Interleukin-18 (IL-18) can regulate osteoblast and osteoclast function. We have identified, using cDNA microarray technology, that IL-18 expression is increased in UMR 106-01 rat osteoblastic cells in response to parathyroid hormone (PTH) treatment. Confirmation of these data using real-time reverse transcription-PCR showed that steady-state levels of IL-18 mRNA increased by 2 h (3-fold), peaked by 4 h (10-fold), and had diminished after 12 h (4.4-fold) and that this regulation was via the protein kinase A signaling pathway and did not involve activation of the PKC signal cascade. PTH regulation of IL-18 was confirmed at the protein level, and analysis of differentiating primary rat calvarial osteoblasts verified that both IL-18 mRNA and protein are regulated by PTH in primary rat osteoblasts. Promoter reporter assays revealed that PTH regulated the upstream IL-18 promoter and induced the exon 1 containing 1.1-kb IL-18 mRNA transcript in primary osteoblast cells. The in vivo physiological role of IL-18 in the anabolic actions of PTH on bone was then assessed using IL-18 knock-out mice. Female IL-18 null mice and wild-type littermate controls were injected with vehicle or 8 μg/100 g of human 1–38 PTH for 4 weeks. In IL-18 knock-out animals the anabolic effect of PTH (determined by bone mineral density changes in the proximal tibia) was abolished in trabecular bone but not in the cortical component. These data characterize the PTH regulation of IL-18 expression in osteoblastic cells and suggest that this cytokine is involved in the anabolic actions of PTH.

Parathyroid hormone (PTH) is an 84-amino acid peptide hormone that is produced in the parathyroid gland and acts peripherally on the kidney and bone, and indirectly on the intestine, to maintain serum calcium homeostasis. PTH is critical to the regulation of bone metabolism and depending on the administration regime can induce either an anabolic (1, 2) or catabolic (3) response in bone. Continuous administration of PTH, as is mimicked by hyperparathyroidism, produces a catabolic response in bone (3) via increased osteoclastogenesis and results in a decrease in bone mineral density (BMD). In comparison, intermittent exposure of animals and humans to PTH results in an anabolic response in bone that is characterized by increased BMD (1, 2).

PTH acts on osteoblastic cells in the bone microenvironment via a seven-transmembrane-domain G-protein-coupled cell surface receptor, the parathyroid hormone receptor 1 (4). Interaction of the PTH ligand with its receptor activates several intracellular signaling pathways within osteoblast cells, including PKA, calcium, PKC, and ERK (5). Activation of these signaling cascades results in changes in gene expression that ultimately determine the biological response of the cells. However, the specific switch in signaling and gene expression that directs either a catabolic or anabolic response to PTH is currently unknown. To date several molecules and mechanisms have been identified as being important in the anabolic actions of PTH on bone: they include insulin-like growth factor-I (6, 7), c-Fos (8), cbfa-1 (RUNX2) (9), sclerostin (10), and β-arrestin 2 (11, 12). However, a full understanding of the mechanisms driving the anabolic actions of PTH remains to be elucidated so that more effective treatments can be developed to treat skeletal disease where bone replacement is a clinical necessity.

Interleukin-18 (IL-18), originally known as interferon-γ-inducing factor, is best known for its role in the immune system where it has pro-inflammatory actions and acts together with IL-12 to promote interferon-γ release from T cells and a subsequent Th1 immune response (13). IL-18 can also induce Th2 immune responses in a number of disease states (13). Most recently IL-18 has been reported to regulate the homeostasis of energy intake and insulin sensitivity (14). IL-18 is a secreted protein that acts in a paracrine or autocrine mechanism on a multisubunit cell surface receptor. The IL-18 receptor, like the IL-1 receptor, is a heterodimeric receptor containing two sub-

precipitation; pQCT, peripheral quantitative computed tomography; 8-Br-cAMP, 8-bromo-cAMP; CREB, cAMP response element-binding protein; KO, knock-out; WT, wild type.
units, α and β. Interleukin-18 receptor α, previously known as IL-1R-related protein, binds IL-18 at relatively low concentrations, whereas the IL-18Rβ chain does not bind IL-18 but rather binds to the IL-18-IL-18Rα complex generating what is believed to be a high affinity complex (15).

