Parathyroid Hormone-induced E4BP4/NFIL3 Down-regulates Transcription in Osteoblasts*

Ibrahim C. Ozkurt and Sotirios Tetradis

From the Division of Diagnostic and Surgical Sciences, UCLA School of Dentistry, Los Angeles, California 90095-1668

Parathyroid hormone (PTH), a major regulator of bone metabolism, activates the PTHR1 receptor on the osteoblast plasma membrane to initiate signaling and induce transcription of primary response genes. Subsequently, primary genes with transcriptional activity regulate expression of downstream PTH targets. We have identified the adenovirus E4 promoter-binding protein/nuclear factor regulated by IL-3 (E4bp4) as a PTH-induced primary gene in osteoblasts. E4BP4 is a basic leucine zipper (bZIP) transcription factor that represses or activates transcription in non-osteoblastic cells. We report here that PTH rapidly and transiently induced E4bp4 mRNA in osteoblastic cells and that this induction did not require protein synthesis. PTH also induced E4BP4 protein synthesis and E4BP4 binding to a consensus but not to a mutant E4BP4 response element (EBPRE). E4BP4 overexpression inhibited an EBPRE-containing promoter-reporter construct, whereas PTH treatment attenuated activity of the same construct in primary mouse osteoblasts. Finally, E4BP4 overexpression inhibited PTH-induced activity of a cyclooxygenase-2 promoter-reporter construct. Our data suggest a role for E4BP4 in attenuation of PTH target gene transcription in osteoblasts.

Received for publication, December 12, 2002, and in revised form, May 9, 2003
Published, JBC Papers in Press, May 12, 2003, DOI 10.1074/jbc.M212652200

* The research was supported by National Institutes of Health Grant R01-DE13316. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be part by the payment of page charges. This article must therefore be solely to indicate this fact.

‡ To whom correspondence should be addressed: Division of Diagnostic and Surgical Sciences, UCLA School of Dentistry, Los Angeles, CA 90095-1668. Tel.: 310-825-5712; Fax: 310-206-6485; E-mail: sotirist@dent.ucla.edu.

The abbreviations used are: PTH, parathyroid hormone; RDA, representational difference analysis; bZIP, basic leucine zipper; IL-3, interleukin-3; E4BP4, E4 promoter-binding protein/nuclear factor regulated by IL-3; EBPRE, E4BP4 response element; MOB, mouse osteoblast; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcriptase; FMS, fms-like tyrosine kinase; DTT, di-thiothreitol; EMSA, electrophoretic mobility shift assay; CHX, cycloheximide; ROS, rat osteosarcoma; ActD, actinomycin D; cox-2, cyclooxygenase-2; ATF, activating transcription factor; TBP, TATA binding protein.
Northern analysis, the E4bp4 (12) were used. pcDNA 3.1 expression vector downstream of the cytomegalovirus promoter. The resulting amplification product was purified and subcloned into the pcDNA 3.1 expression vector downstream of the cytomegalovirus promoter (Invitrogen, San Diego, CA). To generate E4bp4 cDNA probe for Northern analysis, the E4bp4 cDNA insert was excised, gel-purified, and labeled with [32P]dCTP by random primer labeling technique.

**Total and Nuclear Protein Extraction**—MOB cells were treated accordingly and then washed twice with ice-cold 1× phosphate-buffered saline. For total protein extracts, cells were resuspended for 30 min in 1 ml of triple detergent lysis buffer containing 50 mM Tris-CI, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/ml PMSF, 1 μg/ml aprotinin, 1% Nonidet P-40, 0.5% deoxycholate, and 1× protease inhibitors. Lysates were spun at 15,000 g for 15 min at 4°C, and supernatants were used for Western blot assays. Nuclear proteins were prepared as previously described (17). Cells were resuspended in 400 μl of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA) supplemented with freshly added 1 mM DTT, 0.5 mM PMSF, and 1× protease inhibitor. After incubating for 30 min at 4°C on a rocking platform, nuclear lysates were centrifuged at maximum speed for 10 min in a microcentrifuge at 4°C, and the supernatants containing the nuclear proteins were collected. Protein concentrations were determined by the Lowry method.

