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14. ABSTRACT
During the last year of support (from July 1, 2007 to June 30, 2008), our studies have made significant progresses in all aspects of the study: i) we demonstrate that PTH increases ATF4 expression and activity and ATF4 is required for PTH induction of Ocn expression in osteoblasts. ATF4 is a novel downstream target of PTH signaling in osteoblasts; ii) we show that ATF4 is required for the anabolic actions of PTH on bone in vivo, these results were orally presented at the 2007 ASBMR (American Society for Bone and Mineral Research) annual meeting; and iii) We also shows that TFIIAγ increases osteoblast-specific gene expression by facilitating ATF4-Runx2 interactions; Taken together, these data further strongly support our original hypothesis and the specific aims. Two peer-reviewed research papers and two national meeting abstracts are generated from this study during this period of support. In the next year of support, we will: i) determine if ATF4 is required for the anabolic actions of PTH on bone in greater detail; ii) determine if ATF4 is required for PTH regulations of cell proliferation and apoptosis in vitro and in vivo; and iii) determine the role of ATF4-Runx2 interactions in PTH-induced osteoblast function.

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This progress report covers research from the period 04/01/07-06/30/08

Introduction

Osteoporosis is a bone disease that affects a large numbers of both men and women including many of our service women and men now in the Armed Forces and VA patients in the United States. It causes a significant amount of morbidity and mortality in patients and is often diagnosed after a fracture occurs. Reducing the risk of osteoporotic and associated fractures of these patients will greatly improve their life quality and survival. Parathyroid hormone (PTH) is the most potent anabolic treatment of osteoporosis currently available. It not only dramatically improves bone mass, but also restores bone microarchitecture and increases bone diameter. All of these mechanisms contribute to increasing bone strength and reducing the risk for fractures. However, the molecular mechanism whereby PTH increases bone formation remains largely unknown. Our central hypotheses in this study are: 1) PTH activates ATF4 by promoting its phosphorylation and protein-protein interactions with Runx2; and 2) ATF4 mediates the anabolic actions of PTH on bone. The long-term goal of this study has been to elucidate the molecular mechanisms underlying the anabolic actions of PTH on bone. Two Specific Aims have been proposed to determine the functional relationships between ATF4 and PTH actions on bone: 1) determine the mechanism whereby PTH regulates ATF4 transcriptional activity; 2) establish whether the anabolic actions of PTH require ATF4 in vivo. Studies determine if ATF4 is required for the anabolic actions of PTH in vivo using ATF4-deficient mice. PTH anabolic activity is evaluated in wild type and Atf4−/− mice. PTH effects are measured using standard biochemical and histomorphometric criteria.

Body

Task 1: To determine the mechanism by which PTH regulates ATF4 and Runx2 transcriptional activity (1-36 months). In order to determine the role of ATF4 in PTH actions in osteoblasts, we examined effects of PTH on ATF4 expression and activity as well as the requirement for ATF4 in the regulation of Ocn by PTH (see P1). PTH elevated levels of ATF4 mRNA and protein in a dose and time-dependent manner (P1-Fig. 1A-C). This PTH regulation requires transcriptional activity, but not de novo protein synthesis (P1-Fig. 2A and B). PTH also increased binding of nuclear extracts to OSE1 DNA (P1-Fig. 4A-C). PTH stimulated ATF4-dependent transcriptional activity mainly through PKA with a lesser requirement for PKC and MAPK/ERK pathways (P1-Fig. 5A-C). PTH stimulation of Ocn expression was lost by siRNA downregulation of ATF4 in MC-4 cells (P1-Fig. 6A and C) and in Atf4−/− bone marrow stromal cells (BMSCs) (P1-Fig. 7C). Collectively, these studies for the first time demonstrate that PTH increases ATF4 expression and activity and that ATF4 is required for PTH induction of Ocn expression in osteoblasts. Thus, ATF4 is a novel downstream target of PTH actions in osteoblasts. We defined a novel molecular mechanism mediating ATF4-Runx2 interactions (see P2). We identified general transcription factor IIAY (TFIIAY) as a Runx2-interacting factor in a yeast two-hybrid screen. Immunoprecipitation assays confirmed that TFIIAY interacted with Runx2 in osteoblasts and when coexpressed in COS-7 cell or using purified GST-fusion proteins (P2-Fig. 1A-C). Chromatin immunoprecipitation (ChIP) assay of MC3T3-E1 (clone MC-4) preosteoblast cells showed that in intact cells TFIIAY was recruited to the region of the osteocalcin promoter previously shown to bind Runx2 and ATF4 (P2-Fig. 2). A small region of Runx2 (aa 258-286) was found to be required for TFIIAY binding (P2-Fig. 1D). While TFIIAY interacted with Runx2, it did not activate Runx2 (P2-Fig. 3A and B). Instead, TFIIAY bound to and activates ATF4 (P2-Fig. 3C-H). Further, TFIIAY together with ATF4
and Runx2 stimulated osteocalcin promoter activity (P2-Fig. 5B) and endogenous mRNA expression (P2-Fig. 5A). siRNA silencing of TFIIAγ markedly reduced levels of endogenous ATF4 protein and Ocn mRNA in osteoblastic cells (P2-Fig. 6). Overexpression of TFIIAγ increased levels of ATF4 protein (P2-Fig. 7). TFIIAγ significantly prevented ATF4 degradation (P2-Fig. 8). Thus, TFIIAγ functions as a bridging protein linking ATF4 and Runx2. Current study in the project laboratory is determining if PTH regulates the expression of TFIIAγ in osteoblasts.

In addition, expression vectors harboring mutations of potential phosphorylation sites within ATF4 molecule have been successfully generated. The effects of these mutations on ATF4 transcriptional activity and its ability to activate Runx2 as well as on PTH response are being determined in the project laboratory.

**Fig. 1.** ATF4 deficiency severely impairs the anabolic effects of PTH on femurs. A, two-dimensional reconstruction from µCT scan of distal femurs from 5-day-old wt, Atf4<sup>+/−</sup> and Atf4<sup>−/−</sup> mice with and without intermittent PTH for 28 d are shown. B, quantitative analysis of effects of PTH on bone volume/tissue volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular space (Tb.Sp), and cortical thickness (Cort. Th). *P<0.05 (veh vs. PTH), †P<0.05 (PTH/veh-wt vs. PTH/veh-Atf4<sup>−/−</sup>).

**Task 2: To establish whether the anabolic actions of PTH require ATF4 (6-48 months).** In vivo, we examined if ATF4 is required for the anabolic actions of PTH on bone using an Atf4<sup>−/−</sup> mouse model. Five-day-old wt, Atf4<sup>+/−</sup>, and Atf4<sup>−/−</sup> mice were given daily subcutaneous injections of vehicle (saline) or hPTH(1-34) (60 ng/g body weight) for 28 days. In wt mice, µCT analyses of femurs show PTH significantly increased BV/TV, Tb.N, and Tb.Th in wt femurs by 4.4-fold, 1.7-fold, and 50%,
respectively, and decreased Tb.Sp by 1.5-fold (P<0.05, veh vs. PTH). In contrast, PTH only elevated BV/TV, Tb.N, and Tb.Th in Atf4−/− mice by 1.2-fold, 69%, and 12.6%, respectively, and decreased Tb.Sp by 56%. The PTH fold stimulations for all trabecular parameters were significantly decreased in Atf4−/− mice relative to wt mice (P<0.05, PTH/veh-wt vs. PTH/veh-Atf4−/−)(Fig. 1). PTH increased Cort.Th in wt femurs by 70% (P<0.05, veh vs. PTH), which was significantly reduced to 21% in Atf4−/− mice (P<0.05, PTH/veh-wt vs. PTH/veh-Atf4−/−)(Fig. 1). PTH similarly affected all trabecular and cortical parameters in Atf4−/+ and Atf4+/− mice (Fig. 1). For this reason, subsequent experiments only compared the PTH effects between wt and Atf4−/− mice. Histological analyses show that PTH displayed potent anabolic effects on tibiae, vertebrae, and calvariae, which were significantly reduced in Atf4−/− mice) (Fig. 2). At the molecular level, PTH markedly increased levels of osteocalcin (Ocn) and bone sialoprotein (Bsp) mRNA of long bones as measured by quantitative real-time RT/PCR. This increase was significantly reduced in the absence of ATF4 (Fig. 3B and C). In contrast, level of c-Fos was not altered PTH or ATF4 deficiency. Thus, ATF4 is required for the PTH anabolic actions in bone.

Fig. 2. PTH-stimulated bone formation in tibiae, vertebrae, and calvariae is significantly diminished in Atf4−/− mice. Mice were treated as in Fig. 1. A-E, tibiae; F-J, lumber vertebrae; K-O, calvariae. Representative H&E stained section are shown. Data represent mean±S.D. *P<0.05 (veh vs. PTH), #P<0.05 (PTH/veh-wt vs. PTH/veh-Atf4−/−).
Fig. 3. **ATF4 deficiency reduces PTH-induced osteoblast differentiation in vivo.** Mice were treated as in Fig. 1. Total RNAs were isolated from tibiae and analyzed by quantitative real-time RT-PCR using specific primers for *Atf4*, *Ocn*, *Bsp*, and *c-Fos* mRNAs, which were normalized to *Gapdh* mRNA. Data are presented as mean ± SD. *P*<0.05 (veh vs. PTH), ‡*P*<0.05 (PTH/veh-wt vs. PTH/veh-Atf4−/−).

### Key Research Accomplishments

- ATF4 expression vectors that contain mutations of potential phosphorylation sites within ATF4 molecule have been constructed. The effects of these mutations on ATF4 transcriptional activity and its ability to activate Runx2 as well as the PTH response are being determined in the project laboratory.
- PTH increases ATF4 expression and activity and ATF4 is required for PTH induction of *Ocn* expression in osteoblasts. Therefore, ATF4 is a novel downstream target of PTH signaling in osteoblasts (see P1).
- TFIIAγ increases osteoblast-specific gene expression by facilitating ATF4-Runx2 interactions (see P2).
- We have successfully established several in vivo assays for osteoblast activity and bone formation, including H&E staining, in vivo osteoblast proliferation assay (BrdU staining of bone tissue sections), in vivo bone formation assay (calcein labeling), and in vivo apoptosis assay of bone tissues. Using these assays, our preliminary study shows that ATF4 is required for the anabolic actions of PTH on bone in vivo (see A1).
Reportable Outcomes

Peer-reviewed papers:

Abstracts:

Conclusion

During the last year of support, our studies establish that: i) PTH increases ATF4 expression and activity and ATF4 is required for PTH induction of Ocn expression in osteoblasts. ATF4 is a novel downstream target of PTH signaling in osteoblasts; ii) ATF4 is required for the anabolic actions of PTH on bone in vivo; iii) TFIIAγ increases osteoblast-specific gene expression by facilitating ATF4-Runx2 interactions; and iv) ATF4 mutation constructs have been generated and several in vivo bone formation assays have been successfully developed.

The knowledge obtained from these studies will significantly enhance our understanding of the molecular mechanism underlying the actions of PTH in osteoblasts and bone and define new potential therapeutic targets for improved treatment of osteoporosis and other metabolic bone diseases.