In addition to being expressed in hemopoietically derived cells of the immune system, including Kupffer cells, macrophages, dendritic cells, T cells, and B cells, IL-18 is broadly expressed by mesenchymal cells, including intestinal epithelial cells, chondrocytes, and osteoblasts (16). Following the identification of IL-18 production by osteoblasts it was shown that IL-18 could inhibit osteoclast bone resorption indirectly in vitro by inducing T-cell production of granulocyte macrophage-colony stimulating factor (16, 17). Subsequently, both subunits of the IL-18 receptors have been identified on both osteoblastic (18) and osteoclastic cells (19), and a direct effect of IL-18 on both these cell types has been demonstrated. Specifically, IL-18 can increase osteoprotegerin expression in osteoblast cells without altering receptor activator of NF-κB ligand production (18), which suggests that a direct mechanism for inhibiting osteoclast production independent of T cells also exists. In addition to inhibiting bone resorption, IL-18 has an anabolic effect on osteoclastic cells (20). These functional data have been restricted to in vitro assays, and the specific in vivo actions of IL-18 are still unknown, however, the generation of an IL-18 transgenic mouse, which overexpresses IL-18 in the bone marrow cavity, showed anatomical and functional changes in the bone microenvironment, including decreased trabecular bone turnover and altered cortical structure (21), suggesting that IL-18 can regulate bone metabolism in vivo.

We performed microarray analysis on rat osteosarcoma (UMR 106-01) cells, which had been treated with 10⁻⁸ M PTH-(1–34) and identified a number of genes that were previously unknown to be regulated by PTH (22). Included in the list of novel PTH-regulated genes we identified was IL-18. In the present investigations we have characterized the expression profile of IL-18 in osteoblast cells following PTH treatment and identified that this is a primary response acting through the PKA signal transduction pathway. In addition we have used IL-18 knock-out mice to explore the physiological importance of IL-18 in the anabolic actions of PTH.

**Experimental Procedures**

**Chemicals**—Synthetic human PTH amino acids 1–38 (hPTH-(1–38)) were purchased from Bachem (Torrance, CA). The inhibitors H-89 and GF109203 and 1,25-dihydroxyvitamin D₃ were purchased from Calbiochem (San Diego, CA). Rat PTH amino acids 1–34 (rPTH-(1–34)), PGE₂, 8-Br-cAMP, phorbol 12-myristate 13-acetate, and cycloheximide were obtained from Sigma. The anti-rat affinity-purified IL-18 antibody was purchased from R & D Systems.

**Cell Culture**—UMR 106-01 cells were cultured in Eagle’s minimum essential medium (MEM) supplemented with 5% fetal bovine serum. For the indicated experiments the cells were seeded at 1.2 × 10⁴ cells/cm² in 100-mm dishes in the same medium and then changed to serum-free medium the following day for 24 h prior to the addition of the indicated reagents. Primary rat osteoblastic cells were obtained from neonatal rat calvariae by sequential digestion with collagenase and trypsin as previously described (23). These cells were grown in MEM with 10% fetal bovine serum from day 0 to 6 when they reach confluence. At day 7 the medium was changed to BGJb medium containing 10% fetal bovine serum, ascorbic acid (50 µg/ml), β-glycerolphosphate (10 mM). 24 h prior to the indicated time points the cells were serum-starved then treated with 10⁻⁸ M PTH for the indicated times.

**Analysis of mRNA Abundance by Real-time RT-PCR**—Cells and tissues were harvested at the indicated time points after hormone treatment. Total RNA was isolated using Tri Reagent (Sigma) followed by an RNase kit (Qiagen). TaqMan™ Reverse transcription kit (Applied Biosciences) was used to reverse transcribe mRNA into cDNA. PCR was then performed on an Opticon real-time RT-PCR machine (MJ Research) using SYBR® Green PCR Core kit (Applied Biosciences). Analysis of each sample was performed twice for each experiment, and the data in the figures report average values of three experiments ± S.E. For the UMR 106-01, rats β-actin was used as an internal control (5′-TCCTAGGGGACGGTTACTTCTGTTG-3′ and 5′-CGACTCATCGTACTTCTGTT-3′). For the femoral samples and primary osteoblastic cells, rat glyceraldehyde-3-phosphate dehydrogenase (5′-AACCCATCCCCATTTCCAGG-3′ and 5′-CGACTCATCGTACTTCTGTT-3′) was used as the internal control. Specific primer sequences for rat IL-18 and interleukin converting enzyme were designed using Primer 3: interleukin converting enzyme (5′-CGGACCTGTTGGCTATGTGTTCT-3′ and 5′-CAAGCTCCAGATTCTGCCC-3′) and IL-18 (5′-CAACCCGGCAGTAACTGGAGCA-3′ and 5′-CGTTCGGCTTCCGTGATAT-3′).

