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Parathyroid Hormone Bone Anabolic Action Requires Cbfa1/Runx2-Dependent Signaling

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The Cbfa1/Runx2 (referred to herein as Cbfa1) transcription factor has been shown to be essential for osteoblast differentiation and bone formation during embryogenesis. PTH given intermittently is a proven bone anabolic agent. Here, we investigated whether PTH regulates the expression and/or activity of Cbfa1 in osteoblastic cells and in a rat metatarsal organ culture assay. PTH was found to regulate Cbfa1 mRNA in the rat osteosarcoma cell line UMR106 in a concentration-dependent manner. The effect of PTH was mimicked by forskolin, an activator of adenylate cyclase leading to the protein kinase A pathway. PTH administered intermittently for 5 d *in vivo* was found to stimulate Cbfa1 protein in the rat proximal tibiae metaphysis. To demonstrate PTH regulation of Cbfa1 activity, a construct containing six tandem Cbfa1 binding elements fused to luciferase was shown to be rapidly stimulated in response to PTH. This stimulation preceded the effects on mRNA regulation and resulted from a protein kinase A-mediated increase in Cbfa1 activity. Finally, using a neonate rat metatarsal organ culture system, we demonstrated dose-dependent anabolic responsiveness to PTH and to Cbfa1 overexpression from an adenoviral construct. We further showed that Cbfa1 antisense oligonucleotides that blocked adenoviral Cbfa1-induced anabolic effects in this organ culture model also abolished the PTH-mediated anabolic increase. These findings suggest a requirement for Cbfa1 in mediating the anabolic effects of PTH. Thus, regulation of Cbfa1 expression or activity is an important mechanism by which PTH controls osteoblast function. (*Molecular Endocrinology* 17: 423–435, 2003)

PTH IS A POTENT modulator of bone metabolism (1). In experimental animals and patients with osteoporosis, intermittent administration of PTH increases bone mass by stimulating *de novo* bone formation (2–7). At a cellular level, this increase in bone mass is associated with an increased activity of existing osteoblasts and an increased differentiation of osteoblast precursors or recruitment of lining cells to active osteoblasts (8, 9). The molecular mechanisms that mediate these osteoblastic effects of PTH remain to be elucidated. Marker genes that typically signal the bone formation process (such as extracellular matrix proteins, osteocalcin, osteopontin, collagen type 1, and alkaline phosphatase) have been well studied (9–12). Presently, a number of other genes have been shown to be regulated by PTH *in vivo* or *in vitro* (13–19). Although these genes have been associated with PTH actions, there are limited data demonstrating their requirement in bone formation or in PTH anabolic action. We propose that the PTH-induced anabolic effect on bone is likely to involve the control of a master regulatory gene(s) whose expression and activity governs osteoblast function.

The recent discovery of Cbfa1 (Runx2, OSF2, AML3, PEBP2αa) as a master regulatory gene in the osteoblast is of great interest. Cbfa1 is a member of the runt family of transcription factors (20) whose expression is absolutely required for the differentiation of osteoblastic precursors and in modulating the activity of mature osteoblasts (21–24). Targeted deletion of the Cbfa1 gene resulted in a complete lack of skeleton and absence of functional osteoblasts due to maturational arrest (25, 26). Specific expression of a dominant-negative Cbfa1 in mature osteoblasts resulted in severe reduction in bone formation and bone mass due to a decrease in osteoblast activity (27). Overexpression of Cbfa1 by the type I collagen promoter in a transgenic mouse produced defective osteoblasts that had clear effects on the skeleton, demonstrating the need for proper regulation of this gene in maintaining bone homeostasis (28, 29). Additionally, several *in vitro* experiments have demonstrated that Cbfa1 is a major regulator of the osteoblast phenotype and is necessary for osteoblast-specific expression of genes such as osteocalcin, collagen type 1, osteopontin, and bone sialoprotein (24, 25, 27).

Abbreviations: AP-1, Activator protein 1; ASO, antisense oligonucleotide; BMP, bone morphogenetic protein; FBS, fetal bovine serum; β-gal, β-galactosidase; GAPDH, glyceraldehyde phosphate dehydrogenase; GFP, green fluorescent protein; HRP, horseradish peroxidase; Luc, luciferase; MT model, rat neonate metatarsal organ culture model; OSE, osteoblast-specific element; PKA, protein kinase A; PKC, protein kinase C; Scr, scrambled Cbfa1 sequence; SMAD, Sma and Mad-related proteins.

Based on *in vitro* data and the phenotypes of mice with altered Cbfa1 expression, it seems likely that the expression and regulation of Cbfa1 activity in osteoblastic lineage cells might be important in determining bone formation induced by anabolic agents. At the present time, there are limited data on the expression and regulation of Cbfa1 by PTH. In this work, we evaluated the role of Cbfa1 in PTH action. We show that PTH regulates expression of Cbfa1 in a time- and dose-dependent manner. We found that regulation occurred both at the Cbfa1 mRNA level, and at the posttranslational level to increase the activity of Cbfa1 protein. The dose-dependent activity on mRNA and protein expression suggests an anabolic function for PTH at lower doses. However, higher doses of PTH decreased Cbfa1 mRNA and protein levels. Using the rat neonate metatarsal organ culture model (MT model) (30), PTH-mediated increases in endochondral ossification were also found to be dose dependent. Finally, a Cbfa1-specific antisense oligonucleotide (ASO) that blocks adenoviral Cbfa1-induced bone anabolic activity in this model, completely reversed the PTH anabolic effect. Collectively, this study points to a critical role for Cbfa1 in mediating the bone anabolic effects of PTH.

RESULTS

PTH Regulation of Cbfa1 Expression in Rat UMR106 Cells

We initially examined the ability of PTH to regulate Cbfa1 mRNA levels in the rat osteoblast cell line UMR106, which is known to express PTH receptors and is capable of responding to PTH by stimulating intracellular cAMP levels (31). Treatment of UMR106 cells with the fully active PTH (1–38) revealed a concentration-dependent and time-dependent regulatory profile (Fig. 1). The dose-dependent regulation, 24 h after PTH treatment showed that low levels of PTH (0.05–5.0 nM) stimulated Cbfa1 mRNA up to 4-fold, whereas higher levels of PTH (50 nM) had little effect or decreased expression of Cbfa1 mRNA (Fig. 1A). A time course study using 5 nM PTH demonstrated maximal stimulation of Cbfa1 mRNA at 24 h, whereas a high dose of PTH (1000 nM) resulted in a decrease of Cbfa1 mRNA at 12 h (Fig. 1B). These effects were predominantly mediated by the protein kinase A (PKA) signaling pathway, as the adenylate cyclase activator forskolin, showed a stimulatory effect on Cbfa1 mRNA after 24 h at 100 nM but showed inhibition of Cbfa1 mRNA at the higher 10 μM dose (Fig. 1C). Western blot analysis of Cbfa1 protein from UMR106 nuclear extracts of cells treated with different concentrations of PTH, for 24 h, confirmed the biphasic regulation seen at the RNA level (Fig. 2). Thus, the effects on Cbfa1 mRNA and protein expression in response to PTH are dose dependent and include both up-regulation and down-regulation of Cbfa1 depending on the concentration of PTH used.

In Vivo Stimulation of Cbfa1 Protein by PTH

To determine if the regulation of Cbfa1 by PTH could occur *in vivo*, we dosed Sprague Dawley rats with 80 μg/kg PTH 1–38 for 5 d. Transverse sections of proximal tibiae obtained from these rats 1 h after d 5 of injection show increased intensity of Cbfa1 nuclear staining using a Cbfa1-specific antibody compared with vehicle control (Fig. 3). This increased nuclear staining suggests increased Cbfa1 expression *in vivo* after an anabolic PTH treatment schedule.

PTH Regulation of Cbfa1 Activity in Rat UMR106 Cells

We next wanted to determine if the activity of Cbfa1 was modified in response to PTH treatment. We used a reporter construct consisting of 6 tandem Cbfa1 binding elements (6XOSE) cloned in front of an osteocalcin minimal promoter and fused to the luciferase (Luc) reporter gene (Fig. 4A). This construct, which is highly responsive to Cbfa1 protein (23), was transfected into UMR106 cells, and stable transfectants were isolated. Based on the stimulation of Cbfa1 mRNA seen at 24 h, we initially treated with PTH or 8-bromo-cAMP for 24 h. As shown in Fig. 4B, the 6XOSE Luc reporter was stimulated in a dose-responsive manner. An initial time course study revealed that PTH stimulation of the Cbfa1 response element was faster than 24 h, resulting in a large increase in Luc activity at 4 h and gradually decreasing over time to approximately 2-fold at 48 h. In fact, PTH induced stimulation as early as 1 h after treatment with a maximal activation seen at 4 h (Fig. 4C). To show that the stimulation of the 6XOSE construct by PTH was not specific to a particular UMR106 clone, we also examined transient transfectants of UMR106 with the 6XOSE/Luc construct as well as with a mutant 6XOSE/Luc construct, which was previously shown to poorly respond to Cbfa1 (23). As shown in Fig. 5A, after 4 h of PTH treatment, Luc activity was increased approximately 10-fold when the wild-type 6XOSE construct was transfected into the cells, whereas the mutant 6XOSE was only marginally increased. Moreover, a stable 6XOSE/Luc clone of the ROS17/2.8 cell line responded nearly identically to the UMR106 clone used for the majority of these experiments (data not shown). Evidence that stimulation of the PKA/cAMP pathway appeared to be responsible for Cbfa1 protein activation was shown by the ability of 8-bromo-cAMP to stimulate the Cbfa1 response element (Figs. 4B and 5B) in a dose-responsive manner similar to PTH (Fig. 5C). Additional evidence of the PKA pathway involvement was seen with the use of PTH analogs, which either signal both the PKA and protein kinase C (PKC) pathways (PTH 1–38), or just either the PKA (PTH