**Western Blot Analysis**—Fifty micrograms total or nuclear proteins were combined with equal volume of 2× loading buffer, boiled for 5 min, separated by SDS-PAGE (5% stacking at 20 mA and 10% resolving gel at 40 mA), and electrotransferred onto a nitrocellulose membrane overnight at 22 V and 4°C. Membranes were stained with 0.1% Ponceau S (w/v) in 5% acetic acid (v/v) to determine equal loading. Then membranes were blocked for 1 h with 5% nonfat milk in PBS-T (0.5% Tween), incubated with a polyclonal E4BP4 antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, catalog number SC-9549) at 1:100 dilution in 5% nonfat milk/PBS-T for 1–2 h at room temperature, washed with PBS-T, and incubated with anti-goat IgG-horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnologies) in 5% nonfat milk/PBS-T for 1 h. Following incubation, membranes were washed three times with PBS-T and processed with ECL reagent (Amer sham Biosciences, Piscataway, NJ) according to the manufacturer’s instructions and autoradiography.

**Electrophoretic Mobility Shift Assay**—Oligonucleotides (Invitrogen) carrying an E4BP4 consensus response element (EBPRE, 5′-GGGGA-TCGCTTATGCGAACTGC-3′) or EBPRE with single nucleotide mutations, 5′-GGGGA-TCGCTTATGCGACGC-3′ or EBPRE-2, 5′-GGGGA-TCGCTTATGCGATCC-3′ were used (12). In vitro protein synthesis was performed using the TNT Quick-couple Transcription/Translation kit (Promega Corp., Madison, WI) following the manufacturer’s protocol. Nuclear or in vitro-synthesized proteins were incubated with probes in the presence of EMA buffer (25 mM Hepes, 50 mM KCl, 0.1 mM MgCl2, 0.1 mM EGTA, 0.1 mM dNTPs, 0.1 μM probe, 0.1 μg/ml poly(dI-cI), 1 mM DTT, 0.5 mM PMSE) at room temperature for 30 min. Reaction mixtures were resolved by non-denaturing 4% PAGE in 10× TBE at 135 V for 1.5 h. The gel was fixed with 10% methanol, 10% acetic acid for 10 min, dried onto Whatman 3MM paper, and used to expose x-ray film. For supershift experiments, proteins were incubated with 1.5 μl of antibody for 20 min at room temperature prior to loading onto gel.

**Transfection**—Three tandem wild type or mutant EBPREs, described earlier, were subcloned into the pTK-luciferase vector (pTK-LUC, ATCC, Manassas, VA) upstream of the basal thymidine kinase promoter to generate pTK-EBPRE, pTK-EBPRE-m1, and pTK-EBPRE-m2 luciferase reporter constructs. MOB cells were plated in 24-well plates at 50–60% confluence and were co-transfected with 75 ng/well pcDNA3.1-E4BP4 expression vector or with an equimolar amount of pcDNA3.1 empty vector and 75 ng/well pTK-EBPRE, pTK-EBPRE-m1, or pTK-EBPRE-m2 reporter construct utilizing LipofectAMINE Plus and the manufacturer’s protocol (Invitrogen). 2–4 days later luciferase assay was performed with the protocol provided by the supplier (Promega). Protein concentrations were determined by Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) using microplate reader (Multiscan, Fisher, Pittsburgh, PA).

**Statistics**—Statistical differences between two groups were determined with Student’s t test. Comparisons among groups were performed by analysis of variance, and statistical differences were detected with a Bonferroni post hoc test. All experiments were performed at least three times.

**RESULTS**

**PTH Induced E4bp4 Gene Expression in the Absence of Protein Synthesis in MC3T3-E1 Osteoblastic Cells**—Using RDA to identify osteoblastic primary response genes induced by PTH (11), we cloned a DNA fragment that corresponded to E4bp4 (data not shown). To confirm that E4bp4 was a PTH-induced primary response gene, MC3T3-E1 cells were treated with 10 μg/ml CHX for 30 min followed by 10 nM PTH for 90 min. Total RNA was extracted, and Northern blot analysis was performed using the RDA-isolated cDNA fragment as a probe. E4bp4 was constitutively expressed in CHX-treated cells and was markedly induced by PTH treatment (Fig. 1A).
primers demonstrated that PTH induced E4BP4 mRNA in both osteoblastic cell lines with a peak at 2 h (Fig. 1B). DNA sequencing confirmed that the PCR product had the previously published E4bp4 sequence (accession number NM_017373, National Institutes of Health data base).

**PTH Induced E4bp4 Expression in Primary Mouse Osteoblasts and Calvariae—**MC3T3-E1 and ROS 17/2.8 cells are immortalized cell lines that retain a pre-osteoblastic/osteoblastic phenotype. To test if PTH induces E4bp4 gene expression in primary osteoblastic cells and normal bone, MOB cells or calvariae organ cultures were treated with 10 nM PTH for various times, total RNA was extracted, and Northern blot analyses were performed. PTH-induced E4bp4 mRNA expression in both MOB cells and cultured calvariae peaked at 2 h and declined thereafter (Fig. 2).