**Western Blot Analysis**—At the indicated time points, cells were lysed in 500 µl of cell lysis buffer (20 mM of Tris-HCl (pH 8.0), 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride), incubated on ice for 20 min, and microcentrifuged for 10 min to remove cell debris prior to freezing. 40 µg of total cell lysate was subjected to 10% SDS-polyacrylamide electrophoresis under reducing conditions at 150 V for 1 h. The protein was then electro transferred to polyvinylidene difluoride filter then incubated at 4 °C overnight with 0.2 µg/ml anti-rat IL-18 antibody in 5% milk, 20 mM Tris-HCl, pH 7.6, 137 NaCl, and 0.1% Tween 20. Subsequently the filters were incubated for 1 h at room temperature with a mouse anti-goat IgG horseradish peroxidase conjugate (Sigma), and the signal was detected using an enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences).

**Northern Blot Analysis**—Total RNA was isolated from primary rat calvarial osteoblastic cells that had remained untreated or were treated for 4 h with rPTH-(1–34) at the indicated days of in vitro culture. Total RNA was also collected from the adrenal gland, spleen, and pancreas of a 4-week-old male Sprague-Dawley rat. RNA (20 µg) was fractionated by electrophoresis on a 1.5% agarose, 2.2 M formaldehyde gel in 40 mM MOPS, pH 7.0, 10 mM sodium acetate, 1 mM EDTA running buffer. The gel was then blotted overnight to Zeta-Probe membranes (Bio-Rad) and UV cross-linked. The filters were incubated for 1 h at 65 °C in prehybridization solution (0.25 M Na₂HPO₄, pH 7.2, and 7% SDS). Fresh prehybridization solu-
tion was then added along with 10⁶ cpm/ml of random-primed [α-³²P]dCTP-labeled IL-18 cDNA probe and incubated overnight at 65 °C. The following day, the filters were washed twice for 30–60 min each time with 40 mM Na₂HPO₄, pH 7.2, and 5% SDS at 65 °C, and twice with 40 mM Na₂HPO₄, pH 7.2, and 1% SDS at 65 °C for 30–60 min each time. Bound radiolabeled probe was then imaged using a PhosphorImager.

**Transient Transfections and Chloramphenicol Acetyltransferase Activity Analysis of IL-18 Promoter**—The IL-18 1–3 and 1–4 promoter constructs were cloned into pSV0-CAT vector after PCR amplification from the original P1D1 construct (generously supplied by Dr. M. Tone) (24). Following PCR amplification the constructs were cloned into the TOPO vector (Invitrogen) using TA cloning. The primers and locations are as follows: construct 1–3... -1587 from the start site, forward primer = cataagaaatgcaatgatacttagca; construct 1–4... -1137 from the start site, forward primer = tggattcaaccttctcctctcct; the reverse primer = cctcttttgtgtgatgcagc. The constructs were then cut from the TOPO vector using HindIII digestion and ligated into pSVO-CAT reporter plasmid (Promega, Madison, WI). The IL-18 2–1 construct was released from the P1D8 construct (24) with HindIII and ligated directly into the pSVO-CAT vector. The constructs were verified by sequencing at the University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School core DNA automatic sequencing facility. The plasmid DNAs were transiently transfected into cells using Lipofectamine 2000 (Invitrogen). Briefly, cells were plated at 4 × 10⁶ cells/well in 6-well plates in MEM containing 10% fetal bovine serum. The following day, the cells were transfected with 1 μg of DNA and 5 μl of Lipofectamine 2000 per well in 1 ml of serum-free MEM. After 16 h, 1 ml of MEM containing 10% fetal bovine serum was added. After 24 h, the cells were treated with either control or PTH-containing media for 6 h. CAT activity was measured by reacting 50 μl of cell lysate in duplicate in a 100-μl reaction volume consisting of final concentrations of 250 μM n-butyrylcoenzyme A and 23 mM [¹⁴C]chloramphenicol (0.125 μCi/assay). The values were normalized to protein as determined by the Bradford dye binding method (Bio-Rad). A standard curve using purified CAT was performed in every experiment to determine the linear range of the enzyme assay.