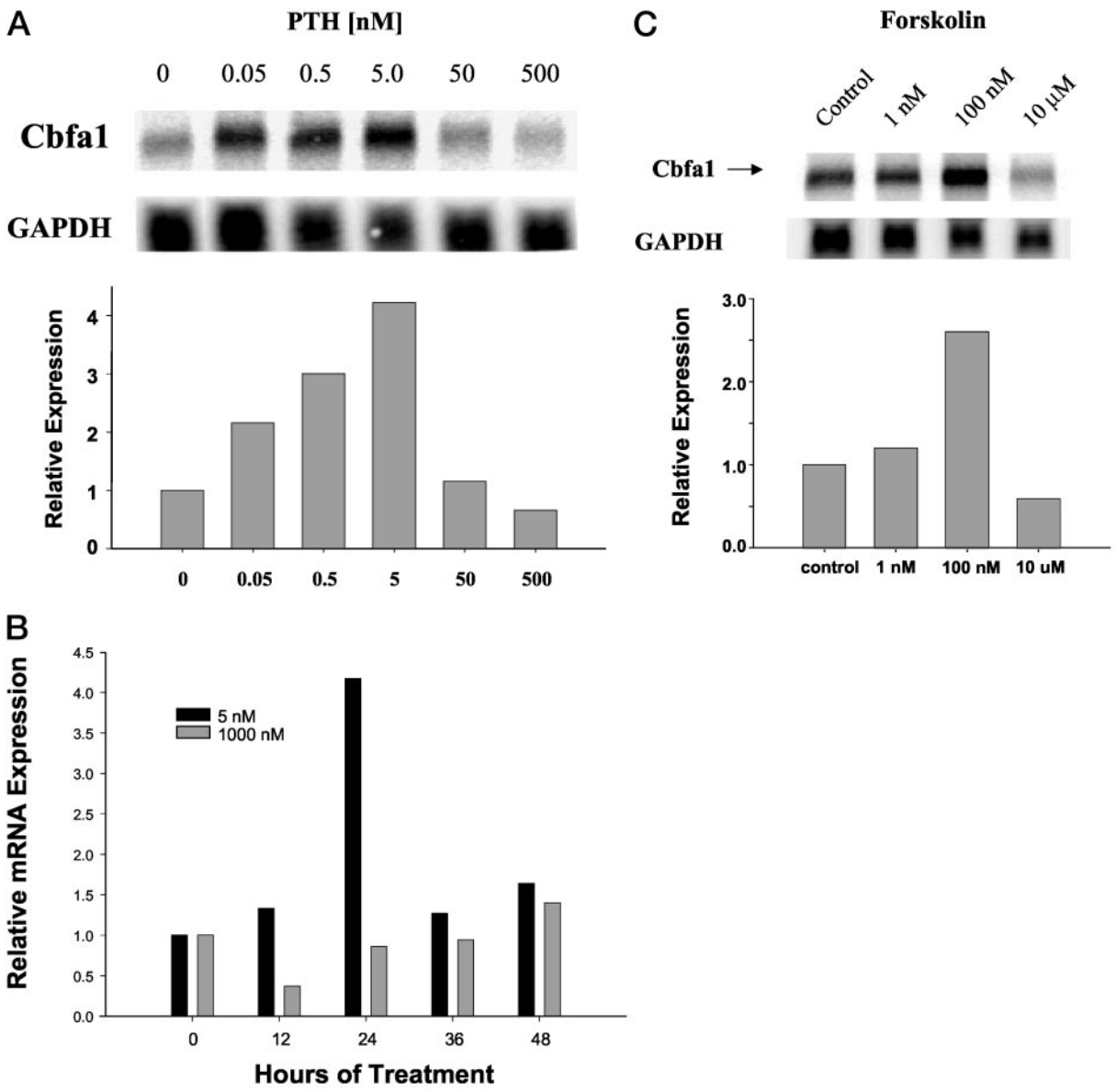

Fig. 1. PTH Regulates Cbfa1 mRNA in UMR106

A, UMR106 cells were treated for 24 h with the concentrations (nM) of PTH 1–38 indicated. The *top band* resulted from hybridization with a rat Cbfa1 probe, whereas a rat GAPDH cDNA probe (*bottom band*) was used as a control for amount of mRNA loaded. The graphic representation of the amount of Cbfa1 mRNA was derived from PhosphorImager quantitation to calculate relative intensity of the Cbfa1 bands normalized to GAPDH. This represents one of four different experiments in which an approximate 4-fold stimulation of Cbfa1 mRNA was seen at the 0.5–5.0 nM concentration range. B, UMR106 cells were treated for up to 48 h with either 5 nM (*black bar*) or 1 μM (*gray bar*) PTH 1–38 before RNA was extracted and analyzed by Northern blot. The relative expression was derived from PhosphorImager quantitation of the Cbfa1 bands and GAPDH bands from each mRNA source. C, UMR106 cells were treated with the PKA activator, forskolin for 24 h, and the RNA blot was hybridized to Cbfa1 and GAPDH probes as described above. Note the increase in Cbfa1 mRNA with 100 nM forskolin and decrease with 10 μM.

1–31) or PKC (PTH 3–34) pathways (32, 33) (Fig. 5D). The PKA inhibitor, H89 (34), was also used to show that the 6XOSE stimulation by PTH was almost entirely mediated through a PKA-dependent mechanism (Fig. 5E). Interestingly, whereas 10 nM of PTH was sufficient to maximally stimulate Cbfa1 activity (Fig. 5, A and C), higher concentrations did not appreciably reduce this stimulation, which contrasts with what was observed for Cbfa1 mRNA and protein expression (Figs. 1A and 2).

Bone Anabolic Activity of PTH in a Rat Metatarsal Organ Culture Model

To assess a functional role of Cbfa1 in PTH signaling in a more biologically relevant setting, we employed

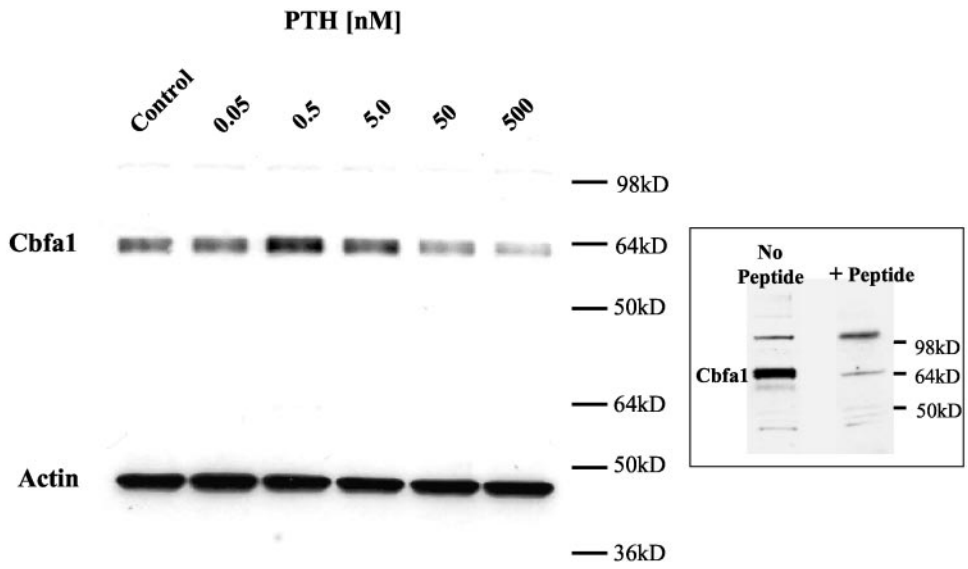

Fig. 2. PTH Regulates Cbfa1 Protein Levels

UMR106 cells were treated with various concentrations (shown in nM) of PTH for 24 h. Nuclear extracts were isolated and nuclear proteins analyzed by Western blot analysis using antibody specific for the N-terminal 26 amino acids of the osteoblast-specific form of Cbfa1. The blot was reanalyzed with antibody to actin to measure equivalency of extracts loaded onto the gel. Specificity of the Cbfa1 antibody is shown by the near-total disappearance of the correct size Cbfa1 band when the antibody is preincubated with the immunizing Cbfa1 peptide before Western analysis (*inset*).