**PTH Induction of E4BP4 Protein Synthesis in MOB Cells—**To determine if PTH induced E4BP4 protein synthesis, MOB cells were treated with 10 nM PTH for 0–6 h, and total or nuclear proteins were extracted. Western immunoblot analysis indicated that PTH induced E4BP4 protein expression in MOB cells that peaked between 2 and 4 h and declined thereafter (Fig. 4). Similar results were obtained with both total and nuclear proteins, although nuclear proteins demonstrated a delayed peak and decline (Fig. 4, compare A and B). Recombinant E4BP4 protein was used as a control (lane R, Fig. 4A).

**PTH Induced Nuclear Protein Binding to a Consensus E4BP4 Response Element (EBPRE)—**EMSAs were performed to test the ability of in vitro synthesized E4BP4 protein to bind consensus or either of two mutant EBPREs. E4BP4 bound with high, moderate, and low affinity to wild type EBPRE, to EBPRE-m1, and to EBPRE-m2, respectively (Fig. 5A). In vitro synthesized luciferase control showed no binding to the DNA probes.

To investigate potential PTH-induced binding to the consensus or mutant EBPREs, EMSAs utilizing nuclear proteins from PTH-treated MOB cells and the EBPRE probes were performed. Untreated cells showed a low binding of nuclear proteins to the consensus EBPRE. Treatment with 10 nM PTH increased this binding that peaked at 2 and 4 h and declined thereafter (Fig. 5B). In vitro synthesized luciferase control showed no binding to the DNA probes.
suggesting specific binding of the nuclear proteins to the wild type probe (Fig. 5C).

**E4BP4 Protein Is Part of the PTH-induced Binding to a Consensus EBPRE**—Basal E4BP4 mRNA and protein levels were very low, whereas PTH treatment significantly induced E4bp4 gene expression. To test if the PTH-induced binding to the consensus EBPRE was due to an existing or to a newly synthesized protein, such as E4BP4, MOB cells were pre-treated with 3 μg/ml CHX for 30 min and then treated with 10 nM PTH for 0–6 h. Nuclear extracts were utilized for EMSAs. In the absence of CHX, PTH induced binding to the EBPRE. However, CHX abolished this PTH effect suggesting that the PTH-induced binding was due to newly synthesized proteins (Fig. 6A).

To confirm that E4BP4 was part of the PTH-induced complexes, we utilized a polyclonal antibody against E4BP4 protein. First we determined that the antibody was able to super-shift in vitro synthesized E4BP4 protein. E4BP4 or luciferase proteins were incubated with EBPRE probes and then with E4BP4 antibody or a nonspecific antibody (Nurr1 antibody at the same concentration, Santa Cruz Biotechnologies, catalog number SC-990). The E4BP4 antibody supershifted the entire E4BP4/EBPRE binding complex, whereas the nonspecific antibody had no effect (Fig. 6B). Similarly, the E4BP4 antibody supershifted part of the PTH-induced nuclear extract-EBPRE complex, whereas the nonspecific antibody had no effect (Fig. 6C). Interestingly, not all of the PTH-induced complexes were supershifted, in contrast to the in vitro synthesized E4BP4 protein (Fig. 6C). Because the consensus EBPRE is very similar to the consensus C/EBPβ binding site (18), we tested the possibility that C/EBPβ is part of the PTH-inducible binding to the EBPRE. Indeed, a C/EBPβ antibody supershifted part of the PTH-induced nuclear extract-EBPRE complex (Fig. 6D).

**E4BP4 Inhibited Promoter Activity in Osteoblasts**—To study the regulatory role of E4BP4 on osteoblastic gene expression, MOB cells were co-transfected with pcDNA3.1-E4BP4 or empty vector and with pTK-Luc, pTK-EBPRE, pTK-EBPRE-m1, or pTK-EBPRE-m2 reporter constructs. E4BP4 overexpression had no effect on the basal thymidine kinase promoter activity. However, E4BP4 strongly inhibited pTK-EBPRE activity by 92% (Fig. 7A). When mutant EBPREs were inserted upstream of the thymidine kinase promoter, E4BP4 overexpression inhibited promoter activity to a lesser degree (pTK-EBPRE-m1 by 59% and pTK-EBPRE-m2 by 39%), in parallel to the ability of E4BP4 to bind these mutant sites, as described above.

Because E4BP4 overexpression inhibited luciferase activity of the pTK-EBPRE reporter construct, we tested the ability of PTH treatment to affect this construct. MOB cells were transfected with pTK-Luc, pTK-EBPRE, pTK-EBPRE-m1, or pTK-EBPRE-m2 reporter constructs and then treated with PTH for 12 h. PTH treatment caused a statistically significant attenuation of the promoter activity by 25%, compared with the untreated control (Fig. 7B). In contrast, PTH treatment had no statistical effect on the pTK-EBPRE-m1 or pTK-EBPRE-m2 constructs (Fig. 7B).

