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Anabolic Actions of Parathyroid Hormone during Bone Growth Are Dependent on c-*fos*

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PTH has anabolic and catabolic actions in bone that are not clearly understood. The protooncogene c-*fos* and other activating protein 1 family members are critical transcriptional mediators in bone, and c-*fos* is up-regulated by PTH. The purpose of this study was to examine the mechanisms of PTH and the role of c-*fos* in PTH-mediated anabolic actions in bone. Mice with ablation of c-*fos* (−/−) and their wild-type (+/+) and heterozygous (+/−) littermates were administered PTH for 17 d. The +/+ mice had increased femoral bone mineral density (BMD), whereas −/− mice had reduced BMD after PTH treatment. PTH increased the ash weight of +/+ and +/−, but not −/−, femurs and decreased the calcium content of −/−, but not +/+ or +/−, femurs. Histomorphometric analysis showed that PTH increased trabecular bone volume in c-*fos* +/+, +/− vertebrae, but, in contrast, decreased trabecular bone in −/− vertebrae. Serum calcium levels in +/+ mice were greater than those in −/− mice, and PTH increased calcium in −/− mice. Histologically, PTH resulted in an exacerbation of the already widened growth plate and zone of hypertrophic chondrocytes but not the proliferating zone in −/− mice. PTH also increased calvarial thickness in +/+ mice, but not −/− mice. The c-*fos* −/− mice had lower bone sialoprotein and osteocalcin (OCN), but unaltered PTH-1 receptor mRNA expression in calvaria, suggesting an alteration in extracellular matrix. Acute PTH injection (8 h) resulted in a decrease in osteocalcin mRNA expression in wild-type, but unaltered expression in −/−, calvaria. These data indicate that c-*fos* plays a critical role in the anabolic actions of PTH during endochondral bone growth. (*Endocrinology* 143: 4038–4047, 2002)

PTH IS AN 84-amino acid hormone that increases extracellular ionized calcium concentrations through its actions in kidney and bone. PTH acts directly on the skeleton to promote calcium release from bone and on the kidney to enhance calcium reabsorption by binding to its receptor, the PTH/PTHrP receptor (PTH-1R) (1). PTH has both anabolic and catabolic actions in bone; but the mechanisms of these actions are not well understood. Anabolic actions of PTH have been reported to be dependent on an intermittent regimen, using the N-terminal peptide of PTH (2), on cAMP stimulation (3), and may be associated with an inhibition of osteoblast apoptosis (4). Beyond this, the key mediators responsible for anabolic actions of PTH are unclear. Recent interest in the promising therapeutic use of PTH to treat osteoporosis emphasizes the need to better understand the pathways of PTH action (5). Furthermore, evidence is accumulating that PTH is anabolic during growth (6, 7), but little is known of the mechanisms involved. More recently, ablation of PTH was found to result in diminished cartilage matrix mineralization, suggesting that PTHrP is not the only cartilage mediator during development (8, 9).

PTH binds to the PTH-1R on osteoblasts and activates protein kinase A and protein kinase C pathways (1). PTH also regulates alkaline phosphatase, collagenase, IGF-1, osteopontin, and bone sialoprotein (BSP) (1). At the transcriptional level, PTH increases the levels of c-*fos* *in vitro* and *in vivo* in osteoblastic cells (10, 11). The immediate early gene c-*fos* is the cellular homolog of v-*fos*, originally isolated from FBJ and FBR-murine sarcoma viruses (12). It is a member of the activating protein 1 (AP-1) transcription factor complex and part of a multigene family consisting of Fos-related (*fos*B, *fra*-1, and *fra*-2) and Jun-related (c-*jun*, *jun*B, and *jun*D) factors. The protooncogene c-*fos* is a key regulator of bone cell growth and differentiation, affecting both osteoblast and osteoclast lineages during normal development and bone diseases (13). Inactivation of c-*fos* causes the bone-remodeling disease osteopetrosis, which is characterized by impaired osteoclastic bone resorption, resulting in a net increase in skeletal mass (14). In contrast, when c-*fos* is overexpressed in tissues, bone tumors develop that are typically chondroblastic osteosarcomas, containing large amounts of neoplastic bone with foci of cartilage (13). Overexpression of the AP-1 family members, ΔFosB and *fra*-1, results in increased bone formation, which leads to osteosclerosis (15, 16). Furthermore, it was shown that the osteopetrosis of c-*fos* mutant mice can be rescued by overexpression of the *fra*-1 transgene (17). Detectable expression of c-*fos* is present in the perichondrial growth regions of the cartilaginous skeleton in the mouse embryo (18). The absence of c-*fos* causes a diminished zone of proliferating chondrocytes at the epiphyseal growth plate. Additionally, formation of calvarial tumors only at the parieto-occipital sutures where cartilage is present during development (19) emphasizes that cells with chondrogenic capacities are affected by c-*fos*. PTH-(1–34) as low as 1 pM is capable of stimulating c-*fos* expression (11). This is a valuable finding, because normal circulating PTH values are in this

Abbreviations: AP-1, Activating protein 1; BMD, bone mineral density; BSP, bone sialoprotein; DEXA, dual energy x-ray absorptiometry; H&E, hematoxylin and eosin; hPTH, human PTH; OCN, osteocalcin; PTH-1R, PTH/PTHrP receptor.

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range, and PTH has anabolic actions when administered in such low doses. As intermittent administration of PTH stimulates bone formation, and PTH up-regulates c-*fos*, the anabolic effects of PTH may be mediated by c-*fos*. The purpose of this study was to determine whether the effects of PTH on endochondral bone formation are dependent on c-*fos*.

Materials and Methods

c-fos mutant mice

Mice (B6/129) heterozygous for the *Fos*^{tm1Pa} deletion mutation were obtained from The Jackson Laboratory (Bar Harbor, ME). Neonatal mouse pups were typed at d 4 to distinguish homozygous (−/−), heterozygous (+/−), and wild-type (+/+) mice. A PCR protocol using two pairs of primers were used according to the method described by Johnson *et al.* (20). Primer 022 (5′-CAA CGC CGA CTA CGA GGC GTC AT-3′) and primer 023 (5′-CAA GTG TGC ACG CGC TCA GAC AA-3′) amplify a 299-bp endogenous band from *fos*, primer 009 (5′-TAA AAC GCA CGG GTG TTG GGT-3′) and primer 024 (5′-CCC CTG CGA GTC ACA CCC CAG-3′) amplify a 190-bp band from the junction fragment of *neo* and *fos*. Standard PCR protocols were performed as previously described (20). A breeding colony was established using heterozygote mice to provide littermate controls.