References

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Appendices

Two peer-reviewed research papers: P1, P2
Three national meeting abstracts: A1, A2
Parathyroid Hormone Increases Activating Transcription Factor 4 Expression and Activity in Osteoblasts: Requirement for Osteocalcin Gene Expression

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PTH is an important peptide hormone regulator of calcium homeostasis and osteoblast function. However, its mechanism of action in osteoblasts is poorly understood. Our previous study demonstrated that PTH activates mouse osteocalcin (Ocn) gene 2 promoter through the osteoblast-specific element 1 site, a recently identified activating transcription factor-4 (ATF4)-binding element. In the present study, we examined effects of PTH on ATF4 expression and activity as well as the requirement for ATF4 in the regulation of Ocn by PTH. Results show that PTH elevated levels of ATF4 mRNA and protein in a dose- and time-dependent manner. This PTH regulation requires transcriptional activity but not de novo protein synthesis. PTH also increased binding of nuclear extracts to osteoblast-specific element 1 DNA. PTH stimulated ATF4-dependent transcriptional activity mainly through protein kinase A with a lesser requirement for protein kinase C and MAPK/ERK pathways. Lastly, PTH stimulation of Ocn expression was lost by silent interfering RNA down-regulation of ATF4 in MC-4 cells and Atf4−/− bone marrow stromal cells. Collectively, these studies for the first time demonstrate that PTH increases ATF4 expression and activity and that ATF4 is required for PTH induction of Ocn expression in osteoblasts. (Endocrinology 93: 0000–0000, 2008).

First Published Online January 10, 2008

Abbreviations: ActD, Actinomycin D; ATF4, activating transcription factor 4; BMSC, bone marrow stromal cell; CHX, cycloheximide; CRE, cAMP response element; CREB, CRE binding protein; FBS, fetal bovine serum; FSK, forskolin; GMSA, gel mobility shift assay; MC-4, MC3T3-E1 subclone 4; mOG2, mouse Ocn gene 2; mt, mutant; OCN, osteocalcin; OSE1, osteoblast-specific element-1; PKA, protein kinase A; PKC, protein kinase C; PMN, peritoneal neutrophil; PTH1R, PTH-1 receptor; RSK2, ribosomal kinase 2; Runx2, Runt-related transcription factor-2; siRNA, small interfering RNA; wt, wild type.

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cooperative interactions with OSE1 and OSE2 (also known as nuclear matrix protein 2 binding site) sites in the promoter (19, 20, 25). ATF4 activity is negatively regulated by factor inhibiting activating transcription factor-4-mediated transcription (42). Factor inhibiting activating transcription factor binds to ATF4 and represses its activity and bone formation in vivo. Although Atf4 mRNA is ubiquitously expressed, ATF4 protein preferentially accumulates in osteoblasts (34). This accumulation is explained by a selective reduction of proteasomal degradation in osteoblasts.

The purpose of this study was to determine the effects of PTH on ATF4 expression and activity and evaluate whether ATF4 mediates PTH induction of Ocn expression in osteoblasts.

Materials and Methods

Reagents

Tissue culture media and fetal bovine serum were obtained from HyClone (Logan, UT); [32P]dCTP (300 Ci/mmol) and α-[32P]dCTP (300 Ci/mmol) were purchased from GE Healthcare (Piscataway, NJ). Other reagents were obtained from the following sources: H89, forskolin (FSK), GF109203X, phorbol 12-myristate 13-acetate (PMA), cycloheximide (CHX), actinomycin D (ActD), and mouse monoclonal antibody against β-actin from Sigma (St. Louis, MO); Ubiquitin (Madison, WI); and U10124 from Calbiochem (La Jolla, CA). PTH (1–34) from Bachem (Torrance, CA), antibodies against ATF4, Runx2, and horseradish peroxidase-conjugated mouse or goat IgG from Santa Cruz (Santa Cruz, CA). All other chemicals were of analytical grade.

Cell cultures

Mouse MC3T3-E1 subclone 4 (MC-4) cells were described previously (43, 44) and maintained in ascorbic acid-free MEM, 10% FBS, 1% penicillin/streptomycin, and 1 mM β-mercaptoethanol g of nuclear extracts. ATF4 mediates PTH induction of Ocn expression in osteoblasts.

Preparation of nuclear extracts and gel mobility shift assay (GMSA)

Nuclear extracts were prepared and GMSA were conducted as previously described (43). Each reaction contained 1 μg of nuclear extracts. The DNA sequences of OSEI oligonucleotides used for GMSA were as follows: wild-type (wt): TGC TTA CAT CAG AGA GCA; mutant (mt): TGC TTA gta CAG AGA GCA.

Western blot analysis

Twenty micrograms of nuclear extracts were fractionated on a 10% SDS-PAGE gel and transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The membrane was blocked in 5% nonfat milk in Tris-buffered saline/Tween 20 buffer, probed with antibodies against Atf4 (1:1000) followed by incubation with secondary antibodies conjugated with horseradish peroxidase (1:5000); and visualized using an enhanced chemiluminescence kit (Pierce, Rockford, IL). Finally, blots were stripped two times in buffer containing 65 mm Tris Cl (pH 6.8), 2% sodium dodecyl sulfate, and 0.2% (vol/vol) β-mercaptoethanol at 65 C for 15 min and reprobed with β-actin antibody (1:5000) for normalization.

RNA isolation and reverse transcription

Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Gaithersburg, MD) according to the manufacturer’s protocol. Reverse transcription was performed using 2 μg of denatured RNA and 100 pmol of random hexamers (Applied Biosystems, Foster, CA) in a total volume of 25 μl containing 12.5 U MultiScribe reverse transcriptase (Applied Biosystems) according to the manufacturer’s instructions.

Quantitative real-time PCR

Quantitative real-time PCR was performed on an iCycler (Bio-Rad, Minneapolis, MN) using a SYBR Green PCR core kit (Applied Biosystems) and cDNA equivalent to 10 ng RNA in a 50-μl reaction according to the manufacturer’s instructions. The DNA sequences of mouse primers used for real-time PCR were: Atf4, 5′-GAC GAT GTC TTT ACC-3′ (forward), 5′-TAG TGA ACA GAC GCT GCC CCT A-3′ (reverse), Ocn, 5′-TAG TGA ACA GAC GCT GCC CCT A-3′ (forward), 5′-TCTGAGGCGTCTTCAAGCATAGT-3′ (reverse); Phtr1, 5′-GAT GCG GAC GAT GTC TTT ACC-3′ (forward), 5′-GCT GGC CTA ATA CTA CCT CC-3′ (reverse); Col1(I), 5′-AGA TGG TAC ACA ACA TCC GCA GCC-3′ (forward), 5′-TCC ACT GCT CTC GCC TCT GCC A-3′ (reverse); Opn, 5′-CCA ATG AAA GTC ATG ACA AAA C-3′ (forward), 5′-CTG CAT GAT CAT GCG GCC CCT GGC TGG AC-3′ (reverse). For all primers the amplification was performed as follows: initial denaturation at 95 C for 10 min followed by 40 cycles of 95 C for 15 sec and 60 C for 30 sec. Melting curve analysis was used to confirm the specificity of the PCR products. Six samples were run for each primer set. The levels of mRNA were calculated by the ΔCT method (46). Atf4, Ocn, Col1(I), Phtr1, and Opn mRNAs were normalized to Gapdh mRNA.

Northern blot

Twenty micrograms of total RNA was fractionated on 1.0% agarose-formaldehyde gels and blotted onto nitrocellulose paper. The mouse Atf4 cDNA inserts were excised from plasmid DNA with the appropriate restriction enzymes and purified by agarose gel electrophoresis before labeling with α-[32P]dCTP using a random primer kit (Roche Molecular Biochemicals, Indianapolis, IN). Hybridizations were performed as previously described using a Bellco Auto blot hybridization oven (47). Same blots were reprobed with [32P]-labeled cDNA to IRS mRNA for loading (48).

Small interfering RNA (siRNA)-MC-4 cells, which contain high levels of Atf4 mRNA, were seeded at a density of 25,000 cells/cm². After 24 h, cells were transfected with mouse Atf4 siRNA (sense: 5′-GAG CAU UCC UUC AGU UUA GUU 3′; antisense: 5′-CUA AAC UAA AGG AAG GCU CUU 3′) (49) or negative control siRNA (low GC, catalog no. H89251; Invitrogen) using LipofectAMINE 2000 (Invitrogen). After 48 h, cells from three identically treated dishes were pooled and harvested for total RNA, followed by quantitative real-time RT-PCR analysis for Ocn, Ocn, Col1(I), and Ocn siRNAs. The second set of mouse Atf4 siRNAs was purchased from Ambion (Austin, TX; catalog no. AM16704, ID 160775 and 160776) and used to confirm the results using the first set of Atf4 siRNA.

Atf4-deficient mice

Breeding pairs of mice heterozygous for Atf4 (Swiss Black mouse background) were obtained from Dr. Randall J. Kaufman (the Howard Endocrinology, April 2008, 93(4):0000–0000
These mice were originally developed by Dr. Tim M. Townes (University of Alabama at Birmingham) and were used to generate Atf4 wild-type (Atf4/+/+), heterozygous (Atf4/+/-), and homozygous mutant (Atf4/-/-) embryos/pups for this study. Original reports describing the phenotype of Atf4 homozygote-null mutants used the identical strain of mice (50). PCR genotyping was performed on tail DNA using a cocktail of three primers (TOWNES-1: 5'-AGC AAA ACA AGA CAG CAG CCA CTA-3', TOWNES-2: 5'-GTT TCT ACA GCT TCC TCC ACT CTT-3', and TOWNES-3: 5'-ATA TTG CTG AAG AGC TTG GCGGC-3') obtained from the laboratories of Dr. Randal J. Kaufman. A 700-bp DNA PCR product was amplified from Atf4/+/+ mouse tail DNA and a 900-bp product from wild-type mice (see Fig. 7A). The genotype of each mouse established by PCR of tail genomic DNA was confirmed by Western blotting of calvaria cell lysates and anti-ATF4 antibody. A breeding colony was established using heterozygote mice to provide littermate controls. All animal studies were approved by the Animal Care Committee of the Veterans Affairs Pittsburgh Healthcare System.

**FIG. 1.** PTH increases levels of ATF4 expression in osteoblasts. A, Effect of PTH on Atf4 mRNA. MC-4 cells were seeded at a density of 50,000 cells/cm² in 35-mm dishes and cultured in 10% FBS medium overnight. Cells were then treated with various concentrations of PTH for 6 h. For each group, total RNA (20 µg/lane) was loaded for Northern hybridization using cDNA probes for mouse Atf4 mRNA and 18S rRNAs (for normalization). B, Effect of PTH on ATF4 proteins (dose response). MC-4 cells were treated with indicated concentrations of PTH for 6 h and nuclear extracts were prepared for Western blot analysis for ATF4. C, Effect of PTH on ATF4 proteins (time course). MC-4 cells were treated with 10⁻¹⁰ M PTH for indicated time (h). Experiments were repeated three to four times, and qualitatively identical results were obtained.

