DEVELOPMENTAL BIOLOGY 175, 166–176 (1996)
ARTICLE NO. 0104

Peptide (PTHrP) Results in Abnormal Postnatal Bone Development

Norio Amizuka,*,†,‡ Andrew C. Karaplis,*,§ Janet E. Henderson,*,§ Hershey Warshawsky,¶ Mark L. Lipman,*,‖ Yutaka Matsuki,** Sadakazu Ejiri,‡ Mikako Tanaka,†† Naoya Izumi,‡ Hidehiro Ozawa,‡ and David Goltzman*,†,‡‡

**Department of Medicine, ¶Department of Anatomy and Cell Biology, and ‡‡Department of Physiology, McGill University, Montreal; †Calcium Research Laboratory, Royal Victoria Hospital; and §Division of Endocrinology and ‖Division of Nephrology, Sir Mortimer B. Davis-Jewish General Hospital, Lady Davis Institute for Medical Research, Montreal, Quebec, Canada; and **Periodontology, ††1st Prosthodontics, and ‡1st Department of Oral Anatomy, Niigata University School of Dentistry, Niigata, Japan*

Although apparently phenotypically normal at birth, mice heterozygous for inactivation of the gene encoding parathyroid hormone-related peptide (PTHrP) develop haplotype insufficiency by 3 months of age. In addition to histologic and morphologic abnormalities similar to those seen in homozygous mutants, heterozygous animals demonstrated alterations in trabecular bone and bone marrow. These included metaphyseal bone spicules which were diminished in volume, irregularly distributed, and less well developed than those seen in age-matched controls as well as bone marrow, which contained an inordinate number of adipocytes. A substantial reduction in PTHrP mRNA was detected in heterozygous tissue, while circulating parathyroid hormone (PTH) and calcium concentrations were normal. Thus, while a physiologic concentration of PTH was capable of maintaining calcium homeostasis, it was incapable of compensating for PTHrP haploinsufficiency in developing bone. In normal animals, both PTHrP and the PTH/PTHrP receptor were expressed predominantly in chondrocytes situated throughout the proliferative zone of the tibial growth plate. In the metaphysis, the PTH/PTHrP receptor was identified on osteoblasts and preosteoblastic cells situated in the bone marrow, while PTHrP was expressed only by osteoblasts. These observations indicate that postnatal bone development involves susceptible pathways that display exquisite sensitivity to critical levels of PTHrP and imply that the skeletal effects of PTH are influenced by locally produced PTHrP. Moreover, identification of both the ligand and its N-terminal receptor in metaphyseal osteoblasts and their progenitors suggests an autocrine/paracrine role for the protein in osteoblast differentiation and/or function. Impairment in this function as a consequence of PTHrP haploinsufficiency may critically influence the course of bone formation, resulting in altered trabecular architecture and perhaps low bone mass and increased bone fragility.

INTRODUCTION

Parathyroid hormone-related peptide (PTHrP)[1] is an oncoprotein involved in the pathogenesis of malignancy-associated hypercalcemia (Strewler *et al.*, 1987; Burtis *et al.*, 1987; Suva *et al.*, 1987). The sequences of cDNAs and genes encoding this peptide have been determined (Mangin *et al.*, 1988; Yasuda *et al.*, 1989a,b; Karaplis *et al.*, 1990). The homology in the NH_2-terminal region of parathyroid hormone (PTH) and PTHrP facilitates their interaction at a common target tissue receptor (Jüppner *et al.*, 1991; Abou-Samra *et al.*, 1992) and results in similar activities with respect to bone metabolism and systemic calcium homeostasis (Rodan *et al.*, 1983; Horiuchi *et al.*, 1987; Kemp *et al.*, 1987; Rabbani *et al.*, 1988; Thompson *et al.*, 1988) Unlike PTH, whose expression is localized to parathyroid cells, PTHrP

[1] Abbreviations used: PTHrP, parathyroid hormone-related peptide; PTH, parathyroid hormone; RT-PCR, reverse transcriptase–polymerase chain reaction; SEM, scanning electron microscopy; TEM, transmission electron microscopy; ALPase, alkaline phosphatase; PT cell, parathyroid hormone target cell.

0012-1606/96 $18.00

is produced in various normal cells and tissues, including keratinocytes, heart, smooth muscle, many endocrine cells, and lactating mammary glands (Merendino *et al.*, 1986; Thiede and Rodan, 1988; Thiede *et al.*, 1990; Drucker *et al.*, 1989; Kramer *et al.*, 1991; Deftos *et al.*, 1993). In parallel with the broad distribution of PTHrP, the PTH/PTHrP receptor has been reported to be widespread in various tissues (Ureña *et al.*, 1993), despite the fact that it had previously been considered to be localized only to the classical PTH target tissues of bone and kidney. This widespread distribution of ligand and receptor suggests that PTHrP may act in an autocrine and/or paracrine mode (Goltzman *et al.*, 1989; Orloff *et al.*, 1989). Evidence to support such a mechanism of action has been provided by studies with antisense inhibition of PTHrP synthesis *in vitro* (Kaiser *et al.*, 1992, 1994), by experiments in transgenic mice which overexpress PTHrP (Wysolmerski *et al.*, 1994; Vasavada *et al.*, 1994), and by studies of PTHrP gene inactivation in mice via homologous recombination (Karaplis *et al.*, 1994; Amizuka *et al.*, 1994). All these approaches have provided evidence for a role for PTHrP in normal cell proliferation and differentiation in addition to its role in cancer.

A central role for PTHrP in skeletal development has been demonstrated in mice homozygous for PTHrP gene inactivation. We have previously examined the dyschondroplastic abnormalities and altered endochondral bone formation in these animals (Karaplis *et al.*, 1994; Amizuka *et al.*, 1994) that culminate in their death in the immediate peripartum period. In contrast, heterozygous PTHrP null mice are phenotypically normal at birth. Surprisingly, however, these animals develop dysmorphic features, similar to those observed in homozygotes, that became readily apparent by 3 months of age. In the present study we examined these mice biochemically and morphologically for evidence of a PTHrP haploinsufficient phenotype.

MATERIALS AND METHODS

Generation of Mice Heterozygous for PTHrP Gene Inactivation

Mice carrying a null mutation of the PTHrP gene were generated using the technique of homologous recombination (Karaplis *et al.*, 1994). Wild-type litter mates and fetuses heterozygous for the mutation were identified by Southern blot analysis of genomic DNA and used for subsequent analysis. For staining and visualization of skeletons, mice were dissected and stained with alizarin red S, as previously described (Karaplis *et al.*, 1994).

Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) for PTHrP

Kidneys from 3-month-old wild-type (+/+) and heterozygous (+/−) litter mates were snap-frozen and total RNA was extracted from 20 mg of tissue using the RNeasy Total RNA kit (Qiagen, Chatsworth, CA). For first-strand cDNA synthesis, 5 μg of total RNA was primed with oligo(dT) and reverse transcribed using SuperScript II RNase H-reverse transcriptase (Gibco BRL, Gaithersburg, MD).

Quantitative PCR was then applied to precisely determine the amount of glyceraldehyde-6-phosphate dehydrogenase (GAPDH) transcripts in each sample as described previously (Lipman *et al.*, 1994). Subsequently, PCR was performed to amplify PTHrP transcripts using the dilutions of normal (cDNA/competitive template (c.t.) of 1.0) and heterozygote cDNA (cDNA/c.t. of 1.0) that had been shown by densitometric scanning to contain equal amounts of GAPDH transcripts. Sense (OC1: 5′-GCT GTG TCT GAA CAT CAG CTA CT-3′) and antisense (OC2: 5′-GAT CCC AAT GCA TTT ACA GTA TG-3′) primers were used to amplify the endogenous 531-bp PTHrP fragment. The thermal cycling parameters consisted of denaturing at 94°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 1 min for 35 cycles. Ten-microliter reaction aliquots were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

Determination of Plasma Calcium and Plasma PTH Concentrations

Plasma was harvested from heparinized blood obtained by cardiac puncture from anesthetized wild-type and heterozygous mice. Calcium was determined by autoanalyzer (Technicon Laboratories, New York, NY) and PTH with an immunoradiometric assay that detects both intact PTH (1–84) and N-terminal PTH (1–34) in rodents (Nichols Institute, San Clementi, CA).