In vivo PTH administration

Four-day-old c-*fos* mutant, heterozygous, and wild-type mice were administered once daily a sc injection of either human PTH-(1–34) [hPTH-(1–34); 2 μg/ml; Bachem, Torrance, CA] to deliver an adjusted final dose of 0.05 μg/g body weight or an equal volume (average volume, 135 μl) of vehicle (0.9% sodium chloride; Abbott Laboratories, Chicago, IL) for 17 d. In addition, there were noninjected age-matched controls. It was previously shown by Rihani-Bisharat *et al.* (6) that PTH-(1–34) has anabolic effects in a similar model system during growth. Mice were killed 48 h after the last injection, and tibiae, femurs, calvariae, vertebrae, and kidneys were collected. One tibia and selected calvariae were fixed in formalin, decalcified in 10% EDTA, and processed for histology. One femur was used for dual energy x-ray absorptiometry (DEXA), bone ashing, and biochemical analysis. RNA was isolated from calvariae, kidneys, and femurs using guanidinium isothiocyanate for analysis by Northern blot as previously described (21). Vertebrae were used for histomorphometric analysis. Acute actions of PTH were also evaluated with 50-μl injections of 0.09% saline solution alone (vehicle) or vehicle containing 20 μg hPTH-(1–34), sc, over the calvaria as previously described (22). Noninjected controls were also evaluated. Mice were killed 8 h after PTH administration, and RNA was isolated from calvaria as previously described (21). Animal procedures were approved by the University committee on the use and care of animals at University of Michigan.

Northern blot analysis

Northern blot analysis was performed as previously described (23). Briefly, total RNA was quantitated by spectrophotometry, and 10 μg electrophoresed on 1.2% agarose-formaldehyde gels, then transferred to nylon membranes (Duralon UV, Stratagene, La Jolla, CA) and UV cross-linked. The nylon membranes were hybridized with a cDNA probe encoding OCN (24), BSP (25), PTH-1R (26), and type X collagen (Col X) (27). Counts per minute were obtained from an Instant Imager (Packard Instrument Co., San Diego, CA), and blots were exposed to Kodak X-OMAT film (Eastman Kodak Co., Rochester, NY) at −70 C for autoradiography. Blots were stripped and reprobed with cDNA probe for 18S rRNA to control for RNA loading (23).

DEXA

Femurs were isolated and dissected free of soft tissue, and bone mineral density (BMD) (grams per square centimeter), was determined using DEXA as previously described (28). Areal BMD was measured with pDEXA Sabre (Norland, Fort Atkinson, WI) using Sabre Small Animal Research software (version 2.2.4). High-resolution scans were performed at 1 mm/sec, with a resolution of 0.1 × 0.1 mm.

Gross evaluation

The length and width of the femurs were measured using an electronic digital caliper. The femoral width was recorded at the narrowest mid diaphysis region of the femur. Femurs were evaluated by microradiographic analysis (Faxitron X-Ray Corp., Wheeling, IL). Body weights were recorded every 3 d.

Bone ashing and biochemical analysis

Bone ashing was performed as previously described (29). Briefly, femurs were isolated, dried at 110 C overnight, and weighed. Bones were then ashed at 800 C for 4 h, weighed again, dissolved in 500 μl 6 N HCl, and calcium content was determined by colorimetric determination with cresolphthalein complexone (Sigma, St. Louis, MO). Total calcium was expressed as a percentage of ash weight.

To determine the serum calcium concentration, blood samples were obtained from 23-d-old mice at death, serum was separated by centrifugation, and total calcium was determined by colorimetric assay with the cresolphthalein complexone method (Sigma). Serum OCN levels were determined by RIA according to the manufacturer's protocol (Biomedical Technologies, Stoughton, MA).

Histological evaluation

The tibiae were trimmed of musculature and fixed in 10% formalin at 4 C. They were decalcified in 10% EDTA (pH 7.4) for 10 d and embedded in paraffin. Longitudinal sections of tibias were cut at 5 μm and stained with hematoxylin and eosin (H&E) for histological evaluation. Calvariae were fixed in 10% formalin at 4 C and decalcified in 10% EDTA (pH 7.4) for 7–8 d. Calvariae were then bisected perpendicular to the sagittal suture through the central portion of the parietal bones, parallel to lamboidal and coronal sutures, and embedded in paraffin to obtain sections of a standard area according to the method described by Zhao *et al.* (30). Five-micron-thick sections were cut and stained with H&E. The vertebrae were isolated, dissected free of soft tissue, and used for histomorphometric analysis. Vertebrae were fixed in 10% formalin at 4 C, decalcified in 10% EDTA (pH 7.4) for 10 d, and embedded in paraffin. Longitudinal sections were cut at 5 μm and stained with H&E. Histomorphometric analyses of the lumbar vertebrae were performed using a computer-assisted bone histomorphometric analyzing system (Image-Pro Plus version 4.0, Media Cybernetics, Silver Spring, MD).

Statistical analysis

The results of multiple experiments were analyzed using *t* test or ANOVA, followed by a Tukey-Kramer multiple comparison test with the Instat 2.1 biostatistics program (GraphPad Software, Inc., San Diego, CA). Uninjected controls (typically fewer than one in four mice) were analyzed *vs.* vehicle-injected controls with no qualitative or quantitative differences noted. The Northern blot analyses are presented with a representative assay in addition to a plot of data from multiple assays including their statistical evaluation.

Results

PTH differentially affected skeletal parameters in femurs of c-fos wild-type and mutant mice

The c-*fos* −/− mice had significantly decreased femur length and width compared with c-*fos* +/+ and +/− mice (Fig. 1, A and B). PTH treatment did not alter femur length or width in +/+ or +/− mice. In contrast, PTH-(1–34) treatment significantly decreased femur length and width in c-*fos* −/− mice. The c-*fos* knockout mice had reduced body weight *vs.* wild-type mice on d 4 (2.60 ± 0.08 *vs.* 3.13 ± 0.06 g; $P < 0.05$) and d 23 (5.8 ± 0.09 *vs.* 9.2 ± 0.23 g; $P < 0.05$), but PTH treatment did not alter the body weights within any genotype group on d 23.