**FIG. 2.** Effects of CHX/ActD treatment on PTH induction of Atf4 mRNA. MC-4 cells were treated with vehicle or 10 µg/ml CHX (A) or ActD (B) in the absence or presence of PTH for 6 h. Atf4 and Gapdh mRNAs were determined by quantitative real-time RT-PCR analysis. Experiments were repeated three times, and qualitatively identical results were obtained. *, P < 0.05 [control (ctrl) vs. PTH]; #, P < 0.05 (CHX vs. CHX/PTH); ?, P < 0.05 (CHX vs. CHX/PTH). To determine the effect of PTH on ATF4-dependent transcriptional activity, MC-4 cells were transiently transfected with the pGL3Luc plasmid, which contains the osteocalcin promoter linked to a luciferase gene. The cells were then treated with PTH for 6 h, and luciferase activity was measured. The results show that PTH increased the expression of the osteocalcin promoter in a dose-dependent manner. Experiments were repeated three to four times, and qualitatively identical results were obtained. *, P < 0.05 [control (ctrl) vs. PTH].
protein synthesis inhibitor CHX alone induced response genes such as by 4-fold, which is typically observed in immediate early dose-dependently increased levels of isolated for Northern blot analysis. As shown in Fig. 1A, PTH (from 10^(-13) to 10^(-7) m) for 6 h. One microgram of each nuclear extract was incubated with end-labeled double-stranded OSE1 (TGC TTA CAT CAG AGA GCA) and analyzed by electrophoresis on 4% polyacrylamide gels. DNA binding to labeled wild-type OSE1 probe was analyzed in the presence of 25- to 50-fold molar excesses of cold wt (lanes 6 and 7) or mt (lanes 4 and 5) OSE1 (TGC TTA gta CAG AGA GCA) by GMSA using 1 μg of nuclear extracts from PTH-treated MC-4 cells. B, Binding site specificity. Labeled wt (lanes 1–3) and mt (lanes 4–6) OSE1 probes were incubated with 1 μg nuclear extracts from MC-4 cells with and without PTH treatment. C, The nuclear complex binding OSE1 contains ATF4. Labeled wild-type OSE1 probe was incubated with 1 μg nuclear extracts from PTH-treated MC-4 cells in the presence of normal control IgG (lane 3), ATF4 antibody (lane 4), Runx2 antibody (lane 5), CREB antibody (lanes 4 and 5) OSE1 (TGC TTA gta CAG AGA GCA) by GMSA using 1 μg of nuclear extracts from PTH-treated MC-4 cells with or without PTH (10^(-13) to 10^(-7) m) for 6 h. As shown in Fig. 2A, the effect of PTH on Atf4 mRNA expression, MC-4 cells were treated with increasing concentrations of PTH (from 10^(-13) to 10^(-7) m) for 6 h, and total RNA was isolated for Northern blot analysis. As shown in Fig. 1A, PTH dose-dependently increased levels of Atf4 mRNAs with a significant stimulatory effect first detected at a concentration of 10^(-10) m. Western blot analyses using nuclear extracts from MC-4 cells with and without PTH treatment show that PTH also dose-dependently elevated the levels of ATF4 protein with maximal stimulation at 10^(-10) m. Measurable stimulation of ATF4 protein was observed 1 h after PTH addition with maximal induction occurring at 3 h and lasting for at least 5 h (Fig. 1A, B and C). PTH similarly increased Atf4 and Ocn mRNA expression in mouse primary bone marrow stromal cells (BMSCs) (see Fig. 7, B and C). To assess the molecular mechanisms of PTH stimulation of Atf4 mRNA expression, MC-4 cells were treated with and without inhibitors of translation and transcription in the presence and absence of PTH (10^(-7) m) for 6 h. As shown in Fig. 2A, the protein synthesis inhibitor CHX alone induced Atf4 mRNA by 4-fold, which is typically observed in immediate early response genes such as Fra-2 (15). The PTH-stimulation of Atf4 mRNA was not blocked by CHX treatment, suggesting that de novo protein synthesis is not necessary for the PTH regulation. In contrast, the transcription inhibitor ActD completely abolished the PTH-stimulated Atf4 mRNA induction (Fig. 2B), suggesting that the PTH effect requires transcription.

Results

**PTH increases ATF4 expression in MC-4 cells**

To determine the effect of PTH on Atf4 mRNA expression, MC-4 cells were treated with increasing concentrations of PTH (from 10^(-13) to 10^(-7) m) for 6 h, and total RNA was isolated for Northern blot analysis. As shown in Fig. 1A, PTH dose-dependently increased levels of Atf4 mRNAs with a significant stimulatory effect first detected at a concentration of 10^(-10) m. Western blot analyses using nuclear extracts from MC-4 cells with and without PTH treatment show that PTH also dose-dependently elevated the levels of ATF4 protein with maximal stimulation at 10^(-10) m. Measurable stimulation of ATF4 protein was observed 1 h after PTH addition with maximal induction occurring at 3 h and lasting for at least 5 h (Fig. 1A, B and C). PTH similarly increased Atf4 and Ocn mRNA expression in mouse primary bone marrow stromal cells (BMSCs) (see Fig. 7, B and C). To assess the molecular mechanisms of PTH stimulation of Atf4 mRNA expression, MC-4 cells were treated with and without inhibitors of translation and transcription in the presence and absence of PTH (10^(-7) m) for 6 h. As shown in Fig. 2A, the protein synthesis inhibitor CHX alone induced Atf4 mRNA by 4-fold, which is typically observed in immediate early response genes such as Fra-2 (15). The PTH-stimulation of Atf4 mRNA was not blocked by CHX treatment, suggesting that de novo protein synthesis is not necessary for the PTH regulation. In contrast, the transcription inhibitor ActD completely abolished the PTH-stimulated Atf4 mRNA induction (Fig. 2B), suggesting that the PTH effect requires transcription.

**PTH increases ATF4-dependent transcriptional activity in osteoblasts**

The effect of PTH on ATF4-dependent transcriptional activity was evaluated in two osteoblast cell lines and primary mouse bone marrow stromal cells. Cells were transiently transfected with wt or mt p4OSE1-luc, an artificial promoter containing four copies of wt or mt OSE1 (a specific ATF4-binding element) fused to a -34 to +13 minimal mOG2 promoter, and pRL-SV40, a renilla luciferase normalization plasmid. After 42 h, cells were treated with PTH (10^(-7) m) for 6 h followed by dual-luciferase assay. Firefly luciferase activity was normalized to renilla luciferase activity as a control for transfection efficiency. As shown in Fig. 3A, PTH stimulated ATF4-dependent OSE1 activity by 17-, 2.7-, and 2.8-fold in MC-4, UMR106–01, and primary BMSCs (P < 0.05, control vs. PTH), respectively. This PTH response was completely lost with the introduction of a 3-bp point mutation in the OSE1 core sequence (from TTACATCA to TTAGTACA). (Note that there are no additional OSE1 sites in the upstream region of the mOG2 promoter.) Figure 3B shows that PTH stimulated ATF4-dependent transcriptional activity in a dose-dependent manner with a significant stimulatory effect first detected at a concentration of 10^(-10) m. This is consistent with our previous study that examined effects of PTH on endogenous Ocn mRNA (33). Time-course studies revealed...
that the earliest effect of PTH stimulation was seen within 1 h and peaked at 5–6 h (Fig. 3C).

**PTH increases ATF4 binding to OSE1 DNA**

To determine whether PTH increases ATF4 binding to OSE1 DNA, we performed GMSA using nuclear extracts from MC-4 cells with and without $10^{-7}$ M PTH for 6 h. Consistent with our previous observation (33), nuclear extracts from PTH-treated MC-4 cells exhibited increased binding to intact OSE1 oligonucleotides (Fig. 4A, lanes 2 and 3), and this binding was significantly reduced by the addition of 25- and 50-fold molar excesses of unlabeled wt OSE1 oligonucleotides (Fig. 4A, lanes 4 and 5) but not by unlabeled mt OSE1 oligonucleotides (Fig. 4A, lane 6 and 7). In contrast, GMSA using labeled mt OSE1 oligonucleotides as probes showed that both basal and PTH-induced binding activity was abolished by the same 3-bp point mutation (Fig. 4B, lanes 4–6). The same mutation also abolished PTH activation of 647- and 116-bp mOG2 promoter fragments and 4OSE1 (33) (Fig. 3A). Importantly, PTH-increased binding to OSE1 was supershifted with an anti-ATF4 antibody (Fig. 4C, lanes 4). In contrast, normal IgG or antibodies against Runx2, CREB, ATF1, and Fra-1 did not significantly supershift the PTH-stimulated band (Fig. 4C, lanes 3–8). Taken together, these studies demonstrate that ATF4 is a component of the PTH-stimulated DNA-protein complex associating with OSE1.

[Note that PTH treatment did not alter binding of Runx2 to OSE2 DNA in the mOG2 promoter in GMSA (33).]

**Protein kinase A (PKA) is the major signaling pathway mediating the PTH response**

To identify signaling pathways mediating PTH activation of ATF4 transcriptional activity, we examined the effects of various inhibitors or activators. As shown in Fig. 5A, H89, a selective inhibitor of the PKA pathway, completely abolished PTH-stimulated ATF4 transcriptional activity ($P < 0.05$, control vs. PTH). GF109203X, a specific inhibitor of the protein kinase C (PKC) pathway, significantly decreased the PTH stimulation. U0126, a specific inhibitor of MAPK, partially suppressed PTH stimulation. As shown in Fig. 5B, FSK, a well-known activator of PKA, increased ATF4 activity in the absence of PTH in a dose-dependent manner. In combination with PTH, the effect of FSK was not additive, indicating that the PKA pathway was maximally stimulated. PMA, a PKC activator, did not significantly affect the PTH-induced ATF4 activity at a concentration range of 0.1–5 $\mu$M. A higher concentration of PMA (20 $\mu$M) slightly increased PTH-stimulated ATF4 activity without changing the basal activity (Fig. 5C). Taken together, these results indicate that PKA is the major pathway mediating PTH activation of ATF4 in osteoblasts with PKC and MAPK/ERK pathways playing lesser roles in the PTH response. The concentrations of the inhibitors or activators used in this study are in the range reported to selectively affect the relevant pathways (33, 51–53). We found no evidence of toxicity; compounds did not reduce cell DNA or protein under the current condition (data not shown).
**PTH-dependent induction of Ocn gene expression requires ATF4**

We used two separate approaches to establish the requirements for ATF4 in the regulation of Ocn gene expression by PTH. First, we examined whether ATF4 is necessary for PTH induction of Ocn mRNA expression in osteoblasts by knocking down endogenous Atf4 transcripts using siRNA. MC-4 cells, which express high levels of Atf4 mRNA, were transiently transfected with Atf4 siRNA or negative control siRNA (Invitrogen) using LipofectAMINE 2000 according to the manufacturer’s instructions. This siRNA specifically targets mouse Atf4 (49). As shown in Fig. 6A, quantitative real-time RT-PCR analysis showed that Atf4 siRNA (20 and 40 nM) efficiently reduced the levels of Atf4 mRNA by 57 and 71%, respectively. In contrast, the negative control siRNA did not reduce the Atf4 mRNA (Fig. 6B). As shown in Fig. 6C, the basal level of Ocn mRNA was reduced greater than 70% by Atf4 siRNA (P < 0.05, control vs. Atf4 siRNA). Importantly, PTH-stimulated Ocn mRNA was completely abolished in Atf4 siRNA group relative to the control siRNA group. Conversely, Col1(I) mRNA was not altered by Atf4 siRNA or PTH (Fig. 6D). Similar results were obtained when a different set of Atf4 siRNAs was used in MC-4 cells (data not shown).