Histological and Ultrastructural Analyses

Wild-type and heterozygous mice were perfused via the left ventricle with a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.067 *M* phosphate buffer (pH 7.4) for 10 min. The femora and tibiae of these mice were then dissected free and maintained in the same solution overnight at 4°C. For analysis by scanning electron microscopy (SEM), specimens of long bones were split and soft tissues eliminated by immersion in hypochlorite prior to postfixation with 1% OsO_4, dehydration, and critical point drying. Visualization was with a Hitachi S-570 microscope operated at 20 kV. Specimens for light and transmission electron microscopy (TEM) were processed as described previously (Warshawsky and Moore, 1967).

Histomorphometric Analysis

For histomorphometric analysis, 3-month-old mice heterozygous (+/−) for ablation of the PTHrP gene and wild-type (+/+) littermates were injected intraperitoneally with 10 μg/100 g body weight calcein (Wako Pure Chemicals, Osaka, Japan) and sacrificed by cervical dislocation 24 hr later. Tibiae were cleaned and kept in 70% ethanol for 5 days at 4°C, stained according to Villaneuva (1974; Villanueva and Lundin, 1989) and dehydrated in graded ethanol prior to embedding in methyl methacrylate (Wako). Polymerized blocks were ground to the midpoint of the longitudinal axis and analyzed using a confocal laser-scanning microscope (LSM-GB200; Olympus, Tokyo, Japan) and a Hewlett Packard Vecta 396/25 equipped with GB200 3D imaging software. The number (TbN/mm^2) of trabeculae as well as the trabecular volume [BV/TV(%)] was measured in the tibial metaphysis of eight wild-type and seven heterozygous animals. Statistical analysis was by Student's *t* test.

Radioautographic Studies

Chondrocyte proliferation was evaluated *in vivo* using [^{3}H]-thymidine labeling as described previously (Amizuka *et al.*, 1994). *In vivo* binding studies using iodinated PTH and PTHrP were performed as previously reported (Rouleau *et al.*, 1986, 1988, 1990). Synthetic [Tyr^{36}] PTHrP (1–36)-NH_2 (Peninsula Laboratories, Belmont, CA) and PTH (1–34) (Bachem Corp., Torrance, CA) were labeled with ^{125}I to a specific activity of 300 μCi/μg using the lactoperoxidase technique (DeMay *et al.*, 1985). ^{125}I-labeled peptides were injected either alone (experimental animals) or with a 500-fold excess of unlabeled hormone (control animals).

Immunocytochemistry for PTHrP, Type II Collagen, and Chondroitin Sulfate

Anesthetized normal mice were perfused with a solution of 2% paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.4) for 10 min. Femora and tibiae were then dissected free and additionally fixed in the same solution for 4 hr at 4°C. Specimens were decalcified in 10% EDTA for 1 week, embedded in OCT compound, and frozen by immersion in liquid nitrogen. Cryostat sections (10 μm thick) were collected on poly-L-lysine-coated glass slides (Polyscience Inc., Warrington, PA) and then treated with a mixture of 95% methanol and 0.3% H_2O_2 for 30 min to inhibit endogenous peroxidase.

PTHrP immunocytochemistry was performed as described previously (Amizuka *et al.*, 1994) using polyclonal antisera raised against synthetic PTHrP (1–34) (17, 36) or PTHrP (87–110). Type II collagen and chondroitin sulfate immunocytochemistry were performed on longitudinal frozen sections of femora and tibiae of wild-type and heterozygous littermates as previously described (Amizuka *et al.*, 1994).

In Situ Hybridization of PTHrP, PTH/PTHrP Receptor, and Alkaline Phosphatase (ALPase)

For *in situ* hybridization to localize mRNAs encoding PTHrP, PTH/PTHrP receptor, and ALPase, femora and tibiae were harvested from wild-type mice and Sprague–Dawley rats as described and embedded in paraffin after decalcification. Paraffin sections (4–6 μm thick) on poly-L-lysine-coated glass slides were deparaffinized and rehydrated in decreasing concentrations of ethanol prior to rinsing with PBS. Sections were treated with 4% paraformaldehyde in 0.1 *M* phosphate buffer for 15 min, with proteinase K (10 μg/ml) in 10 m*M* Tris–HCl (pH 8.0) at 37°C for 15 min, and then with 0.2 *M* HCl for 10 min. Acetylation was achieved by incubating for 10 min with 0.25% acetic anhydride in 0.1 *M* triethanolamine (pH 8.0).

Sense and antisense cRNA probes for PTHrP were obtained respectively by transcription of a 620-bp fragment of rat PTHrP cDNA and a 537-bp fragment of the hPTHrP gene (Kaiser *et al.*, 1992) using digoxigenin-labeled UTP (Boehringer Mannheim, Mannheim, Germany). Sense and antisense cRNA probes for the PTH/PTHrP receptor were obtained from cDNA (kindly provided by Dr. Abdul Abou-Samra, Massachusetts General Hospital, Boston, MA) employing T7 and SP6 RNA polymerase, respectively. Antisense cRNA for ALPase was prepared from a 2.5-kb fragment, rAP54 (kindly provided by Dr. Gideon A. Rodan, Merck, Sharp and Dohme Laboratories, West Point, PA), inserted into the *Eco*RI site of pBS(+) (Stratagene, La Jolla, CA). Probes were reduced in length to an average of 300 bases, a size appropriate for hybridization in tissues (Cox *et al.*, 1984).

A hybridization mixture composed of 50% formamide, 10 m*M* Tris–HCl, pH 7.6, 100 μg tRNA, 1× Denhardt's solution, 10% dextran sulfate, 600 m*M* NaCl, 0.25% SDS, and 1 m*M* EDTA was preheated for 10 min at 90°C. cRNAs were adjusted to a concentration of 0.1–1.0 μg/ml (determined by the protocol of Boehringer Mannheim) and denatured by heating at 90°C for 2–3 min before applying to the sections. After hybridizing overnight at 50°C, sections were washed with 50% formamide in 2× SSC at 50–55°C for 30 min and treated at 37°C with a solution of 10 m*M* Tris–HCl (pH 8.0), 0.5 *M* NaCl, and 1 m*M* EDTA (TNE). Non-specific binding of probes was reduced by RNase A treatment (20 μg/ml in TNE solution) at 37°C for 30 min.

Digoxigenin-labeled sections were preincubated with 2% blocking agent and incubated for 45–60 min with alkaline phosphatase-conjugated sheep anti-digoxigenin antibody at a dilution of 1:1000. Visualization was achieved using nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate.

For double-labeled *in situ* hybridization, radioactive sense and antisense cRNA probes for the PTH/PTHrP receptor were prepared from the cloned cDNA using [^{35}S]UTP (Amersham Canada, Oakville, ON). Digoxigenin-labeled probes, prepared as described above, were used for PTHrP. After reduction of the average probe length to 300 bases, radioactive antisense probes (5–10 × 104 cpm/μl) and digoxigenin-labeled probes were added together in the same hybridization mixture. Sections were then incubated with the hybridization mixture as described above. Immunodetection of digoxigenin-labeled probes was performed, as described above, prior to detection of radioactivity by radioautography. For radioautography, sections were dipped in Kodak NTB-2 liquid emulsion and exposed for 2 weeks in the dark at 4°C. Sense probes were used as controls.

RESULTS

Gross Morphology and Molecular and Biochemical Characteristics of Heterozygous Mice

Mice with only one functional allele for PTHrP displayed no obvious abnormalities until 3 months postpartum, at which time they developed short, blunt snouts relative to their wild-type litter mates. Undecalcified preparations of the heads of these animals, stained with alizarin red, revealed incisal malocclusion due to reduced protrusion of the maxilla. We therefore examined the possibility that the heterozygous animals were displaying a PTHrP haploinsufficient phenotype; i.e., two copies of the gene are required for a normal phenotype.