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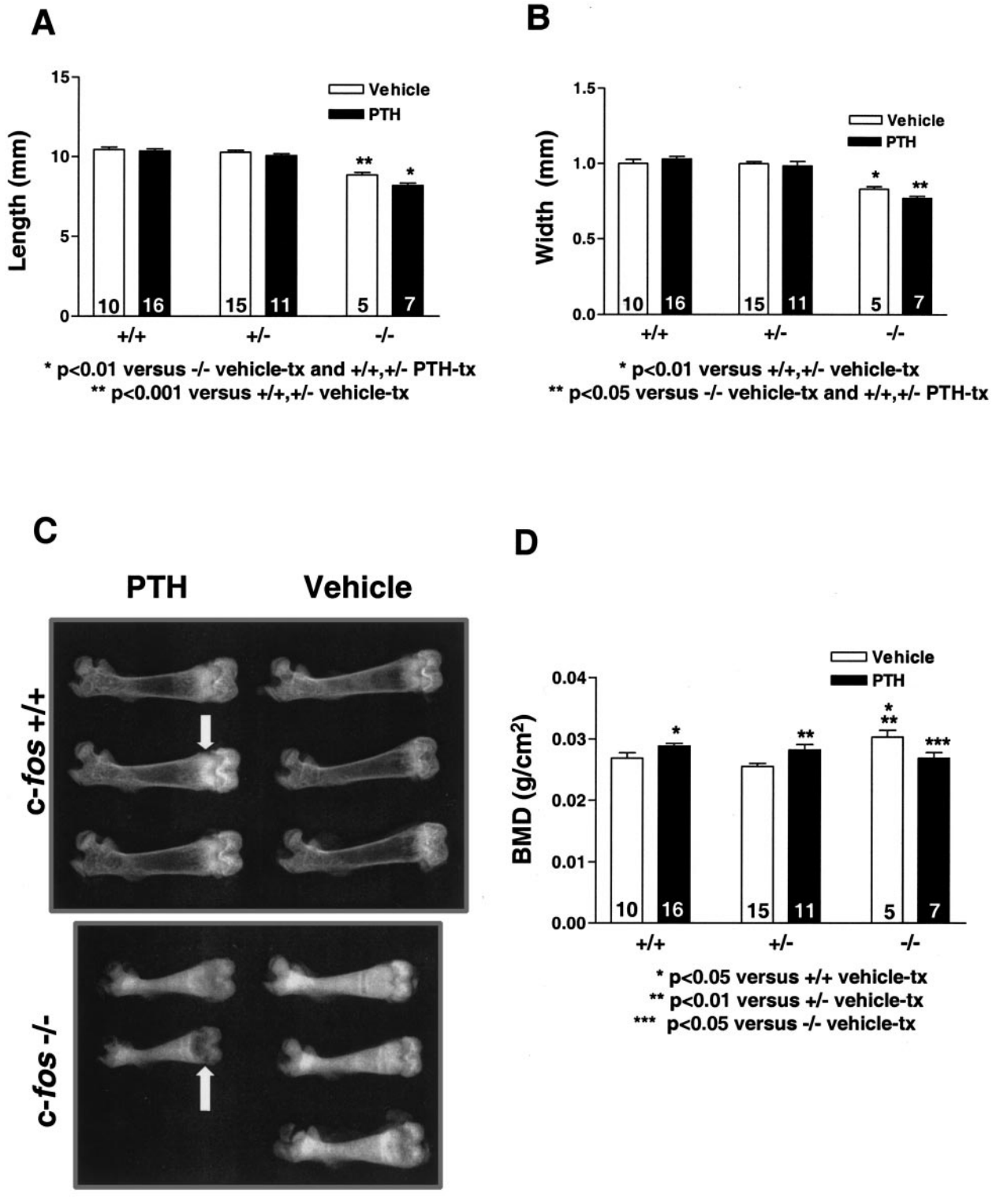

FIG. 1. Effects of PTH on femurs of c-*fos* normal and mutant mice. The c-*fos* −/− mice had reduced femur length (A) and width (B) compared with c-*fos* +/+ and +/− mice. PTH treatment decreased femur length and width in c-*fos* −/− mice, but not in +/+ or +/− mice. C, Microradiographic images of femurs from vehicle and PTH-treated mice. Femurs from c-*fos* −/− mice displayed a club-shaped morphology compared with their wild-type littermates. The femurs from c-*fos* +/+ mice treated with PTH had increased radioopacity in the regions of metaphyseal bone (*arrow*). In contrast, radiolucencies were detected in PTH-treated c-*fos* −/− mice in the same region (*arrow*). Intermittent PTH treatment during growth exaggerated the club-shaped morphology of the c-*fos* −/− mouse femurs. D, BMD determined by DEXA was higher in femurs from c-*fos* −/− mice than c-*fos* +/− and +/+ mice. PTH treatment resulted in an increase in BMD in c-*fos* +/+ and c-*fos* +/− mice, whereas it resulted in a reduction in BMD in c-*fos* −/− mice. Data are expressed as the mean ± SEM. The number of animals per group is indicated *on bar plots*.

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Microradiographs of femurs from c-*fos* +/+ mice demonstrated increased radioopacity in the regions of metaphyseal bone of femurs from mice treated with PTH-(1–34) (Fig. 1C). In contrast, radiolucencies were detected in the PTH-treated c-*fos* −/− mice in the same region. As previously reported, c-*fos* −/− mice had club-shaped femurs with a broader epiphysis and metaphysis, and their bone marrow spaces were occluded with matrix (14). Intermittent PTH-(1–34) treatment during growth (4–23 d) exaggerated this club-shaped phenotype.

To determine the effects of PTH in c-*fos* mutant mice, BMDs of femurs were measured using DEXA (Fig. 1D). The femoral BMDs of c-*fos* −/− mice on d 23 was increased *vs.* that of c-*fos* +/− and c-*fos* +/+ mice associated with their osteopetrosis. PTH treatment resulted in an increase in BMD in c-*fos* +/+ and c-*fos* +/− mice, in contrast to a reduction in c-*fos* −/− mice.

PTH differentially affected growth plate width and histomorphometry of vertebral and calvarial bone

Histological analysis of tibias from different genotypes revealed important differences (Fig. 2). In the c-*fos* +/+ mice, PTH treatment increased the zone of proliferating chondrocytes and increased trabeculation in the metaphysis of tibiae compared with those in vehicle-treated mice, but did not have the same effect in c-*fos* −/− mice. The growth plate and the zone of the hypertrophic and proliferating chondrocytes in vehicle-treated c-*fos* −/− tibiae was wider than that in c-*fos* +/+ mice. In addition, PTH treatment in c-*fos* −/− mice resulted in an even wider growth plate, with an increase in the zone of hypertrophic chondrocytes, but not in the zone of proliferating chondrocytes. This suggests that PTH increased the zones of proliferating and hypertrophic chondrocytes by c-*fos*-dependent and -independent mechanisms, respectively.