ChIP Assays—ChIP analyses were performed using a modification of the Upstate Biotechnology procedure. Briefly, DNA and associated proteins were cross-linked by incubating cells in medium without fetal bovine serum containing 0.8% formaldehyde at 37 °C for 10 min. Cells were washed in ice-cold phosphate-buffered saline containing 1:25 dilution of protease inhibitor mixture (Roche Applied Science) and 1 mM phenylmethylsulfonyl fluoride, scraped, sonicated to generate 400- to 1000-bp DNA fragments, and then diluted 10-fold. 2-ml aliquots were preclared at 4 °C for 1 h with 80 μl of a salmon sperm DNA/protein A-agarose 50% gel slurry and immunoprecipitated at 4 °C overnight using the following antibodies from Santa Cruz Biotechnology: anti-rabbit IgG (sc-2027) or anti-Runx2 (sc-10758). Immune complexes were collected, washed sequentially in low salt, high salt, lithium chloride, and then followed by two washes in Tris/EDTA buffers. Protein-DNA complexes were eluted with ChIP elution buffer (1% SDS and 100 mM NaHCO₃) and heated at 65 °C for 12 h to reverse cross-linking. Supernatant obtained from sonicated chromatin without antibody was used as the input control. Following treatment with proteinase K for 1 h at 45 °C, the DNA was purified and resuspended in 50 μl of Tris-EDTA buffer, and 4-μl aliquots (triplicate samples for each treatment group) were used for PCR. PCR primers used for analysis of the IL-18 promoter were the following: 5’-GGCGTTTCTGCGTCTTATATA-3’ (sense: -356 bases upstream from the transcription start site) and 5’-GCTTTGATTTCGAGTCAATTTG-3’ (antisense: -226 bases upstream from the transcription start site) that flanked the RUNX2 site and produced an amplicon of 130 bp. The conditions for semi-quantitative PCR amplification were as follows: denaturing at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 15 s. The number of cycles used for a primer set was 31. Products were run on a 2% agarose gel and stained with ethidium bromide.

**In Vivo Injections of PTH into Mice**—IL-18 null mice on the DBA/1 background were constructed as previously described (25). A breeding colony was established using heterozygous mice to obtain knock-out and wild-type littermate control animals. The femur and tibia of 31-day-old IL-18 null and wild-type littermate control mice were harvested and subjected to peripheral quantitative computed tomography (pQCT) analysis. In addition 7- to 8-week-old IL-18 knock-out and littermate control animals were injected subcutaneously 5 of 7 days for 4 weeks with either hPTH-(1–38) dissolved in saline (8 μg/100 g) or vehicle. The animals were euthanized with CO₂, and the femur and tibia were harvested and fixed in 70% ethanol for pQCT analysis. The animal protocol was approved by Robert Wood Johnson Medical School Animal Care and Use Committee.

**Ex Vivo pQCT for Mouse Tibia**—The total, trabecular, and cortical/subcortical BMD of the proximal tibia were evaluated ex vivo using an XCT Research SA (Stratec Medizintechnik, Pforzheim, Germany). A two-dimensional scout scan of the tibia was run for a length of 10 mm. The pQCT scan was initiated 1.4 mm distal from the proximal epiphysis of the tibia in the area of the secondary spongiosa. The scan was 1-mm thick with a voxel size of 90 μm. Using an iterative algorithm, soft tissue (density below 223 mg/cm³) was automatically removed. The density of the remaining tissue was reported as total bone density (milligrams/cm³). The outer 55% of the bone (cortical/subcortical bone density) was peeled away in a concentric fashion to determine trabecular bone density (milligrams/cm³).

**Statistical Analysis**—Data are expressed as the means ± S.E. Where indicated, data were analyzed by one-tailed unpaired t test using Prism4 GraphPad software.

**RESULTS**

**PTH Stimulates Production of IL-18 mRNA and Protein in Osteoblast-like Cells**—We have previously performed a microarray screen of PTH-treated UMR 106-01 cells to identify novel genes regulated by PTH (22). IL-18 was identified as a novel PTH-regulated gene, and this result has been confirmed by real-time RT-PCR analysis of PTH-treated UMR 106-01 cells in the present study. The increase in steady-state levels of mRNA started 2 h post PTH treatment, peaked at 4 h, and
returned to base-line 24 h after treatment (Fig. 1A). In addition, the steady-state levels of interleukin converting enzyme mRNA, the protease responsible for cleavage of pro-IL-18 into an active enzyme, had an increasing trend, which paralleled the changes seen in IL-18 mRNA (Fig. 1A). This change in steady-state levels of IL-18 mRNA produced in response to PTH treatment was translated into an increase in IL-18 protein (Fig. 1B). The increase in IL-18 protein was delayed compared with that seen in the mRNA occurring 8 h after PTH treatment and then remaining elevated for up to 48 h (Fig. 1B). In addition to the time-dependent effect of PTH on the IL-18 protein the effect of the hormone was also dose-dependent (Fig. 1C).