Using RT-PCR, we first compared the relative amounts of PTHrP mRNA transcripts in kidney tissue removed from 3-month-old wild-type and heterozygous littermates. The cDNAs to be compared were standardized to contain equal quantities of GAPDH RT-cDNA. This was accomplished by coamplification of a constant amount of GAPDH competitive template and serially diluted cDNAs (Fig. 1A). The dilutions of normal and heterozygote cDNA that gave equal band intensities (determined by scanning densitometry) were subsequently used to amplify PTHrP cDNA. As shown in Fig. 2B, the PTHrP transcript level was much reduced in the heterozygote kidney compared to that of the wild-type tissue, suggesting that gene dosage did indeed exist. In contrast to this striking reduction in the level of tissue PTHrP

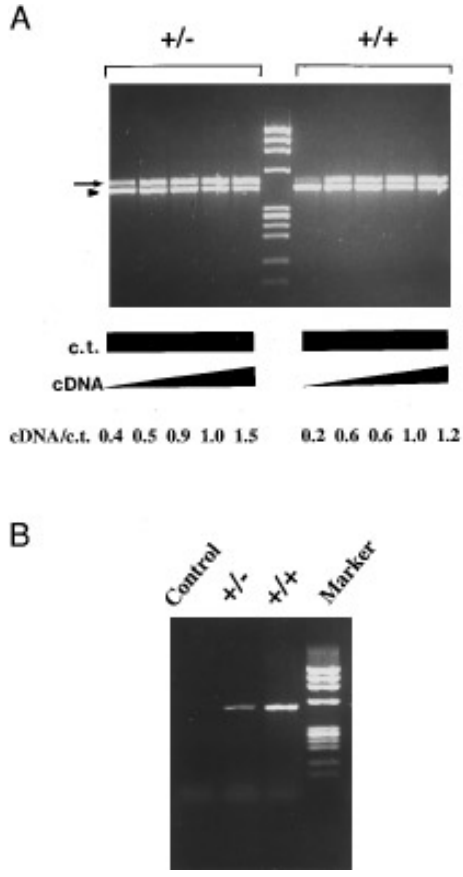


FIG. 1. Comparative analysis of PTHrP mRNA transcripts in 3-month-old animals by RT-PCR. (A) One nanogram of GAPDH competitive template (c.t.) was coamplified with serial twofold dilutions of reverse-transcribed cDNA derived from wild-type (+/+) or heterozygous (+/−) kidneys. The top band represents the cDNA amplification product (arrow), while the bottom band is derived from amplification of the c.t. (arrowhead). (Bottom) The results of densitometric scanning of the respective bands are shown. (B) cDNA calibrated to contain identical GAPDH transcript equivalents (giving a ratio 1.0 cDNA/c.t. in both cases) was used to amplify PTHrP transcripts. The band intensity of the amplification product from the heterozygous kidney compared to that from the wild-type kidney provided a ratio of 0.3. Control, no cDNA added. The molecular weight marker is a ϕX174DNA–*Hae*III digest.

transcripts, heterozygous mice displayed normal concentrations of plasma calcium (1.94 ± 0.09 m*M;* normal, 1.97 ± 0.11 m*M;* each value is the mean ± SEM of six determinations) and of plasma PTH (33.7 ± 13.9 pg/ml; normal, 37.9 ± 11.1 pg/ml; each value is the mean ± SEM of six determinations).

Histological Alterations of Long Bones in Heterozygous Mice

Given the dysmorphic features and reduced level of PTHrP mRNA in the heterozygotes, we examined the long bones of these animals histologically for further evidence of haploinsufficiency. Using SEM, the three-dimensional structure of the femur of a 3-month-old heterozygote (Fig. 2B) was compared with that of its wild-type littermate (Fig. 2A). Although the overall size of the distal portion of these bones was similar, the epiphyseal head was smaller and the epiphyseal cartilage reduced in size in the heterozygotes. In the metaphysis of the wild-type mouse, bone spicules were of uniform size and extended parallel to the longitudinal axis, whereas those of the heterozygote were not oriented longitudinally and were of variable thickness and length. These alterations were prominent in 3-month-old heterozygotes but of minor consequence in 1.5-month-old mice. There were no apparent abnormalities in cortical bone at either time point.

Longitudinal histological sections (Figs. 2C and 2D) of tibiae and femora from heterozygous mice revealed fewer mixed spicules, which also had an abnormal orientation, in metaphyseal bone. In addition, an inordinate number of adipocytes were observed in the bone marrow of these 3-month-old mice compared with that in their wild-type litter mates (compare Figs. 2C and 2D). Nonmarrow cells in the intertrabecular regions occasionally also contained lipid droplets. At 1.5 months there were fewer adipocytes in the marrow and lipid droplets were not seen in nonmarrow cells in this region.

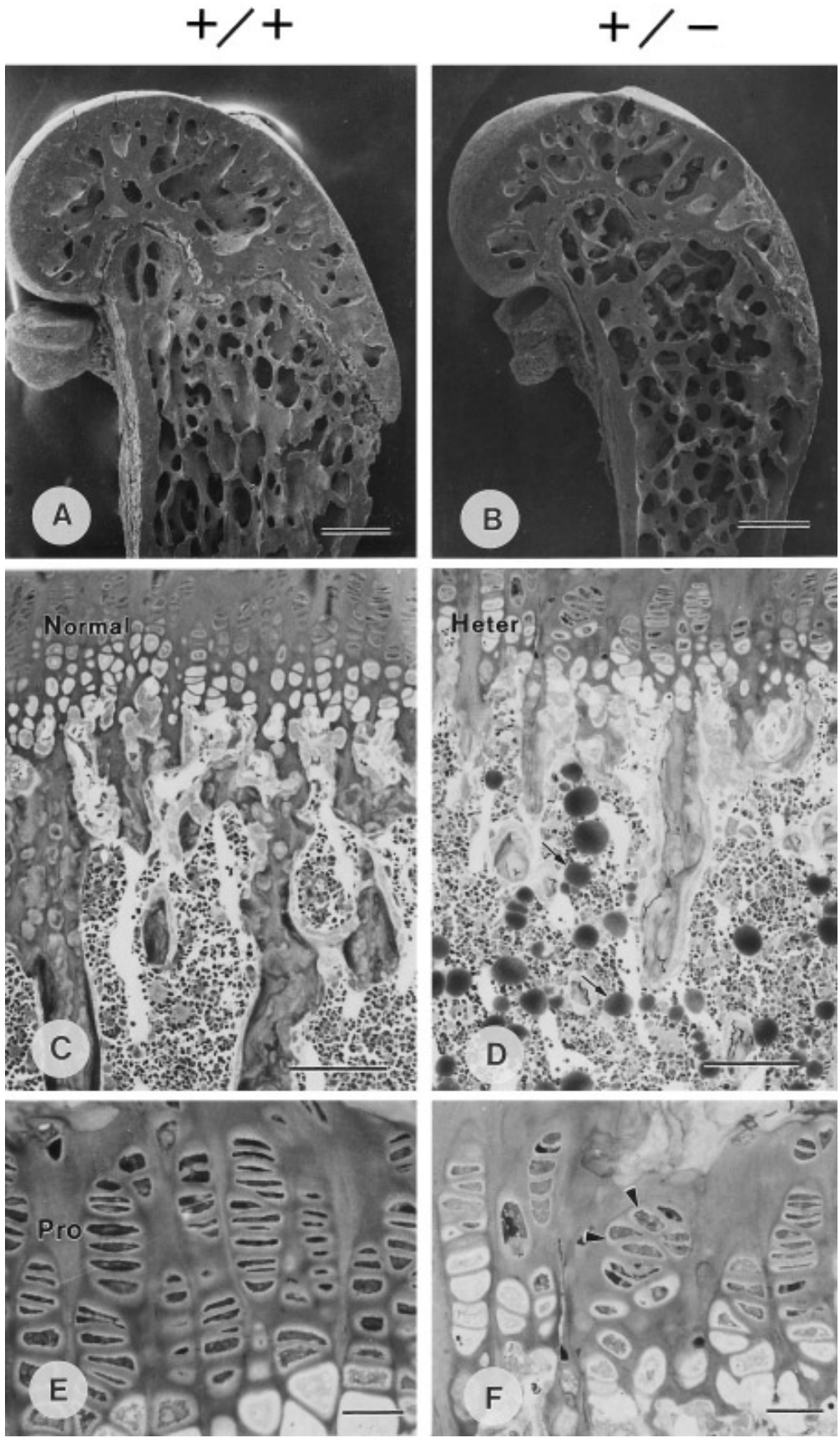
In the growth plate of the heterozygotes, clusters of chondrocytes disrupting the orderly columns were often observed (compare Figs. 2E and 2F), resulting in distortion of the longitudinal cartilage partitions. This local failure of chondrocytes to assemble the correct cartilage matrix architecture most probably accounts for the abnormal distribution and size of mixed spicules observed under SEM in the metaphyseal bone tissue of the heterozygotes.