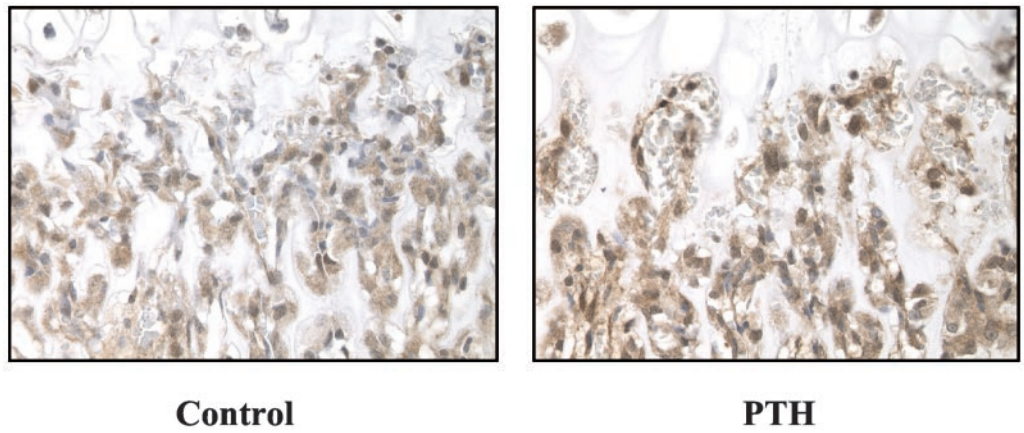

Fig. 3. PTH Increases Nuclear Staining of Cbfa1 Protein in the Rat Tibiae

Sprague Dawley rats were treated with PTH (80 μg/kg·d; sc injection for d 5) or vehicle control. Rat proximal tibiae were harvested at 1 h after d 5 of injection. Transverse section of representative proximal tibia (5 μm thick) were prepared for immunohistochemical evaluation with the same N-terminal peptide polyclonal antibody to Cbfa1 used for Western analysis. Image shown is original $\times$225 and is representative of the staining seen in sections from five different paired PTH- and control-treated animals.

an organ culture model that is capable of responding to bone anabolic agents (35). This model allows for the appropriate interplay between various cell types in their natural matrix microenvironment. In this model, neonatal rat metatarsal bones are removed and individually cultured in a 96-well plate (30). The length of the central, mineralized bone in the putative diaphyseal region of the metatarsal is quantitatively imaged at the beginning of the 7-d culture period, and then again at the end of the 7-d incubation period to measure relative growth with treatment compared with vehicle control (see *inset* to Fig. 6A). Increase in mineralized bone over the 7 d of incubation is defined as bone anabolic activity in this model. We found that PTH at a concentration of 1 and 10 nM had a reproducible and significant stimulatory effect on these bones with about a 3-fold increase in growth compared with vehicle control (Fig. 6A). At higher PTH concentrations, the stimulatory effect was reduced, and at levels of 100 nM and greater, there was no significant stimulation of the metatarsals. This low dose-dependent induc-

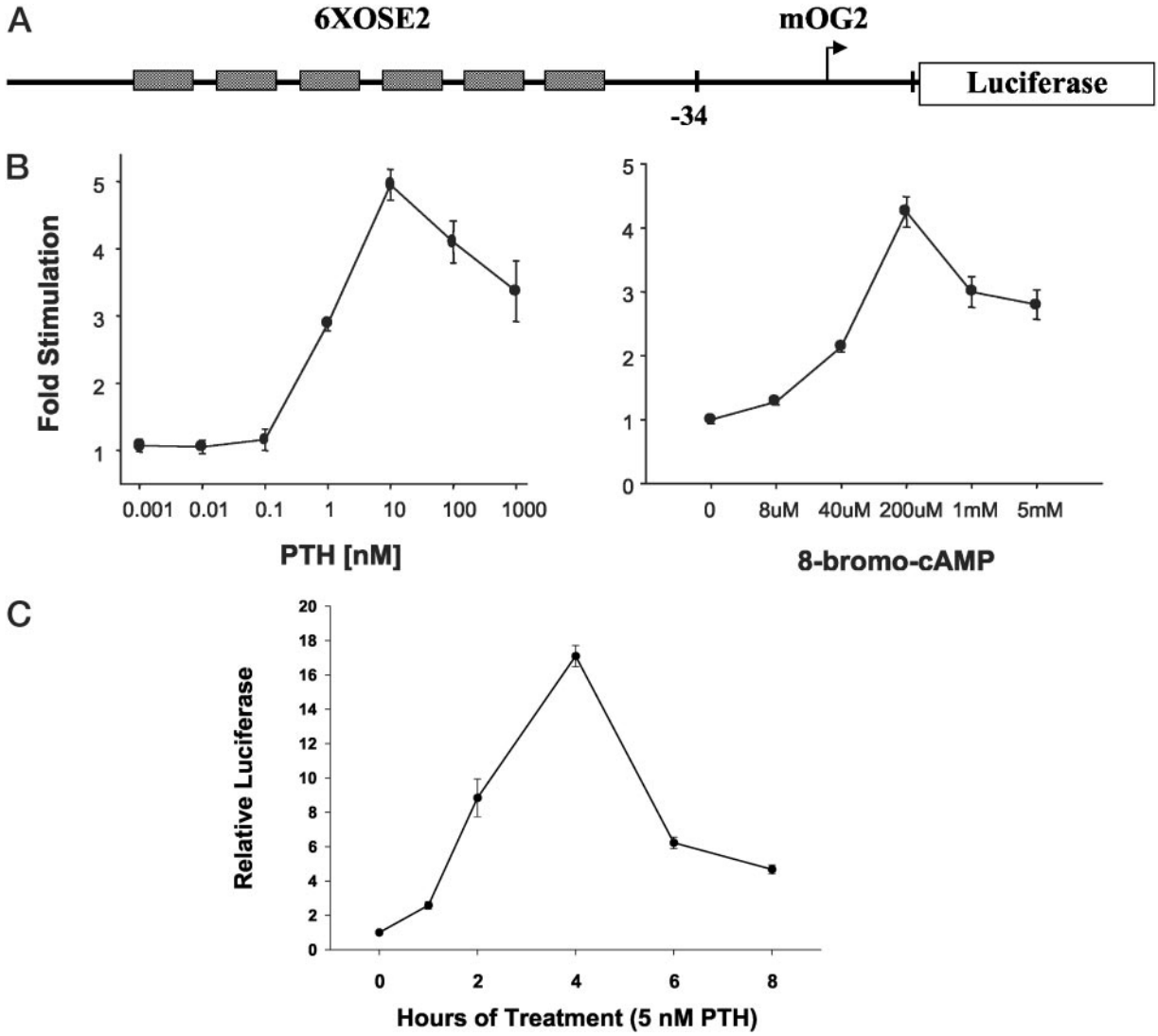

Fig. 4. The Cbfa1 6XOSE Response Element Is Activated by PTH and PKA Stimulation

A, Diagrammatic representation of the 6XOSE response element/Luc construct used to measure relative Cbfa1 activity. Luc is regulated by a minimal osteocalcin promoter (mouse OG2) and six copies of the Cbfa1 response element (OSE); core sequence = AACCACA. B, UMR106 cells stably transfected with the 6XOSE/Luc construct were treated for 24 h with various concentrations of PTH (*left panel*) or 8-bromo-cAMP (*right*). *Error bars* represent SEM, n = 4 for each concentration. C, A time-course of treatment with 5 nM PTH in UMR106 cells stably transfected with the 6XOSE/Luc demonstrated maximal activation at 4 h. *Error bars* represent SEM, n = 4 for each concentration.

tion of bone formation activity is consistent with the PTH doses used to increase Cbfa1 mRNA in UMR106 cells.

Various concentrations of PTH 3–34, PTH 1–38, and a PKA-specific inhibitor H-89 (34) were used to study the role of PKA *vs.* PKC pathways in mediating these anabolic changes in the rat neonate metatarsal model. Statistically significant ($P < 0.05$) activity compared with vehicle control was observed at 1 nM PTH 1–38, and this activity was significantly reduced by cotreatment with 50 nM H-89 (Fig. 6B). In contrast, PTH 3–34 failed to show significant anabolic activity at 10 nM and 100 nM concentrations (Fig. 6B). The 10-fold higher dose of PTH (3–34) used was based on its relative affinity to the PTH receptor type I (36). Interestingly, H-89 cotreatment did not completely reverse the PTH-induced bone anabolic activity as it did in the 6XOSE reporter assay (refer to Fig. 5C). To more fully understand the role of PKA (*vs.* PKC) in mediating this activity, we used a form of PTH, previously described by Takasu *et al*. (36, 37). This form of PTH $1–28^{(Gly\ 1,\ Arg\ 19)}$ is devoid of any PKC activity and binds the PTH receptor type I with similar affinity as the PTH 1–38. PTH $1–28^{(Gly\ 1,\ Arg\ 19)}$ induces a dose-dependent bone anabolic activity in the MT model which is similar to that of PTH 1–38 (refer to Fig. 6C). Collectively, these results using PKA-specific forms of PTH and PKA-specific inhibitors indicate that the dose-