**PTH Induction of IL-18 Occurs in Primary Calvarial Osteoblasts**—To more fully assess the physiological importance of the PTH induction of IL-18 we isolated primary osteoblasts from neonatal rat calvarial cells and assessed the ability of PTH to induce IL-18 as the cells differentiated. These cultures are routinely performed in the laboratory and follow a well characterized gene expression profile associated with primary rat osteoblast differentiation (26). Interestingly, the basal expression of IL-18 in the osteoblast increased progressively as the cells entered differentiation and mineralization (Fig. 2A), a common characteristic of PTH-regulated genes. In addition, primary osteoblastic cells in all three phases of development, proliferation, differentiation, and mineralization showed an increase in both steady-state mRNA (Fig. 2B) and protein (Fig. 2C) of IL-18 in response to PTH treatment.

**PTH Induction of IL-18 Is a PKA-dependent Primary Response**—Binding of PTH to its seven-transmembrane G-protein-coupled cell surface receptor results in activation of both the PKA and PKC intracellular signaling pathways. Thus, to investigate which of these pathways, PKA or PKC, is responsible for activation of the IL-18 gene, different chemical activators and inhibitors of these pathways were used.

The cell-permeable cAMP analogue 8-Br-cAMP increased IL-18 mRNA levels equivalent to that seen in cells treated with PTH alone. Similarly, pre-treatment of the osteoblast cells with the selective PKA inhibitor H-89 prevented the PTH induction of IL-18 mRNA (Fig. 3A). However, use of the PKC activator, phorbol 12-myristate 13-acetate, did not change IL-18 mRNA levels, and pretreatment of the cells with the PKC inhibitor GF109203 did not alter the PTH induction of IL-18 mRNA (Fig. 3A). These data indicate that the PTH-induced
activation of the IL-18 gene occurs via the PKA pathway and does not require signaling through PKC.

To determine if the induction of IL-18 by PTH was a primary response or if it required de novo synthesis of an intermediate protein, the UMR 106-01 cells were treated with PTH in the presence or absence of the protein synthesis inhibitor, cycloheximide. Cycloheximide did not influence the PTH induction of IL-18 (Fig. 5B) indicating that de novo protein synthesis is not required in this process and that induction of IL-18 is a direct effect of PTH signaling to the IL-18 gene.

IL-18 Is a Target Gene for PGE2—In addition to PTH, PGE2, 1,25(OH)2 vitamin D3 are also involved in regulating bone metabolism via the osteoblast and to determine whether IL-18 may be a target gene of these agents, their ability to regulate IL-18 mRNA was assessed. Primary rat osteoblastic cells were serum-starved for 1 day, then treated with either 1α,25(OH)2 vitamin D3 or PGE2 for various time periods. RNA was harvested, and the IL-18 mRNA levels were analyzed by real-time RT-PCR (Fig. 4). PGE2 is a mediator of bone metabolism and regulates cell behavior via one of four membrane G-protein-coupled receptors. PGE2 activation of its receptors, PE2 and PE4, stimulates the PKA signal transduction pathway in osteoblast cells via coupling to Gs proteins and cAMP (27). It was not surprising that PGE2 induced expression of IL-18 mRNA in a very similar profile to that seen with PTH (Fig. 4A). However 1,25(OH)2 vitamin D3, a lipid-soluble hormone that regulates gene transcription via the vitamin D nuclear hormone receptor, did not appear to regulate expression of IL-18 in these cells (Fig. 4B).

**PTH Transcriptional Regulation of the IL-18 Gene**—The rodent IL-18 gene is regulated by two promoters (24), which produce transcripts that contain indistinguishable protein coding regions and translate identical IL-18 peptides (Fig. 5A). The two IL-18 mRNA transcripts differ in the length of their 5’-untranslated regions due to transcription initiating from alternative promoters that generate transcripts of 1.1 and 0.9 kb. In the rat these two promoters are differentially regulated in a tissue-specific manner, the 1.1-kb transcript is expressed in the adrenal gland, and the 0.9-kb one is expressed in the spleen (28). To identify the specific IL-18 transcript that is produced by rat osteoblastic cells, Northern blot analysis was performed on mRNAs isolated from primary rat calvarial osteoblastic cells grown in culture for 5 or 13 days. Rat adrenal and spleen mRNAs were used as positive controls for the two different IL-18 mRNA transcripts. The primary rat calvarial osteoblastic cells expressed an IL-18 mRNA transcript both basally and in response to PTH treatment that was the same size as that seen in the adrenal gland indicating that in osteoblastic cells it is the more distal promoter (often referred to as promoter 1) that regulates expression of IL-18 (Fig. 5B).