In keeping with the abnormalities seen in homozygous mutants, chondrocytes of 3-month-old heterozygotes contained numerous dispersed glycogen granules throughout their cytoplasm and [^{3}H]thymidine incorporation was slightly but significantly decreased (3.03 ± 0.09% versus 3.38 ± 0.09%; mean ± SEM of six determinations in two experiments, $P < 0.05$). In contrast, their capacity to express matrix-associated proteins appeared to be unaltered. Immunostaining for type II collagen and chondroitin sulfate showed similar patterns of distribution in normal and heterozygous animals, as did *in situ* hybridization for ALPase mRNA (data not shown).

As shown in Fig. 3, histomorphometric analysis of the proximal tibial metaphysis of wild-type (+/+) and heterozygous (+/−) mice revealed a significant reduction in trabecular bone volume although the numbers of trabeculae were not significantly different.

Localization of PTHrP and the PTH/PTHrP Receptor

In light of our observations documenting the existence of a haploinsufficient phenotype in heterozygous PTHrP mutants and to assess the role played by PTHrP in their skeletal


+/+
+/−
A
B
Normal
Heter
C
D
Pro
E
F

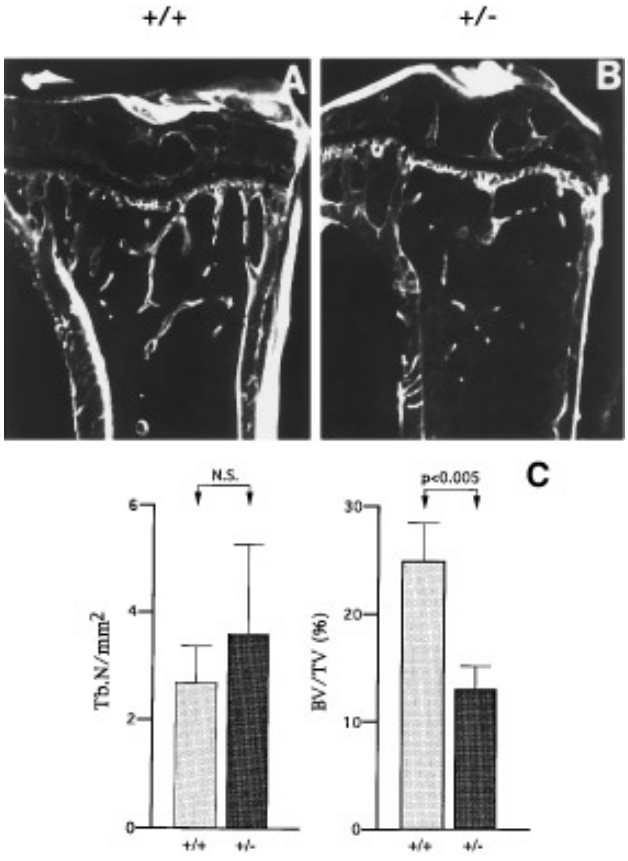


FIG. 3. Confocal laser microscopy of the tibial metaphysis of 3-month-old wild-type (+/+) and heterozygous (+/−) mice demonstrating bone formation surfaces represented by calcein (white) labeling. In wild-type tibiae (A), trabeculae are well formed and run continuously parallel to the longitudinal axis of the bone while those of heterozygous mice (B) form disorganized clusters with no apparent longitudinal alignment. Less calcein deposition is seen in the heterozygous bone. (C) The results of histomorphometric analysis of the tibial metaphysis of wild-type (+/+) and heterozygous (+/−) animals. Bars represent the mean ± SD of measurements for eight wild-type and seven heterozygotes for trabecular bone area expressed as a percentage of total metaphyseal area [BV/TV (%)] (right) and for number of trabeculae/mm^2 of metaphyseal area [Tb.N/mm^2] (left).

abnormalities, we examined the distribution of both the ligand and its receptor in bone tissue from normal (+/+) and heterozygous (+/−) animals. As the results from these localization studies were similar in both groups of animals, only the results from normal animals have been shown.

Both PTHrP mRNA (Fig. 4A) and protein (Fig. 4B) were expressed most intensely by plump osteoblasts on mixed spicules close to the epiphyseal plate. Flattened osteoblasts (bone lining cells) in the epiphysis and diaphysis demonstrated weaker reactivity, as did some superficial osteocytes. Osteoclasts revealed neither gene expression nor immunoreactivity for PTHrP. No reactivity was evident in sections treated with a sense probe for *in situ* hybridization (Fig. 4C) or with preadsorbed serum for immunocytochemistry (Fig. 4D).

PTH/PTHrP receptor gene expression was distributed mainly in the proliferative zone of the epiphyseal growth plate, including the uppermost region of the hypertrophic zone, but not in the lower hypertrophic zone (Fig. 5A). In bone tissue, mature osteoblasts on the bone surface and nonmarrow ''preosteoblastic'' cells (previously called PT cells by us) located in the intertrabecular region expressed mRNA encoding the PTH/PTHrP receptor (Fig. 5B). PT cells are characterized by having minimal rough endoplasmic reticulum, many vesicles, abundant glycogen granules, and scattered Golgi apparatus (Rouleau *et al.*, 1988, 1990). A similar distribution of binding of both ^{125}I-labeled PTHrP and ^{125}I-labeled PTH to both mature osteoblasts and PT cells was observed by light microscope radioautography after *in vivo* injection (Fig. 5C). Using electron microscope radioautography, iodinated ligand was found to bind to the surface of both chondrocytic and osteoblastic cell types.

Double-labeled *in situ* hybridization for PTHrP and its receptor confirmed that PTHrP mRNA is expressed in the metaphysis mainly in osteoblasts (Fig. 6A), whereas mRNA encoding the PTH/PTHrP receptor is expressed in both osteoblasts and nonmarrow cells between the spicules (Figs. 6A and 6B).

DISCUSSION

Our studies have demonstrated that mice heterozygous for targeted ablation of the PTHrP gene demonstrate haploinsufficiency, that is, a single copy of the PTHrP gene is inadequate for the development of a normal phenotype. Morphologically, the defects in heterozygotes appear somewhat similar to those seen in mice homozygous for PTHrP gene ablation (Amizuka *et al.*, 1994), although the alterations in trabecular bone structure and the infiltration of

FIG. 2. (A and B) Scanning electron micrographs of femora from normal (A) and heterozygous (B) littermates. The overall sizes of these femora are similar. However, in the metaphysis of the normal femur mixed spicules are aligned in parallel along the longitudinal axis, whereas in the metaphysis of heterozygotes, mixed spicules are irregularly distributed and not oriented longitudinally. (C–F) Longitudinal sections of tibiae from normal (C, E) and heterozygous (D, F) littermates. Mixed spicules in the tibia of the heterozygote (D) are less well developed compared to those of normal mice (C). Numerous adipocytes (arrows) can be seen in the bone marrow of the heterozygote. In the epiphyseal growth plate of the normal tibia (E), chondrocyte columns extend parallel to the longitudinal axis, whereas in the heterozygotes (F) clusters of chondrocytes (arrowheads) are seen distorting the intercolumnar septa. Pro, proliferative zone. Bar, (A, B) 0.5 mm; (C, D) 100 μm; and (E, F) 20 μm.

