGGGACC-3', for P2- and P3-containing sequences, 5'-CTG-CAGCTTTAGCCCCGACTTG-3' for P1-specific sequence, and 5'-TGGCCATGAAGACAATGTAGTGGACG-3' 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 (45-s denaturation at 95°C , 20-s 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, San Diego, CA), and characterized by DNA sequencing. Secondary structure analysis of 5' UTRs of P2-specific transcripts was performed with Squiggles (GCG package, University of Wisconsin).

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 (27) was inserted in BamHI-PstI-digested pSKb, a derivative of pBluescript SK+ (Stratagene, La Jolla, CA) in which the XbaI site has been converted into a BglIII site. The insert was excised from pSKb as a BamHI-BglIII fragment and inserted in the polylinker of the promoterless luciferase plasmid pXP2 (29). To construct P2-luciferase recombinant pXP2-P2, a 1.1-kb SacI fragment (containing P2 se-

quences -959 to +101), whose ends were rendered blunt with T4 polymerase, was inserted into the SpeI site of pSKb. Insert orientation was confirmed by restriction digestion and DNA sequencing. The insert was excised as a BamHI-BglIII 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-BglIII fragment was excised and inserted in the polylinker of pXP2.

Cell culture and transient transfections. SaOS-2 cells (ATCC) were propagated in McCoy 5A media + 10% fetal bovine serum. HOS and