Several transcription factors have been shown to influence PTH regulation of different osteoblastic genes, including the transcription factors RUNX2, CREB, and AP-1 (5). In vitro CAT reporter assays were performed using the proximal and
distal IL-18 promoters to further investigate the specific transcriptional response sites involved in the PTH regulation of the IL-18 gene. Fig. 5C shows schematic diagrams of the IL-18 promoter deletion constructs that were generated from the distal IL-18 promoter and indicate the location of the RUNX2 and AP-1 binding sites. The −1587 distal IL-18 promoter, which contains both RUNX2 and AP-1 DNA binding domains, was responsive to PTH treatment and had a 3-fold increase in CAT activity compared with the SVO CAT construct (Fig. 5D). Deletion of the AP-1-containing fragment of the distal IL-18 promoter did have a small effect on the basal promoter activity in this context. However, removal of the AP-1 binding site did not alter the PTH responsiveness of the distal IL-18 promoter, because the −1137 promoter also exhibited a 3-fold increase in CAT activity following PTH treatment, suggesting that the PTH regulation of the rodent IL-18 gene may be directed through the RUNX2 binding site in the distal promoter. ChIP analysis of the control and PTH-treated UMR cells revealed that RUNX2 was bound to the distal IL-18 promoter in both conditions (Fig. 5E). Interestingly there was no increase in RUNX2 binding to the distal IL-18 promoter in response to PTH treatment. Transcriptional activity in response to PTH treatment of the proximal IL-18 promoter, which contains an AP-1 binding site, was also evaluated in this system. The proximal IL-18 promoter did have a higher basal activity in the CAT assay (Fig. 5D) despite the fact that there was no detectable transcript of this size by Northern blot analysis (Fig. 5B) but did not show PTH responsiveness. This high level of basal CAT activity due to the proximal IL-18 promoter was unexpected and likely reflects the loss of negative regulation that can result when DNA is removed from its chromatin context, as can occur in this assay system.

IL-18 is Required for the in Vivo Anabolic Action of PTH in Trabecular Bone of the Tibia—To explore the importance of IL-18 in the in vivo setting of physiological bone growth and remodeling we undertook experiments with IL-18 knockout (KO) mice. Initially, 31-day-old male and female KO and wild-type (WT) litter mate control mice were sacrificed, and the BMDs of their left proximal tibiae were analyzed by pQCT. No significant difference between the BMD of WT and KO animals was found in any regions measured at this age, indicating that IL-18 is not required for the growth of developing long bones in either male or female mice (Table 1). To investigate the involvement of IL-18 in the in vivo anabolic actions of PTH, 7- to 8-week-old female mice were treated daily with 8 μg/100 g of PTH or vehicle for 4 weeks. At the end of the treatment period the proximal tibia was dissected, and BMD was analyzed using pQCT. There was no significant difference in the total, cortical, or trabecular BMD of the vehicle-treated WT or KO animals. WT female mice responded to the PTH treatment and had a significantly
higher total, trabecular, and cortical BMD in the proximal tibia compared with their vehicle-treated controls (Fig. 6). In the IL-18 KO animals increases in BMD in response to PTH treatment were significantly higher when total BMD of the proximal tibia was measured; however, there was a trend toward this increase being lower than that seen in WT animals. When the cortical and trabecular components of the KO animals were assessed independently, PTH treatment only increased BMD in the cortical bone, and there was no significant increase in trabecular BMD with PTH treatment (Fig. 6).