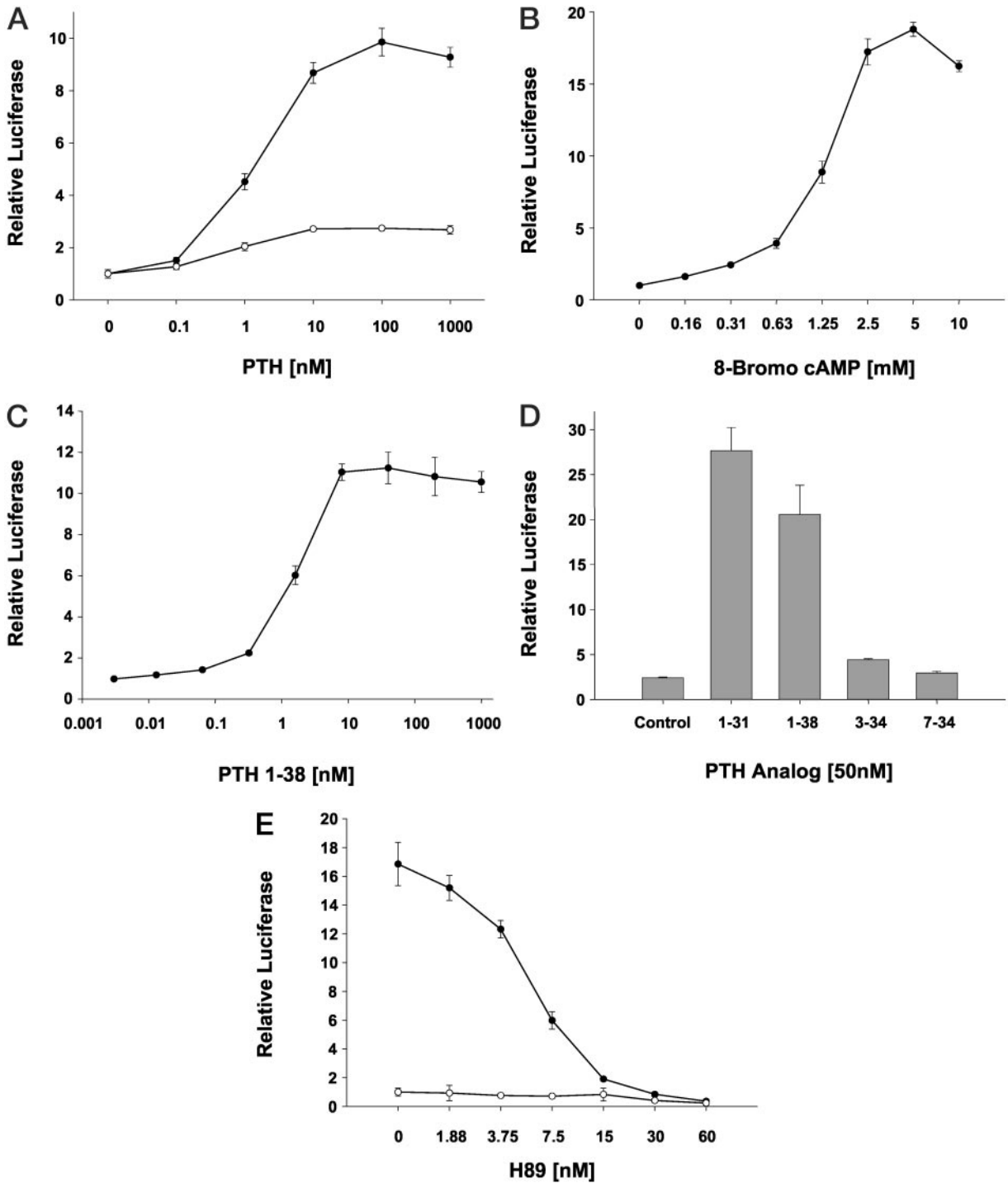

Fig. 5. Acute Activation of the Cbfa1 6XOSE Response Element

A, Transient transfections of UMR106 cells with the wild-type 6XOSE/Luc (*closed circles*) or the mutant 6XOSE/Luc (*open circles*) were left overnight before treatment with PTH at the indicated concentrations for 4 h. *Error bars* represent SEM, n = 4 for each concentration. B, 8-Bromo-cAMP dose-dependent regulation of Cbfa1 activity in the stable UMR106 6XOSE/Luc transfectant after 4 h. C, PTH dose-responsive (5× dilutions) activation of Cbfa1 after 4 h treatment in stable 6XOSE/Luc transfectants. D, Different PTH analogs were used at a concentration of 50 nM each on the stable 6XOSE/Luc transfected UMR106 cells for 4 h. PTH 1–38 is considered fully active, PTH 1–31 lacks the ability to activate PKC and both PTH 3–34 and PTH 7–34 lack the ability to activate PKA (32, 33). E, The PKA-specific inhibitor H89 was added to cells 1 h before the addition of 50 nM PTH 1–38 for a 4-h incubation. *Closed circles* represent data from cells treated with 50 nM PTH 1–38 and H89; *open circles* represent treatment with H89 in the absence of PTH.

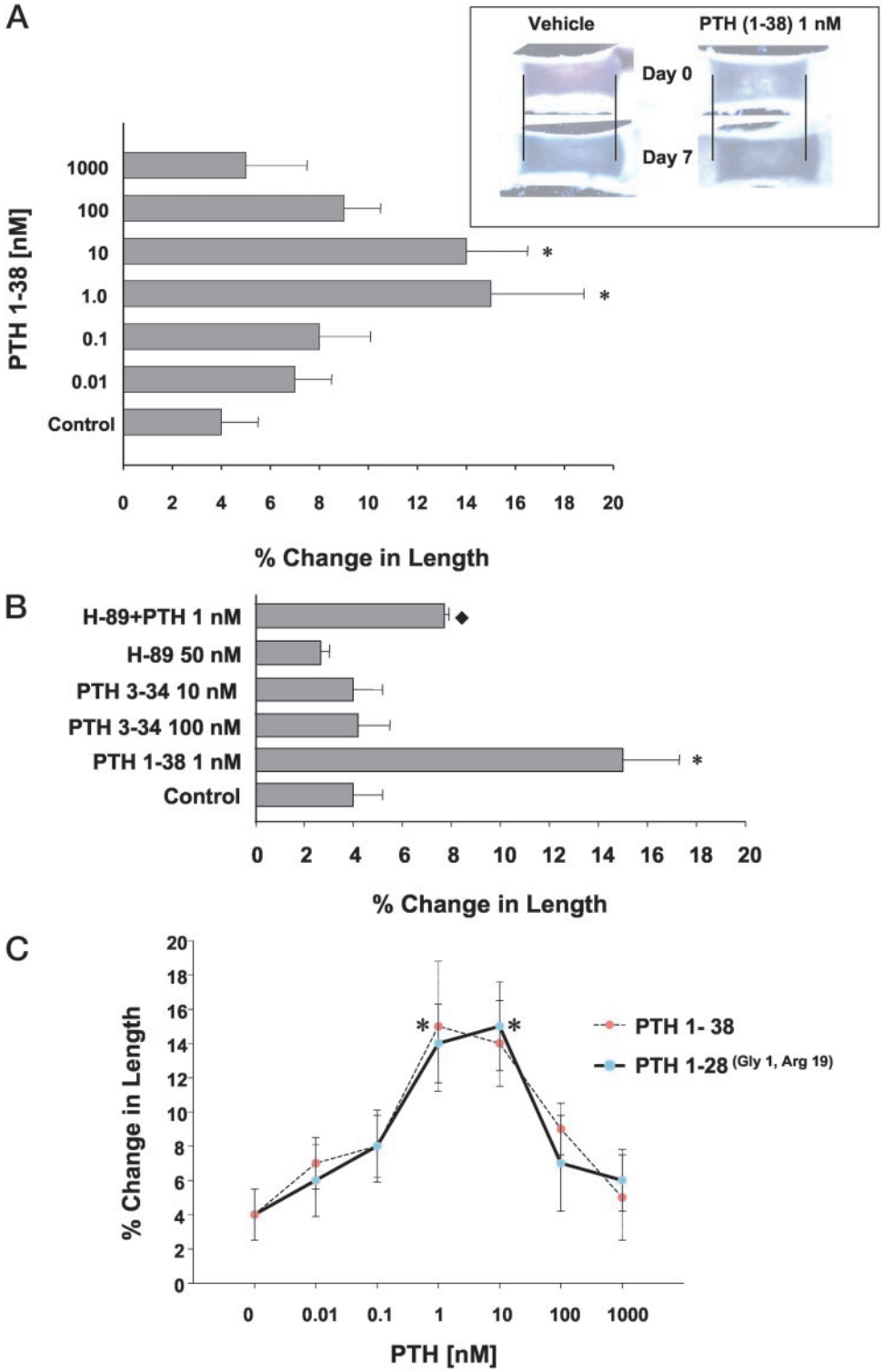

Fig. 6. PTH and Cbfa1 Can Stimulate Metatarsal Bone Growth

A, Rat neonate metatarsals were treated daily with PTH 1–38 for 7 d, and bone anabolic activity was measured as described in *Materials and Methods*. Data are expressed as the percentage of change in length of the central mineralized bone from d 0–7. Only the 1 nM and 10 nM doses were significantly different (∗, $P < 0.05$) from control. The *inset* depicts phase contrast images of the central mineralized metatarsal bone with the lines drawn to border this region at d 0. Note the greater length in mineralized bone with PTH treatment. B, PTH 1–38 or the PKA defective PTH analog 3–34 were used to dose metatarsals. Only PTH 1–38 showed significant (∗) stimulation over control, but the PKA inhibitor H89 significantly (◆) reduced the PTH 1–38 stimulation. C, Metatarsals were treated with the PKA-specific PTH analog 1–28 $^{(Gly\ 1,\ Arg\ 19)}$ and showed significant stimulation at the 1 and 10 nM doses (∗, $P < 0.05$). Data from metatarsals treated with PTH 1–38 are superimposed for comparison.

dependent bone anabolic activity of PTH is largely driven by its PKA specific signaling component.

Bone Anabolic Activity of Cbfa1 in the Rat Metatarsal Organ Culture Model

To investigate the consequences of Cbfa1 overexpression in this model, we infected the metatarsals with an adenoviral construct that expresses Cbfa1 protein. We tested the activity of this adenoviral Cbfa1 construct and found that it markedly stimulated the 6XOSE/Luc construct (but not the mutated 6XOSE/Luc) transfected into U2OS or C2C12 cells, both of which express reduced amounts of endogenous Cbfa1 protein compared with UMR106 (data not shown). A dose-responsive stimulation of metatarsal bone growth was found after Cbfa1 adenovirus was applied each day for 7 d (Fig. 7A). A control adenoviral construct expressing only β-galactosidase (β-gal) had no significant effect on metatarsal bone growth. Western blot analysis of protein lysates from several pooled infected metatarsals using an antibody to Cbfa1 revealed the full-length Cbfa1 protein expressed from the adenoviral construct (Fig. 7B). The parallel infection of metatarsals with the control adenovirus was unable to produce a detectable Cbfa1 band.