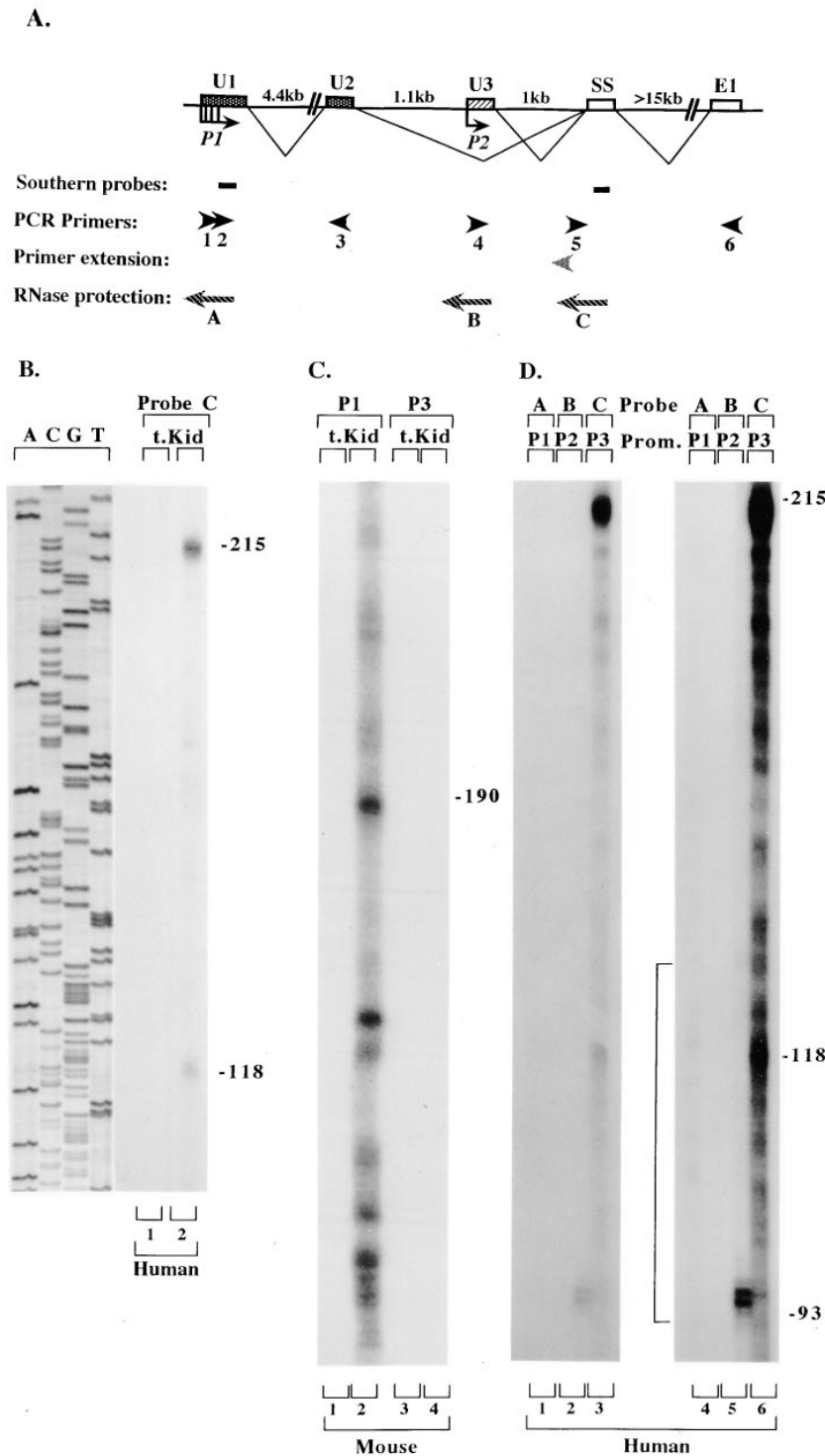


Figure 1. Detection of a third human PTHR gene promoter. (A) Schematic representation of the 5' end of the human PTHR gene. UTR exons U1, U2, and U3 are indicated along with the signal sequence exon (SS) and the first extracellular domain exon E1. Splicing patterns of transcripts expressed from P1 and P2 promoters are shown up to E1. Probes used for Southern analysis, primers used for PCR (numbered 1-6) 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; reference 25), and a probe extending 1 kb 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) 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).

U2OS cells (ATCC) were cultured in DMEM + 10% fetal calf serum. HK-2 cells (30) were propagated in keratinocyte serum-free medium + 5 ng/ml epidermal growth factor, and 50 μ g/ml bovine pituitary extract (GIBCO), and MCF-7 cells were propagated in Eagle's MEM + 10% fetal bovine serum. Transient transfections were performed with HOS cells (50% confluent) grown in 12-well plates. Cells were transfected with 1 μ g of luciferase reporter plasmid and 250 ng of β -galactosidase expression vector p610AZ (31) using Lipofectin (GIBCO), according to the manufacturer's instructions. Media were changed 12 h later, and cells were harvested in 100 μ l of Reporter Lysis Buffer (Promega) 72 h after transfection. Luciferase assays were performed according to instructions (Promega), and β -galactosidase assays to normalize for transfection efficiency were performed as previously described (32).

Results

Identification of a major PTHR promoter, P3, active in human but not mouse kidney. We have previously identified two promoters, P1 and P2 (Fig. 1 A), which control human PTHR transcription (27). While these two promoters are conserved between human and mouse (18, 25), expression of P1 in the human kidney is weak, whereas in the mouse, P1 activity accounts for at least 90% of renal transcripts (25, 26). 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 human genomic probe extending upstream of signal sequence exon SS (Fig. 1 A, *Probe C*) protected a 118-b fragment. This corresponds to splicing of SS to the 5' untranslated region exons (5' UTRs) of transcripts expressed from P1 or P2 (Fig. 1 B, lane 2). The probe also protected an abundant, larger fragment of 215 b, 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. 1 C, 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 suggested that P3 activity accounts for the majority of PTHR transcripts in the human kidney. This was 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. P3 is the major human renal promoter, and P2 activity accounts for most of the remaining transcripts detected (Fig. 1 D, lanes 1–3). Indeed, under these conditions P1-specific transcripts were detected only after prolonged exposure (Fig. 1 D, lane 4).

P3 initiation sites were mapped by primer extension analysis using a primer (Fig. 1 A) whose 5' end lies 70 bp upstream of the 5' end of SS. Two products of 140 and 147 b were detected (Fig. 2, *Kid*), which correspond to a novel fusion exon combining a 5' exon of 210–217 bp in length (designated U4) and the previously identified SS exon. The 5' UTR contains an upstream open reading frame, suggesting that the transcript is subjected to translational regulation. These results are summarized in Fig. 3.