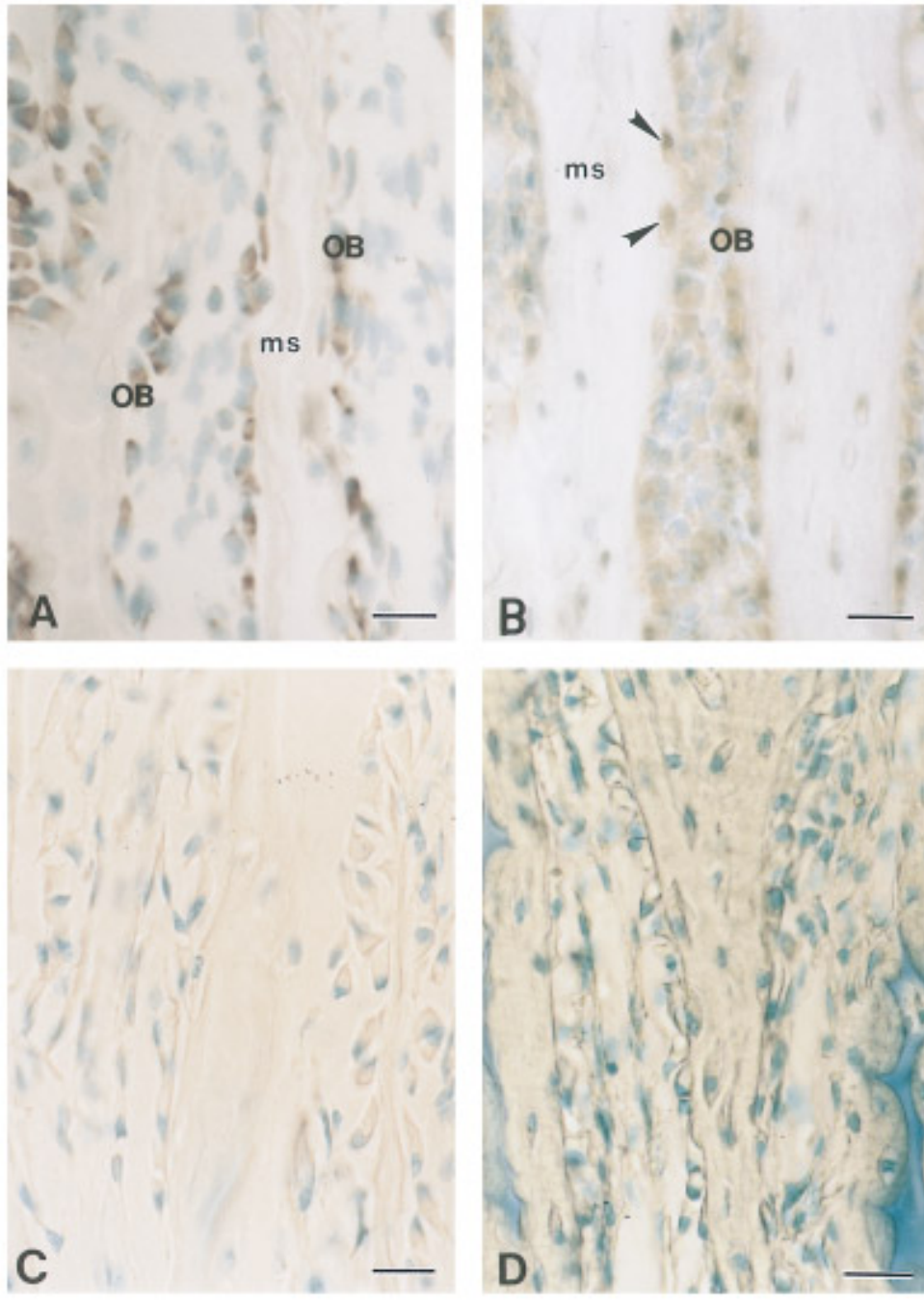
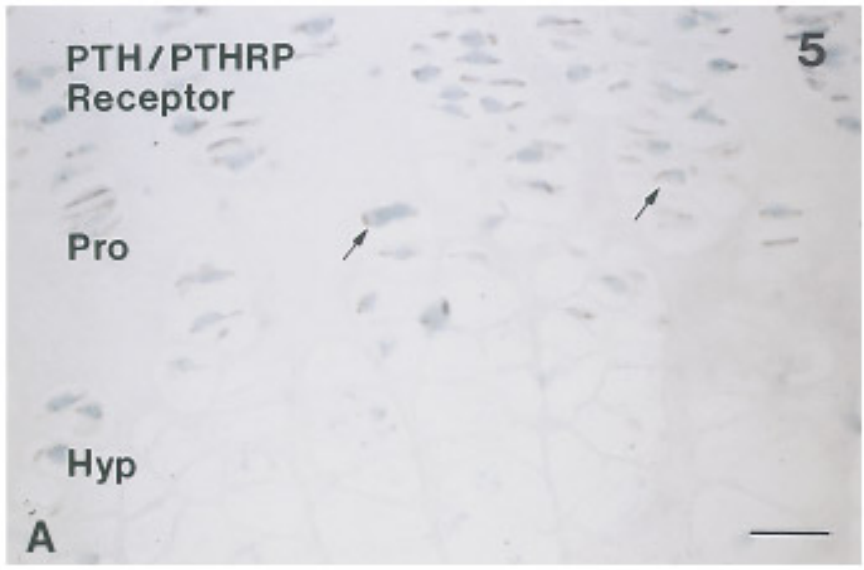
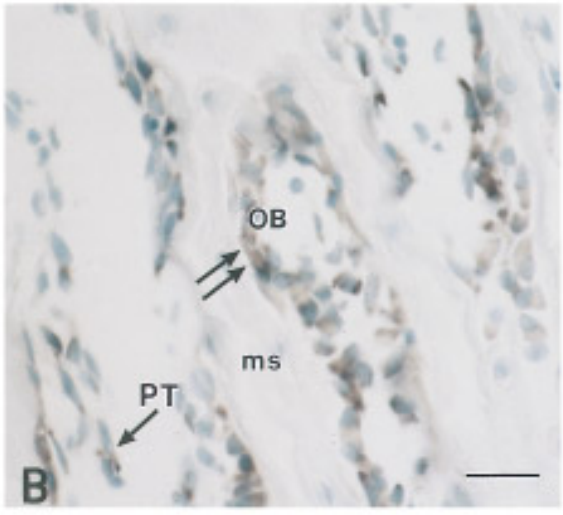


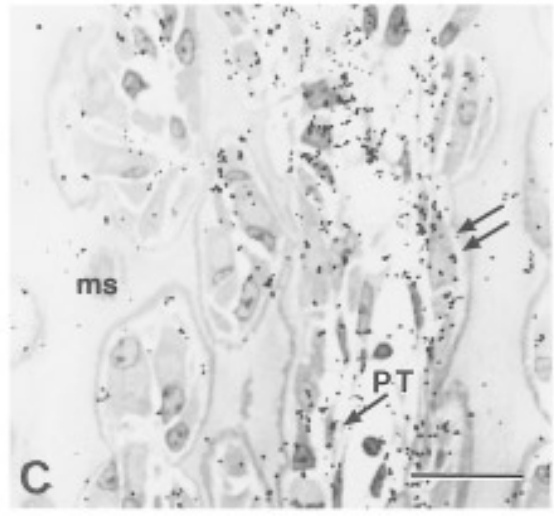
FIG. 4. Localization of PTHrP by *in situ* hybridization (A) and by immunocytochemistry (B) in the metaphyseal bone tissue. (A) Using an antisense riboprobe, hybridization signals of PTHrP (brown color) are observed mainly in osteoblasts (OB) on the mixed spicules (ms) of the normal metaphysis. (B) PTHrP immunoreactivity (light brown color) is also seen mainly in osteoblasts (OB). However, a faint reaction can also be detected in some osteocytes (arrowheads) in the superficial layer of the bone matrix. No signals were observed using a sense riboprobe (C) or if the antiserum was preadsorbed with 100 μg/ml of PTHrP-(1-34) (D). All sections are counterstained with methyl green. All bars, 20 μm.

