Abstract

The parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor (PTHR) functions in skeletal development and mediates an array of other physiological responses modulated by PTH and PTHrP. PTHR 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 PTHR transcripts in the adult. No P3 activity was detected in mouse kidney, indicating that renal PTHR 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 PTHR gene expression during skeletal development. Our results indicate that human PTHR 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 PTHR gene transcription and mRNA splicing are developmentally regulated. Moreover, our data indicate that renal and nonrenal PTHR gene expression are tightly coordinated in humans. (J. Clin. Invest. 1998. 102:958–967.) Key words: gene transcription • differential splicing • renal development • calcium homeostasis • tissue-specific promoter

Introduction

Signaling through the parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor (PTHR) 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 PTHRs expressed in kidney and bone. Stimulation of renal and osseous PTHRs 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 PTHR is widely expressed. In the rat, the PTHR 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 PTHR in skeletal development has been highlighted by studies of Jansen-type metaphyseal chondrodysplasia, a rare form of dwarfism associated with hypercalcemia despite normal circulating levels of PTH and PTHrP. Mutations in transmembrane domains of the PTHR 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 PTHR null mice displayed similar phenotypes, with reduced proliferation and accelerated maturation of chondrocytes associated with accelerated ossification.

The PTHR 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 PTHR 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 PTHR 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 PTHR transcription in 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 PTHR transcription in 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 PTHR transcription in 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 PTHR transcription in 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).
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 (10⁵ 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-ethanesulfonic acid (pH 6.4), 0.4 mol/liter sodium acetate, and 1 mmol/liter ethylene-diaminetetraacetic acid), 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′-CTATCGGAGCCCAGCGGTCC-3′), which is complementary to sequences starting 61 bp upstream of the SS exon, was labeled for 1 h at 72°C with [α-32P]-GTP using T4 polynucleotide kinase. One tenth of this reaction was incubated with 10 µg of either adult human kidney total RNA or yeast RNA 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 run 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 RNAsin (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× DNeasy buffer (Promega) containing 0.5 U of DNeasy 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 MgCl₂, 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′-AGGAGGAATTCATGTCATCCACCAGGC-3′ as the forward primer (primer 1) and 5′-GACGTACATCTGACATCCACCACGC-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′-AGTGTGTTGCTTGATCAGCCTACA-3′ (primer 2) and reverse primer 5′-GCTCCGGCTGGCAACT-TCAGTGC-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′-CACCACACTAGGATCTCTGGTG-3′, and the reverse primer used was 5′-GATTGTCTGTATCTCCTGTCG-3′. For β-actin, the forward primer was 5′-GCAGTTGCACT-CTCCTGACG-3′ and the reverse primer was 5′-GGCATGGTGGAGCACC-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′-AGGAGGAATTCAGGTAGGACCCG-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) in 5% formamide and 5 µl of Taq DNA polymerase. The forward primer was 5′-GGCATGGTGGAGCACC-3′ (primer 3), 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× NET (100 mM Tris-HCl [pH 7.5], 0.9 M NaCl, 6 mM EDTA), 10× Denhardt’s, 0.5% SDS, 10 mg/ml denatured salmon sperm DNA at 62°C, and then hybridized in 6× NET, 5× Denhardt’s, 0.5% SDS, 1 mg/ml denatured salmon sperm DNA with 10⁶ cpm of end-labeled oligonucleotides for 5 h at 62°C. Oligonucleotides used for probing were: 5′-CCATAGGGCCCGTTCGGTACGG-3′, for P2- and P3-containing sequences, 5′-CTGGACGTCTAGGCGGTCC-3′, for P1-specific sequence, and 5′-GGCATGGTGGAGCACC-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 pCR2 (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 BglII site. The insert was excised from pSKb as a BamHI-BglII fragment and inserted in the polylinker of the promoterless luciferase plasmid pXP2 (29). To construct P2-luciferase recombinant pXP2-P2, a 1.1-kb Sacl fragment (containing P2 sequences –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-BglII fragment and subcloned into pXP2. To construct pXP2-P3, a 1.1 kb NcoI-AvrII fragment (2843 to 1245) 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.

Cell culture and transient transfections. SaOS-2 cells (ATCC) were propagated in McCoy 5A media + 10% fetal bovine serum. HOS and
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 μg/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 μg 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 μl 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. 1A), 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. 1A, 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. 1B, 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. 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 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. 1D, lanes 1–3). Indeed, under these conditions P1-specific transcripts were detected only after prolonged exposure (Fig. 1D, lane 4).

P3 initiation sites were mapped by primer extension analysis using a primer (Fig. 1A) 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.

P2 and P3 promoters lie within a CpG island. The sequence of human genomic DNA between exons U3 and SS (Fig. 3B) 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 0.12 (Fig. 3A; reference 27). In contrast, a 1.1-kb region around human P3 contains only 11 CpG dinucleotides and a CpG/GpC ratio of 0.12 (Fig. 3A; 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. 3B). 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-
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-
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 (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.

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.

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 AGGCCG 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
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/PTHrP 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.
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 ex-
pression would increase the inhibitory effects of PTH on renal
reabsorption of sodium, bicarbonate, and phosphate through
its downregulation of Na+/H+ and Na+/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 PTHRs would se-
lectively 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 recep-
tor 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 de-
fects 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 osteo-
blast 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 devel-
opment (43). Taken together, these results suggest that, de-
spite 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 con-
served. 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 osteo-
blast-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 evi-
dence for alternative splicing was found in fetal tissues. The
splice acceptor contains the sequence UAGG, which corre-
sponds to the consensus YAGG for AG acceptor sites. The
corresponding splice donor sequence, GCGGGG, diverges
from the consensus GURAGU (44, 45). However, this se-
quence 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
5’ UTRs can inhibit translational initiation (47, 48). Further stud-
ies will have to be performed to assess the impact of the
shorter 5’ UTR on translation of PTHR gene transcripts.
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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References