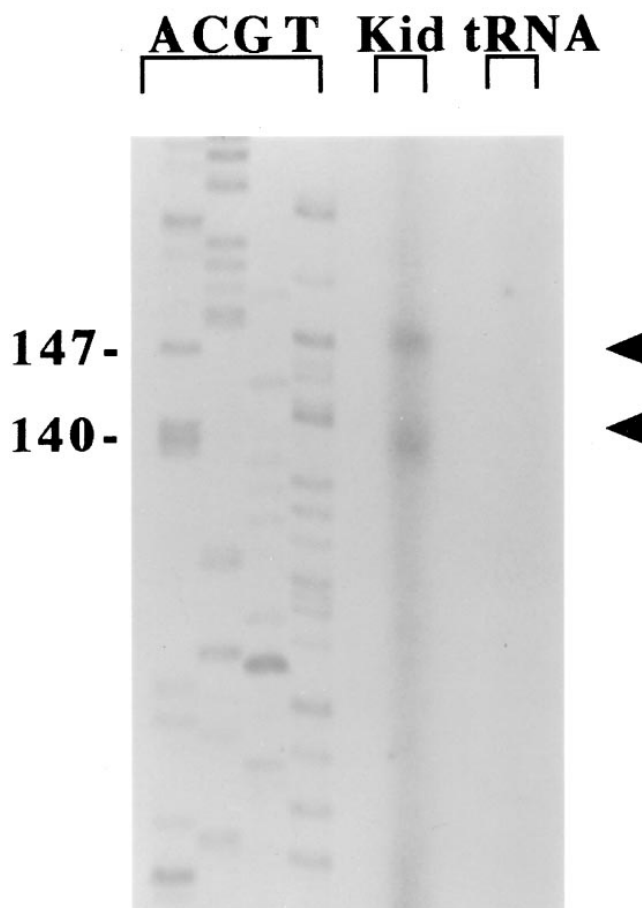
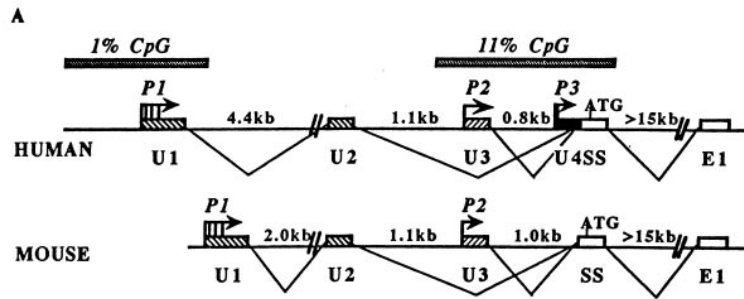


Figure 2. Identification of P3 promoter initiation sites by primer extension analysis. Primer extension was performed with a primer whose 5' end lies 70 bp upstream of the 5' end of SS (see Fig. 1 A). Two extension products of 140 and 147 b are detected using human kidney total RNA but not with a tRNA control. The sequencing ladder used as molecular weight markers is shown on the left.

P2 and P3 promoters lie within a CpG island. The sequence of human genomic DNA between exons U3 and SS (Fig. 3 B) revealed that P2 and P3 lie within the same CpG island. CpG dinucleotides occur at a frequency of 11 per 100 bp, with a CpG/GpC ratio of approximately 1 in 1.3 kb of human sequence extending from 300 bp upstream of U3 to SS (Fig. 3, A and B; reference 27). In contrast, a 1.1-kb region around human P1 contains only 11 CpG dinucleotides and a CpG/GpC ratio of 0.12 (Fig. 3 A; reference 27). Similar to other CpG island promoters (33), P3 lacks a discernible TATA box, is relatively (G + C)-rich (65%) between –250 and +100, and contains a number of Sp1 sites (Fig. 3 B). In the mouse, activity of the major renal promoter P1 is largely restricted to kidney and is weak or inactive in bone and cartilage (26, 27, and unpublished results). The major human renal promoter, P3, resembles the (G + C)-rich P2 promoter, which functions in a number of tissues in the mouse (25). Therefore, we analyzed the expression patterns of the three human promoters, in particular to determine if P3 functions in tissues other than kidney.

P3 is widely expressed in human tissues and cell lines. Expression of PTHR coding sequence and of P2- and P3-specific transcripts was examined by semiquantitative RT-PCR analysis us-



B

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GTCGGCGGGC CTCTCGGAA CCGGGGGAG TCGCCAGCC CGCGCCCTC GGCTCGGTGG CTTTTTTGGA AACTTGCAA -632
TGTTCGTA GAGAGAAAG GGGAGGGAG GGAGCGAGG AGTGACGAA ACGGAGCTTG GGGCCGCTGG AAGAAGTAG -552
GCCAAGGCC GGGGAGCTAG AGACGGACTG ACAGACAGCC AGACCGACAG AGCGTCGGGG CCGCTGCCCC GAGCCGCACA -472
GGCCCAAGGG GCTCTGCAG GATGAGGAA GGGGTGCGG AGCGCGCTGC CAGGGTCTG GATCTCAGG AGGCCGAAC -392
CGGGGCTGG CCGCCGAAC ACCTAAGGGC TCAGTGTGGC TGCAAAATTG AGATCGCAC CCCTAAGTGC ACGCCCGCC -312
CGGCTCAGAA CCGCCCCCTC GCCTGGCCCT GACTCCCTAC CCCGAAAGTC GGCGAGCTAA AAATAACAGG TTCCTGCGC -232
CCCGCCGAG ACCCGGACC CGACCCCTCC CCCGCCCTC CCCCCACTG GGCGTGGGG GAAGCCACAG CTCCCATTTC -152
CCCAAAAGAA AAAAAAGAA AGAAAGAAAG AAAGAAAGAA AAGCGGCGC GGGAGGGGG GGGGGGGCG GCGGGGGCG -72
GGGGGCGCG CATATGGATG TGATTTCTTC GCTCCGAGGC AGACGGCCG GTCGCAGCG GCTTGGCGCC GCGCCCGCC +9
CCCTCCGCT CTCTCCCTC CCCTCCTGC CCTCCCTCC TCCTCCTTT GCTCCTCTC GTCGCTGCT CCGCCCTCAGC +89
GGATGCCCC CGCCGGGCC CCGGCGCTC GGGCGCCGG ACAGCGGGT CCCATAGCC GGGGCCTGG CCGGCGGCC +169
AGCCTGACC AGCTCTGCAC CCCTACCAC CACCAGGCC GCGCGCGCG GCTGCCCGA GGGACCGCC CTTAGCGGT +249
GCGATGGG ACCCGCCGA TCACCCCG CCGTGCCTC CTGCTCTGT GCCCTGCTC CAGCTCCGA CCGCTGGTG +329
AGTCCCCCG
  