To further establish the requirement for ATF4 in the PTH response, primary BMSCs were isolated from wt and Atf4<sup>−/−</sup> mice (Fig. 7A) and treated with or without PTH (10<sup>−7</sup> M) for 6 h followed by RNA preparation and quantitative real-time PCR analysis. As shown in Fig. 7B, minimal Atf4 mRNA was detected by real-time RT/PCR in the Atf4<sup>−/−</sup> BMSCs. Consistent with the results of experiments with MC-4 cells, PTH significantly stimulated Atf4 mRNA in wt BMSCs (P < 0.05, control vs. PTH), but this induction was completely lost in cells from Atf4<sup>−/−</sup> mice (Fig. 7B). As shown in Fig. 7C, PTH significantly increased Ocn mRNA in wt BMSCs, which was abolished in Atf4<sup>−/−</sup> BMSCs (P > 0.05, control vs. PTH). The basal level of Ocn mRNA was also significantly reduced in Atf4<sup>−/−</sup> BMSCs relative to wt cells (P < 0.05, wt vs. mt). In contrast, PTH did not increase Opn mRNA in wt or mt BMSCs (P > 0.05, control vs. PTH) (Fig. 7D). However, the level of Opn mRNA was increased in Atf4<sup>−/−</sup> cells (P < 0.05, wt vs. mt), indicating that ATF4 may function as a negative regulator of Opn expression (Fig. 7D). In addition, the levels of Pth1r mRNA were not significantly changed by either Atf4 deficiency or PTH, suggesting that PTH signaling is intact in the absence of ATF4 (Fig. 7E). Taken together, these data clearly establish that ATF4 is required for PTH induction of Ocn mRNA in primary BMSCs.

**Discussion**

This study examined actions of PTH on ATF4 expression and activity in osteoblasts. Using the Ocn gene as a model system for studying PTH-dependent transcription, we found the following: 1) PTH rapidly induces Atf4 expression in MC-4 cells and mouse primary bone marrow stromal cells in a time- and dose-dependent manners; 2) PTH increases in vitro binding of ATF4 to OSE1 DNA; 3) PTH dramatically activates ATF4 transcriptional activity mainly through the PKA pathway; 4) PTH stimulation of Ocn gene expression requires ATF4 because it is abolished by Atf4 siRNA in MC-4 cells and is not seen in ATF4-deficient BMSCs. Col-

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**Fig. 6.** ATF4 siRNA blocks PTH stimulation of Ocn expression. A and B, MC-4 cells were transiently transfected with Atf4 siRNA (A) or negative control (Ctrl) siRNA (B). After 48 h, total RNA was prepared for quantitative real-time RT-PCR analyses for Atf4 mRNA, which was normalized to Gapdh mRNA. C and D, MC-4 cells were transiently transfected with 40 nM Atf4 siRNA or negative control siRNAs. After 42 h, cells were treated with and without 10<sup>−7</sup> M PTH for 6 h followed by RNA preparation and quantitative real-time RT-PCR analyses for Ocn and Col1(I) mRNAs, which were normalized to the Gapdh mRNAs. *, P < 0.05 (ctrl vs. PTH); #, P < 0.05 (ctrl siRNA vs. Atf4 siRNA). Data represent mean ± SD. Experiments were repeated three times, and qualitatively identical results were obtained.
lectively, this study establishes that ATF4 is a novel downstream target of PTH actions in osteoblasts. It is well documented that PTH signals mainly through the PKA pathway. In the present study, we show that PKA inhibition completely blocked PTH stimulation of ATF4 activity. Furthermore, activation of the PKA pathway by FSK dramatically increased ATF4 activity in the absence of PTH. However, when combined with PTH, the effect of FSK was not additive. These results strongly suggest that PKA is the major pathway for PTH to activate ATF4 because each agent (i.e., FSK or PTH) maximally stimulates the same pathway, making additional ATF4 activation impossible. Inhibition of the PKC pathway also resulted in a significant reduction in PTH-induced ATF4 activity (data not shown), but PKC activation by PMA failed to activate both basal or PTH-induced ATF4 activity. Thus, PKC is partially required for PTH activation of ATF4. Lastly, inhibition of the MAPK/ERK pathway led to partial inhibition of the PTH stimulation. These three pathways are also required for PTH induction of both Ocn mRNA and 1.3-kb mOG2 promoter activity as previously described (33), further supporting our hypothesis that ATF4 mediates PTH induction of Ocn gene expression.

A recent study showed that ATF4 mediates β-adrenergic induction of Rankl mRNA expression via direct binding to the upstream OSE1 site in the Rankl promoter in osteoblasts (54). However, PTH stimulation of Rankl expression was not reduced in the absence of ATF4, suggesting that this catabolic action of PTH is independent of this transcription factor. Phosphorylation seems to be critical for ATF4 to elicit its function in osteoblasts and bone. A PKA phosphorylation site (serine 254) within the ATF4 molecule was recently shown to mediate β-adrenergic induction of Rankl mRNA expression in osteoblasts (54). In addition, ATF4 is phosphorylated at serine 251 by ribosomal kinase 2 (RSK2), the kinase inactivated in Coffin-Lowry syndrome, an X-linked mental retardation disorder associated with skeletal manifestations (29). Because RSK2 is an immediate downstream target of MAPK/ERK that is activated by PTH signaling (8, 9), PTH may in part activate ATF4 via the MAPK/ERK/RSK2 pathway. It remains to be determined whether the PKA and/or RSK2 phosphorylation sites are involved in the PTH activation of ATF4.

One of the major downstream factors for PTH signaling is CREB, the cAMP response element binding protein. Actions of CREB are mediated through cAMP response elements (CREs) in the regulatory regions of target genes. PTH phosphorylates CREB at serine 133. This phosphorylation event stimulates the binding of CREB to the CRE and is required for CREB to activate transcription of target genes. Through this classical pathway, PTH rapidly induces transcription of immediate-early response genes including those encoding activator protein-1 family members such as c-Fos, c-Jun, Fra-1, Fra-2, and FosB (10, 14, 15, 52–57). Although CREB was shown to binding to the OSE1 site (29), overexpression of CREB was unable to activate OSE1-dependent transcription activity of the mOG2 promoter in vitro (29), suggesting...
that this site is not a major functional site for CREB. Furthermore, the OSE1 binding activity stimulated by PTH was not supershifted by an anti-CREB antibody. Instead, this complex clearly contains ATF4 protein (Fig. 4C). Thus, we were unable to obtain any evidence for the involvement of CREB in the PTH response. However, our results do not exclude the possibility that PTH/CREB activates Atf4 mRNA transcription via CREB binding to potential CRE sites in the Atf4 promoter.

PTH induction of immediate-early response genes occurs very rapidly (minutes to hours) and lasts for several hours. This PTH response is usually independent upon the presence of de novo protein synthesis but requires active cellular transcription. The time-course experiments in the present study indicate that PTH induction of Atf4 occurs within 1 h of PTH addition and peaks after 3–6 h. Furthermore, this regulation depends on active cellular transcription and does not require de novo protein synthesis. Therefore, ATF4 may be considered as an additional PTH early response gene.

ATF4-deficient mice as well as humans with mutations in RSK2, an ATF4 activating kinase, exhibit striking deficits in bone formation and osteoblast activity. Because ATF4 is required for osteoblast function and bone formation in vivo, and as shown herein, ATF4 is a novel downstream target of PTH/CREB. ATF4 may be considered as an additional PTH early response gene.

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General Transcription Factor IIα-γ Increases Osteoblast-specific Osteocalcin Gene Expression via Activating Transcription Factor 4 and Runt-related Transcription Factor 2

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ATF4 (activating transcription factor 4) is an osteoblast-enriched transcription factor that regulates terminal osteoblast differentiation and bone formation. ATF4 knock-out mice have reduced bone mass (severe osteoporosis) throughout life. Runx2 (runt-related transcription factor 2) is a runt domain-containing transcription factor that is essential for bone formation during embryogenesis and postnatal life. In this study, we identified general transcription factor IIα-γ (TFIIA-γ) as a Runx2-interacting factor in a yeast two-hybrid screen. Immunoprecipitation assays confirmed that TFIIA-γ interacts with Runx2 in osteoblasts and when coexpressed in COS-7 cells or using purified Runx2 protein and TFIIA-γ precipitates Runx2 and causes ATF4 degradation. This study shows that a general transcription factor, TFIIA-γ, regulates osteoblast-specific gene expression through interactions with two important bone transcription factors ATF4 and Runx2.

Skeletal integrity requires a balance between bone-forming cells (osteoblasts) and bone-resorbing cells or osteoclasts. Imbalance between bone formation and resorption results in metabolic bone diseases such as osteoporosis. Multipotent mesenchymal cells proliferate and differentiate into osteoblasts that synthesize and deposit the mineralizing extracellular matrix of bone. Osteoblast activity is regulated by a number of growth factors and hormones, including bone morphogenetic proteins, insulin-like growth factor 1, basic fibroblast growth factor 2, parathyroid hormone, tumor necrosis factor-α, and extracellular matrix signals (1–9). Runx2 is a runt domain-containing transcription factor identified as a transcriptional activator of osteoblast differentiation and the master gene for bone development in vitro and in vivo (10–14). Runx2 knock-out mice die at birth and completely lack both skeletal ossification and mature osteoblasts (10, 12). Runx2 haplo-insufficiency causes the skeletal disorder, cleidocranial dysplasia, a disease characterized by defective endochondral and intramembranous bone formation. Runx2 is expressed in mesenchymal condensations during early development at E11.5 and acts as an osteoblast differentiation factor (13).

ATF4 (activating transcription factor 4), also known as CREB2 (cAMP-response element-binding protein 2) (15) and Tax-responsive Enhancer Element B67 (TAXREB67) (16), is a member of the activating transcription factor cAMP-response element-binding protein family of leucine zipper factors that also includes cAMP-response element-binding protein 2 (CREB2) (15) and Tax-responsive element modulator (CREM)2 ATF1, ATF2, ATF3, and ATF4 (17–21). These proteins bind to DNA via their basic region and dimerize via their leucine domain to form a large variety of homodimers and/or heterodimers that allow the cell to coordinate signals from multiple pathways (17–21). An in vivo role for ATF4 in bone development was established using Atf4-deficient mice (22). ATF4 is required for expression of osteocalcin (Ocn) and bone sialoprotein (Bsp) as demonstrated by a dramatic reduction of their mRNAs in Atf4−/− bone (22). ATF4 activates Ocn transcription through direct binding to the OSE1 site of the mOG2 promoter. In addition, ATF4 interacts with Runx2 in osteoblasts or when coexpressed in COS-7 cells. ATF4 and Runx2 cooperatively regulate Ocn transcription through interactions with OSE1 (osteoblast-specific element 1)

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2The abbreviations used are: CREM, cAMP-response element modulator; TFIIA-γ, transcription factor IIα-γ; Chip, chromatin immunoprecipitation; GST, glutathione S-transferase; WB, Western blot; IP, immunoprecipitation; FBS, fetal bovine serum; RT, reverse transcription; siRNA, small interfering RNA; aa, amino acids; CHX, cycloheximide; VDR, vitamin D receptor.
and OSE2 (osteoblast-specific element 2, also known as nuclear matrix protein 2 or NMP2-binding site) sites in the promoter (23–25).