The adenoviral construct was engineered with a green fluorescent protein (GFP) tag downstream from the Cbfa1 gene using an internal ribosomal entry site to generate a bi-cistronic message. This allowed visualization of the infected regions within these treated metatarsals by following GFP fluorescence over time. In the metatarsals infected with the Cbfa1 adenoviral construct, we found a dose-dependent GFP signal (as early as 3 d) in the periosteum as well as in the putative diaphyseal region of the metatarsal (Fig. 7D).

To further assess the effects of Cbfa1 on metatarsal bone growth, ASO specifically targeted against rat Cbfa1 (22), were used to treat metatarsals along with an optimal dose (1 μl/ml) of Cbfa1 adenovirus. The Cbfa1 ASO blocked metatarsal stimulation by the Cbfa1 adenovirus, but a control oligonucleotide of scrambled Cbfa1 sequence (Scr) had no effect on this stimulation (Fig. 7A). In addition, the ASO or Scr oligos did not affect the growth of the β-gal adenovirus treated metatarsals.

PTH Bone Anabolic Activity in the Rat Metatarsal Organ Culture Model Is Cbfa1 Dependent

We have previously shown that in addition to PTH, another anabolic agent, bone morphogenetic protein (BMP)-4, increased metatarsal bone length and depended on Cbfa1 expression for activity (30). To test the role of Cbfa1 in mediating PTH-induced bone anabolic activity in the metatarsal model, we pretreated ASO to Cbfa1 along with 10 nM PTH. Cbfa1 ASO (but not Cbfa1 Scr) completely blocked the PTH stimulation of metatarsal growth, suggesting a Cbfa1-dependent mechanism (Fig. 8). These results thus implicate a functional requirement for Cbfa1 expression in the bone anabolic activity induced by PTH.

DISCUSSION

We have demonstrated that PTH, through activation of the PKA pathway, regulates the expression and activity of Cbfa1, and this effect is required for PTH-induced bone anabolic activity in a metatarsal bone organ culture. PTH was able to maximally increase 6XOSE/Luc activity from endogenous Cbfa1 protein in 4 h. It is likely that these early effects of PTH are mediated through stimulation of PKA to posttranslationally activate Cbfa1. Earlier reports have implicated specific signaling pathways, such as MAPK and PKA in modulating the activation function of Cbfa1 protein (38, 39).

The increase in Cbfa1 transcriptional activity as measured by the 6XOSE appears to be maximal after 4 h of PTH stimulation. A similar PTH-induced early increase ($\sim$4 h) has been reported for the osteocalcin promoter (40), a gene that is directly regulated by Cbfa1 (24). However, there is a delayed effect (24 h) on the regulation of Cbfa1 mRNA, which is very dependent on the concentration of PTH. Thus, whereas lower concentrations of PTH (5×10^{-11} to 5×10^{-9} M) stimulate expression of Cbfa1 mRNA and protein, higher concentrations of PTH tend to decrease their levels. It is noteworthy that in the metatarsal study PTH anabolic activity is only seen at the lower concentrations of PTH and suggests that the functional consequences of PTH-regulated Cbfa1 expression are important. It is likely that a combination of effects on Cbfa1 expression and on Cbfa1 activation are at play in determining the eventual anabolic or catabolic response to a given PTH dosing regimen *in vivo*. Results from the PTH1–28$^{(Gly\ 1,\ Arg\ 19)}$-treated metatarsals clearly indicate that the effects of PTH are largely dependent on the PKA pathway stimulated by cAMP accumulation via the G_s protein-coupled PTH receptor.

Previous work by others has shown a connection between PTH and activation of Cbfa1 in the regulation of the MMP-13 promoter (41–43). These researchers showed that a cooperative interaction of Cbfa1 and activator protein (AP)-1 at adjacent binding sites on the promoter is necessary for promoter induction by PTH. PTH was found to increase DNA binding activity of AP-1, whereas no increase in binding of Cbfa1 was apparent. The regulation of MMP-13 transcription by PTH required Cbfa1 but did not involve the direct activation of Cbfa1, only the increased DNA binding activity of AP-1. Recently, however, it was reported that stimulation of the MMP-13 promoter by PTH was blocked by PKA inhibitors but not other pathway inhibitors (39). That study demonstrated that a Gal4 DNA binding domain fused to the AD3 activation domain of Cbfa1, containing a consensus PKA site (see Ref. 44), was phosphorylated and transcriptionally activated by PTH. Thus, in addition to an increase in AP-1 binding, it was proposed that PTH induced a PKA-dependent transactivation of Cbfa1. It remains to be proven that PKA-dependent phosphorylation is responsible for native Cbfa1 activation, but our results would be consistent with that mechanism. Accordingly, our results

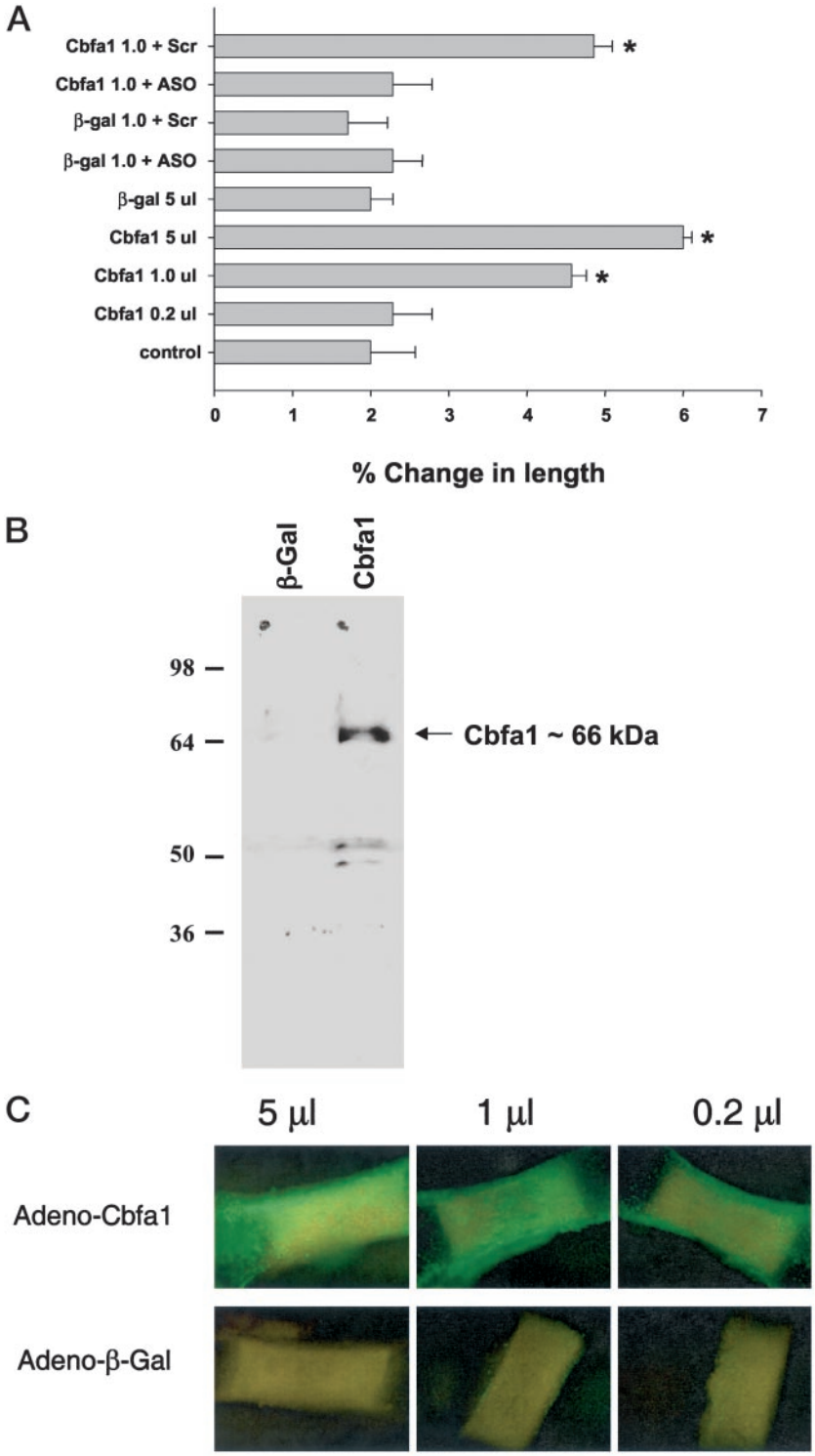

Fig. 7. Adenoviral Expression of Cbfa1 Stimulates Metatarsal Bone Growth and Is Blocked by Antisense Oligonucleotides to Cbfa1

A, Metatarsals were infected with adenoviral Cbfa1 at a volume of 0.2–5.0 μl/ml of growth medium (Cbfa1 adenovirus = 1.6×10^{12} viral particles (VP)/ml). As a control, an adenovirus expressing β-gal was used to infect metatarsals (Ad β-gal, 1.4×10^{12} VP/ml). Note that expression of adenoviral Cbfa1 at the two higher doses resulted in significant anabolic activity (*, $P < 0.05$). ASO to Cbfa1 or Scr was added 1 h before adenoviral treatment each day. B, Western blot from protein extracts of metatarsals showing intact full-length Cbfa1 protein (66 kDa) is expressed from the 1 μl/ml dose of the Cbfa1-adenoviral construct but not from the control adenovirus C, GFP fluorescence from the adenoviral Cbfa1 construct shows dose-dependent periosteal and chondrocytic expression of the adenovirus after 3 d dosing.