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Figure 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. P3 initiation sites are indicated by arrowheads, with the upstream site designated as +1. Consensus Sp1 sites are indicated, and 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 (*italics*) of the PTHR coding sequence.

ing specific primers (see Fig. 1 A). 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. Amplified PTHR cDNA sequences were transferred to membranes and probed with internal oligonucleotides that were distinct from PCR primers (Fig. 4). PCR sequencing was also used to confirm that the amplified products corresponded to expected PTHR cDNA fragments (data not shown). As expected, the highest levels of expression of PTHR coding sequences were found in kidney (Fig. 4 A, lane 14), with lower levels detected in spleen and bowel (Fig. 4 A, lanes 16 and 18). Lower levels of expression were detected in human osteoblast-like osteosarcoma cell lines HOS (Fig. 4 A, lane 6), SaOS-2 (lane 10), and U2OS (lane 8), in cultures of human skin fibroblasts (lane 12), the renal epithelial cell line HK-2 (lane 4), and 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 levels and not to variable RNA concentrations in RT reactions. This is supported by Northern analyses of PTHR transcripts showing a wide variation of expression levels in different rat and human tissues (8, 16, data not shown).

Consistent with results obtained in the mouse (25), P1 expression was restricted to kidney (Fig. 4, lane 14). P2-specific transcripts were detected in all human tissues and cell lines expressing PTHR coding sequences, consistent with P2 activity in the mouse (25). However, cDNA sequences amplified from

HOS cell extracts were shorter than the predominant products amplified from other tissues and cell lines (Fig. 4, lane 6; see below). P3-specific transcripts were detected in all PTHR-positive tissues and cell lines, except MCF-7, HK2, and U2OS (Fig. 4 D, 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, we found 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.

P3 sequences drive expression of a heterologous gene in transiently transfected HOS cells. PTHR promoter-luciferase reporter recombinants were constructed to analyze the function of promoter sequences in driving expression of heterologous reporter genes (Fig. 5 A). Transient transfections were performed in HOS cells, which were shown to express P2- and P3-specific transcripts (Fig. 4). Consistent with this observation, both P2 and P3 promoter sequences drove expression of luciferase activity in HOS cells, whereas little or no activity of P1 was observed (Fig. 5 B). These results confirm the capacity of sequences in the P3 region to function as a promoter in driving expression of heterologous genes.

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 FA human fetus (Fig. 6). PTHR cod-