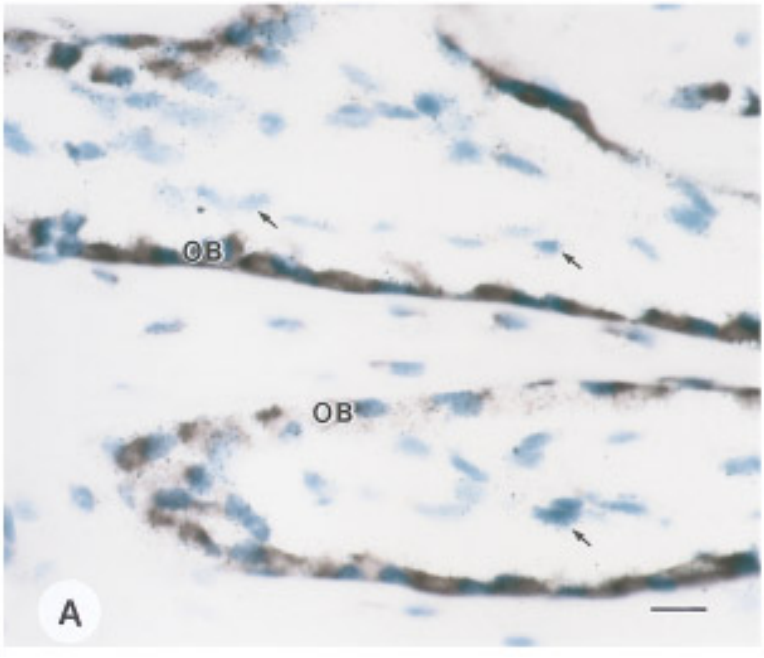
FIG. 5. PTH/PTHrP receptor mRNA detected by *in situ* hybridization and protein detected by binding of radioligand. (A) Hybridization signals for the PTH/PTHrP receptor (brown color, arrows) are detected mainly in chondrocytes of the proliferative zone (Pro) but not in the hypertrophic zone (Hyp) of the epiphyseal plate. In the tibial metaphysis (B) osteoblasts (OB, double arrows) as well as preosteoblastic PT cells (PT) express mRNA encoding PTH/PTHrP receptor. (C) Light microscope radioautograph demonstrating ^{125}I-labeled PTHrP binding in the tibial metaphysis. Silver grains representing hormone binding are localized to differentiated osteoblasts (double arrows) and to PT cells and their processes (PT) seen in the intertrabecular spaces between the mixed spicules (ms). All bars, 20 μm.

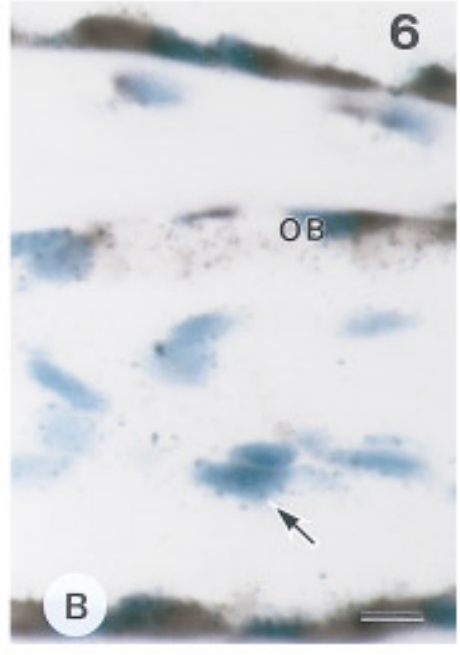
FIG. 6. Double-labeled *in situ* hybridization of PTHrP and the PTH/PTHrP receptor in metaphyseal bone. (A) PTHrP mRNA (brown color) expressed mainly in osteoblasts (OB) located on mixed spicules, whereas mRNA encoding the PTH/PTHrP receptor (indicated by fine black silver grains) is found both on osteoblasts and nonmarrow cells including preosteoblasts (arrows). A higher magnification of A is provided in B. Sections are counterstained with methyl green. Bar in A, 10 μm, and in B, 5 μm.


PTH/PTHRP
Receptor
5
Pro
Hyp
A


OB
ms
PT
B


ms
PT
C


OB
OB
A


6
OB
B

the bone marrow and intertrabecular spaces by adipocytes are skeletal abnormalities characteristic of the heterozygotes. Furthermore, the defects in homozygous animals occur in the fetus and result in the death of these dyschondroplastic mice at birth (Karaplis *et al.*, 1994). In contrast, the abnormalities in heterozygotes are not evident at birth and are of minor consequence at 1 and 1/2 months of age. Such gene dosage effects, appearing at a later stage in the heterozygotes, could be explained on the basis of decreased levels of PTHrP in tissues at the precise site and/or at the precise time it is required (Fisher and Scambler, 1994). It would therefore appear that absolute levels of PTHrP are critical for normal bone integrity not only in the fetus but also in the postnatal skeleton.

Cellular abnormalities were clearly evident in the epiphyseal growth plate of 3-month-old heterozygous mice in which altered chondrocyte proliferation was observed. Despite retention of reasonable differentiated function, as indicated by the relatively normal distribution of type II collagen, chondroitin sulfate, and ALPase, the spatial organization of the growth plate was substantially altered. This appeared to be the primary cause of the distorted and disorganized mixed spicules in the metaphysis of heterozygotes. Therefore, it is evident from the current findings that PTHrP is critically important for normal development of the growth plate after birth.

The presence of PTHrP and PTH/PTHrP receptors which we demonstrated in osteoblastic cells, together with the abnormalities of trabecular bone which we observed in heterozygous PTHrP-deficient animals, indicates that PTHrP also plays an essential role in osteoblast differentiation and the subsequent maintenance of normal trabecular architecture. The alterations in trabecular bone and the abundance of adipocytes in the bone marrow suggest that the PTHrP-deficient heterozygote manifests a form of osteopenia. A direct relationship exists between the prevalence of adipocytes in bone marrow tissue and impaired osteogenic cell differentiation resulting in osteopenia. Such a relationship has been reported with increasing age and in some pathological states (osteoporosis) and experimental conditions (weightlessness, ovariectomy, immobilization) (Meunier *et al.*, 1971; Jee *et al.*, 1983; Wronski *et al.*, 1986; Minaire *et al.*, 1974). This osteopenia can result not only from defective function of mature osteoblasts but also from a decrease in the number of osteoprogenitor cells. Given that the same pluripotent stromal stem cells in the bone marrow compartment can give rise to adipocytes and osteoprogenitor cells (Owen, 1985), the increased number of adipocytes in the marrow space of PTHrP heterozygous mice could be due to altered stem cell differentiation as a consequence of PTHrP haploinsufficiency. It becomes essential, therefore, to understand the mechanism whereby PTHrP influences this common pluripotent precursor cell.

The demonstration of PTHrP production by osteoblastic cells, which also possess the PTH/PTHrP receptor, has important implications for the actions of PTH in bone. Previously, the efficacy of circulating PTH in influencing bone was considered only in terms of the ambient concentration of the hormone and perhaps the pulsatile nature of its secretion. Our studies and those of others (Lee *et al.*, 1993, 1994) now indicate that the capacity of PTH to act in bone must also be considered in terms of its interaction with receptors on osteoblasts which may be occupied to a variable extent by endogenously produced PTHrP, which itself can act as an autoregulatory factor.