Histomorphometric analysis of vertebrae supported the c-*fos*-dependent PTH effects in trabecular bone as well. PTH treatment resulted in an increase in trabecular bone volume in c-*fos* +/+ and +/− mice, but a decrease in c-*fos* −/− mice (Fig. 3).

When histological sections of calvariae were compared, calvariae from c-*fos* +/+ and +/− mice had a greater width than those of c-*fos* −/− mice (Fig. 4). PTH treatment increased the width of the calvariae in c-*fos* +/+ and +/− mice, but did not alter c-*fos* −/− calvariae. The c-*fos* +/+ and +/− calvariae had more osteocytes than those in c-*fos* −/− mice, and PTH treatment resulted in an increase in the number of osteocytes in c-*fos* +/+ and +/− calvariae, but did not alter osteocyte number in c-*fos* −/− calvariae. Interestingly, in separate *in vitro* assays with primary calvarial osteoblasts, there was no alteration in osteoblast proliferation in response to PTH in cells from c-*fos* +/+ or −/− mice (data not shown).

PTH differentially altered ash weight and calcium content of femurs

The ash weight and calcium content of femurs from all genotypes were also determined. The ash weight of femurs from c-*fos* +/+ and c-*fos* +/− mice was significantly higher than c-*fos* −/− mice (Fig. 5). PTH treatment resulted in an increase in ash weight of femurs from c-*fos* +/+ and +/−, but not c-*fos* −/−, mice. When the total calcium was calculated as a percentage of ash weight, the calcium content of c-*fos* −/− femurs was higher than that in c-*fos* +/+ and +/− mice ($P < 0.05$). PTH treatment did not alter calcium/ash weight in c-*fos* +/+ and +/− mice, whereas it was significantly decreased in c-*fos* −/− femurs. This suggests that PTH increases physiological mineralization in normal mice, but the mineralization program in response to PTH is dysregulated in the absence of c-*fos*.

PTH differentially altered serum calcium and OCN levels

To determine the impact of the c-*fos* mutation and PTH administration on normal and c-*fos* −/− serum parameters, blood samples were obtained at death (48 h after the last injection). Serum calcium concentrations were significantly lower in c-*fos* −/− mice *vs.* c-*fos* +/+ and +/− mice. Serum calcium levels in c-*fos* +/+ mice were unchanged after PTH treatment, but were significantly increased in c-*fos* −/− mice (Fig. 6). Serum OCN levels were significantly lower in c-*fos* −/− mice and were unchanged by PTH administration. Other studies indicate elevated OCN levels with PTH administration (31, 32). The lack of alteration by PTH in this study may be due to the low dose of PTH administered, the short administration period (3 wk), and/or the fact that serum was collected 48 h after the last injection.

Extracellular matrix molecules, but not PTH-1R, were altered in c-fos mutant mice

To evaluate candidate genes that may be responsible for the demonstrated phenotype, Northern blot analyses of whole tissues were performed. The c-*fos* −/− mice had less steady state BSP and OCN expression in calvaria than c-*fos* +/+ mice (Fig. 7A). In contrast, there were no significant differences in PTH-1R mRNA expression in calvaria or kidneys of c-*fos* +/+ or c-*fos* −/− mice, and type X collagen (Col X) was elevated in femurs of c-*fos* −/− mice.

To evaluate the short-term effects of PTH, 20 μg hPTH-(1–34) were injected sc over the calvaria and Northern blot analysis of whole calvaria performed. Short-term PTH administration (8 h) resulted in a decrease in OCN and no change in PTH-1R mRNA expression in calvaria of +/+ mice, whereas there was no alteration in gene expression for OCN or PTH-1R in −/− mice (Fig. 7B). Although PTH is traditionally considered to increase OCN expression, studies of PTH administration to osteoblasts in culture indicate that PTH decreases OCN expression (33), and hence, differences may exist in the duration and mode of administration.

Discussion

The findings in this study using c-*fos* mutant mice support the hypothesis that the anabolic effects of PTH-(1–34) during growth depend on a functional c-*fos* gene. Once-daily PTH administration increased BMD and trabecular bone in wild-type mice. In contrast, PTH treatment resulted in opposite effects of reduced BMD and trabecular bone in c-*fos* mutant mice, suggesting that c-*fos* has an important mechanistic role

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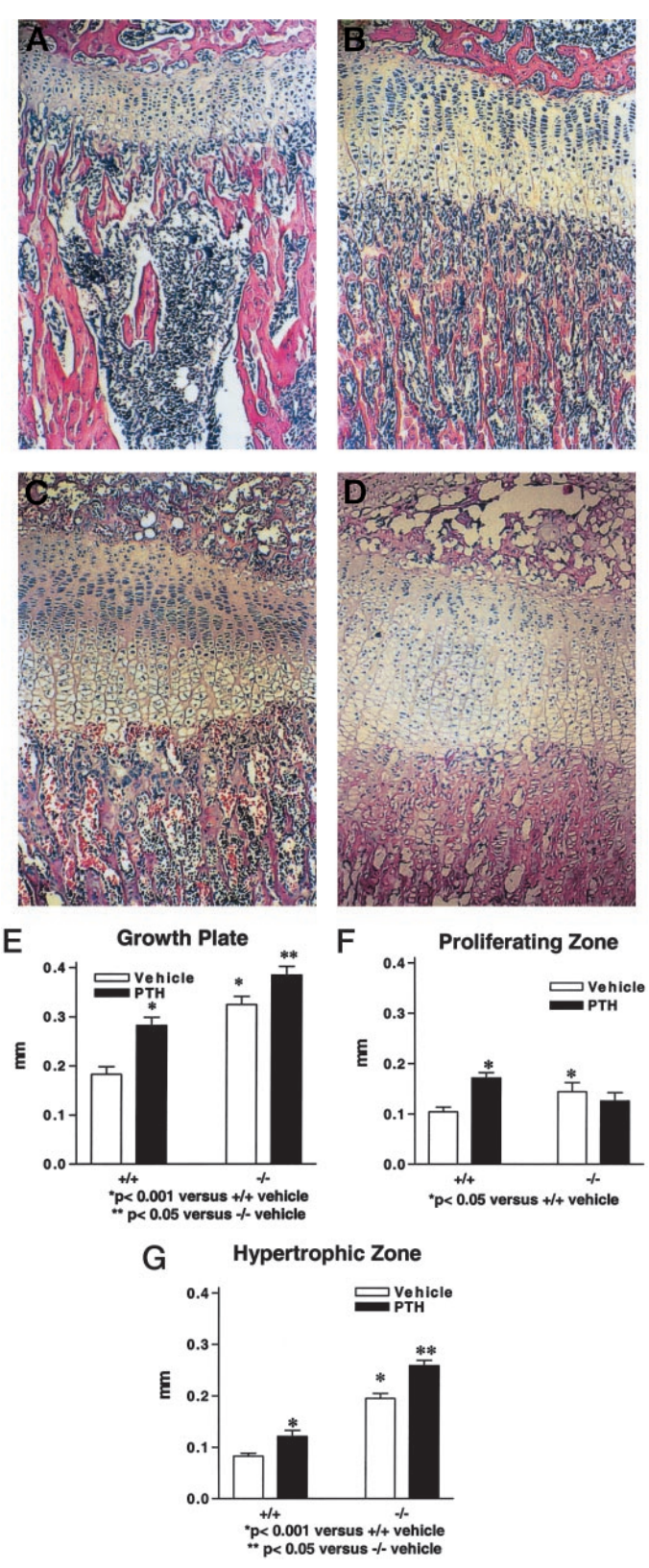