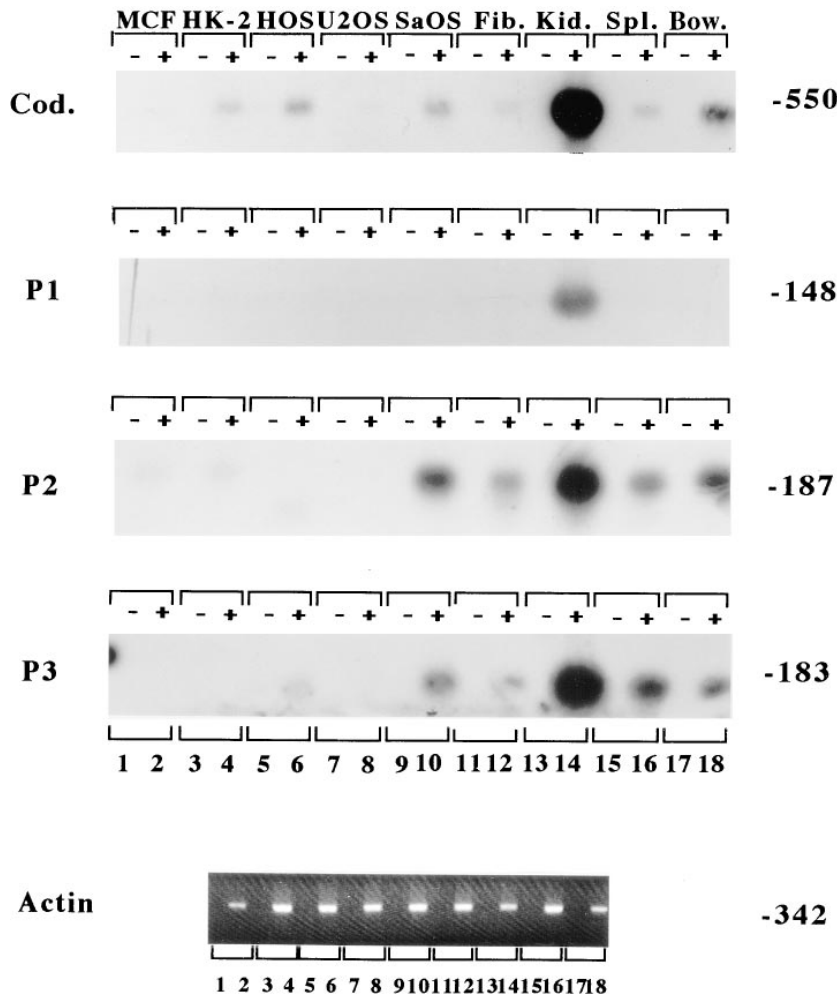


Figure 4. Tissue-specific expression of PTHR transcripts in adult human tissues and cell lines. Promoter-specific expression was analyzed by RT-PCR using PCR primer combinations diagrammed in Fig. 1 A. P1-specific sequences were amplified using primers 1 and 6, followed by amplification with primers 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. 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 RT products of kidney RNA (data not shown), whereas reactions performed with P1-specific primers were amplified beyond the linear range (see Methods for details of PCR conditions). Amplifications were performed on total RNA from MCF-7 breast carcinoma cells (*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.

ing sequences were detected by RT-PCR in RNA from liver, long bone, calvaria, muscle, and kidney (Fig. 6, 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. 6, lane 12). Consistent with its broad 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 with adult kidney RNA (Fig. 6, lane 12). Essentially identical results were obtained with tissues from a 17.5-week 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. 6, 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. These results also indicate that P2 is the major PTHR promoter active at midgestation, and moreover that the onset of expression of P1 and P3 in kidney occurs after 19 weeks FA.

Differential splicing of P2-specific transcripts in adult, but not fetal tissues. The shorter P2-specific RT-PCR product amplified from HOS cells (Fig. 4) was further investigated by subcloning and sequence analysis. The shorter fragment arose

from differential splicing between U3 and SS exons using donor and acceptor sites distinct from those previously identified (Fig. 7). The sequence AGGCGG is present at both the donor and acceptor sites, preventing the unambiguous determination of the splice junction (Fig. 7, *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. Structural analyses suggest that the differentially spliced 5' UTR forms a less stable hairpin loop which liberates the ATG of the PTHR open reading frame from secondary structure (data not shown).

The cell and tissue specificity of the alternative splice was probed with an oligonucleotide that encompasses the alternative splice junction (Fig. 7 A) and detects both long and short cDNA fragments under the hybridization conditions used. The blot revealed the existence of shorter products amplified as expected from HOS cells (Fig. 6 B, lane 6), and from skin fibroblasts, kidney, and spleen (Fig. 7 B, lanes 12, 14, and 16), but not from MCF-7, HK-2, U2OS, SaOS-2, or bowel even after extended exposure (Fig. 7 B, lanes 2, 4, 8, and 18; and data not shown). No evidence for alternative splicing was observed in 18- or 19-week FA tissue samples, including kidney, bone, and calvaria (Fig. 7 C), 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