**DISCUSSION**

In the present study we have demonstrated that PTH can regulate the expression of IL-18 in osteoblastic cells. PTH rapidly increased the expression of IL-18 in both osteoblastic cell lines and primary osteoblastic cell cultures, and the use of selective signaling activators and inhibitors confirmed that IL-18 gene expression was regulated through the PKA signal transduction pathway. PTH has been reported to activate both the PKA and PKC intracellular signaling pathways in osteoblastic cells, however only IGBP-5 and transforming growth factor-β are known to be regulated via the PKC pathway in these cells (29, 30). The majority of PTH-regulated genes, for example, osteocalcin (31), c-Fos (32), collagenase-3 (33), IL-6 (34), receptor activator of NF-κB ligand, and osteoprotegerin (35), are regulated completely through the PKA signal transduction pathway. The results presented here reveal that IL-18 is another PTH-stimulated osteoblast gene that is regulated via PKA signaling.

PTH activation of the cAMP signal transduction pathway results in activation of several transcription factors, including cAMP response element-binding protein (CREB), activator protein-1 (AP-1), and RUNX2 (5). Analysis of the 1.5-kb distal (P1) PTH-responsive IL-18 promoter region identified both an AP-1 and RUNX2 consensus-binding site. Removal of the AP-1 binding site did not alter the PTH activation of the promoter, which suggests that RUNX2 contributes to the PTH induction of the IL-18 distal promoter. The lack of involvement of the AP-1 transcription binding site in regulating the PTH-responsive IL-18 promoter is not surprising given that several of the AP-1 transcriptional partners are immediate-early gene family members that are rapidly and transiently induced in response to both mitotic stimuli and PTH (36, 37). Because blockade of de novo protein synthesis by cycloheximide treatment did not alter the PTH induction of IL-18 mRNA, this suggested that the immediate-early gene family members were unlikely to regulate IL-18 mRNA induction. These results reveal that PTH regulation of the IL-18 gene differs from that of the well characterized collagenase-3 gene. PTH induction of collagenase-3 transcription involves cooperation of both the AP-1 and RUNX2 binding sites (38) and as such requires de novo protein synthesis.

Although the precise molecular mechanisms involved in the anabolic actions of PTH have not been fully elucidated, several proteins have been implicated in the process. The insulin-like growth factor-1 KO mice have provided initial in vivo evidence that this growth factor is required for the PTH-mediated increase in BMD in growing mice (6) and for periosteal mineral apposition in mature animals (7). The early PTH response gene c-fos has also been implicated in the anabolic actions of PTH in

**TABLE 1**

**Influence of IL-18 on the BMD of the proximal tibia of 31-day-old mice**

|                | Proximal tibia BMD |                  |                  |
|----------------|--------------------|------------------|------------------|
|                | Males      | Females      | Males      | Females      |
| Total BMD      | 262.8 ± 4.4 | 252.3 ± 4.4  | 261 ± 6.8  | 260.5 ± 4.4  |
| Trabecular BMD | 266.3 ± 27.1 | 246 ± 20.6   | 250 ± 10.9 | 255 ± 5.2    |
| Cortical/subcortical BMD | 259.9 ± 5.1 | 257.1 ± 9.6  | 271 ± 5.1  | 264.9 ± 4.0  |

**FIGURE 6. IL-18 is required for the anabolic actions of PTH in the trabecular bone of mice.** 7- to 8-week-old female IL-18 KO mice or their littersmates were injected subcutaneously with human 1–38 PTH (8 μg/100 g body weight, 5 of 7 days for 4 weeks. Femur and tibia were excised and fixed in 70% ethanol prior to the assessment of their BMD by pQCT. *, p < 0.05; **, p < 0.01; #, p < 0.001 significantly different from control.
endochondral bone growth in mice (8) along with the master regulator of gene expression in osteoblasts, RUNX2/cbfa-1. Specifically, osteoblast expression of RUNX2 protein has been shown to increase in vivo in response to intermittent PTH injections and is required for an anabolic PTH response in in vitro cultures of metatarsal bone (9). Most recently, expression of sclerostin was shown to be negatively regulated by PTH treatment both in osteoblast and osteocyte cell lines and in vivo (10, 39). These observations suggest that the molecular mechanisms driving PTH-induced bone growth are complex and that, in addition to the positive regulation of cytokines and growth factors, inhibition of negative regulators is also important.

The clinical significance of the anabolic actions of PTH on bone are well recognized, and teraparatide (synthetic hPTH-(1–34)) is now being used clinically to reverse disease-associated bone loss. However, despite the inclusion of teraparatide in the armamentarium of clinical drugs used to treat diseases involving bone loss, the mechanisms underlying the anabolic actions of PTH have not been fully revealed. To guarantee effective patient care complete elucidation of the molecular mechanisms underlying the anabolic actions of PTH is essential to ensure the optimal clinical use of this peptide hormone.