FIG. 2. Tibiae were sectioned longitudinally and stained with H&E for histological evaluation. A–D, Representative H&E-stained sections; E–G, graphic representation of growth plate measurements. A, c-*fos* +/+ vehicle treated; B, c-*fos* +/+ PTH treated; C, c-*fos* −/− vehicle treated; D, c-*fos* −/− PTH treated; E, total growth plate width; F, width of proliferating zone chondrocytes; G, width of hypertrophic zone chondrocytes. In the c-*fos* +/+ tibias, PTH treatment resulted in an increase in the zone of proliferating and hypertrophic zone chondrocytes compared with tibias from vehicle-treated mice. The growth plate in c-*fos* −/− mice was wider than that in c-*fos* +/+ mice due to an expanded zone of hypertrophic chondrocytes, but there was no alteration in the proliferating chondrocyte zone. Data are expressed as the mean ± SEM (n = 4–5 in −/− groups and 9–11 in +/+ groups).

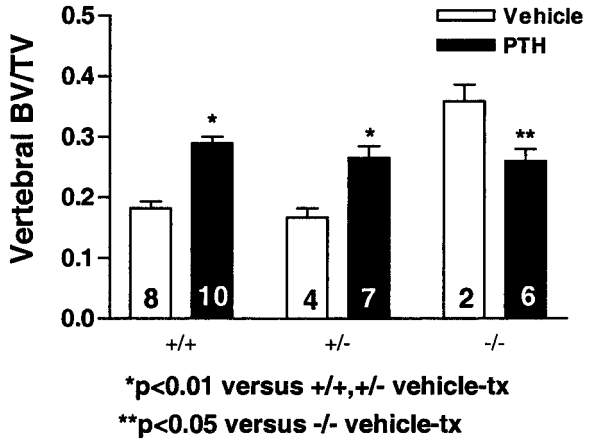

FIG. 3. Effect of PTH on the trabecular bone volume of vertebrae determined by histomorphometric analysis. PTH treatment resulted in an increase in the trabecular bone volume in c-*fos* +/+ and +/− vertebrae, but a decrease in c-*fos* −/− mice. Data are expressed as the mean ± SEM. The numbers of animals per group are indicated *on bar plots*.

in the anabolic effects of PTH. The decrease in BMD after PTH treatment in c-*fos* mutant mice, also supported by the findings of reduced calcium content of femurs after PTH treatment, could be due to the alterations in the mineralization program critical for bone growth and elongation. c-*fos* −/− mice had reduced serum calcium levels compared with c-*fos* +/+ mice, and levels in −/− mice increased after PTH treatment. The PTH-mediated increase in serum calcium levels in c-*fos* −/− mice might be due to enhanced calcium reabsorption from kidney and/or an inability to adequately incorporate calcium into the developing bone. Our findings of equivalent PTH-1R mRNA in the kidneys of c-*fos* +/+ and −/− mice suggest that alterations in c-*fos*-null mice are not due to alterations in organ level expression of the PTH-1R. Interestingly, not only did PTH not have an anabolic effect in the c-*fos*-null mice, but it appeared to have an antianabolic effect. Whether PTH alters the rate of bone formation in this model system is unclear, as parameters of dynamic bone histomorphometry were not evaluated. However, it is possible that PTH acts to inhibit mineralization through a c-*fos*-independent mechanism that is normally obscured by the c-*fos*-dependent anabolic action. In support of this, we recently reported that PTH and PTHrP induced the expression of matrix Gla protein, a potent inhibitor of mineralization (34).

One explanation for the lengthened growth plate and zone of hypertrophic chondrocytes in c-*fos* −/− mice after PTH treatment could be an alteration in the proliferation or maturation of chondrocytes. In addition, the widening of growth plates may be due to altered apoptotic events in c-*fos* −/− mice, as ossification in the epiphyseal growth plate of long

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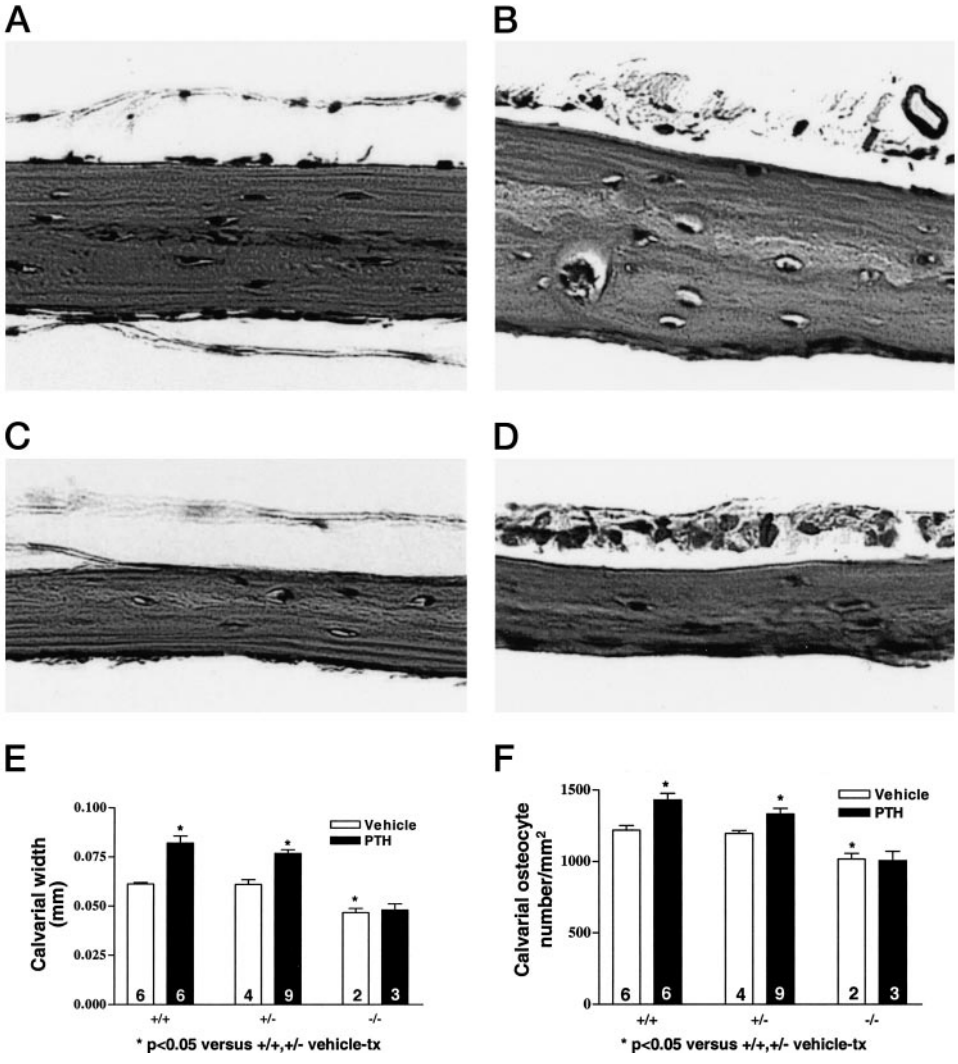