Finally, we tested the ability of E4BP4 to affect activity of endogenous promoters. Computer analysis revealed four EBPRE-like elements (seven of eight nucleotides) within the proximal 963 bp of the cyclooxygenase-2 (cox-2) promoter (the kind gift of Dr. Harvey Herschman, UCLA). MOB cells were co-transfected with a luciferase reporter that contained 1 kb of the cox-2 promoter and with pcDNA3.1-E4BP4 or empty vector. Four days later, cells were treated with vehicle or 10 nM PTH for 3 h, and luciferase activity was measured. As previously described (19), PTH induced cox-2 promoter activity (Fig. 7C).
E4BP4 overexpression inhibited basal luciferase activity, although not at statistically significant levels. More importantly, E4BP4 overexpression significantly inhibited PTH-induced Cox-2 promoter activity.

**DISCUSSION**

PTH has important effects on bone metabolism (1). Osteoblasts are the direct PTH target in bone, yet the molecular mechanisms that mediate the effects of PTH on osteoblastic gene expression remain largely elusive (9). PTH-triggered osteoblast activity begins with the rapid and transient induction of primary response gene expression (9, 10). Subsequently, primary gene products mediate downstream PTH effects (19–22). Among the primary genes induced by PTH are several transcription factors, including the AP-1 transcription factors c-fos and c-jun (23), the nuclear orphan receptors Nurr1 (11) and Nur77 (24) and the transcriptional repressor ICER (16). Here, we show that PTH induced E4BP4, which appears to be a transcriptional repressor in osteoblasts. To the best of our knowledge, this is the first report of osseous localization and PTH regulation of E4bp4 gene expression.

E4BP4 is constitutively present in a variety of tissues, including spleen, prostate, testis, ovary, heart, brain, lung, liver, and skeletal muscle (25, 26). In addition, E4BP4 expression is induced by phytohemagglutinin, thapsigargin, and phorbol ester in T-cells (14, 27), by glucocorticoids in mouse fibroblasts (28), by IL-3 and IL-4 in pro-B lymphocytes (29–31), by oncogenic Ras mutants or by granulocyte-macrophage colony-stimulating factor treatment in pro-B lymphocytes (32), and by overexpression of the tumor suppressor phosphatase and tensin homologue in cancer cells (33).

Similar to other tissues, osteoblastic cell lines and calvariae demonstrated low basal expression of E4bp4 mRNA levels. PTH treatment rapidly and transiently induced E4bp4 gene expression that peaked around 2 h and declined thereafter. In MC3T3-E1 and MOB cells, PTH-induced E4bp4 mRNA levels did not require protein synthesis suggesting that E4bp4 is a primary response gene in PTH-treated osteoblasts. In fact, CHX pretreatment led to sustained E4bp4 mRNA levels following PTH treatment. This is in agreement with the glucocorticoid induction of E4bp4 in mouse fibroblasts, where CHX pre-treatment enhances the induction of E4bp4 mRNA levels (28). Interestingly, our findings contrast the observation that CHX abolishes IL-3-induced E4bp4 expression in pro-B lymphocytes (29). The role of E4bp4 as a primary response gene appears to be cell-specific.

E4BP4 contains a domain that is homologous to the DNA binding domain of the bZIP family of transcription factors, and both endogenous and recombinant E4BP4 bind DNA as a dimer at ATF-like sites (12, 15). High affinity binding of random sequence oligonucleotides revealed the consensus E4BP4 binding site sequence (EBPRE) to be (G/A/T/G/T)A(C/T/G)TA, which is homologous to the CRE consensus site (TCACGTCA (12)).

The EBPRE sequence that we selected for our experiments conforms to the consensus but precludes CREB or ATF binding (12). Nuclear extracts from PTH-treated MOB cells formed complexes with the EBPRE site that we used. The nuclear protein-DNA complexes were completely abolished by pretreatment of the cells with CHX, suggesting that binding was due to newly synthesized, rather than pre-existing, proteins. Part of the PTH-induced nuclear protein-EBPRE complexes contained E4BP4 protein, because it supershifted with an E4BP4 antibody but not with a nonspecific antibody.

E4BP4 antibody did not supershift the whole PTH-induced EBPRE-binding complex. Analysis with the TRANSFAC version 4.0 software (18) revealed that the EBPRE site has close homology to the consensus C/EBPβ binding site. Indeed, a C/EBPβ antibody supershifted a PTH-inducible EBPRE-nuclear protein complex. C/EBPβ is induced by PTH in osteoblastic cells (34). Recognition of the same cis-binding element by both E4BP4 and C/EBPβ points to potentially interesting interactions of these transcription factors for the regulation of target gene expression.