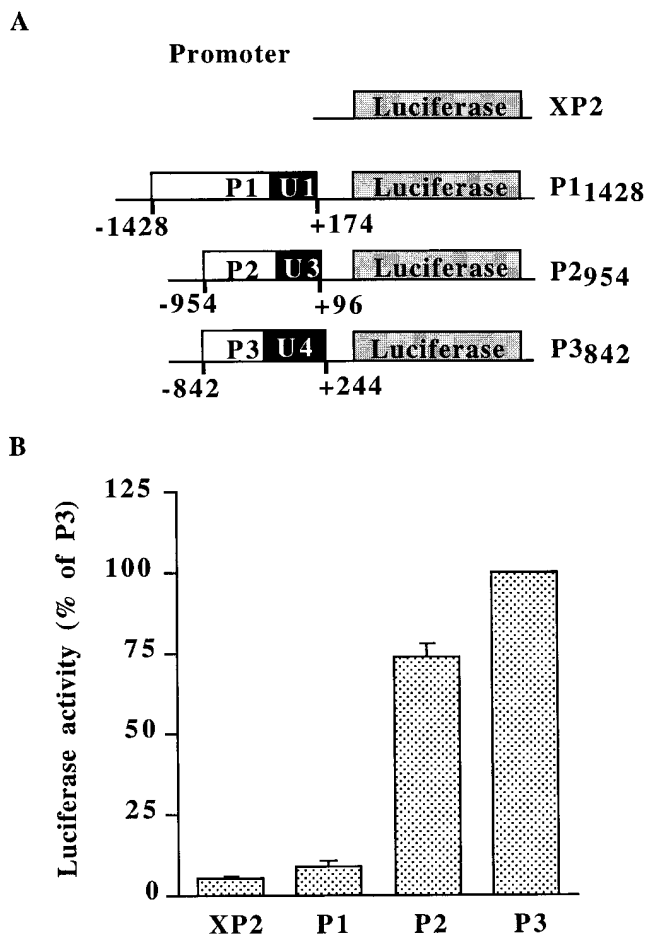


Figure 5. Function of P3 promoter sequences in transiently transfected HOS cells. (A) Luciferase reporter recombinants used in these studies. See Methods for details of plasmid constructions. (B) Results of transient transfections of HOS cells with promoterless luciferase recombinant pXP2 or with recombinants containing P1, P2, or P3 sequences. Relative luciferase activities observed in three to five sets of transfections (\pm SEM) are indicated.

adult but not fetal kidney indicates that the event is developmentally regulated.

Discussion

PTHr gene expression in kidney. P1 activity in the adult mouse accounts for at least 90% of PTHr transcripts in kidney (25, 26). Renal PTHr gene transcription in the mouse is thus largely controlled by kidney-specific regulatory sequences of P1. While P1 is active specifically in adult kidney, it accounts for only a small proportion of renal transcripts (Fig. 1; reference 27). In contrast, the P3 promoter drives the majority of PTHr gene transcription in human adult kidney. P3-specific transcripts are also expressed in a number of other tissues and cell lines, suggesting that common regulatory signals control human PTHr gene expression in renal and nonrenal tissues.

Studies of PTHr mRNA and protein expression in murine kidney revealed a cell-specific distribution of receptor protein and P1- and P2-specific mRNAs (26). Highest densities of

PTHr protein and transcripts were found in peritubular endothelial cells and vascular smooth muscle. 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 (26). The weak activity of P1 in human kidney suggests that either P1 is widely active at low levels, or that the expression pattern of P1-specific transcripts is much more restricted than in mouse. 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 all renal cell types which express the PTHr. However, while P1 activity may be somewhat higher in specific cell types, it is clearly much less active in human than in mouse.

The moderate activity of P2 in murine kidney was found to be restricted to tubular epithelial cells (26). 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), suggesting that P2 transcripts may have similar distributions in mouse and human kidney. The replacement of P1 by P3 as the major human renal promoter driving PTHr gene transcription suggests that the regulatory signals controlling PTHr expression in the kidney may differ between mouse and human. It is not clear whether P3 activity in human kidney simply replaces reduced P1 function in some cell types. The distribution and relative expression levels of PTHr transcripts and protein in different renal cell types may differ between mouse and human. It will therefore be important to determine the cellular distribution of PTHr transcripts and protein in human kidney.

Our results limit the number of potential models for the PTH resistance syndrome pseudohypoparathyroidism type 1b (PHP1b). Previous work suggested that PHP1b arises from a defect in renal PTHr function that is not caused by mutations affecting PTHr sequence or splicing of P1- or P2-specific transcripts (27, 34–37). The broad activity of P3 suggests that PHP1b does not arise from mutations in proximal P3 promoter sequences. A defect in a kidney-specific PTHr gene enhancer could give rise to PHP1b in some patients. However, results point more toward mutations that disrupt function of a kidney-specific transcription factor that drives renal PTHr gene expression, or a defect in a downstream component of the PTH/PTHr signaling pathway, as supported by preliminary mapping studies that exclude the PTHr gene in some families (37).