FIG. 4. The calvariae from vehicle- and PTH-treated c-*fos* mutant and wild-type mice. A–D, Representative H&E-stained sections; E and F, graphic representation of calvarial histological measurements. A, c-*fos* +/+ vehicle treated; B, c-*fos* +/+ PTH treated; C, c-*fos* −/− vehicle treated; D, c-*fos* −/− PTH treated; E, width of calvariae from vehicle- and PTH-treated c-*fos* mutant and littermate mice; F, osteocyte numbers in calvariae. Calvariae from +/+ and +/− mice were wider than those from c-*fos* −/− mice. PTH treatment resulted in an increase in the width of the calvariae in the c-*fos* +/+ and +/− mice, but did not alter calvariae in c-*fos* −/− mice. The c-*fos* +/+ and +/− mice had more osteocytes per unit area than c-*fos* −/− mice. PTH treatment resulted in an increase in the number of osteocytes in c-*fos* +/+ and +/− mice, but did not alter osteocyte number in c-*fos* −/− mice. Data are expressed as the mean ± SEM. The number of animals per group is indicated *on bar plots*.

bones is associated with chondrocyte programmed cell death. Jilka *et al.* (4) explained the anabolic effects of intermittent PTH by evidence that PTH increases the life span of mature osteoblasts *in vivo* by reducing the prevalence of their apoptosis rather than by affecting the generation of new osteoblasts. Furthermore, they suggested that the rate of apoptosis of osteoblastic cells can be a key mechanism for the effects of anabolic agents such as PTH (35). Similarly, PTHrP acts in the growth plate to inhibit the apoptosis of chondrocytes and prevent premature mineralization (36). It is possible that in our model system, systemic PTH effects are overlying local effects of PTHrP in the growing long bone and that the lack of increase in proliferating chondrocytes in the mutant mice treated with PTH as opposed to wild-type mice is due to the inability to inhibit apoptosis in the absence of Fos.

BSP is a bone matrix protein with a high affinity for calcium and is a valuable marker for osteogenic differentiation and bone formation (37, 38). OCN is a noncollagenous protein that is involved in the formation aspect of bone remodeling and is expressed late in the phase of osteoblast differentiation (39). The c-*fos* −/− mice had less steady state BSP and OCN mRNA expression in whole calvaria and reduced serum OCN than c-*fos* +/+ mice. As the vehicle-treated c-*fos* mutant mice had higher bone incorporated calcium and lower serum calcium levels, gene expression of these extracellular matrix molecules were also reduced. A reduction in OCN mRNA was also reported in PTH-1R knockout mice, which would also lack PTH-mediated increases in c-*fos* (40). The c-*fos* mutant mice expressed similar levels of PTH-1R as wild-type mice, but without the c-*fos* gene may be lacking a critical transcription factor for downstream actions of PTH. The increase in growth plate and decrease in BSP and OCN mRNA expression suggest a composition of less differentiated cells in c-*fos* mutant mice. The lack of c-*fos* might also affect the regulation of BSP and OCN gene expression, as both have AP-1 sites in their promoter regions (37, 41). The decrease in OCN mRNA expression after an acute catabolic-like regimen of PTH may be explained by its specific expression during the late phase of osteoblast differentiation. The lack of OCN regulation in the c-*fos*-null mice suggests that PTH-mediated changes in OCN are AP-1 dependent.

The phenotypic and histological features of the c-*fos* mutant models used in this study show similarities and differences when compared with models of PTHrP gene disruption (9) or overexpression (42), PTH gene disruption (8), and

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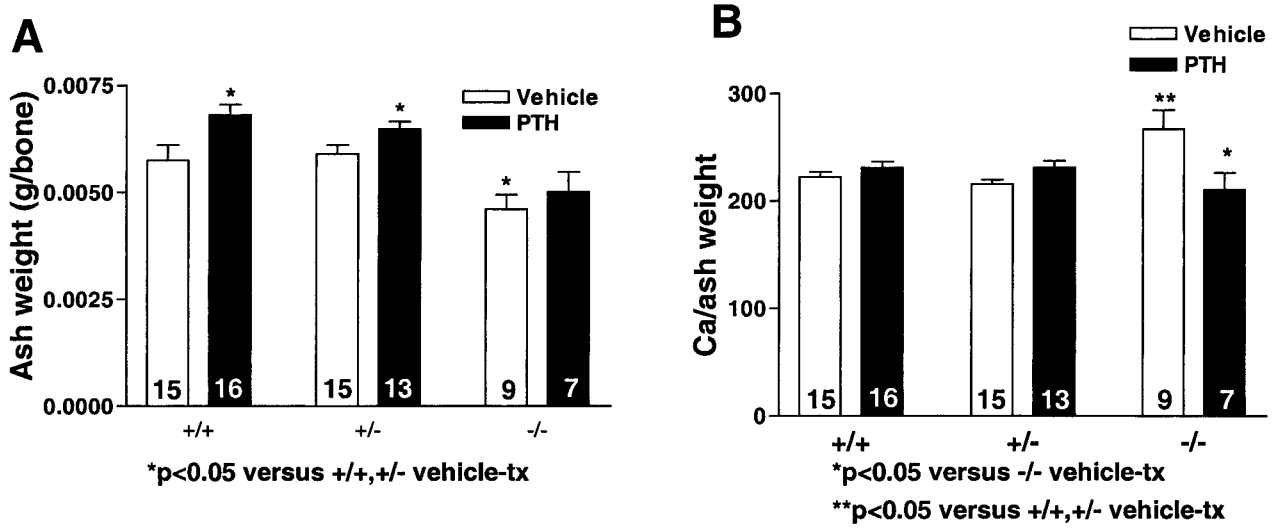