E4BP4 acts as a transcriptional inhibitor in most experimental settings (12, 25, 35–37). However, E4BP4 overexpression transactivates the IL-3 promoter in resting T cells, presumably through binding to a cis element located within –165/–128 bp of the IL-3 promoter (14). Why E4BP4 has such distinct effects on transcriptional activity is not clear. Possibly these effects reflect differences in the cell types used with different coactivators/corepressors present. Interestingly, the E4BP4 binding site in the IL-3 promoter is not a canonical EBPRE (14). Thus, the cis-element sequence or the overall promoter context could also influence the effect of E4BP4 on transcription (15).

The E4BP4 transcription repression domain is localized between amino acids 298 and 363. This domain confers transcription repression function in an E4BP4-GAL4 chimeric protein (38). A possible mechanism for the transcriptional inhibition by E4BP4 is the binding of its repression domain to the TBP-binding protein DR1 (39). DR1 is a co-repressor of both basal and induced transcription that interacts with TBP and prevents the association of TFII B with the TBP-TATA complex (40, 41).

To test if E4BP4 represses or activates gene transcription in c-fos and c-jun (23), the nuclear orphan receptors Nurr1 (11) and Nur77 (24) and the transcriptional repressor ICER (16). Here, we show that PTH induced E4BP4, which appears to be a transcriptional repressor in osteoblasts. To the best of our knowledge, this is the first report of osseous localization and PTH regulation of E4bp4 gene expression.

E4BP4 is constitutively present in a variety of tissues, including spleen, prostate, testis, ovary, heart, brain, lung, liver, and skeletal muscle (25, 26). In addition, E4BP4 expression is induced by phytohemagglutinin, thapsigargin, and phorbol ester in T-cells (14, 27), by glucocorticoids in mouse fibroblasts (28), by IL-3 and IL-4 in pro-B lymphocytes (29–31), by oncogenic Ras mutants or by granulocyte-macrophage colony-stimulating factor treatment in pro-B lymphocytes (32), and by overexpression of the tumor suppressor phosphatase and tensin homologue in cancer cells (33).

Similar to other tissues, osteoblastic cell lines and calvariae demonstrated low basal expression of E4bp4 mRNA levels. PTH treatment rapidly and transiently induced E4bp4 gene expression that peaked around 2 h and declined thereafter. In MC3T3-E1 and MOB cells, PTH-induced E4bp4 mRNA levels did not require protein synthesis suggesting that E4bp4 is a primary response gene in PTH-treated osteoblasts. In fact, CHX pretreatment led to sustained E4bp4 mRNA levels following PTH treatment. This is in agreement with the glucocorticoid induction of E4bp4 in mouse fibroblasts, where CHX pre-treatment enhances the induction of E4bp4 mRNA levels (28). Interestingly, our findings contrast the observation that CHX abolishes IL-3-induced E4bp4 expression in pro-B lymphocytes (29). The role of E4bp4 as a primary response gene appears to be cell-specific.

E4BP4 contains a domain that is homologous to the DNA binding domain of the bZIP family of transcription factors, and both endogenous and recombinant E4BP4 bind DNA as a dimer at ATF-like sites (12, 15). High affinity binding of random sequence oligonucleotides revealed the consensus E4BP4 binding site sequence (EBPRE) to be (G/A/T/G/T)A(C/T/G)TA, which is homologous to the CRE consensus site (TCACGTCA (12)).

The EBPRE sequence that we selected for our experiments conforms to the consensus but precludes CREB or ATF binding (12). Nuclear extracts from PTH-treated MOB cells formed complexes with the EBPRE site that we used. The nuclear protein-DNA complexes were completely abolished by pretreatment of the cells with CHX, suggesting that binding was due to newly synthesized, rather than pre-existing, proteins. Part of the PTH-induced nuclear protein-EBPRE complexes contained E4BP4 protein, because it supershifted with an E4BP4 antibody but not with a nonspecific antibody.

E4BP4 antibody did not supershift the whole PTH-induced EBPRE-binding complex. Analysis with the TRANSFAC version 4.0 software (18) revealed that the EBPRE site has close homology to the consensus C/EBPβ binding site. Indeed, a C/EBPβ antibody supershifted a PTH-inducible EBPRE-nuclear protein complex. C/EBPβ is induced by PTH in osteoblastic cells (34). Recognition of the same cis-binding element by both E4BP4 and C/EBPβ points to potentially interesting interactions of these transcription factors for the regulation of target gene expression.