Finally, our studies may also bear on the issue of whether PTH and PTHrP exert overlapping or discrete roles in modulating skeletal function. PTH has been reported to be capable of modulating not only osteoblast but also chondrocyte function (Koike *et al.*, 1990). However, during embryogenesis, PTHrP and its receptor are believed to be expressed at a much earlier developmental stage than PTH (van de Stolpe *et al.*, 1993). Consequently, it is clearly PTHrP deficiency per se which is responsible for the marked developmental abnormalities observed in the fetus homozygous for PTHrP gene ablation. In that model it is difficult to determine whether PTH could subserve the same functional role in fetal skeletal development as PTHrP, especially inasmuch as recent preliminary studies have suggested that the phenotype obtained in animals homozygous for ablation of the common PTH/PTHrP receptor (Lanske *et al.*, 1994) is qualitatively similar to that occurring in animals homozygous for PTHrP gene ablation. In the present study we have found that in animals heterozygous for PTHrP gene ablation, physiological concentrations of circulating PTH, although capable of maintaining normal calcium homeostasis, were unable to compensate for PTHrP haploinsufficiency. Consequently, PTHrP appears to play a role distinct from PTH in both growth plate cartilage and trabecular bone in the postnatal state. One possible explanation for this observation is that a defect in skeletal progenitor cells was caused by PTHrP deficiency *in utero* prior to parathyroid gland development and only became manifest in the postnatal state. A second possibility is that circulating PTH is unable to permeate and interact with skeletal receptors to which locally produced PTHrP may bind. This seems unlikely in view of the fact that, as determined by radioautography, circulating PTH and PTHrP can bind to the same receptor sites *in vivo* which have now been identified by *in situ* hybridization. A third possibility is that domains of the PTHrP molecule other than those shared with the PTH molecule may subserve unique functions which are important for normal skeletal development. Further studies are required to test these possibilities.

ACKNOWLEDGMENTS

This work was supported by grants from the Medical Research Council (MRC) of Canada to A. C. Karaplis, H. Warshawsky, M. L. Lipman, and D. Goltzman and by a grant to D. Goltzman from the National Cancer Institute of Canada. J. E. Henderson and A. C. Karaplis are recipients of Fellowship and Scholarship Awards from the MRC of Canada, respectively. M. L. Lipman is a recipient of a scholarship from the Kidney Foundation of Canada. We thank Judith Marshall for excellent secretarial assistance and Marie Rou-

leau for invaluable assistance with the radioautographic studies of ^{125}I-labeled PTH and PTHrP binding.

REFERENCES

Abou-Samra, A.-B., Jüppner, H., Force, T., Freeman, M. W., Kong, X. F., Schipani, E., Ureña, P., Richards, J., Bonventre, J. V., Potts, J. T., Jr., Kronenberg, H. M., and Segre, G. V. (1992). Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: A single receptor stimulates intracellular accumulation of both cAMP and inositol triphosphates and increases intracellular free calcium. *Proc. Natl. Acad. Sci. USA* 89, 2732–2736.

Amizuka, N., Warshawsky, H., Henderson, J. E., Goltzman, D., and Karaplis, A. C. (1994). Parathyroid hormone-related peptide (PTHrP)-depleted mice show abnormal epiphyseal cartilage and altered endochondral bone formation. *J. Cell Biol.* 126, 1611–1623.

Burtis, W. J., Wu, T., Bunch, C., Wysolmerski, J. J., Insogna, K. L., Weir, E. C., Broadus, A. E., and Stewart, A. F. (1987). Identification of a novel 17,000-dalton parathyroid hormone-like adenylate cyclase-stimulating protein from a tumor associated with humoral hypercalcemia of malignancy. *J. Biol. Chem.* 161, 7151–7156.

Byers, S., Caterson, B., Hopwood, J. J., and Foster, B. C. (1992). Immunolocation analysis of glycosaminoglycans in the human growth plate. *J. Histochem. Cytochem.* 40, 275–282.

Cox, K. H., Deleton, D. V., Angerer, L. M., and Angerer, R. C. (1984). Detection of mRNA in sea urchin embryos by *in situ* hybridization using asymmetric RNA probes. *Dev. Biol.* 101, 485–502.

Deftos, L. J., Burton, D. W., and Brandt, D. W. (1993). Immunological identification and distribution of parathyroid hormone-related protein polypeptides in normal and malignant tissues. *J. Clin. Invest.* 92, 727–735.

DeMay, M., Mitchell, J., and Goltzman, D. (1985). Comparison of the binding of parathyroid hormone and its amino-terminal fragments to renal and osseous receptors. *Am. J. Physiol.* 249, E437–E446.

Drucker, D. J., Asa, S. L., Henderson, J. E., and Goltzman, D. (1989). The parathyroid hormone-like peptide gene is expressed in the normal and neoplastic human endocrine pancreas. *Mol. Endocrinol.* 89, 1589–1595.

Fisher, E., and Scambler, P. (1994). Human haploinsufficiency—One for sorrow, two for joy. *Nature Genet.* 7, 5–7.

Goltzman, D., Hendy, G. N., and Banville, D. (1989). Parathyroid hormone-like peptide: Molecular characterization and biological properties. *Trends Endocrinol. Metab.* 1, 39–44.

Henderson, J. E., Shustik, C., Kremer, R., Rabbani, S. A., Hendy, G. N., and Goltzman, D. (1990). Circulating concentration of parathyroid hormone-like peptide in malignancy and hyperparathyroidism. *J. Bone Miner. Res.* 5, 105–113.

Henderson, J. E., Amizuka, N., Warshawsky, H., Biasotto, D., Lanske, B. M. K., Goltzman, D., and Karaplis, A. C. (1995). Nucleolar localization of PTHrP enhances survival of chondrocytes under conditions that promote apoptotic cell death. *Mol. Cell. Biol.* 15, 4064–4075.

Horiuchi, N., Caulfield, M. P., Fisher, J. E., Goldman, M. E., McKee, G. R., Reagan, J. E., Levy, J. J., Nutt, R. F., Rodan, S. B., Clemens, T. L., and Rosenblatt, M. (1987). Similarity of synthetic peptide from human tumor to parathyroid hormone in vivo and in vitro. *Science* 238, 1566–1568.

Jee, W. S. S., Wronski, T. J., Morey, E. R., and Kimmel, D. B. (1983). Effects of spaceflight on trabecular bone in rats. *Am. J. Physiol.* 244, R310–R314.

Jüppner, H., Abou-Samra, A-B., Freeman, M., Kong, X. F., Schipani, E., Richards, J., Kolakowski, L. F., Jr., Hock, J., Potts, J. T., Jr., Kronenberg, H. M., and Segre, G. V. (1991). A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide. *Science* 254, 1024–1026.

Kaiser, S., Laneuville, P., Bernier, S. M., Rhim, J. S., Kremer, R., and Goltzman, D. (1992). Enhanced growth of a human keratinocyte cell line induced by antisense RNA for parathyroid hormone-related peptide. *J. Biol. Chem.* 267, 13623–13628.

Kaiser, S., Sebag, M., Rhim, J. S., Kremer, R., and Goltzman, D. (1994). Antisense-mediated inhibition of parathyroid hormone-related peptide (PTHrP) production in a keratinocyte cell line impedes differentiation. *Mol. Endocrinol.* 8, 139–147.

Karaplis, A. C., Yasuda, T., Hendy, G. N., Goltzman, D., and Banville, D. (1990). Gene-encoding parathyroid hormone-like peptide: Nucleotide sequence of the rat gene and comparison with the human homologue. *Mol. Endocrinol.* 4, 441–446.

Karaplis, A. C., Luz, A., Glowacki, J., Bronson, R. T., Tybulewicz, V. L. J., Kronenberg, H. M., and Mulligan, R. C. (1994). Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide (PTHrP) gene. *Genes Dev.* 8, 277–289.

Kemp, B. E., Moseley, J. M., Rodda, C. P., Ebeling, P. R., Wettenhall, R. E. H., Stapleton, D., Diefenbach-Jagger, H., Ure, F., Michelangeli, V. P., Simmons, H. A., Raisz, L. G., and Martin, T. J. (1987). Parathyroid hormone-related protein of malignancy: Active synthetic fragments. *Science* 238, 1568–1570.

Koike, T., Iwamoto, M., Shimazu, A., Nakashima, K., Suzuki, F., and Kato, Y. (1990). Potent mitogenic effects of parathyroid hormone on embryonic chick and rabbit chondrocytes: Differential effects of age on growth, proteoglycan and cyclic AMP responses of chondrocytes to PTH. *J. Clin. Invest.* 85, 626–631.

Kramer, S., Reynolds, F. H., Jr., Castillo, M., Valenzuela, D. M., Thorikay, M., and Sorvillo, J. M. (1991). Immunological identification and distribution of parathyroid hormone-related protein polypeptides in normal and malignant tissues. *Endocrinology* 128, 1927–1937.