FIG. 5. Ash weight and calcium content of femurs. A, Ash weight of femurs expressed as grams of ash per bone; B, calcium per ash weight. PTH treatment resulted in an increase in the ash weight of femurs from c-*fos* +/+ and +/−, but not −/−, mice. When the total calcium was calculated as a percentage of ash weight, the c-*fos* −/− calcium content was higher than that in +/+ and +/− mice. PTH treatment did not alter the calcium/ash weight in c-*fos* +/+ and +/−, whereas it was significantly decreased in c-*fos* −/− mice. Data are expressed as the mean ± SEM. The number of animals per group is indicated *on bar plots*.

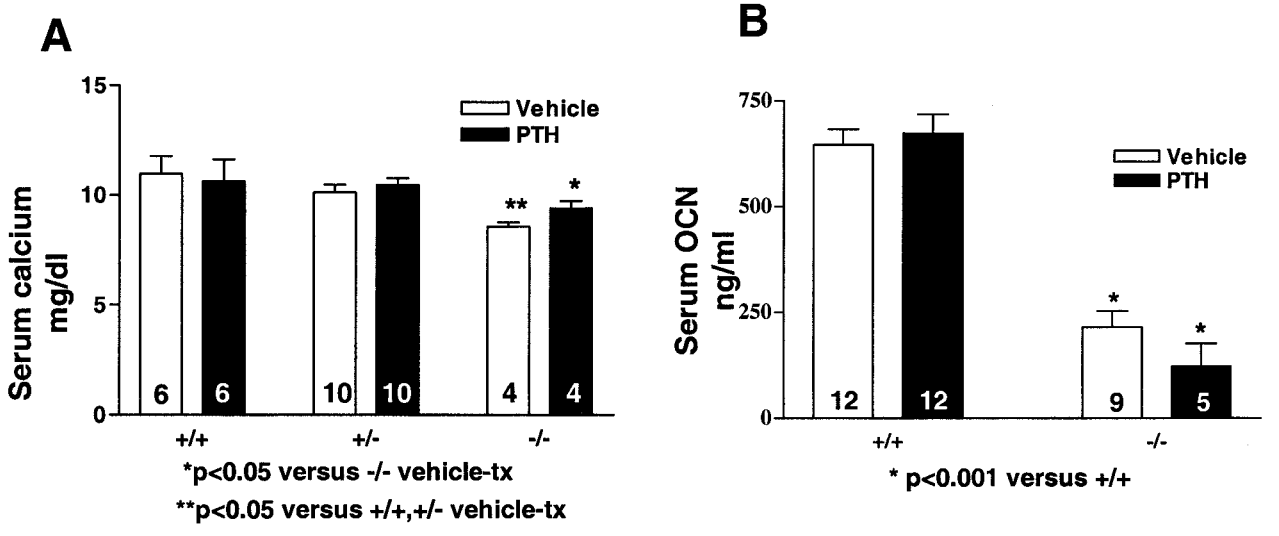

FIG. 6. Effects of PTH on serum calcium and OCN levels. Blood samples were obtained at death (48 h after last PTH injection). A, Serum calcium levels were reduced in c-*fos* −/− mice compared with +/+ and +/− mice. Serum calcium levels were increased in c-*fos* −/− mice after PTH treatment. B, Serum OCN levels were reduced in c-*fos* −/− mice and were not altered by PTH treatment. Data are expressed as the mean ± SEM. The number of animals per group is indicated *on bar plots*.

Jansen's chondrodysplasia (constitutively active PTH-1R) (43–45). When PTHrP is overexpressed in transgenic mice, there is an increase in the zone of proliferating and prehypertrophic chondrocytes embedded in the cartilaginous matrix and a marked widening in growth plates of long bones. In wild-type and heterozygous mice treated with PTH in our study we also noted an increase in proliferating chondrocytes. Disruption of the PTHrP gene results in a lethal skeletal dysplasia characterized by shortened limbs. Karaplis *et al.* (9) showed that there is a decrease in proliferating chondrocytes and an increase in hypertrophic chondrocytes in the growth plate. The balance between proliferating and hypertrophic chondrocytes is suggested to be controlled by a negative feedback mechanism between PTHrP and Indian hedgehog (46, 47). The c-*fos* knockout mice have widened growth plates, with an increase in the hypertrophic zone that is further exaggerated after PTH treatment. Interestingly, the PTH knockout mouse has an increased hypertrophic zone, but no change in the proliferating zone width (8). In Jansen's dysplasia the gross phenotypic features are similar to those seen in PTHrP overexpression and in the disruption of c-*fos*. They all have foreshortened and dumbbell-like shaped long bones, widened growth plates, and alterations in chondrocyte differentiation. Histologically, growth plates are widened in Jansen's dysplasia similar to PTHrP overexpression. In Jansen's dysplasia *vs.* c-*fos* ablation there is an increase in