P2 is the major human PTHr promoter during midgestational fetal development. Although P3-specific transcripts were readily detectable by RT-PCR in adult human tissues and 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. No evidence was found for activity of P1 in 17.5- or 19-week FA kidney (Fig. 6, and data not shown). In contrast, P2-specific transcripts were detected in several fetal tissues, including kidney, long bone, and calvaria (Fig. 6, and data not shown). This indicates that the highly conserved P2 promoter controls PTHr gene expression in kidney at midgestation, and that P1 and P3 function later.

Very little is known about PTHr function in fetal kidney. Although the placenta plays an important role in fetal calcium homeostasis, PTH or PTHrP may control fetal renal tubular function, providing a role for the PTHr expressed in fetal kidney (38). Upregulation 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

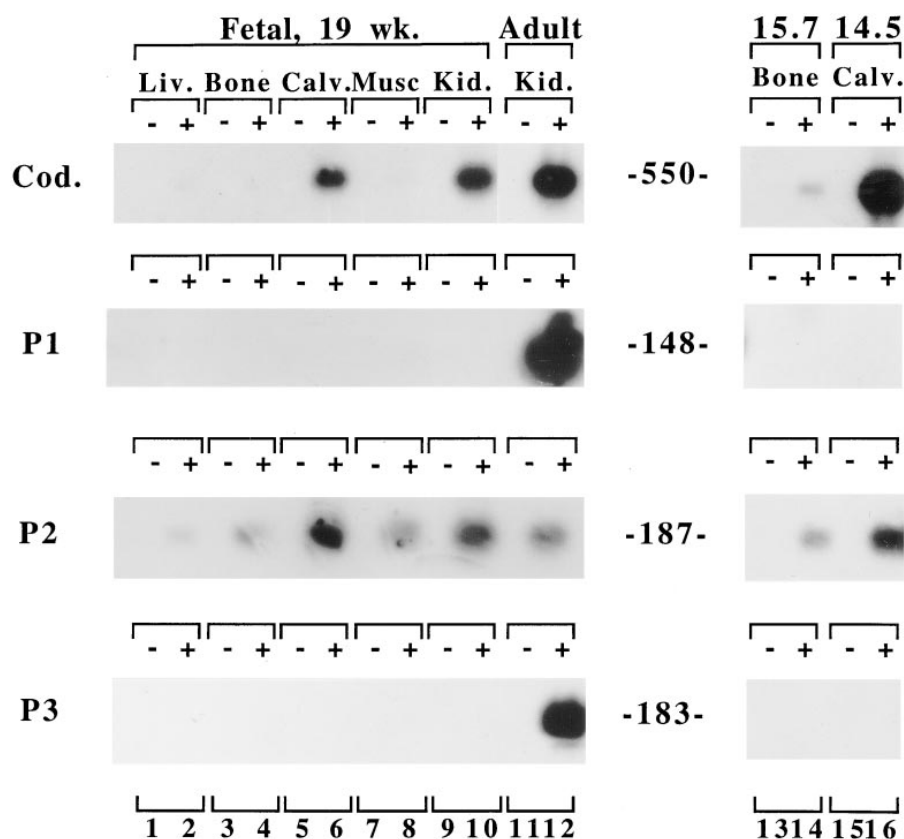


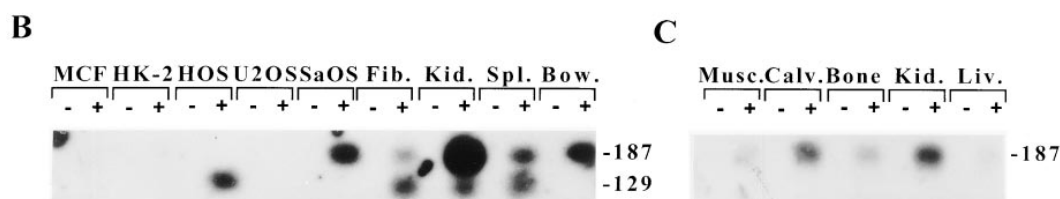
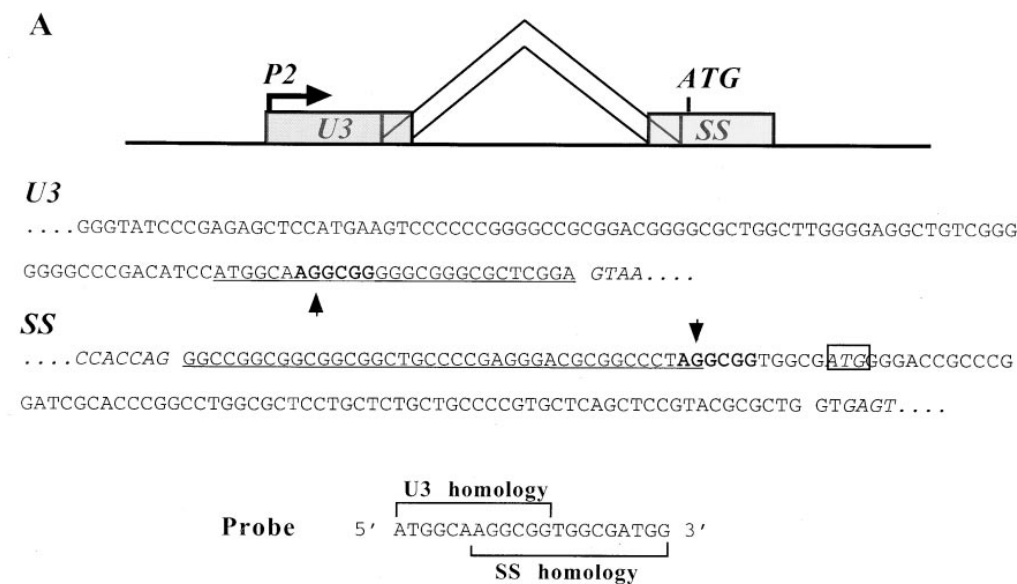
Figure 6. Expression of P2, but not P1 or P3 during the midgestational stage of fetal development. Expression of coding and promoter-specific PTHR sequences was analyzed by RT-PCR using the conditions described in Fig. 4, except that all products were amplified beyond the linear range (see Methods for 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.

largely by the placenta. At this stage, it would be expected that PTHR expression would be stimulated to accommodate the increased function of the kidneys. Stimulation of PTHR expression would increase the inhibitory effects of PTH on renal reabsorption of sodium, bicarbonate, and phosphate through its downregulation of Na^+/H^+ and $\text{Na}^+/\text{phosphate}$ exchangers. Downregulation of both exchangers by PTH is at least partly dependent on stimulation of phospholipase C (39, 40). Studies in renal epithelial LLC-PK1 cells expressing variable levels of receptors has shown that, unlike the response to cAMP, PTH stimulation of the PTHR is strongly dependent on receptor number (41). Thus, increasing the density of PTHR would selectively enhance PTH stimulation of phospholipase C.

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 postconception (13, 14). However, in rodents, kidney development continues after birth (42), 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 (13, 14). 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 (reference 25; and data not shown). Our present results 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 9–12 weeks FA in calvarial bone, and continues throughout development (43). Taken together, these results suggest that, despite 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. Regulation of PTHR gene expression in adult bone is likely to be more complex in human than in mouse, because both P2 and P3 were found to function in the human osteoblast-like cell lines HOS and SaOS-2 (Fig. 4).