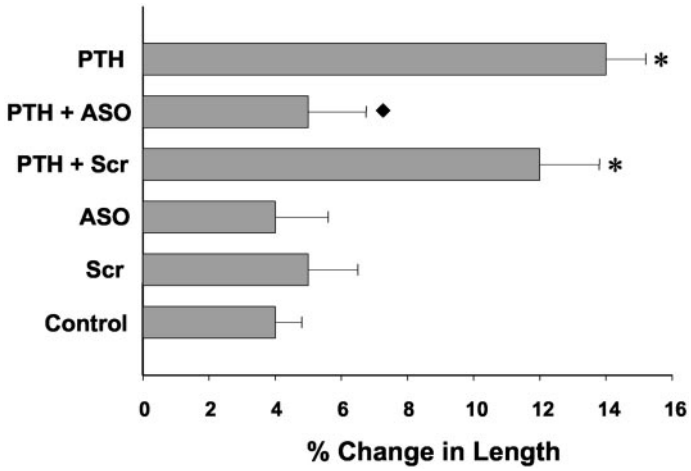

Fig. 8. Cbfa1 Expression Is Necessary for PTH Stimulation of Metatarsal Bone Growth

Metatarsals were treated with 10 nM PTH 1–38 alone, or pretreated with 10 μM rat Cbfa1 ASO or control oligos (Scr). Both oligos alone were found to have no significant effect on metatarsal growth. Statistically significant difference ($P < 0.05$) from control (*) or from PTH treatment (♦).

demonstrate PKA-mediated increases in endogenous Cbfa1 activity as measured by the 6XOSE reporter.

Tintut *et al*. (45) have demonstrated that in the MC3T3 osteoblastic cell line, continuous activation of the cAMP pathway over a period of days can stimulate the degradation of Cbfa1 through activation of the 26S proteasome complex. They noted, however, that reductions of Cbfa1 were not observed before 32 h of treatment, and in fact, greater amounts of Cbfa1 protein were observed with forskolin treatment after 24 h. In our studies, we found that only lower concentrations of PTH or PKA activators increased Cbfa1 message and protein, whereas higher concentrations did not (Fig. 1). It has been reported that continuous administration of PTH at high doses results in a longer period of PTH exposure with a net catabolic effect on bone, whereas intermittent or low PTH concentrations result in an anabolic response (2–4). Under these catabolic conditions, a recent study showed that Cbfa1 mRNA is down-regulated by PTH (46). Hence, it would be interesting to study the relationship between the dose-dependent effect of PTH on Cbfa1 mRNA and the anabolic or catabolic effect on bone.

Other studies have suggested stimulation of Cbfa1 activity by MAPK pathways. Xiao *et al.* (38) demonstrated phosphorylation of Cbfa1 in MC3T3 cells by MAPK kinase (MEK1). In that study, constitutively active MEK1 was able to stimulate osteocalcin mRNA and an osteocalcin promoter/reporter construct through the osteoblast-specific element (OSE) response element found in the osteocalcin promoter. This pathway linked earlier work addressing integrin activation by extracellular matrix, to stimulation of bone markers through Cbfa1 activation (47). Other reports have shown that the bone morphogenetic proteins BMP-2, -4, or -7 are capable of stimulating Cbfa1 expression (24, 48, 49). Whereas new Cbfa1 expression in response to BMP may be responsible for the osteogenic activity of BMP, the kinetics of Cbfa1 induction after BMP treatment suggests that it is more likely the result of an osteogenic processes already underway. The SMAD proteins, which act as transcriptional mediators of BMP and other TGF-β family members, have been shown to interact with Cbfa1 (50, 51). This interaction may provide a potential pathway by which BMP can effectively increase the activity of Cbfa1, although it has recently been shown that the osteogenic actions of Cbfa1 do not absolutely require association with SMAD1/SMAD5 (52). Conversely, it should be noted that our previous work (30) suggests a requirement for Cbfa1 in the ability of BMP-4 to increase metatarsal bone growth.

In conclusion, our findings point to a mechanism involving Cbfa1 by which PTH may regulate osteoblast function. These findings suggest that Cbfa1 is a central target that mediates the anabolic effects of PTH on bone. *In vitro*, the immediate response to PTH appears to be a PKA-dependent modulation resulting in enhanced transactivation of Cbfa1-mediated transcription. Subsequent to this PKA-mediated transactivation effect, PTH regulates Cbfa1 expression in a concentration-dependent manner. This concentration-dependent effect on Cbfa1 expression correlates with the dose-dependent bone anabolic effect as measured by the metatarsal organ culture model. Finally, using specific ASOs targeted against Cbfa1, we show that the PTH-induced anabolic activity is completely reversed in this *ex-vivo* bone model. It remains to be determined how central Cbfa1 is to other pathways of anabolic bone formation.

MATERIALS AND METHODS

Cell Culture

UMR106 cells were maintained in DMEM/F12 (3:1) media supplemented with 10% fetal bovine serum (FBS), 2 mM L-

glutamine, and 1× antibiotic-antimycotic (Invitrogen Corp., Carlsbad, CA). The cells were incubated in reduced serum (0.5% FBS) overnight before PTH treatment for the RNA and protein studies in UMR106. Stable cell lines containing six tandem OSE repeats in front of an osteocalcin minimal promoter fused to Luc (23) were generated in UMR106 cells using Fugene 6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN). The pSV2Neo plasmid was cotransfected for geneticin selection. Clones were maintained in parental culture media containing 500 μg/ml geneticin. All cultures were maintained in a humidified 5% CO_2 atmosphere at 37 C.

RNA Isolation and Northern Analysis of UMR106 Cells

Total RNA from UMR106 cells was isolated by a guanidium extraction and CsCl gradient centrifugation procedure as described in Ref. 53. RNA (25 μg) was loaded onto formaldehyde/agarose (10%/1.2%) gels. After electrophoretic separation, RNA was transferred to Nytran filters (Schleicher & Schuell, Inc., Keene, NH) and hybridized to random primer-labeled cDNA probes using modified Church hybridization conditions (53). Bands were visualized and quantitated by PhosphorImager and ImageQuant software (Molecular Dynamics, Inc., Amersham Biosciences, Sunnyvale, CA). Probes used were rat Cbfa1 (cloned as a PCR product from the DNA binding RUNT domain) and rat glyceraldehyde phosphate dehydrogenase (GAPDH; Ref. 14).

Transient Transfections and Luc Assays

A total of 1.7×10^6 UMR106 cells per 100-mm dish were plated approximately 24 h before being transiently transfected with 15 μg of either the wild-type 6XOSE (above) or a mutant 6XOSE driving Luc (23) using Lipofectamine Plus Reagent (Invitrogen Corp.). Cells were incubated for 3 h in serum-free media, then overnight in 2% FBS media. Cells were then dispersed evenly to a 96-well culture plate in 2% FBS complete media and allowed to adhere overnight. UMR106 stable clones were seeded at 3×10^{-4} cells per well in 96-well plates in 10% FBS complete media. Once adhered, cells were serum starved in 0.5% FBS media overnight. Both transiently transfected and stable clones were treated with human PTH (1–38) (Zeneca Pharmaceuticals, Wilmington, DE) for 4 or 24 h in 0.5% FBS complete media at given concentrations. PKA inhibitor H89 was purchased from Calbiochem (La Jolla, CA). Cells were harvested directly in the wells with 30 μl lysis buffer (Tropix, Inc., Bedford, MA) containing 0.5 mM dithiothreitol. Luc activity was determined by injecting 100 μl Luc reagent (Promega Corp., Madison, WI) per well. Relative light units were detected on a Luminoskan RT (Thermo Labsystems, Franklin, MA) luminometer with a 5-sec read time.

Western Blot Analysis

Nuclear extracts were isolated from treated cells (reduced serum to 0.5% overnight) as described (54) and the protein quantitated using Bio-Rad Laboratories, Inc. (Hercules, CA) protein dye reagent. Acrylamide gels (10% Tris/glycine, Invitrogen Corp.) were run with 20 μg of protein from each sample and electroblotted to nitrocellulose (Invitrogen Corp.). See-Blue protein markers (Invitrogen Corp.) were used to estimate the molecular weight of protein bands. Blots were blocked for at least 45 min in Blotto (TBST, 10 mM Tris-HCl at pH 8.0, 150 mM NaCl, 0.05% Tween-20 plus 5% nonfat dry milk) before incubation with 1:2500 dilution of anti-Cbfa1 specific antibody. The Cbfa1-specific antibody was generated in rabbits (Genosys Biotechnologies, Inc., The Woodlands, TX) with a peptide corresponding to the N-terminal 26 amino acids of Cbfa1: MASNSLFSAVTPCQQSFFWDPSTSRR. To show specificity of antibody for Cbfa1, 5 μg of this peptide were preincubated for 30 min at 30 C with the antibody before hybridization with a nitrocellulose blot of UMR106 protein extract. Serum antibody was IgG purified using the Bio-Rad Laboratories, Inc. (Hercules, CA) Econo-Pac serum IgG DEAE columns, as per manufacturer's instructions. A horseradish peroxidase (HRP)-conjugated anti-rabbit IgG polyclonal (Amersham Pharmacia Biotech, Piscataway, NJ) secondary antibody was used at 1:2000 dilution. Amersham Pharmacia Biotech enhanced chemiluminescence Western blotting reagents were used for signal detection. An actin antibody (SC1616, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at 1:200 dilution, and HRP-conjugated antigoat secondary antibody used at 1:75,000 dilution to show equivalent loading on the Western blot.