One of the most striking characteristics of ATF4 protein is its very short half-life (30–60 min) in many cell types (26). ATF4 is rapidly degraded via a ubiquitin/proteasomal pathway. This degradation requires the presence of the serine residue 219 in the context of DSGXXXS within the ATF4 molecule and its phosphorylation by an unknown kinase. This phosphorylation was shown to be required for subsequent recognition by the SCF\(^{\text{ATF4}}\) and degradation by the 26 S proteasome (27). Although \(\text{ATF4} \) mRNA is ubiquitously expressed, ATF4 protein preferentially accumulates in osteoblasts (28). This accumulation is explained by a selective reduction of proteasomal degradation in osteoblasts. Indeed, inhibition of the ubiquitin/proteasomal pathway by MG115, which blocks the N-terminal threonine in the active site of \(\beta\)-subunit of 26 S proteasomal complex (29, 30), led to ATF4 accumulation and induced \(\text{Ocn} \) mRNA expression in non-osteoblastic cells (28). These observations suggest that modulation of ATF4 stability constitutes an important step to control its protein level and activity and, ultimately, osteoblast-specific gene expression and bone formation.

Transcription factor II A (TFIIA) is a general transcription factor consisting of three subunits designated TFIIA\(\alpha\), TFIIA\(\beta\), and TFIIA\(\gamma\) (31). TFIIA interacts with and stabilizes TFIIID (also known as TBP, TATA box-binding protein) to DNA and activates transcription (32, 33). Although TFIIA was classified as a general transcription factor when it was first identified, more and more evidence shows that this elusive factor may play an important role in the regulation of tissue-specific gene expression via interactions with tissue- or cell type-specific transcription factors (34–36).

The \(\text{Ocn} \) promoter has been the major paradigm for unravelling the mechanisms mediating osteoblast-specific gene expression and defining a number of key transcription factors or cofactors (13, 14, 23–25, 37–41). However, very few studies have focused on how tissue-specific transcription factors interface with general transcriptional initiation factors in osteoblasts. In this study, by using a combination of a yeast two-hybrid system and pulldown assays as well as functional assays, we show that TFIIA\(\gamma\), the smallest subunit (12 kDa) of TFIIA (42), interacts with both Runx2 and ATF4. TFIIA\(\gamma\) delays ATF4 protein degradation and increases its activity. Together with ATF4 and Runx2, TFIIA\(\gamma\) enhances osteoblast-specific \(\text{Ocn} \) gene expression.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Tissue culture media were purchased from Invitrogen and fetal bovine serum from HyClone (Logan, UT). Other reagents were obtained from the following sources: antibodies against TFIIA\(\alpha\), TFIIA\(\gamma\), ATF4, Runx2, and horseradish peroxidase-conjugated mouse or goat IgG from Santa Cruz Biotechnology (Santa Cruz, CA), mouse monoclonal antibody against \(\beta\)-actin from Sigma, and GST antibody from Amersham Biosciences. All other chemicals were of analytical grade.

**Cell Cultures**—Mouse MC3T3-E1 subclone 4 (MC-4) cells were described previously (43, 44) and maintained in ascorbic acid-free \(\alpha\)-modified Eagle’s medium, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin and were not used beyond passage 15. C2C12 myoblasts, a gift from Dr. Daniel Goldman (University of Michigan, Ann Arbor, MI), C3H10T1/2 fibroblasts (American Type Culture Collection), and 3T3-L1 mouse preadipocytes (American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium, 10% FBS. F9 teratocarcinoma cells (American Type Culture Collection) and rat ROS17/2.8 osteosarcoma cells (gift from Dr. Laurie McCauley, University of Michigan School of Dentistry) were grown in modified Eagle’s medium, 10% FBS.

**Yeast Two-hybrid Analysis**—A yeast pLexA-two hybrid system (Clontech) was used to identify proteins that bind to mouse Runx2. A cDNA fragment encoding the aa-263–351 region of Runx2 was subcloned into the BamHI/XhoI sites of pLexA, creating an in-frame fusion with the DNA binding domain of the LexA gene that is controlled by the strong yeast ADH1 promoter. The resultant plasmid pLexA-Runx2 (aa 263–351) was then transformed into a yeast reporter strain (YM4271), and the transformed cells (1 \times 10^9) were mated for 24 h with cells (2.5 \times 10^9) of a pretransformed two-hybrid library made from human brain cDNA. The resultant mating mixture was spread on 20 \times 10-cm plates to select for expression of the \(\text{LEU2} \) and lacZ reporter genes. Approximately 2 \times 10^6 colonies were screened. Sixty four positive colonies were isolated. The prey plasmids were extracted from the positive colonies and the cDNA inserts in the plasmids were amplified by PCR and sequenced. Of the 64 positive colonies, 5 are the full-length TFIIA\(\gamma\) cDNAs, and the rest contained 16 different cDNAs.

**DNA Constructs and Transfection**—p657mO2G2-luc, p657mO2G2OSE1mt-luc, p657mO2G2OSE2mt-luc, p657mO2G2-OSE1 + 2mt-luc, p4OSE1-luc, p4OSE1mt-luc, p6OSE2-luc, p6OSE2mt-luc, pCMV/\(\beta\)-galactosidase, pCMV/ATF4, pCMV/Runx2, pCMV/FLAG-Runx2 and its deletion mutants (aa 1–330, aa 1–286, and aa 258), GST-Runx2 and GST-ATF4 fusion protein expression vectors were described previously (1, 13, 23, 25, 45). The full-length cDNA of human TFIIA\(\gamma\) was cloned by an RT-PCR strategy using total RNA from human Saos2 osteoblastic cells as a template and specific primers (forward, 5’-ATG GCA TAT CAG TTA TAC AGA AA-3’, and reverse, 5’-TTC TGT AGT ATT GGA GCC AGT A-3’). Digested PCR products were purified and subcloned into the NotI/BamHI sites of the pFLAG-5a expression vector (Sigma). Addition of a C-terminal FLAG sequence into the TFIIA\(\gamma\) cDNA facilitates monitoring of expression levels and immunoprecipitation using M2 antibody (Sigma). GST-TFIIA\(\gamma\) fusion protein expression plasmid was constructed by subcloning the full-length TFIIA\(\gamma\) cDNA into the glutathione S-transferase gene fusion vector pGEX-4T-1 (Amersham Biosciences) in correct reading frame. The accuracy of DNA sequences was verified by automated sequencing. The size of expressed proteins was confirmed by Western blot analysis using specific antibodies. For expression and functional studies, cells were plated on 35-mm dishes at a density of 5 \times 10^4 cells/cm^2. After 24 h, cells were transfected with the indicated plasmid DNAs (0.01 μg of pRL-SV40, 0.25 μg of test luciferase reporter, and 1.0 μg of expression plasmids balanced as necessary with \(\beta\)-galactosidase expression plasmid such that the total DNA was constant)
TFII\(\gamma\) Interacts with ATF4 and Runx2

and Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions. After 36 h, whole cell extracts were prepared and used for Western blot analysis or dual luciferase assay using the dual luciferase assay kit (Promega, Madison, WI) on a Veritas\textsuperscript{TM} microplate luminometer (Turner Biosystem, Inc., Sunnyvale, CA). Firefly luciferase activity was normalized to Renilla luciferase activity for transfection efficiency.

**RNA Isolation and Reverse Transcription (RT)**—Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. RT was performed using 2 \(\mu\)g of denatured RNA and 100 pmol of random hexamers (Applied Biosystem, Foster, CA) in a total volume of 25 \(\mu\)l containing 12.5 units of MultiScribe reverse transcriptase (Applied Biosystem, Foster, CA) according to the manufacturer’s instructions.

**Regular PCR**—Regular PCR was performed on a 2720 Thermal Cycler (Applied Biosystem, Foster, CA), using 2.5 \(\mu\)l of the cDNA (equivalent to 0.2 \(\mu\)g of RNA) and AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA) in a 25-\(\mu\)l reaction according to the manufacturer’s instructions. The DNA sequences of primers used for PCR were as follows: mouse/rat TFII\(\gamma\), 5\'-ATG GCA TAT CAG TTA TTA AGA AAT ACA-3' (forward), 5\'-GTT ATT TTT ACC ATC ATC GAC CCA GCT T-3' (reverse); mouse/rat Hprt, 5\'-ATG GCT TGG CCA GTG CCT CAG A-3' (forward), 5\'-GCT CTG GAG TGG AAG ACA GAA CCA C-3' (reverse); mouse/rat Hprt, 5\'-GTT GAG ATG ACAA GAG AAA GAC CAC C-3' (reverse); mouse/rat Hprt, 5\'-GTT GAG ATG ACA TCT TCT CCA CC-3' (forward), 5\'-AGC GAT GAT GAA CCA CCA GGT T-3' (reverse). For all primers the amplification was performed as follows: initial denaturation at 95 °C for 30 s followed by 31 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s and extension at 72 °C for 7 min. The amplified PCR products were run on a 1.2% agarose gel and visualized by ethidium bromide staining.

**Quantitative Real Time PCR**—Quantitative real time PCR was performed on an iCycler (Bio-Rad) using a SYBR\textsuperscript{®} Green PCR core kit (Applied Biosystem, Foster, CA) and cDNA equivalent to 10 ng of RNA in a 50-\(\mu\)l reaction according to the manufacturer’s instructions. The DNA sequences of primers used for real time PCR were as follows: mouse Ocn, 5\'-TAG TGA ACA GAC TCC GGC GCT A-3' (forward), 5\'-TGG AGG CGG TCT TCA AGC CAT-3' (reverse); mouse and rat ATF4, 5\'-ATG GCT TGG CCA GTG CCT CAG A-3' (forward), 5\'-GCT CTG GAG TGG AAG ACA GAA CCA C-3' (reverse); mouse and rat ATF4, 5\'-GGC TGC TGC CTT CCT CCG ATG T-3' (reverse); mouse and rat TFII\(\gamma\), 5\'-TGG GGA ACA GAC TGC TTC AAG AGA GCC TT-3' (forward); 5\'-TTC CTG ACT CTC TCT GGC AAT GCT G-3' (reverse); mouse Ocn, 5\'-TGG TGA ATA GAC TCC GGC GCT ACC T-3' (forward), 5\'-GCC GGC GAA AGA CAA TGT CGG CCG-3' (reverse); rat Bsp, 5\'-GGC TGG AGA TGC AGA GGG CAA GCC-3' (forward), 5\'-TGG TGG TGG TGC CTT TGG AAG CCG-3' (reverse); rat Ocn, 5\'-TGG TGA ATA GAC TCC GGC GCT ACC T-3' (forward), 5\'-GCC GGC GAA AGA CAA TGT CGT CCGG-3' (reverse). For all primers the amplification was performed as follows: initial denaturation at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Melting curve analysis was used to confirm the specificity of the PCR products. Six samples were run for each primer set. The levels of mRNA were calculated by the \(\Delta CT\) method (46). Ocn, Bsp, TFII\(\gamma\), osteopontin (Ocn), and ATF4 mRNAs were normalized to 18 \(S\) rRNA mRNA.