In the present study, genetically modified mice, which lack IL-18, were used to investigate the importance of this cytokine in normal bone development and to explore the involvement of IL-18 in the anabolic actions of PTH on bone. These studies revealed that the absence of IL-18 does not alter basal BMD in 1- or 7- to 8-month-old animals. However, IL-18 did selectively influence the anabolic bone actions of PTH. Despite the fact that there was a significant increase in the total BMD of the IL-18 KO animals in response to PTH treatment there was an overall trend toward a reduced anabolic PTH response of whole bone in the IL-18 KO animals compared with WT animals. When the BMD of the cortical and trabecular bone was assessed independently the PTH anabolic response of the IL-18 KO animals only occurred in the cortical bone compartment. Previous in vitro data reporting the functional effects of IL-18 on both osteoblastic and osteoclastic cells suggested in vivo loss of IL-18 would result in increased bone resorption, loss of osteoblast proliferation, and thus potentially a generalized phenotype of osteopenia. However, loss of IL-18 did not influence the basal BMD of mice, suggesting that in normal physiological bone remodeling, other cytokines can compensate for the lack of IL-18. Interestingly, the data reported here indicate that loss of IL-18 can influence bone remodeling under defined conditions, specifically lack of IL-18 alters the PTH anabolic response in mice in a site-specific manner such that increased bone formation only occurs in the cortical bone and not in the trabecular bone. These observations suggest that PTH may be driving unique molecular processes that influence cortical and trabecular bone growth independently. Differences in cortical and trabecular bone response to anabolic PTH treatment regime have been previously reported (12, 40, 41). In these reports it has always been trabecular bone that has consistently responded to anabolic PTH treatment, and cortical bone has shown variable effects. However, interpretation of the site-specific nature of PTH-induced bone formation in cortical and trabecular bone has been challenging due to the differences in treatment dose, genetic background, age, sex, and hormonal status of the animals used.

A complete explanation of the reported site-specific effects of PTH relies on delineation of the molecular process (either remodeling or modeling) that is occurring at these different anatomical locations within the bone microenvironment. Initially, evidence suggested that the anabolic actions of PTH induced a modeling response involving direct stimulation of bone formation through the activation of quiescent bone lining cells (2, 42, 43) and the lengthening of osteoblast life span (44). However, the recent use of anti-resorptive agents either prior to, or in conjunction with, PTH treatment in osteoporotic patients shows a significant blunting of the in vivo PTH anabolic response (45, 46). These results suggest that PTH potentially involves a remodeling response that requires either an initial resorptive phase or signaling from osteoclastic cells prior to the induction of bone formation. The existence of different bone-forming mechanisms (remodeling versus modeling) in cortical and trabecular bone would explain the observations of the present study and would suggest that IL-18 is an essential cytokine involved in the actions of PTH on trabecular bone but not on cortical bone. In vitro experimental data on the actions of IL-18 on bone cells suggests that the required molecular mechanisms are present to differentially regulate modeling and remodeling processes. For instance, in vitro experiments show that IL-18 has an anabolic effect on osteoblast cells (20), suggesting that production of this cytokine alone could produce a modeling response in vivo. Additionally IL-18 had been shown in vitro to indirectly inhibit the formation of osteoclasts via T-cell production of granulocyte macrophage-colony stimulating factor (17) and increase osteoblast production of osteoprotegerin (18) revealing that mechanisms also exist to regulate PTH-induced remodeling events in vivo. These inhibitory actions of IL-18 on osteoclastic resorption and the fact that IL-18 is a later stage PTH-induced gene suggest that IL-18 may be necessary to regulate the pulse of osteoclastic resorption that is required for the in vivo anabolic actions of PTH on bone (45, 46).

Divergent effects on trabecular and cortical bone in response to PTH have been previously reported in β-arrestin-2 KO mice (12). In that case, PTH-induced cortical bone formation was greater in the KO animals compared with the WT animals, and it was suggested that differential signaling responses in osteoblasts on the periosteal and endosteal surfaces were responsible for the divergent effects reported.

In summary our findings reveal IL-18 as a PTH-regulated gene in osteoblastic cells and suggest that this cytokine may be important in the anabolic actions of PTH on trabecular bone. Given the divergent effects that PTH has on the cortical and trabecular bone of IL-18 KO animals, this is a potentially valuable model to further investigate the molecular processes occurring in these compartments in response to intermittent PTH treatment.

REFERENCES

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