E4BP4 acts as a transcriptional inhibitor in most experimental settings (12, 25, 35–37). However, E4BP4 overexpression transactivates the IL-3 promoter in resting T cells, presumably through binding to a cis element located within –165/–128 bp of the IL-3 promoter (14). Why E4BP4 has such distinct effects on transcriptional activity is not clear. Possibly these effects reflect differences in the cell types used with different coactivators/corepressors present. Interestingly, the E4BP4 binding site in the IL-3 promoter is not a canonical EBPRE (14). Thus, the cis-element sequence or the overall promoter context could also influence the effect of E4BP4 on transcription (15).

The E4BP4 transcription repression domain is localized between amino acids 298 and 363. This domain confers transcription repression function in an E4BP4-GAL4 chimeric protein (38). A possible mechanism for the transcriptional inhibition by E4BP4 is the binding of its repression domain to the TBP-binding protein DR1 (39). DR1 is a co-repressor of both basal and induced transcription that interacts with TBP and prevents the association of TFII B with the TBP-TATA complex (40, 41).

To test if E4BP4 represses or activates gene transcription in...
osteoblasts, we overexpressed E4BP4 in resting MOB cells transfected with a reporter construct carrying a basal promoter under the control of three tandem consensus EBPREs. E4BP4 strongly inhibited the chimeric promoter activity (92%), whereas it had no effect on the basal promoter. When mutant EBPREs were used, E4BP4 inhibition closely paralleled its ability to bind to the mutant sites.

When MOB cells, transfected with the reporter construct containing three tandem consensus EBPREs, were treated with PTH, a statistically significant (25%) attenuation of promoter activity was observed. This suggests that PTH not only induces binding of E4BP4 to EBPREs but also that PTH regulates E4BP4-responsive promoters. The inhibition of the same promoter by E4BP4 overexpression was substantially greater than the inhibition by PTH treatment. A possible explanation is that the pcDNA-E4BP4 vector forces much higher E4BP4 expression than the induction by PTH treatment. Another possible explanation is that, in addition to E4BP4, PTH induces transcription activators that compete for EBPRE binding. The resulting promoter regulation will reflect the competition between transcription activators and repressors. Indeed, Fig. 6D shows that, in addition to E4BP4, PTH induces C/EBPβ binding to EBPRE.

PTH inhibits transcription of osteoblastic genes, including both primary (19) and late response genes (20, 42, 43). The catabolic effects of continuous PTH treatment appear to be associated with decreased expression of many of the genes involved in bone formation and increased expression of genes involved in bone resorption (10). Because E4BP4 appears to be a transcriptional inhibitor in osteoblasts, E4BP4 expression could potentially mediate some of the inhibitory effects of PTH on target gene transcription.

One PTH target gene is cox-2, which is rapidly and transiently induced by PTH through activation of the cAMP-PKA pathway (19). Although induction of cox-2 transcription does not require new protein synthesis, the return to basal transcription does. This suggests that PTH simultaneously induces cox-2 and a transcriptional inhibitor. A possible mode of action for this inhibitor would be competition for promoter binding and transcriptional repression. E4BP4 is an ideal candidate for cox-2 repression, because activation of ATF/CREB would induce transcription, whereas E4BP4 synthesis would compete for binding and would attenuate promoter activity (44). Moreover, computer analysis revealed four EBPRE-like sites (seven out of eight nucleotides) within the cox-2 promoter at positions −833/−826, −683/−676, −59/−52, and −34/−27. Thus, we tested the ability of E4BP4 protein to inhibit basal and, more importantly, PTH-induced cox-2 promoter activity. Indeed, E4BP4 overexpression caused an 80% inhibition of the PTH-induced cox-2 promoter.

The possibility that E4BP4 is part of a tightly regulated system of transcriptional activators and repressors has precedent in the molecular control of circadian rhythms (12, 15, 25, 36). E4bp4 expression follows a circadian pattern in the suprachiasmatic nucleus, the pineal gland, and the liver (37, 45). Furthermore, E4bp4 mRNA levels are in opposite phase with the expression of dbp, hlf, and tef genes that also oscillate with a daily rhythm in a number of tissues (45). E4bp4, dbp, hlf, and tef genes are members of the proline- and acidic residue-rich family of proteins and recognize identical promoter elements (15). Thus the involvement of proline- and acidic residue-rich proteins as reciprocal regulators of gene expression controlling circadian rhythms was hypothesized (45).