Western Blot Analysis—Cells were washed with cold 1× phosphate-buffered saline and lysed in 1× Passive Buffer (Promega, Madison, WI) at room temperature for 20 min. Lysates were clarified by centrifugation (20 min, 13,000 \(\times\) g, 4 °C). Protein concentrations were determined by the method developed by Bio-Rad. Twenty \(\mu\)g of total protein were fractionated on a 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Schleicher & Schuell). The membrane was blocked in 5% nonfat milk in Tris-buffered saline/Tween 20 (TBST) buffer, probed with antibodies against TFII\(\gamma\) (1:200), TFII\(\alpha\) (1:1000), ATF4 (1:1000), Runx2 (1:1000), Fra-1 (1:1000), GST (1:5000), or M2 (1:2000) followed by incubation with anti-goat-mouse or -rabbit antibodies conjugated with horseradish peroxidase (1:5000) and visualized using an enhanced chemiluminescence kit (Pierce). Finally, blots were stripped two times in buffer containing 65 mM Tris-Cl, pH 6.8, 2% SDS, and 0.7% (v/v) \(\beta\)-mercaptoethanol at 65 °C for 15 min and re-probed with \(\beta\)-actin antibody (1:5000) for normalization.

**Immunoprecipitation**—GST, GST-TFII\(\gamma\), GST-ATF4, and GST-Runx2 fusion proteins were purified using the Bulk GST purification module kit (Amersham Biosciences) according the manufacturer’s instructions. Whole cell extracts (500 \(\mu\)g), nuclear extracts (200 \(\mu\)g), or GST fusion proteins (1 \(\mu\)g) were pre-cleaned twice with 50 \(\mu\)l of protein A/G-agarose beads (Stratagene, La Jolla, CA) for 30 min followed by pelleting of beads. The protein A/G-agarose beads were blocked with 10 \(\mu\)g/ml bovine serum albumin in 1× phosphate-buffered saline for 1 h before use to reduce nonspecific binding of proteins. Five \(\mu\)g of respective antibody was added and incubated for 2 h at 4 °C with gentle rocking. The immune complexes were collected by addition of 30 \(\mu\)l of protein A/G-agarose beads and incubation for 1 h at 4 °C followed by centrifugation. Precipitates were washed five times with 1× washing buffer (20 mM HEPES, pH 7.6, 50 mM KCl, 1 mM dithiothreitol, 0.25% Nonidet P-40, 5 mM NaF, 1 mM EGTA, 5 mM MgCl\(_2\), 0.25 mM phenylmethylsulfonyl fluoride), and the immunoprecipitated complexes were suspended in SDS sample buffer and analyzed by SDS-PAGE followed by Western blot analysis using the indicated antibodies.

**ChIP Assays**—ChIP assays were performed as described previously (41) using a protocol kindly provided by Dr. Dwight Towler (Washington University) (47). After sonication, the amount of chromatin was quantified using the PicoGreen double-stranded DNA quantitation assay (Molecular Probes) according to the manufacturer’s instructions. The equivalent of 10 \(\mu\)g of DNA was used as starting material (input) in each ChIP reaction with 2 \(\mu\)g of the appropriate antibody (TFII\(\gamma\), or control rabbit IgG). Fractions of the purified ChIP DNA (5%) or inputs (0.02–0.05%) were used for PCR analysis. The reaction was performed with AmpliTaq Gold DNA polymerase (Applied Biosystems) for 35 cycles of 60 s at 95 °C, 90 s at 58 °C, and 120 s at 68 °C. PCR primer pairs were generated to detect DNA segments located near the Runx2-binding site at -131/131 (primers P1 and P2), ATF4-binding site at -55/-48 (primers P3 and P4) in mouse osteocalcin gene 2 (mOG2) proximal promoter, or the Runx2-binding site located between -370 and -42 in the proximal mouse Runx2 promoter region (primers
control siRNA (low GC, catalog number 12935-200, Invitrogen) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instruction. After 36 h, total RNA was harvested for quantitative real-time RT-PCR analysis for TFIIAγ, Ocn, Bsp, Opn (osteopontin), and Atf4 mRNAs. A second set of mouse TFIIAγ siRNAs (sense, AUG ACA ACA CUG UGC UAU AUU; antisense, UAU AGC ACA GUG UUG UCA UUU) was designed in the project laboratory and used to confirm the results using the first set of TFIIAγ siRNA.

Statistical Analysis—Results were expressed as means ± S.D. Student’s t test was used to test for differences between two groups. Differences with a p < 0.05 was considered as statistically significant.

RESULTS

TFIIAγ Interacts with Runx2 and ATF4—A yeast pLexA two-hybrid system (Clontech) was used to identify proteins that bind to mouse Runx2. cDNA fragments encoding several C-terminal regions of Runx2 were subcloned into the BamH1/XhoI sites of pLexA, creating in-frame fusions with the DNA binding domain of the LexA gene that is controlled by the strong yeast ADH1 promoter. Preliminary experiments using relatively larger regions of Runx2 (aa 232–391, aa 232–428, and aa 232–517) as baits were not successful because of their inability to autoactivate the lacZ reporter gene in yeast. In contrast, by using the aa 263–351 region of Runx2 as a bait, we identified TFIIAγ, a general transcriptional factor involved in the initiation step of eukaryotic transcription, as a Runx2-interacting factor. A diagram and a picture of a positive colony are shown in Fig. S1.

To verify the TFIIAγ-Runx2 interaction identified by yeast two-hybrid system, we conducted pulldown assays. COS-7 cells were transiently transfected with expression vectors for FLAG-TFIIAγ, Runx2, and ATF4 (a recently identified Runx2-interacting factor). After 36 h, whole cell extracts were prepared for immunoprecipitation (IP) assay using a TFIIAγ antibody followed by Western blot analysis for Runx2 and ATF4. As seen in Fig. 1A (lane 2), Runx2 protein was present in a TFIIAγ anti-

P5 and P6) (48), and the mOG2 gene region (+177/+311) (primers P7 and P8) (see Fig. 2A and Table 1). The PCR products were separated on 3% agarose gels and visualized with ultraviolet light. All ChIP assays were repeated at least three times.

siRNA—ROS17/2.8 osteoblast-like cells, which contain high levels of TFIIAγ protein, were transfected with mouse TFIIAγ siRNA kit (Santa Cruz Biotechnology) or negative
body immunoprecipitate. Interestingly, anti-TFII\(\alpha\) antibody also immunoprecipitated ATF4. Reciprocal IPs showed that both Runx2 and ATF4 antibodies immunoprecipitated the FLAG-tagged TFII\(\alpha\) (Fig. 1A, lanes 4 and 6). To determine whether TFII\(\alpha\) can interact with Runx2 and ATF4 in osteoblasts, nuclear extracts from ROS17/2.8 cells that express high levels of Runx2, ATF4, and TFII\(\alpha\) were immunoprecipitated with anti-TFII\(\alpha\) antibody followed by Western blot analysis for Runx2, ATF4, or Fra-1 (a member of AP1 family). Results show that both Runx2 and ATF4 but not Fra-1 proteins were present in anti-TFII\(\alpha\) immunoprecipitates (Fig. 1B, lane 2). Reciprocal IPs showed that antibodies against Runx2 or ATF4 but not Fra-1 immunoprecipitated TFII\(\alpha\) in ROS17/2.8 cells (Fig. 1B, lanes 4, 6, and 8). Normal control IgG failed to significantly pull down Runx2, ATF4, or TFII\(\alpha\) in either COS-7 cells or osteoblasts. Taken together, these studies confirm that TFII\(\alpha\) interacts with Runx2 and ATF4 in osteoblasts or when coexpressed in COS-7 cells.

Although Runx2 and ATF4 interact in osteoblasts, IP assays using purified GST fusion proteins failed to show a direct physical interaction between ATF4 and Runx2 (25), suggesting that accessory factors may be involved in their interactions. To determine whether TFII\(\alpha\) directly interacts with Runx2 or ATF4 in the absence of other nuclear proteins, we mixed GST or GST-TFII\(\alpha\) with GST-ATF4 or GST-Runx2 fusion proteins purified from *Escherichia coli*, followed by IP and Western blot analysis. As shown in Fig. 1C, both GST-Runx2 and GST-ATF4 proteins mixed with GST-TFII\(\alpha\) were immunoprecipitated by anti-TFII\(\alpha\) antibody (lanes 1 and 2). Anti-Runx2 or anti-ATF4 antibody was unable to immunoprecipitate GST protein mixed with GST-Runx2 (Fig. 1C, lane 3) or GST-ATF4 (lane 4). Reciprocal IPs show that GST-TFII\(\alpha\) was immunoprecipitated by both anti-Runx2 or anti-ATF4 antibodies (Fig. 1C, lanes 6 and 7) but not by normal control IgG (lane 5). These results demonstrate that TFII\(\alpha\) directly binds to both Runx2 and ATF4.

As a first step to identify the TFII\(\alpha\) binding domain, FLAG-Runx2 deletion mutant expression vectors (wild type aa 1–528, aa 1–330, aa 1–286, and aa 1–258) were transfected into COS-7 cells because of the high transfection efficiency. Nuclear extracts were prepared 36 h later, mixed with equal amounts of nuclear extracts of ROS17/2.8 (which contain large amounts of endogenous TFII\(\alpha\)), and immunoprecipitated using anti-TFII\(\alpha\) antibody followed by Western blot analysis for Runx2 (M2 antibody). As shown in Fig. 1D, deletion of Runx2 from aa 528 to aa 286 did not reduce TFII\(\alpha\) binding. However, further deletion from aa 286 to aa 258 completely abrogated TFII\(\alpha\)-Runx2 complex formation. These data clearly demonstrate the following: (i) endogenous TFII\(\alpha\) can interact with overexpressed FLAG-Runx2 proteins *in vitro*; and (ii) the aa 258–286 region of Runx2 is required for TFII\(\alpha\) binding. Interestingly, this same region is required for ATF4-Runx2 interactions (25).

