CCAAT/enhancer-binding protein δ activates the Runx2-mediated transcription of mouse osteocalcin II promoter

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Abstract

CCAAT/enhancer-binding proteins (C/EBPs) are involved in the regulation of cell proliferation, differentiation, and control of metabolic function. Although the roles of C/EBPs in osteoblasts are largely unknown, both C/EBPβ and -δ have been shown to enhance rat osteocalcin promoter activity through the synergistic activation of Runx2 at the C/EBP element. Here we show that in the mouse, C/EBPδ increases the expression of osteocalcin whereas C/EBPβ does not. This increased expression was found to occur at the transcriptional level, as demonstrated by the increased transcriptional activity from mouse osteocalcin II (OG2) promoter by C/EBPδ. Although we found three putative C/EBP sites in the −637/±34 region of the OG2 promoter, none of these sites showed binding activity with *in vitro* translated C/EBP proteins. Notably, we show that C/EBPδ physically interacts with Runx2 and that C/EBPδ overexpression increases binding between the Runx2–C/EBPδ complex and the OSE2 element, a critical osteoblast-specific *cis*-acting element in the OG2 promoter. Consistent with these DNA binding data, a mutation in OSE2 abrogated the stimulatory effect of C/EBPδ on this promoter activity. Finally, chromatin immunoprecipitation analysis in MC3T3-E1 cells showed *in vivo* occupancy of the OG2 promoter by Runx2 and C/EBPδ. In conclusion, C/EBPδ was found to regulate mouse osteocalcin OG2 promoter activity indirectly by interacting with Runx2 in the context of the OSE2 element and this subsequently resulted in the cooperative activation of the OG2 promoter.

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Introduction

The CCAAT/enhancer-binding proteins (C/EBPs) are members of the basic leucine zipper (bZIP) transcription factor family (Landschulz *et al.* 1989), which are involved in the regulation of several biological processes such as inflammatory and immune responses, cell proliferation and differentiation, and in the control of metabolic function (Cao *et al.* 1991, Darlington *et al.* 1998, Poli 1998). First isolated from rat liver, six isoforms of C/EBPs have been identified (Lekstrom-Himes & Xanthopoulos 1998), most of which are expressed in liver, spleen and adipose tissues. C/EBP family proteins share strong amino acid similarity in their C-terminal regions, which contain motifs for DNA binding and a leucine zipper dimerization domain (Wedel & Ziegler-Heitbrock 1995). Consequently, each family member can homo- or heterodimerize and bind to a common DNA consensus sequence for transcriptional activation (Cao *et al.* 1991). C/EBP isoforms are expressed at high levels in adipocytes and are induced during adipogenesis (Cao *et al.* 1991). C/EBPα and C/EBPβ function as transcriptional activators and play major roles in adipocyte differentiation and in the regulation of gene expression in liver and other tissues (Wedel & Ziegler-Heitbrock 1995, Mandrup & Lane 1997, Tanaka *et al.* 1997). C/EBPα has been demonstrated to regulate the differentiation of preadiopocytes to adipocytes (Samuelsson *et al.* 1991, Umek *et al.* 1991, Lin & Lane 1994) and convert fibroblasts into adipocytes (Freytag *et al.* 1994). Moreover, determined myoblasts with no inherent adipogenic potential can be induced to transdifferentiate into mature adipocytes by the ectopic expression of C/EBPα and peroxisome proliferator-activated receptor γ, the latter being another key adipogenic transcription factor (Hu *et al.* 1995). C/EBPδ has also been identified to control adipogenesis and to mediate acute phase response to inflammatory stimuli (Wedel & Ziegler-Heitbrock 1995, Mandrup & Lane 1997, Tanaka *et al.* 1997).

Little is known of the roles of C/EBPs in osteoblasts. In the basal state, osteoblasts constitutively express C/EBPβ and -δ (Thomas *et al.* 1996, Gutierrez *et al.* 2002), whereas C/EBPα has not been detected in bone

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tissue (Gutierrez *et al.* 2002). C/EBPδ has been identified as a principal cAMP-activated regulator of insulin-like growth factor (IGF)-I gene transcription in osteoblasts via its binding to the HS3D site (Umayahara *et al.* 1997). In addition, the enhanced expression of both C/EBPβ and -δ potentiated IGF-I promoter activity in response to prostaglandin E_2 (McCarthy *et al.* 2000*a*). Expression of interleukin-6 is also regulated by C/EBPβ by cross-coupling with estrogen receptor and nuclear factor-kappaB (Stein & Yang 1995). Although the targeted deletions of C/EBPβ or -δ did not result in skeletal abnormalities (Tanaka *et al.* 1995, 1997, Sterneck *et al.* 1997), these results suggest that C/EBP factors regulate expression of genes involved in bone formation or resorption.