E4BP4 induction is associated with inhibition of cell apoptosis. E4BP4 overexpression promotes survival of IL-3-dependent pro-B lymphocytes in the absence of IL-3 treatment. In combination with the observation that IL-3 induces E4BP4 expression in these cells, E4BP4 was hypothesized to play a pivotal role in IL-3-mediated pro-B survival (29). However, no gene targets that might mediate the E4BP4 effects on cell survival have been identified. Interestingly, apoptosis inhibition has been proposed as a possible mechanism for the anabolic effects of PTH. PTH inhibits osteoblast and osteocyte apoptosis in normal and osteopenic mice in vivo and dexamethasone-induced osteoblast apoptosis in vitro (46, 47). It is conceivable that E4BP4 induction may participate in the PTH-mediated inhibition of apoptosis.

In summary, we demonstrate that PTH induced expression of the transcription factor E4BP4 in osteoblastic cells as a primary response gene. Furthermore, PTH induced binding of nuclear E4BP4 protein to EBPRE and regulated the activity of an EBPRE-containing promoter. E4BP4 inhibited chimeric and endogenous promoters in osteoblasts, suggesting that E4BP4 might mediate the inhibitory effects of PTH on target gene transcription.

Acknowledgment—We thank Dr. Jeanne Nervina for providing insightful comments and reviewing the manuscript.
REFERENCES