To determine whether, in intact cells, TFII\(\alpha\) is associated with the endogenous *osteocalcin* gene 2 (*mOG2*) promoter region that has been shown to bind Runx2 and ATF4, we performed the chromatin immunoprecipitation (ChIP) assay using MC3T3-E1 (clone MC-4) preosteoblast cells. After shearing, soluble chromatin was immunoprecipitated with either an antibody against TFII\(\alpha\) or control IgG. The positions and sequences of primers used for PCR analysis of ChIP DNAs are shown in Fig. 2A and Table 1. As shown in Fig. 2B, the PCR bands amplified with primers P1/P2 and P3/P4 and corresponding to ChIP DNAs immunoprecipitated with TFII\(\alpha\) antibody revealed that TFII\(\alpha\) specifically interacts with chromatin fragments of the proximal *mOG2* promoter that contain Runx2- or ATF4-binding sites. Furthermore, TFII\(\alpha\) antibody

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**TABLE 1**

<table>
<thead>
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<th>Oligonucleotide name</th>
<th>Sequence</th>
</tr>
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<tr>
<td>P1</td>
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</tr>
<tr>
<td>P2</td>
<td>AGGGGATCTGCCCAGGACATAAT</td>
</tr>
<tr>
<td>P3</td>
<td>CAGCACGATCTTTTTGTTGAC</td>
</tr>
<tr>
<td>P4</td>
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<tr>
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</tr>
<tr>
<td>P8</td>
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</table>
TFIIAγ Interacts with ATF4 and Runx2

TFIIAγ Increases ATF4 but Not Runx2-dependent Transcriptional Activity—To determine whether TFIIAγ increases Runx2- and ATF4-dependent transcriptional activity, we measured the ability of TFIIAγ to stimulate transcription of p6OSE2-luc, a reporter plasmid containing 6 copies of the Runx2-binding element OSE2 upstream of a minimal 34-bp mOG2 promoter (13, 43, 49) or p4OSE1-luc, a reporter plasmid that contains four copies of OSE1 (a specific ATF4-binding element) upstream of a minimal 34-bp mOG2 promoter (22, 25). For these studies, we used C3H10T1/2 fibroblasts because they contain undetectable levels of both endogenous Runx2 and ATF4 proteins (28, 49). As shown in Fig. 3A, as expected, Runx2 alone increased OSE2 transcriptional activity by 11-fold. This stimulation was abolished in the 6OSE2mt-luc in which the OSE2 core sequence was mutated (25) (Fig. 3B). Although we have shown above that TFIIAγ interacts with Runx2, TFIIAγ transfection did not activate basal or Runx2-dependent OSE2 transcription (Fig. 3A). As shown in Fig. 3C, ATF4 activated OSE1 activity about 2-fold (p < 0.01, $\beta$-galactosidase versus ATF4). Although TFIIAγ alone was unable to activate OSE1 activity, unexpectedly, when coexpressed with ATF4, it dramatically increased OSE1 activity 5-fold above ATF4 alone. This stimulation was abolished in 4OSE1mt-luc, in which the OSE1 core sequence was mutated from TTACATCA to TTAGTACA in the reporter plasmid (45) (Fig. 3D). Note: TFIIAγ, Runx2, or ATF4 failed to activate a minimal 34-bp mOG2 promoter that contains a TATA box (23, 50) (Fig. 3E). Fig. 3F shows that TFIIAγ activated ATF4 transcription activity in a dose-dependent manner in C3H10T1/2 cells. TFIIAγ similarly stimulated ATF4-directed OSE1 activity in C2C12 myoblasts (3-fold) and COS-7 cells (4.3-fold) (Fig. 3, G and H).

TFIIAγ Expression in Different Cell Lines—The levels of TFIIAγ mRNAs and proteins were determined in different cell lines by RT-PCR and Western blot analysis, respectively. As shown in Fig. 4, Western blot analysis shows that TFIIAγ protein was expressed at high levels in osteoblastic cells (MC-4

also immunoprecipitated a Runx2-binding site-containing chromatin fragment of the proximal Runx2 promoter (primers P5/P6). In contrast, TFIIAγ antibody failed to immunoprecipitate a chromatin fragment of mOG2 gene that contains no Runx2- or ATF4-binding sites (primers P7/P8). Taken together, these data show that TFIIAγ is recruited to a chromatin fragment of the mOG2 promoter that was previously demonstrated to be bound by Runx2 and ATF4 in osteoblasts (13, 22).
TFIIAγ Interacts with ATF4 and Runx2

Silencing of TFIIAγ Markedly Reduces Levels of Endogenous Ocn and Bsp mRNAs and ATF4 Protein in Osteoblasts—To determine whether TFIIAγ is required for the endogenous Ocn mRNA expression in osteoblasts, we knocked down the endogenous TFIIAγ transcripts by siRNA. ROS17/2.8 osteoblast-like cells, which express high levels of TFIIAγ and Ocn and Bsp mRNAs, were transiently transfected with TFIIAγ siRNA reagent from Santa Cruz Biotechnology according to the manufacturer’s instructions. This siRNA is a pool of three specific 20–25-nucleotide siRNA targeting both mouse and rat TFIIAγ.

As shown in Fig. 6A, quantitative real time RT-PCR analysis showed that levels of TFIIAγ mRNA were efficiently reduced by TFIIAγ siRNA in a dose-dependent manner. The level of Ocn mRNA was reduced greater than 50% by TFIIAγ siRNA (p < 0.01, control versus TFIIAγ siRNA). Interestingly, Bsp mRNA, another ATF4 downstream target gene (22), was also reduced by 50% (p < 0.01, control versus TFIIAγ siRNA). This inhibition was specific because levels of Opn and Atf4 mRNAs were not reduced by TFIIAγ siRNA. In contrast, as shown in Fig. 6B, levels of all these mRNAs were not reduced by the negative control siRNA (Invitrogen). Although Atf4 mRNA was not altered by TFIIAγ siRNA, the level of endogenous ATF4 protein was significantly reduced by silencing TFIIAγ in osteoblasts (Fig. 6C). Similar results were obtained when a different set of TFIIAγ siRNA was used (Fig. S2).

Overexpression of TFIIAγ Increases the Levels of ATF4 Protein—The above studies clearly demonstrated that TFIIAγ increased ATF4-dependent transcription activity and Ocn gene expression probably by targeting ATF4 protein. To further study the mechanism of this regulation, we determined the effect of TFIIAγ overexpression on the levels of ATF4 protein. C3H10T1/2 cells, which express undetectable level of endogenous ATF4 protein (28), were transiently transfected with 1.0 μg of ATF4 expression plasmid and increasing amounts of TFIIAγ expression plasmid (0, 0.5, 1, and 2 μg). After 36 h, cells were harvested for Western blot analysis. As shown in Fig. 7A, overexpression of TFIIAγ in C3H10T1/2 cells increased the levels of ATF4 protein in a dose-dependent manner. This increase in ATF4 protein was specific because levels of Runx2 were not altered by TFIIAγ. TFIIAγ similarly elevated levels of ATF4 protein in COS-7 cells (Fig. 7B). Next, we determined if TFIIAγ could increase the levels of endogenous ATF4 proteins in osteoblasts. ROS17/2.8 cells were transiently transfected with indicated amount of TFIIAγ expression vector. Western blot analysis shows that TFIIAγ dose-dependently increased levels of endogenous ATF4 protein in ROS17/2.8 cells (Fig. 7C). Similar results were obtained in MC-4 cells (Fig. 7D). Interestingly, overexpression of TFIIAγ did not increase the levels of Atf4 mRNA in all these cells examined (bottom, Fig. 7A–D). Taken collectively, TFIIAγ markedly increased levels of ATF4 proteins in osteoblasts and non-osteoblasts.

TFIIAγ Increases ATF4 Protein Stability—Lassot et al. (51) recently showed that acetylation p300 markedly increased the levels of ATF4 protein and ATF4-dependent transcriptional activity by inhibiting ATF4 protein degradation via a proteasomal ubiquitin pathway. As an initial step to determine whether TFIIAγ alters ATF4 protein stability, C3H10T1/2 cells were...
transiently transfected with ATF4 expression vector in the presence of β-galactosidase, TFIIAγ, or Runx2 expression vectors. After 36 h, cells were treated with 50 μg/ml of protein synthesis inhibitor cycloheximide (CHX) (i.e. to completely block de novo protein synthesis) and harvested at different time points of CHX addition (0, 0.5, 1, and 3 h) followed by Western blot analysis for ATF4 and Runx2. This technique has been widely used to study protein stability (51). As shown in Fig. 8A, in the absence of TFIIAγ overexpression, ATF4 protein was rapidly degraded and almost undetectable on Western blot by 3 h after CHX addition, which is consistent with a previous study (51). However, overexpression of TFIIAγ greatly delayed the degradation process with the levels of ATF4 protein only slightly reduced by 3 h after CHX addition. In contrast, levels of Runx2 protein were not affected by TFIIAγ (Fig. 8B).

**DISCUSSION**

This study identifies TFIIAγ as a bridging molecule between Runx2, ATF4, and the transcription machinery in osteoblasts. Although Runx2 and ATF4 interact in osteoblasts or when coexpressed in COS-7 cells, IPs using purified GST fusion proteins were unable to demonstrate a direct physical interaction between ATF4 and Runx2 (25). Thus, accessory factors are likely involved in bridging these two molecules. Several lines of evidence support that TFIIAγ may be a factor linking Runx2 and ATF4. (i) TFIIAγ forms complexes with both Runx2 and ATF4 in osteoblasts and when coexpressed in COS-7 cells. (ii) The same region of Runx2 (i.e. aa 258–286) is required for both TFIIAγ–Runx2 and ATF4–Runx2 interactions. (iii) Purified GST-TFIIAγ fusion protein directly binds to both purified GST–Runx2 and GST–ATF4 fusion proteins. (iv) Overexpression of TFIIAγ in 10T1/2 cells dramatically enhances endogenous Ocn gene expression and the 657-bp mOG2 promoter activity in the presence of ATF4 and Runx2. (v) siRNA knockdown of TFIIAγ mRNA markedly reduces osteoblast-specific Ocn and Bsp expression.

Accumulating evidence establishes that ubiquitin-proteasome pathways control osteoblast differentiation and bone formation. For example, the proteasome inhibitors epoxomicin and proteasome inhibitor-1, when administered systemically to mice, strongly stimulated bone volume and bone formation rates by greater than 70% after only 5 days of treatment (52). Although the mechanism of this regulation remains unclear, critical bone transcription factors seem to be targets for the ubiquitin-proteasomal pathway. Zhao and co-workers (52, 53) recently showed that Smurf1, an E3 ubiquitin-protein isopeptide ligase, accelerated Runx2 ubiquitin-proteasomal degradation and inhibited osteoblast differentiation and bone forma-
TFIIAγ Interacts with ATF4 and Runx2

![Diagram](image)

**FIGURE 6.** TFIIAγ siRNA blocks endogenous Ocn mRNA expression in osteoblastic cells. ROS17/2.8 osteoblast-like cells were transiently transfected with TFIIAγ siRNA (A) or negative control (Ctrl) siRNAs (B). After 36 h, total RNA or whole cell extracts were prepared for quantitative real time RT-PCR analysis for TFIIAγ, Ocn, Bsp, Opn, and ATF4 mRNAs which were normalized to the 18S rRNA mRNAs or Western blot analysis for ATF4, TFIIAγ, and β-actin (C and D). *, p < 0.01 (control versus siRNA). Data represent mean ± S.D. Experiments were repeated three times with similar results.

**TABLE 1.** TFIIAγ siRNA blocks endogenous Ocn mRNA expression in osteoblastic cells.

<table>
<thead>
<tr>
<th>siRNA Treatment</th>
<th>Ocn mRNA</th>
<th>Bsp mRNA</th>
<th>Opn mRNA</th>
<th>ATF4 mRNA</th>
</tr>
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<tbody>
<tr>
<td>Ctrl siRNA</td>
<td>1.0</td>
<td>0.9</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>-TFIIAγ siRNA</td>
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<td>-β-actin siRNA</td>
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</tr>
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</table>

In osteoblasts, bone transcription factors such as Runx2 and ATF4 directly bind to specific DNA sequences in their target gene promoters. Experiments show that TFIIAγ greatly inhibits ATF4 degradation. TFIIAγ siRNA decreases ATF4 stability in osteoblasts. Lassot et al. (51) recently found that ATF4 is similarly stabilized by cofactor p300, a histone acetyltransferase. p300 inhibits ATF4 ubiquitination and degradation through interaction with the ATF4 N terminus. Interestingly, this stabilization does not require either the acetyltransferase activity of p300 or the serine residue 219 in the context of DSGXXXS within ATF4 molecule that is known to be required for ATF4 degradation via the SCFβTrCP and the 26 S proteasome (51).