Immunohistochemistry Analysis of Cbfa1 Protein in Rat Femur

Four-week-old female Sprague-Dawley rats (Harlan, Indianapolis, IN) were maintained on a 12-h light, 12-h dark cycle at 22 C with *ad libitum* access to food of 1.2% Ca, 0.9% P in content (TD5001, Teklad, Madison, WI). Rats were sc injected daily with vehicle (20 mM NaH_2PO_4, 0.9% NaCl, and 2.13 g/liter mannitol), or PTH (1–38) 80 μg/kg·d for 5 d. Animals were killed at 1 h after the last dose.

Immunohistochemical staining was conducted on 5-μm thick, decalcified proximal tibial sections with a polyclonal antibody to Cbfa1 (see *Western Blot Analysis* section above) using an indirect immunoenzymatic Streptavidin detection method (Vector Laboratories, Inc., Burlingame, CA). Briefly, tissue sections were deparaffinized and rehydrated through a series of graded alcohols to distilled water. Sections were washed three times in PBS containing Brij 35 (0.1%). A PBS buffer wash was included after application of each histological reagent unless otherwise noted. An avidin/biotin blocking kit (Vector Laboratories, Inc.) was applied to block nonspecific binding, followed by preincubation in 10% normal goat serum for 30 min at room temperature. The Cbfa1 antibody at 1:300 was applied for 60 min at room temperature in a humidified chamber. The secondary antibody was applied for 30 min and endogenous peroxidase activity was blocked with 3% hydrogen peroxide in PBS for 30 min. A PBS buffer wash was performed and a Streptavidin HRP-conjugated labeling complex was applied for 30 min. Cbfa1 expression was visualized with a diaminobenzidine chromogen substrate (Sigma-Aldrich Corp., St. Louis, MO). Sections were counterstained in Mayer's Hematoxylin (Sigma-Aldrich Corp.), dehydrated, and mounted for subsequent light microscope evaluation.

Rat Neonate Metatarsal Culture

Newborn Sprague Dawley rats (Harlan, Indianapolis, IN) were killed at d 0 and the metatarsals were surgically isolated and placed in BGJ6 media (Life Technologies, Inc., Rockville, MD) without serum containing 10% antibiotic/antimycotic solution (Life Technologies, Inc.). Metatarsals were cultured for 7 d in a 96-well round bottom Petri dish (Nunclon, Nunc Products, Roskilde, Denmark) under 5% CO_2 at 37 C (see Ref. 30). Fresh media containing treatments or vehicle were replaced every 24 h. The metatarsals were imaged under a light microscope at the start of the 7-d incubation and changes in endochondral ossification were quantified at the end of the 7-d incubation period using an Image Pro analysis software. Data are shown as a percent change from d 0–7, performed in quadruplicate.

For treatment, wells were replaced with fresh media containing PTH vehicle (150 mM NaCl, 1 mM HCl, 0.1% BSA), and PTH analogs at the doses indicated. PTH analog $1–28^{(Gly\ 1,\ Arg\ 19)}$ was purchased from AnaSpec, Inc. (San Jose, CA). Fresh media containing the appropriate amounts of PTH analogs were provided every 24 h for 7 d. Adenoviral-Cbfa1 and adenoviral-β-gal (control) treatment was done daily with the indicated volumes of adenovirus stocks. For antisense oligomer assays, metatarsals were pretreated for 1 h with 10 μM rat Cbfa1/Runx2 antisense oligonucleotides (ASO) that have been described previously

(22). As a control, scrambled sequence oligonucleotides (Scr) of the ASO were used at the same concentration. For protein analysis, a set of 12 individual MT cultures treated with 1 μl/ml of Adeno-β-gal or Adeno-Cbfa1 for 7 d were boiled in a 6× SDS-PAGE loading dye and run on a 1.2% Tris-Glycine gel and transferred to a nitrocellulose membrane. Western blot analysis was performed as described earlier using antibodies raised against the N terminus of Cbfa1.

Adenoviral Cbfa1

Human Cbfa1 cDNA (amino acids 1–528 with a *Bgl*II linker) was cloned into the *Bgl*II site of the pQBI-AdCMV5-IRES-GFP vector (Quantum Biotechnologies, Montréal, Canada). Recombinant adenovirus was generated and amplified in QBI-293A cells using the Adeno-Quest kit and methods (Quantum). GFP positive viral plaques were screened for Cbfa1 expression by Western analysis. The control β-gal adenovirus used was the QBI-Infect+ virus with a lacZ gene expressed from the cytomegalovirus promoter (Quantum). U2OS, C2C12, and C3HT101/2 cells transfected with 6XOSE/Luc were infected with recombinant adenovirus to verify functional Cbfa1 expression. Amplified viral preparations were purified using the Viraprep filtration system (Virapur, Carlsbad, CA).

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REFERENCES

1. Potts JT, Bringhurst FR, Gardella T, Nussbaum S, Segre G, Kronenberg H 1995 Parathyroid hormone: physiology, chemistry, biosynthesis, secretion, metabolism, and mode of action. In: DeGroot LJ, ed. Endocrinology. 3rd ed., vol. 2. Philadelphia: W. B. Saunders; 920–966
2. Hock JM, Fonseca GJ, Raisz LG 1988 Human parathyroid hormone (1–34) increases bone mass in ovariectomized and orchidectomized rats. Endocrinology 122:2899–2904
3. Hock JM, Gera I 1992 Effects of continuous and intermittent administration and inhibition of resorption on the anabolic response of bone to parathyroid hormone. J Bone Miner Res 7:65–72
4. Dempster DW, Cosman F, Parisien M, Shen V, Lindsay R 1993 Anabolic actions of parathyroid hormone on bone. Endocr Rev 14:690–708
5. Cosman F, Lindsay R 1998 Is parathyroid hormone a therapeutic option for osteoporosis? A review of the clinical evidence. Calcif Tissue Int 62:475–480
6. Jerome CP, Johnson CS, Vafai HT, Kaplan KC, Bailey J, Capwell B, Fraser F, Hansen L, Ramsay H, Shadoan M, Lees CJ, Thomsen JS, Mosekilde L 1999 Effect of treatment for 6 months with human parathyroid hormone (1–34) peptide in ovariectomized Cynomolgus monkeys (*Macaca fascicularis*). Bone 25:301–309
7. Neer RM, Arnaud CD, Zanchetta JR, Prince R, Gaich GA, Reginster J-Y, Hodsman AB, Eriksen EF, Ish-Shalom S, Genant HK, Wang O, Mitlak BH 2001 Effect of parathyroid hormone (1–34) on fractures and bone mineral density in postmenopausal women with osteoporosis. N Engl J Med 344:1434–1441
8. Hock JM, Onyia JE, Miller B, Hulman J, Herring J, Chandrasekhar S, Harvey AK, Gunness M 1994 Anabolic PTH targets proliferating cells of the primary spongiosa in young rats, and increases the number differentiating into osteoblasts. J Bone Miner Res 9:S412 (Abstract)
9. Schmidt IU, Dobnig H, Turner RT 1995 Intermittent parathyroid hormone treatment increases osteoblast number, steady state messenger ribonucleic acid levels for osteocalcin, and bone formation in tibial metaphysis of hypophysectomized female rats. Endocrinology 136:5127–5133
10. McClelland P, Onyia JE, Miles R, Tu Y, Liang J, Harvey AK, Chandrasekhar S, Hock J, Bidwell JP 1998 Intermittent administration of parathyroid hormone (1–34) stimulates matrix metalloproteinase-9 (MMP-9) expression in rat long bone. J Cell Biochem 70:391–401
11. Noda M, Yoon K, Rodan GA 1988 Cyclic AMP-mediated stabilization of osteocalcin mRNA in rat osteoblast-like cells treated with parathyroid hormone. J Biol Chem 263: 18574–18577
12. Noda M, Rodan GA 1989 Transcriptional regulation of osteopontin production in rat osteoblast-like cells by parathyroid hormone. J Cell Biol 108:713–718
13. Lee K, Deeds JD, Chiba S, Un-No M, Bond AT, Segre GV 1994 Parathyroid hormone induces sequential c-fos expression in bone cells *in vivo*: *in situ* localization of its receptor and c-fos messenger ribonucleic acids. Endocrinology 134:441–450
14. Onyia JE, Bidwell J, Herring J, Hulman J, Hock JM 1995 *In vivo*, human parathyroid hormone fragment (hPTH 1–34) transiently stimulates immediate early response gene expression, but not proliferation, in trabecular bone cells of young rats. Bone 17:479–484
15. Pollock JH, Blaha MJ, Lavish SA, Stevenson S, Greenfield EM 1996 *In vivo* demonstration that parathyroid hormone and parathyroid hormone-related protein stimulate expression by osteoblasts of interleukin-6 and leukemia inhibitory factor. J Bone Miner Res 11:754–759
16. Liang JD, Hock JM, Sandusky GE, Santerre RF, Onyia JE 1999 Immunohistochemical localization of selected early response genes expressed in trabecular bone of young rats given hPTH 1–34. Calcif Tissue Int 65:369–373
17. Miles RR, Sluka JP, Santerre RF, Hale LV, Bloem L, Boguslawski G, Thirunavukkarasu K, Hock JM, Onyia JE 2000 Dynamic regulation of RGS2 in bone: potential new insights into parathyroid hormone signaling mechanism. Endocrinology 141:28–36
18. Miles RR, Sluka JP, Halladay DL, Santerre RF, Hale LV, Bloem L, Thirunavukkarasu K, Galvin RJ, Hock JM, Onyia JE 2000 ADAMTS-1: a cellular disintegrin and metalloprotease with thrombospondin motifs is a target for parathyroid hormone in bone. Endocrinology 141:4533–4542
19. Swarthout JT, D'Alonzo RC, Selvamurugan N, Partridge NC 2002 Parathyroid hormone-dependent signaling pathways regulating genes in bone cells. Gene 282:1–17
20. Speck NA, Terryl S 1995 A new transcription factor family associated with human leukemias. Crit Rev Eukaryot Gene Expr 5:337–364
21. Banerjee C, Hiebert SW, Stein JL, Lian JB, Stein GS 1996 An AML-1 consensus sequence binds an osteoblast-specific complex and transcriptionally activates the osteocalcin gene. Proc Natl Acad Sci USA 93:4968–4973
22. Banerjee C, McCabe LR, Choi J-Y, Hiebert SW, Stein JL, Stein GS, Lian JB 1997 Runt homology domain proteins in osteoblast differentiation: AML3/CBFA1 is a major component of a bone specific complex. J Cell Biochem 66:1–8
23. Ducy P, Karsenty G 1995 Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene. Mol Cell Biol 15:1858–1869
24. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G 1997 Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 89:747–754
25. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T 1997 Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89:755–764

26. Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GWH, Beddington RSP, Mundlos S, Olsen BR, Selby PB, Owen MJ 1997 Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89: 765–771
27. Ducy P, Starbuck M, Priemel M, Shen JH, Pinero G, Geoffroy V, Amling M, Karsteny G 1999 A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. Genes Dev 13:1025–1036
28. Liu W, Toyosawa S, Furuichi T, Kanatani N, Yoshida C, Liu Y, Himeno M, Narai S, Yamaguchi A, Komori T 2001 Overexpression of Cbfa1 in osteoblasts inhibits osteoblast maturation and causes osteopenia with multiple fractures. J Cell Biol 155:157–166
29. Geoffroy V, Kneissel M, Fournier B, Boyde A, Matthias P 2002 High bone resorption in adult aging transgenic mice overexpressing Cbfa1/Runx2 in cells of the osteoblastic lineage. Mol Cell Biol 22:6222–6233
30. Krishnan V, Ma YL, Moseley JM, Geiser AG, Friant S, Frolik CA 2001 Bone anabolic effects of sonic/Indian hedgehog are mediated by BMP-2/4 dependent pathways in the neonatal rat metatarsal model. Endocrinology 142:940–947
31. Ahlstrom M, Lamberg-Allardt C 1997 Rapid protein kinase A-mediated activation of cyclic AMP-phosphodiesterase by parathyroid hormone in UMR-106 osteoblast-like cells. J Bone Miner Res 12:172–178
32. Fujimori A, Cheng SL, Avioli LV, Civitelli R 1991 Dissociation of second messenger activation by parathyroid hormone fragments in osteosarcoma cells. Endocrinology 128: 3032–3039
33. Jouishomme H, Whitfield JF, Gagnon L, Maclean S, Isaacs R, Chakravarthy B, Durkin J, Neugebauer W, Willick G, Rixon RH 1994 Further definition of the protein kinase C activation domain of the parathyroid hormone. J Bone Miner Res 9:943–949
34. Chijiwa T, Mishima A, Hagiwara M, Sano M, Hayashi K, Inoue T, Naito K, Toshioka T, Hidaka H 1990 Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, *N*-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. J Biol Chem 265: 5267–5272
35. Haaijman A, D'Souza RN, Bronckers ALJ, Goei SW, Burger EH 1997 OP-1 (BMP-7) affects mRNA expression of type I, II, X collagen, and matrix Gla protein in ossifying long bones *in vitro*. J Bone Miner Res 12:1815–1823
36. Takasu H, Guo J, Bringhurst FR 1999 Dual signaling and ligand selectivity of the human PTH/PTHrP receptor. J Bone Miner Res 14:11–20
37. Takasu H, Gardella TJ, Luck MD, Potts JT, Bringhurst FR 1999 Amino-terminal modifications of human parathyroid hormone (PTH) selectively alter phospholipase C signaling via the type 1 PTH receptor: implications for design of signal-specific PTH ligands. Biochemistry 38:13453–13460
38. Xiao G, Jiang D, Thomas P, Benson MD, Guan K, Karsenty G, Franceschi RT 2000 MAPK pathways activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1. J Biol Chem 275:4453–4459
39. Selvamurugan N, Pulumati MR, Tyson DR, Partridge NC 2000 Parathyroid hormone regulation of the rat collagenase-3 promoter by protein kinase A-dependent transactivation of core binding factor a1. J Biol Chem 275: 5037–5042
40. Yu XP, Chandrasekhar S 1997 Parathyroid hormone (PTH 1–34) regulation of rat osteocalcin gene transcription. Endocrinology 138:3085–3092
41. Selvamurugan N, Chou W-Y, Pearman AT, Pulumati MR, Partridge NC 1998 Parathyroid hormone regulates the rat collagenase-3 promoter in osteoblastic cells through the cooperative interaction of the activator protein-1 site and the runt domain binding sequence. J Biol Chem 273: 10647–10657
42. Porte D, Tuckerman J, Becker M, Baumann B, Teurich S, Higgins T, Owen MJ, Schorpp-Kistner M, Angel P 1999 Both AP-1 and Cbfa1-like factors are required for the induction of interstitial collagenase by parathyroid hormone. Oncogene 18:667–678
43. Hess J, Porte D, Munz C, Angel P 2001 AP-1 and Cbfa/runt physically interact and regulate parathyroid hormone-dependent MMP13 expression in osteoblasts through a new osteoblast-specific element 2/AP-1 composite element. J Biol Chem 276:20029–20038
44. Thirunavukkarasu K, Mahajan M, McLarren KW, Stifani S, Karsenty G 1998 Two domains unique to osteoblast-specific transcription factor Osf2/CBFA1 contribute to its transactivation function and its inability to heterodimerize with CbfB. Mol Cell Biol 18:4197–4208
45. Tintut Y, Parhami F, Le V, Karsenty G, Demer LL 1999 Inhibition of osteoblast-specific transcription factor Cbfa1 by the cAMP pathway in osteoblastic cells—ubiquitin/proteasome-dependent regulation. J Biol Chem 274: 28875–28879
46. Ma YL, Cain RL, Halladay DL, Yang X, Zeng Q, Miles RR, Chandrasekhar S, Martin TJ, Onyia JE 2001 Catabolic effects of continuous human PTH (1–38) *in vivo* is associated with sustained stimulation of RANKL and inhibition of osteoprotegerin and gene-associated bone formation. Endocrinology 142:4047–4054
47. Xiao GZ, Wang D, Benson MD, Karsenty G, Franceschi RT 1998 Role of the α(2)-integrin in osteoblast-specific gene expression and activation of the osf2 transcription factor. J Biol Chem 273:32988–32994
48. Tsuji K, Ito Y, Noda M 1998 Expression of the PEBP2αA/AML3/CBFA1 gene is regulated by BMP4/7 heterodimer and its overexpression suppresses type I collagen and osteocalcin gene expression in osteoblastic and nonosteoblastic mesenchymal cells. Bone 22:87–92
49. Lee MH, Javed A, Kim HJ, Shin HI, Gutierrez S, Choi JY, Rosen V, Stein JL, vanWijnen AJ, Stein GS, Lian JB, Ryoo HM 1999 Transient upregulation of CBFA1 in response to bone morphogenetic protein-2 and transforming growth factor β 1 in C2C12 myogenic cells coincides with suppression of the myogenic phenotype but is not sufficient for osteoblast differentiation. J Cell Biochem 73:114–125
50. Hanai J, Chen LF, Kanno T, Ohtani-Fujita N, Kim W-Y, Guo W-H, Imamura T, Ishidou Y, Fukuchi M 1999 Interaction and functional cooperation of PEBP2/CBF with Smads. J Biol Chem 274:31577–31582
51. Zhang Y-W, Yasui N, Ito K, Huang G, Fujii M, Hanai J-I, Nogami H, Ochi T, Miyazono K, Ito Y 2000 A RUNX2/PEBP2aA/CBFA1 mutation diplaying impaired transactivation and Smad interaction in cleidocranial dysplasia. Proc Natl Acad Sci USA 97:10549–10554
52. Nishimura R, Hata K, Harris SE, Ikeda F, Yoneda T 2002 Core-binding factor a1 (Cbfa1) induces osteoblastic differentiation of C2C12 cells without interactions with Smad1 and Smad5. Bone 31:303–312
53. Geiser AG, Letterio JL, Kulkarni AB, Karlsson S, Roberts AB, Sporn MB 1993 Transforming growth factor B1 (TGF-B1) controls expression of major histocompatibility genes in the mouse: aberrant histocompatibility antigen expression in the pathogenesis of the TGF-B1 null mouse phenotype. Proc Natl Acad Sci USA 90:9944–9948
54. Krishnan V, Elberg G, Tsai M-J, Tsai SY 1997 Identification of a novel sonic hedgehog response element in the chicken ovalbumin upstream promoter-transcription factor II promoter. Mol Endocrinol 11:1458–1466