1. Rubin, M. R., Cosman, F., Lindsay, R., and Bilezikian, J. P. (2002) Osteoporos.
2. Jerome, C. P., and Gubler, H. P. (1991) Calcif. Tissue Int. 49, 398–402
3. Jerome, C. P., Colwell, A., Eastell, R., Russell, R. G. G., and Trechsel, U. (1992) Bone Miner. 19, 117–125
4. Strewler, G. J. (2003) J. Clin. Invest. 107, 217–272
5. Juppner, H., Abou-Samra, A., Freeman, M., Kong, X. F., Schipani, E., Richards, J., Kohakowski, L. F. J., Hock, J., Potts, J. T. J., Kronenberg, H. M., and Segre, G. V. (1991) Science 254, 1024–1025
6. Abou-Samra, A. B., Juppner, H., Force, T., Freeman, M. W., Kong, X. F., Schipani, A., Urena, P., Richards, J., Bozoret, J. V., Potts, J. T. J., Kronenberg, H. M., and Segre, G. V. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2732–2736
7. Schipani, E., Karga, H., Karupisu, A. C., Potts, J. T. J., Kronenberg, H. M., Segre, G. V., Abou-Samra, A. B., and Juppner, H. (1993) Endocrinology 132, 2157–2165
8. Fujimori, A., Cheng, S. L., Avidi, L. V., and Civitelli, R. (1992) Endocrinology 129, 29–36
9. Partridge, N. C., Bloch, S. R., and Pearman, A. T. (1994) J. Cell. Biochem. 55, 321–327
10. Swarthout, J. T., D’Alonzo, R. C., Selvanuragah, N., and Partridge, N. C. (2002) Gene (Amst.) 282, 1–17
11. Tetrads, S., Bezuglia, O., and Tsingotjidou, A. (2001) Endocrinology 142, 663–670
12. Cowell, I. G., Skinner, A., and Hurst, H. C. (1992) Mol. Cell. Biol. 12, 3070–3077
13. Chen, W. J., Lewis, K. S., Chandra, G., Cogswell, J. P., Stinnett, S. W., Kadwell, S. H., and Gray, J. G. (1995) Biochim. Biophys. Acta 1264, 388–396
14. Zhang, W., Zhang, J., Kornuc, M., Kwan, K., Frank, R., and Nimer, S. D. (1995) Mol. Cell. Biol. 15, 6055–6063
15. Cowell, I. G. (2002) Bioessays 24, 1023–1029
16. Tetrads, S., Nervina, J. M., Nemoto, K., and Kream, B. E. (1998) J. Bone Miner. Res. 13, 1846–1851
17. Tomasar, G. D., Foster, D. A., Burrell, C. M., and Taffet, S. M. (1999) J. Leukoc. Biol. 66, 183–193
18. Quandt, K., Fresh, K., Karas, H., Wingender, E., and Werner, T. (1995) Nucleic Acids Res. 23, 4878–4884
19. Tetrads, S., Pilebein, C. C., Liu, Y., Herschman, H. R., and Kream, B. E. (1997) Endocrinology 138, 3594–3600
20. Kream, B. E., LaFrancis, D., Petersen, D. N., Woody, C., Clark, S., Rowe, D. W., and Lichtler, A. (1993) Mol. Endocrinol. 7, 399–408
21. Scott, D. K., Brakenhoff, K. D., Clohisy, J. C., Quinn, C. O., and Partridge, N. C. (1992) Mol. Endocrinol. 6, 2153–2159
22. Onyia, J. E., Bidwell, J., Herring, J., Hulman, J., and Hock, J. M. (1995) Bone 17, 479–484
23. Clohisy, J. C., Scott, D. K., Brakenhoff, K. D., Quinn, C. O., and Partridge, N. C. (1992) Mol. Endocrinol. 6, 1834–1842
24. Tetrads, S., Bezuglia, O., Tsingotjidou, A., and Vila, A. (2001) Biochem. Biophys. Res. Commun. 281, 913–916
25. Lai, C. K., and Ting, L. P. (1999) J. Virol. 73, 3197–3209
26. Hulme, D. J., Blair, I. P., Dawkins, J. L., and Nicholson, G. A. (2000) Hum. Genet. 106, 594–596
27. Nishimura, Y., and Tanaka, T. (2001) J. Biol. Chem. 276, 19921–19928
28. Wallace, A. D., Wheeler, T. T., and Young, D. A. (1997) Biochem. Biophys. Res. Commun. 232, 403–406
29. Ikushima, S., Inukai, T., Inaba, T., Nimer, S. D., Cleveland, J. L., and Look, A. T. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2609–2614
30. Chu, C. C., and Paul, W. E. (1998) Mol. Immunol. 35, 487–502
31. Yu, Y. L., Chiang, Y. J., and Yen, J. J. (2002) J. Biol. Chem. 277, 27144–27153
32. Kitahara, R., Kinoshita, T., Miyajima, A., Shinoy, T., Yoshitaka, T., Inukai, T., Ozawa, K., Look, A. T., and Inaba, T. (1999) Mol. Cell. Biol. 19, 2754–2762
33. Unoki, M., and Nakamura, Y. (2001) Oncogene 20, 4457–4465
34. Qin, L., Qui, P., Wang, L., Li, X., Swarthout, J. T., Soteropoulos, P., Tolias, P., and Partridge, N. C. (2003) J. Biol. Chem. 278, 19723–19731
35. Gray, J. R., Siddika, G., Clay, C. W., Stopper, S. W., Haneline, S. A., Lorenz, J. J., Patel, I. R., W Atlas, B. G., Fauron, P. J., Taylor, J. D., and Stot, T. A. (1990) Mol. Cell. Biol. 13, 6678–6689
36. Ishida, H., Ueda, K., Ohka, K., Kanazawa, Y., Hosoi, A., Nakashima, P., Mita, E., Kasahara, A., Sasaki, Y., Hori, M., and Hayashi, N. (2000) J. Virol. 74, 1241–1251
37. Dui, M., Nakajima, Y., Okano, T., and Fukada, Y. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 8085–8094
38. Cowell, I. G., and Hurst, H. C. (1994) Nucleic Acids Res. 22, 59–65
39. Cowell, I. G., and Hurst, H. C. (1996) Nucleic Acids Res. 24, 3607–3613
40. Kim, S., Na, J. G., Hambyse, M., and Reinberg, D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 820–825
41. Castano, E., Gross, P., Wang, Z., Roeder, R. G., and Oegeschlager, T. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7184–7189
42. Fukayama, S., Allan, E. H., Yee, J. A., Gelehrter, T. D., and Martin, T. J. (1992) J. Biol. Chem. 267, 403–406
43. Fukayama, S., Schipani, E., Juppner, H., Lanake, B., Kronenberg, H. M., Abou-Samra, A. B., and Bringhurst, F. R. (1994) Endocrinology 134, 1851–1858
44. Haas, N. B., Cantwell, C. A., Johnson, P. F., and Burch, J. B. (1995) Mol. Endocrinol. 15, 2152–2162
45. Matsui, S., Yamaguchi, S., Matsui, T., Ishida, Y., and Okamura, H. (2001) Genes Dev. 15, 995–1006
46. Jilka, R. L., Weinstein, R. S., Bellido, T., Roberson, P., Parfitt, A. M., and Manolagas, S. C. (1999) J. Clin. Invest. 104, 439–446
47. Chen, H. L., Demiralp, B., Schneider, A., Koh, A. J., Silve, C., Wang, C. Y., and McCauley, L. K. (2002) J. Biol. Chem. 277, 19374–19381
Parathyroid Hormone-induced E4BP4/NFIL3 Down-regulates Transcription in Osteoblasts
Ibrahim C. Ozkurt and Sotirios Tetradis

J. Biol. Chem. 2003, 278:26803-26809.
doi: 10.1074/jbc.M212652200 originally published online May 12, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M212652200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 47 references, 18 of which can be accessed free at
http://www.jbc.org/content/278/29/26803.full.html#ref-list-1