Lanske, B., Karaplis, A. C., Luz, A., Mulligan, R., and Kronenberg, H. M. (1994). Characterization of mice homozygous for the PTH/PTHrP receptor gene null mutation. *J. Bone Miner. Res.* 9, S121.

Lee, K., Deeds, J. D., Bond, A. T., Jüppner, H., Abou-Samra, A-B., and Segre, G. V. (1993). In situ hybridization of PTH/PTHrP receptor mRNA in the bone of fetal and young rats. *Bone* 14, 341–345.

Lee, K., Deeds, J. D., Chibs, S., Un-No, M., Bond, A. T., and Segre, G. V. (1994). Parathyroid hormone induces sequential c-fos expression in bone cells in vivo: In situ hybridization of its receptor and c-fos messenger ribonucleic acid. *Endocrinology* 134, 441–450.

Lipman, M. L., Stevens, A. C., and Strom, T. B. (1994). Heightened intragraft CTL gene expression in acutely reject renal allografts. *J. Immunol.* 152, 5120–5127.

Mangin, M., Webb, A. C., Dreyer, B. E., Posillico, J. T., Ikeda, K., Weir, E. C., Stewart, A. F., Bander, N. H., Milstone, L., Barton, D. E., Francke, U., and Broadus, A. E. (1988). Identification of a cDNA encoding a parathyroid hormone-like peptide from a human tumor associated with humoral hypercalcemia of malignancy. *Proc. Natl. Acad. Sci. USA* 85, 597–601.

Merendino, J. J., Isogna, K. L., Jr., Milstone, L. M., Broadus, A. E., and Stewart, A. F. (1986). A parathyroid hormone-like protein from cultured human keratinocytes. *Science* 231, 388–390.

Meunier, P. J., Aaron, J., Edouard, C., and Vignon, G. (1971). Osteoporosis and the replacement of cell populations of the marrow by adipose tissue: A quantitative study of 84 iliac bone biopsies. *Clin. Orthop. Rel. Res.* 80, 147–154.

Minaire, P., Meunier, P. J., Edouard, C., Bernard, J., Courpron, J., and Bourret, J. (1974). Histological data on disuse osteoporosis: Comparison with biological data. *Calc. Tissue Res.* 17, 57–73.

Orloff, J. J., Wu, T. L., and Stewart, A. F. (1989). Parathyroid hormone-like proteins: Biochemical response and receptor interaction. *Endocrine Rev.* 10, 476–495.

Owen, M. E. (1985). Lineage of osteogenic cells and their relationships to the stromal system. *In* "Bone and Mineral Research" (W. A. Peck, Ed.), Vol. 2, pp. 1–25. Elsevier, Amsterdam.

Rabbani, S. A., Mitchell, J., Roy, D. R., Hendy, G. N., and Goltzman, D. (1988). Influence of the amino-terminus on in vitro biological activity of synthetic parathyroid hormone-like peptides of malignancy. *Endocrinology* 123, 2709–2716.

Rodan, S. B., Isogna, K. L., Vignery, A. M-G., Stewart, A. F., Broadus, A. E., D'Souza, S. M., and Bertolini, D. R. (1983). Factors associated with humoral hypercalcemia of malignancy stimulate adenyle cyclase in osteoclastic cells. *J. Clin. Invest.* 72, 1511–1515.

Rouleau, M. F., Warshawsky, H., and Goltzman, D. (1986). Parathyroid hormone binding in vivo to renal, hepatic, and skeletal tissues of the rat using a radioautographic approach. *Endocrinology* 118, 919–931.

Rouleau, M. F., Mitchell, J., and Goltzman, D. (1988). In vivo distribution of parathyroid hormone receptors in bone: Evidence that a predominant osseus target cell is not the mature osteoblast. *Endocrinology* 123, 187–191.

Rouleau, M. F., Mitchell, J., and Goltzman, D. (1990). Characterization of the major parathyroid hormone target cell in the endosteal metaphysis of rat long bones. *J. Bone Miner. Res.* 5, 1043–1053.

Strewler, G. J., Stern, P. H., Jacob, J. W., Eveloff, J., Klein, R. F., Leung, S. C., Rosenblatt, M., and Nissenson, R. A. (1987). Parathyroid hormonelike protein from human renal carcinoma cells: Structural and functional homology with parathyroid hormone. *J. Clin. Invest.* 80, 1803–1807.

Suva, L. J., Winslow, G. A., Wettenhall, R. E. H., Hammond, R. G., Mosley, J. M., Diefenbach-Jagger, H., Rodda, C. P., Kemp, B. E., Rodriguez, H., Chen, E. Y., Hudson, P. J., Martin, T. J., and Wood, W. I. (1987). A parathyroid hormone-related protein implicated malignant hypercalcemia: Cloning and expression. *Science* 237, 893–896.

Thiede, M. A., and Rodan, G. A. (1988). Expression of a calcium mobilizing parathyroid hormone-like peptide in lactating mammary tissue. *Science* 242, 278–280.

Thiede, M. A., Weir, E. C., Brines, M. L., Burtis, W. J., Ikeda, K., Dreyer, B. E., Garfield, R. E., and Broadus, A. E. (1990). Intrauterine occupancy controls expression of the parathyroid hormone-related peptide gene in preterm rat myometrium. *Proc. Natl. Acad. Sci. USA* 87, 6969–6973.

Thompson, D. D., Seedor, J. G., Fisher, J. E., Rosenblatt, M., and Rodan, G. A. (1988). Influence of the amino-terminus on in vitro biological activity of synthetic parathyroid hormone-like peptides of malignancy. *Proc. Natl. Acad. Sci. USA* 85, 5673–5677.

Ureña, P., Kong, X. F., Abou-Samra, A-B., Jüppner, H., Kronenberg, H. M., Potts, J. T., Jr., and Segre, G. V. (1993). Parathyroid hormone (PTH)/PTH-related peptide receptor messenger ribonucleic acids are widely distributed in rat tissues. *Endocrinology* 133, 617–623.

van de Stolpe, A., Karperien, M., Lowik, C. W. G. M., Jüppner, H., Segre, G. V., Abou-Samra, A-B., de Laat, S. W., and Defize, L. H. K. (1993). Parathyroid hormone-related peptide as an endogenous inducer of parietal endoderm differentiation. *J. Cell Biol.* 120, 235–243.

Vasavada, R., Soifer, N. E., Dann, P., Philbrick, W. M., and Stewart, A. F. (1994). Overexpression of PTHrP in the pancreatic islet of transgenic mice causes fasting and post-prandial hypoglycemia. *J. Bone Miner. Res.* 9, S121.

Villanueva, A. R. (1974). A bone stain for osteoid seams in fresh, unembedded, mineralized sections of bone. *Stain Technol.* 49, 1–8.

Villanueva, A. R., and Lundin, K. D. (1989). A versatile new mineralized bone stain for simultaneous assessment of tetracycline and osteoid seams. *Stain Technol.* 64, 129–138.

Warshawsky, H., and Moore, G. (1967). A technique for the fixation and decalcification of rats incisors for electron microscopy. *J. Histochem. Cytochem.* 15, 524–549.

Wronski, T. J., Walsh, C. C., and Ignaszewski, L. A. (1986). Histologic evidence for osteopenia and increased bone turnover in ovariectomised rats. *Bone* 7, 119–123.

Wysolmerski, J. J., Broadus, A. E., Zhou, J., Fuchs, E., Milstone, L. M., and Philbrick, W. M. (1994). Overexpression of parathyroid hormone-related peptide in the skin of transgenic mice interferes with hair follicle development. *Proc. Natl. Acad. Sci. USA* 91, 1133–1137.

Yasuda, T., Banville, D., Hendy, G. N., and Goltzman, D. (1989a). Characterization of the human parathyroid hormone-like peptide gene: Functional and evolutionary aspects. *J. Biol. Chem.* 264, 7720–7725.

Yasuda, T., Banville, D., Rabbani, S. A., Hendy, G. N., and Goltzman, D. (1989b). Rat parathyroidhormone-like peptide: Comparison with the human homologue and expression in malignant and normal tissue. *Mol. Endocrinol.* 3, 518–525.

Received for publication August 25, 1995
Accepted January 10, 1996