Osteocalcin is a γ-carboxylated protein, which composes up to 15% of the noncollagenous protein in mature bone (Price *et al.* 1976). Moreover, osteocalcin expression is largely restricted to the osteoblasts of bone and the odontoblasts and cementoblasts of teeth (McKee *et al.* 1992). The tissue-specific expression of osteocalcin and its transcriptional control are mainly regulated by Runx2 (Cbfa1/AML3/Pebp2αA), a runt related transcription factor (Ducy & Karsenty 1995, Ducy *et al.* 1997). Runx2 has been shown to activate osteocalcin gene expression by binding to the osteoblast-specific *cis*-acting element (OSE2), located in the osteocalcin promoter (Ducy *et al.* 1997) and its transcriptional activity can be modified by growth factors or other transcription factors (Javed *et al.* 2000, Alliston *et al.* 2001, Jeon *et al.* 2003). Recently, the C/EBP-responsive element was identified in the rat osteocalcin promoter, which resides in close proximity to the Runx2 element (Gutierrez *et al.* 2002), and C/EBPβ and -δ were found synergistically to activate osteocalcin promoter. Although rat and mouse osteocalcin promoters have much in common regarding the regulatory element, they exhibit differences in some aspects of gene regulation. For example, 1,25-dihydroxyvitamin D3 inhibits mouse osteocalcin I and II promoters (OG1, OG2) (Zhang *et al.* 1997), whereas it activates transcription from rat osteocalcin promoter by interacting with a vitamin D responsive element (Price & Baukol 1980, Demay *et al.* 1989, Lian *et al.* 1989).

In this study, we addressed the roles of C/EBPs in the regulation of mouse osteocalcin gene expression. We found that mouse osteoblastic cells constitutively express C/EBPβ and -δ. C/EBPδ enhances mouse osteocalcin gene expression through synergistic cooperation with Runx2, whereas C/EBPβ did not affect its activity. Interestingly, this functional synergism between C/EBPδ and Runx2 was found to be mediated through the OSE2 element of OG2 promoter. We also present evidence that direct interaction between C/EBPδ and Runx2 is crucial in the cooperative activation of the OG2 promoter.

Materials and methods

Materials

Monoclonal anti-C/EBPα, -β, and -δ and anti-Runx2 antibodies were obtained from Santa Cruz Biotechnology, Inc. (San Diego, CA, USA), TRI reagent was from Molecular Research Center (Cincinnati, OH, USA), and western blotting detection reagents and [α-^{32}P]dCTP were from Amersham Biosciences (Piscataway, NJ, USA). Random priming kits and reagents for the luciferase assay were purchased from Promega Corp. (Madison, WI, USA) and nitrocellulose membranes were from Schleicher & Schuell (Dassel, Germany). Lipofectamine Plus was obtained from Invitrogen Corp. (Carlsbad, CA, USA). Oligonucleotides were synthesized by Bioneer Corp. (Chungwon, Korea), and all other chemicals, including tissue culture media, were from Sigma Chemical (St Louis, MO, USA), unless otherwise indicated.

Expression vectors and reporter plasmids

An expression vector for C/EBPα (pSV-SPORT1-C/EBPα) was constructed by isolating full-length C/EBPα cDNA from MSV-C/EBPα by digestion with EcoRI-BssHII, and insertion into the EcoRI/MluI sites of pSV-SPORT vector (Invitrogen). Expression vectors for C/EBPβ and -δ were constructed by ligating EcoRI/BamHI restriction fragments of C/EBPβ or -δ from MSV-C/EBPβ or -δ into pSV-SPORT, giving pSV-SPORT C/EBPβ and -δ respectively. Retroviral vectors for C/EBPβ and -δ were constructed by ligating the EcoRI/BamHI restriction fragment of C/EBPβ or -δ from MSV-C/EBPβ or -δ into pLXSN, giving pLXSN-C/EBPβ and -δ respectively. The expression vector for Runx2 (MASNSL isoform), pcDNA3-til, was provided by Dr Je-Yong Choi (Kyungpook National University, Daegu, Korea). pGST-C/EBPδ containing the whole open reading frame (ORF) of C/EBPδ cloned into pGEX-4T vector (Amersham Biosciences) was obtained from Dr Minho Shong (Chungnam National University, Daejeon, Korea) and has been described (Jung *et al.* 2000). Mouse osteocalcin II (OG2) promoter-luciferase reporter constructs (–1·3 OG2-Luc) containing 1·3 kb (positions –1316 to +13) have been described previously (Zhang *et al.* 1997). A series of deletion mutants of the –1·3 OG2-Luc, namely p657OG2-Luc, p343OG2-Luc, p147OG2-Luc, and p34OG2-Luc, have also been described previously (Ducy & Karsenty 1995). Rat osteocalcin promoter-luciferase reporter constructs (–647 OC-Luc) containing 680 base pairs (position –647 to ± 32) in pGL2 Basic (Promega) have been described in detail (Boudreaux & Towler 1996). Deletion constructs lacking each of the putative C/EBP sites were generated using the 'Quick Change' PCR-based mutagenesis procedure (Stratagene,