Differential splicing of P2-specific transcripts. We also found that transcripts are differentially splicing between U3, and SS gives rise to a shortened 5' UTR. Both long and short UTRs are formed in kidney, spleen, and skin fibroblasts, whereas only the short UTR was detected in HOS cells (Fig. 7). No evidence for alternative splicing was found in fetal tissues. The splice acceptor contains the sequence UAGG, which corresponds to the consensus YAGG for AG acceptor sites. The corresponding splice donor sequence, GCGGGG, diverges from the consensus GURAGU (44, 45). However, this sequence corresponds to a variant that has been described (46) and which is considered to function by interacting with the U1 snRNA. Secondary structure analysis suggests that the shorter UTR has a less stable stem-loop structure in the region of the initiator ATG (data not shown), suggesting that it may be more efficiently translated. Extensive secondary structure in 5' UTRs can inhibit translational initiation (47, 48). Further studies will have to be performed to assess the impact of the shorter 5' UTR on translation of PTHR gene transcripts.



gonucleotide probe used for Southern analysis that corresponds to the fusion between U3 and SS caused by the differential splicing 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.

In summary, we have shown that expression of the PTHR gene in human and in mouse is regulated differently in the adult. The P3 promoter, which is apparently specific to human, drives the majority of renal PTHR gene transcripts and functions in a number of other tissues. P2 promoter activity is apparently well conserved, whereas P1 activity is much weaker in human than in mouse. P2 alone drives PTHR gene expression at midgestation, indicating that factors controlling P2 regulate PTHR gene expression during skeletal development. These results document the complexity of promoter functions that control the developmental- and tissue-specific expression of the human PTHR gene.

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Figure 7. 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 (*italics*) of the PTHR coding sequence is 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 and acceptor sites cannot be determined unambiguously because of the sequence AGGCCG (*bold*) present in both exons. The sequence of the oli-

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