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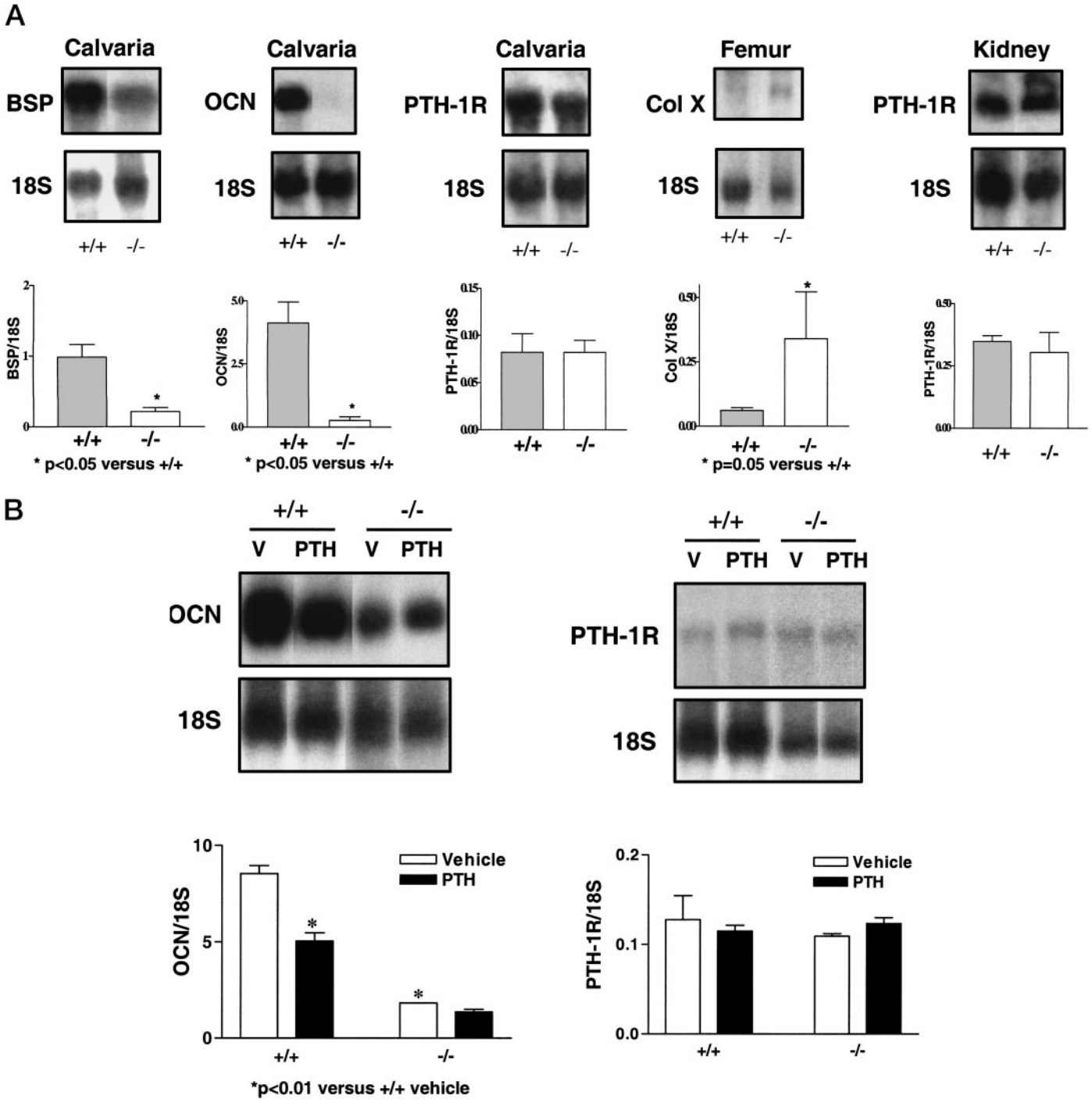

FIG. 7. Autoradiograph of representative Northern blot analysis with graphic representation of multiple samples of (A) BSP, OCN, PTH-1R, and type X collagen (Col X) gene expression in calvaria, femurs, and kidney; and (B) OCN and PTH-1R in calvaria of c-*fos* +/+ and −/− mice 8 h after a single PTH injection. The c-*fos* −/− mice had lower steady-state BSP and OCN and higher Col X expression than the c-*fos* +/+ mice. There were no significant differences in PTH-1R mRNA expression in the calvaria or kidney of c-*fos* +/+ and c-*fos* −/− mice. Injection of PTH resulted in a decrease in OCN mRNA in c-*fos* +/+ calvariae, but no alteration in −/− mice. PTH injection did not alter PTH-1R mRNA in either genotype. Data are expressed as relative values of counts per minute standardized to 18S rRNA and as the mean ± SEM (n = 2–6 mice/group).

the proliferating chondrocytes with a slow conversion of proliferative chondrocytes into hypertrophic chondrocytes and delayed vascular invasion (45). The c-*fos* knockout mice represent a model system that selectively dysregulates one aspect of the PTH and PTHrP signaling pathway. If c-*fos* was the only mediator of PTH and PTHrP action, one would expect a similar phenotype as that of the PTH/PTHrP receptor knockout mice (48). Instead, this model is more complex, as it probably allows some signaling, but totally eliminates osteoclastic activity. Hence, it is difficult to determine

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whether the expanded growth plate is due to the lack of osteoclastic activity or to c-*fos*-independent actions of PTH in the growth plate. That the phenotype of PTH-treated mice is different and in some measures opposite from that of the vehicle-treated mice substantiates that PTH is still active in the growing skeleton and supports the possibility that there are both c-*fos*-dependent and -independent pathways.

Studies with other factors that affect osteoclasts demonstrate similarity with the c-*fos* mutant mice. Mice lacking tartrate-resistant acid phosphatase (Acp5), a protein that increases in the plasma when bone remodeling is active, have osteopetrosis and widened growth plates, with an increase in hypertrophic chondrocytes (49). Bones are shortened, widened, and club-shaped. Mice deficient in gelatinase B, a matrix metalloproteinase that degrades components of the extracellular matrix with high specificity for denatured collagens, have delayed endochondral ossification, with an abnormally large hypertrophic cartilage zone (50). To our knowledge these models have not been treated with PTH, so it is difficult to conclude whether the findings we report here are due only to an alteration in c-*fos* activity or to a more global defect in osteoclasts. However, the lack of altered expression of OCN with acute PTH injection in c-*fos*-null mice suggests a c-*fos*-dependent effect that is independent of osteoclastic activity, as evidenced by the lack of a regulated effect during the short period analyzed. Furthermore, the anabolic regimen of PTH administration in c-*fos* knockout mice was not only ineffective at increasing bone mass, but actually reduced BMD and bone volume, indicating that PTH was biologically active, but in an opposite manner.

The use of a model system evaluating anabolic actions of PTH during endochondral bone growth is different from its prescribed use for patients with osteoporosis in whom the skeleton is mature. Anabolic actions of PTH during bone growth have been reported (6), although much of the focus in endochondral bone growth has been on PTHrP as a local mediator. As many of the processes of endochondral bone growth are recapitulated in bone remodeling and repair, our study provides novel findings that impact the roles of PTH and PTHrP in skeletal metabolism. Interestingly, another recent study found that anabolic actions of PTH during growth are dependent on IGF-I (7). Our study does not preclude a role for IGF-I, as IGF-1 also stimulates c-*fos* signaling. We did not find any alterations in PTH-stimulated IGF-1 mRNA in the c-*fos* knockout mice (data not shown) and hence speculate that Fos could be downstream of IGF-I in the PTH-mediated anabolic actions.

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