TFIIAγ stimulation of Ocn gene transcription is dependent on the presence of both ATF4 and Runx2. As a master regulator of osteoblast differentiation, Runx2 alone is sufficient to activate expression of many osteoblast-specific genes, including Ocn and Bsp, by direct binding to their promoters (13). In contrast, although ATF4 directly binds to the OSE1 site of the mouse Ocn gene and activates OSE1, it alone is not sufficient for activation of the endogenous Ocn gene or the 657-bp mOG2 promoter which contains sufficient information for the bone-specific expression of Ocn in vivo (54). Instead, ATF4 stimulation of Ocn is dependent on the presence of Runx2 as demonstrated by our recent study (25). ATF4 interacts with Runx2 and activates Runx2-dependent transcriptional activity. A recent study shows that SATB2, a nuclear matrix protein that directly interacts with both ATF4 and Runx2, activates osteoblast differentiation and controls craniofacial patterning in vivo (55). This study shows that although TFIIAγ interacts with Runx2, it does not directly activate Runx2. Like ATF4, TFIIAγ alone is not sufficient to activate transcription from either the Ocn gene or the 657-bp mOG2 promoter. In fact, even TFIIAγ and ATF4 together are not sufficient for Ocn gene expression without the presence of Runx2 (Fig. 5). However, in the presence of both ATF4 and Runx2, TFIIAγ greatly activates Ocn gene expression.

General transcription factors were originally defined as such because they were thought to be universally required for transcription. In eukaryotic cells, initiation of transcription is a complex process, which requires RNA polymerase II and many other basal transcription factors and/or cofactors, including TFIIA, TFIIB, TFIID (TBP or TATA box-binding protein), TFIIE, TFIIF, and TFIH (56–59). Binding of TBP to the TATA box is the first step, which is regulated by TFIIA. TFIIA enhances transcription by interacting with TBP and stabilizing its binding to DNA (32, 33). More and more evidence shows that general transcription factors play unique roles in the regulation of tissue-specific gene expression under physiological and pathological conditions. For example, the androgen receptor, via its N-terminal AF1 domain, interacts with basal transcription factors TBP and TFIIA and activates tissue-specific transcription in target tissues and cells (60). Likewise, TAFII17 (a component of the TFIID complex), via specific protein–protein interactions with the vitamin D receptor (VDR), increases osteoclast formation from osteoclast precursors in response to 1,25-dihydroxyvitamin D3 in patients with Paget disease (61). In osteoblasts, bone transcription factors such as Runx2 and ATF4 directly bind to specific DNA sequences in their target gene promoters (i.e. OSE2 or NMP2 and OSE1, respectively) and activate osteoblast-specific gene expression, osteoblast differentiation, and bone formation (10–14, 24, 43). Obviously,
cooperative interactions between osteoblast-specific transcription factors and basal (general) transcriptional machinery are essential for achieving maximal transcription of osteoblast-specific genes. However, little is known about these interactions. Experiments from this study demonstrate that TFIIAγ, which is expressed at high level in osteoblasts, facilitates osteoblast-specific gene expression via two mechanisms. 1) TFIIAγ stabilizes ATF4 and increases the levels of ATF4 proteins. The increased levels of ATF4 further activate Runx2 activity and Ocn transcription (25). 2) Through its ability to directly interact with both ATF4 and Runx2, TFIIAγ could recruit these two critical bone transcription factors to the basal transcriptional machinery and greatly enhance osteoblast-specific gene expression. In support of our observation, Guo and Stein (62) showed that Yin Yang-1 (YY1) regulates vitamin D enhancement of Ocn gene transcription by interfering with interactions of the VDR with both the VDR element and TFIIB. TFIIB interacts with both VDR and YY1 (63). Likewise, Newberry et al. (64) showed that TFIIF (RAP74 and RAP30) mediates Msx2 (a homeobox transcription factor required for craniofacial development) inhibition of Ocn promoter activity. Finally, a recent study showed that TFIIB could directly bind to the transactivation domain of Osterix, another important osteoblast transcription factor (65).

TFIIAγ interacts with ATF4 and Runx2

TFIIA γ Interacts with ATF4 and Runx2

FIGURE 7. TFIIAγ increases the levels of ATF4 protein. C3H10T1/2 (A) and COS-7 (B) cells were transfected with 1 µg of pCMV/ATF4 or pCMV/Runx2 and increasing amounts of FLAG-TFIIAγ expression vector (0, 0.5, 1, 2 µg) followed by Western blotting for ATF4, TFIIAγ, Runx2, and β-actin (top) or RNA preparation and RT-PCR for Atf4 and Hprt mRNA (bottom). ROS17/2.8 (C) and MC-4 (D) cells were transfected with increasing amounts of FLAG-TFIIAγ expression vector (0, 0.5, 1, and 2 µg). Experiments were repeated three times with similar results.

FIGURE 8. TFIIAγ increases ATF4 protein stability. C3H10T1/2 cells were transfected with 1.0 µg of ATF4 (A) or Runx2 (B) expression vector with and without 1.0 µg of TFIIAγ expression vector. After 36 h, cells were treated with 50 µg/ml of protein synthesis inhibitor cycloheximide (CHX) and harvested at different time points (0, 1, and 3 h) followed by Western blot analysis for ATF4 and Runx2. Experiments were repeated three times with similar results.

TFIIA consists of three subunits designated TFIIAα, TFIIAβ, and TFIIAγ. TFIIAα and TFIIAβ are produced by a specific proteolytic cleavage of the αβ polypeptide that is encoded by TFIIA-L (31, 33). TFIIAγ is the smallest subunit with a molecular mass of 12 kDa (42). Although it is encoded by a distinct gene (TFIIA γ), TFIIAγ shares a high degree of homology with TFIIAα and TFIIAβ. Interestingly, TFIIAα activates testis-specific gene expression via interactions with a tissue-specific partner, ACT (activator of CREM in testis) and CREM (34). Likewise, TFIIAα enhances human T-cell lymphotropic virus type 1 gene activation through interactions with the Tax protein, a factor associated with adult enhances human T-cell lymphotropic virus type 1 (HTLV-1) (35, 66). It remains to be determined whether TFIIAα and TFIIAβ can also interact with ATF4 and Runx2 and similarly activate osteoblast-specific gene expression.

It should be noted that although TFIIAγ belongs to the family of general transcription factors, its expression seems to show some tissue or cell specificity. Osteoblastic cells (MC-4 cells and ROS17/2.8), C3H10T1/2 fibroblasts, and L1 preadipocytes express high levels of TFIIAγ proteins. In contrast, the levels of TFIIAγ protein were undetectable in F9 teratocarcinoma cells.
and COS-7 on Western blots. The meaning of this observation remains unknown.

These findings suggest that TFIIAγ is a critical factor regulating ATF4 stability and functions as a molecular linker between ATF4 and Runx2 and the basal transcriptional machinery. TFIIAγ may play a unique role in the regulation of osteoblast-specific gene expression and ultimately osteoblast differentiation and bone formation. A working model is proposed in Fig. 9, which summarizes the role of TFIIAγ in osteoblast-specific mOG2 gene expression. Future study aimed at identifying factors that affect levels and activity of TFIIAγ will allow us to address the functional significance of TFIIAγ in osteoblast function in greater detail.

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REFERENCES

ATF4 Is Required for the Anabolic Actions of PTH on Bone in vivo

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Abstract: Parathyroid hormone (PTH) is a potent stimulator of bone formation and a proven anabolic agent for the treatment of osteoporosis. However, the mechanism whereby PTH increases bone formation remains poorly understood. Activating transcription factor 4 (ATF4) is a critical factor for bone formation during development and throughout postnatal life. This study examined if ATF4 is required for the anabolic actions of PTH on bone using an Atf4-/- mouse model. Five-day-old wt and Atf4-/- mice were given daily subcutaneous injections of vehicle (saline) or hPTH(1-34) (0.04 µg/g body weight) for 28 days. In wt mice, µCT analyses of femurs show that this PTH regimen significantly increased bone volume/tissue volume (BV/TV, 4.3-fold), trabecular thickness (Tb.Th, 50%), trabecular numbers (Tb.N, 1.5-fold), cortical thickness (Cort.Th, 77%), and cross sectional area (CSA, 24%) and decreased trabecular spacing (Tb.Sp, 1.7-fold). These PTH effects were dramatically reduced or completely abolished in the absence of ATF4. Histological analyses show that this PTH regimen significantly increased bone volume/tissue volume (BV/TV, 4.3-fold), trabecular thickness (Tb.Th, 50%), trabecular numbers (Tb.N, 1.5-fold), cortical thickness (Cort.Th, 77%), and cross sectional area (CSA, 24%) and decreased trabecular spacing (Tb.Sp, 1.7-fold). These PTH effects were dramatically reduced or completely abolished in the absence of ATF4. Histological analyses show that PTH displayed potent anabolic effects on tibiae, vertebrae, and calvariae, which were significantly reduced in Atf4-/- mice. At the molecular level, PTH markedly increased levels of osteocalcin (Ocn) and bone sialoprotein (Bsp) mRNA of long bones as measured by quantitative real-time RT/PCR. This increase was completely abolished in the absence of ATF4. This study demonstrates that ATF4 is required for the anabolic actions of PTH on bone in vivo and also suggested that modulation of the levels and activity of ATF4 may have therapeutic significance for the treatment of metabolic bone diseases such as osteoporosis.

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TFIIA, ATF4, and Runx2 Synergistically Activate Osteoblast-specific Osteocalcin Gene Expression

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Abstract:
Runx2, a member of the runt homology domain family of transcription factors, is a master regulator of osteoblast function and bone formation. Mice lacking Runx2 have no mineralized skeleton due to a complete lack of mature osteoblasts. The expression level of Runx2 protein is regulated by a number of factors including BMPs, FGF-2, IGF-1, TNF-α, TGF-β, and PTH, all of which play important roles in osteoblasts and bone both in vitro and in vivo. In addition, the activity of Runx2 protein is positively or negatively modulated through protein-protein interactions. Activating transcription factor 4 (ATF4) is an osteoblast-enriched factor which regulates the terminal differentiation and function of osteoblasts. ATF4 knock-out mice have reduced bone mass and bone mineral density (severe osteoporosis) throughout their life. To identify proteins interacting with Runx2, we used a yeast two-hybrid system and identified TFIIA, a general transcriptional factor, as a Runx2-interacting factor. While pull-down assays confirmed that TFIIA physically interacted with Runx2 when both factors were coexpressed in COS-7 cells, surprisingly, it did not activate or inhibit Runx2-dependent transcriptional activity. In contrast, TFIIA unexpectedly activated ATF4, which we recently identified as a Runx2-interacting protein, in a dose-dependent manner. Deletion analysis found that this activation required the presence of the C-terminal 15 amino acid residues of ATF4 molecule. Finally, TFIIA, ATF4, and Runx2 synergistically stimulated the 0.657-kb mOG2 (mouse osteocalcin gene 2) promoter activity and endogenous osteocalcin mRNA expression. In summary, this study demonstrates a novel mechanism through which bone-specific transcription factors and general transcription factors cooperate in regulating osteoblast-specific gene expression.

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