La Jolla, CA, USA) with the p657OG2-Luc plasmid as a template. The p657ΔC1OG2-Luc mutant construct was generated with the primers 5′-GAAGAGCCTAGCCC ATTGTG-3′ and 5′-GGAGGCATTTTCTCAATTGA GG-3′, the p657ΔC2 OG2-Luc construct with primers 5′-TCCATAAGATCCGGTTGTAGGG-3′ and 5′-AG GGTTCTTGTCTCTAGGGC-3′, the p657ΔC3 OG2-Luc construct with primers 5′-GCCCTAGAGACAAGA ACCCT-3′ and 5′-ACCAACCACAGCATCCTTTG-3′, and the p657ΔOSE2 OG2-Luc construct with primers 5′-GGTGATTGCAGCTGCCT-3′ and 5′-GAGAGCACAGAGTAGCCGAT-3′. The appropriate absence of unwanted mutations was confirmed by sequencing the plasmid. The p6OSE2-Luc and p6OSE2 m-Luc plasmids contain six copies of the wild-type or of the mutant OSE2 sequence of the OG2 promoter respectively, followed by a minimal promoter, which directs luciferase expression (Ducy & Karsenty 1995).

Cell culture

Normal mouse osteoblasts were isolated from 21-day-old fetal mouse calvariae using a well-characterized technique essentially as described previously (Furlan *et al.* 2001). The rat osteogenic sarcoma cell line, ROS 17/2·8 cells, expresses several osteoblastic features, including the production of osteocalcin and other matrix proteins (Majeska *et al.* 1985). ROS 17/2·8 cells were cultured in DMEM/F12 containing 10% heat-inactivated fetal bovine serum (FBS, BioWhittaker Inc., Walkersville, MD, USA). Mouse MC3T3-E1 osteoblastic cells, derived from spontaneously immortalized calvarial cells (Sudo *et al.* 1983) were maintained in DMEM/F12 medium containing 10% FBS. During osteoblastic differentiation studies, MC3T3-E1 cells were cultured in DMEM/F12 medium containing 10% FBS supplemented with 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate. Another mouse osteoblastic cell line, ST2 cells, was maintained in αMEM medium containing 10% FBS. Mouse embryonic mesenchymal cells, C3H10T1/2 (American Type Culture Collection, Manassas, VA, USA), are pluripotent cells that retain an immature, fibroblast-like appearance under standard tissue culture conditions. C3H10T1/2 cells were grown in basal medium of Eagle (BME) containing 10% FBS. The 3T3-L1 preadipocytic cell line was a kind gift from Dr Jae Bum Kim (Seoul National University, Seoul, Korea). 3T3-L1 cells were maintained in an immature state by culturing in DMEM supplemented with 20% FBS and 2·0 mM glutamine.

Retrovirus production and transduction of cell lines

For transient generation of VSV-G pseudo-typed retrovirus, 293T cells were plated in 60-mm diameter dishes ($1·8 \times 10^6$ cells in 3 ml DMEM containing 10% FBS) and allowed to attach overnight. The plasmids pMD-gag-pol and pMD-VSVG (both kindly provided by Dr Richard C Mulligan at Harvard Medical School, Boston, MA, USA) and the retroviral vectors pLXSN, pLXSN-C/EBPβ, or pLXSN-C/EBPδ were introduced into 293T cells (a packaging cell line) using Lipofectamine Plus reagents according to the manufacturer's instructions. Viral supernatant was collected at 48 h after DNA addition, filtered through a 0·45 μm syringe filter (Nalgene, Rochester, NY, USA), and stored at −80 °C. Logarithmically growing MC3T3-E1 or ROS 17/2·8 cells were transduced with pLXSN, pLXSN-C/EBPβ, or pLXSN-C/EBPδ retrovirus by centrifugation at 1200 ***g*** for 90 min at 32 °C. Cells expressing exogenous DNA were selected by G418 (1000 mg/ml for MC3T3-E1 and 400 mg/ml for ROS17/2·8 cells) for 2 to 3 weeks. Drug-resistant colonies were selected and expanded, and the expression of the exogenous genes was confirmed by Western blotting analysis, as described below.

Northern blotting

Total cellular RNA was isolated from cell monolayers using TRI reagent according to the manufacturer's instructions. Samples (20 μg/lane) were separated on 1% formaldehyde agarose gels by electrophoresis, blotted onto nylon membranes, and UV crosslinked. The membranes were then hybridized using ^{32}P-labeled probes prepared using the random primed oligonucleotide method in ULTRAhyb solution (Ambion, Austin, TX, USA) at 42 °C overnight and washed twice in 2 × SSC, 0·1% SDS at 42 °C, followed by one high stringency wash in 0·2 × SSC, 0·1% SDS at 42 °C for 15 min. The following cDNA probes were used: 1·0 kb NcoI fragment of C/EBPα, 1·5 kb EcoRI-XhoI fragment of C/EBPβ, 1·0 kb EcoRI–BamHI fragment of C/EBPδ, 470 bp EcoRI–PstI fragment of osteocalcin, 1·1 kb KpnI fragment of collagen type 1, 1·5 kb full length cDNA of Runx2, 1·3 kb full length cDNA of osteopontin, and 1·9 kb BamHI fragment of rat β-actin.

Western blotting

Cell lysates were prepared by treating cells with lysis buffer (150 mM NaCl, 50 mM Tris–HCl (pH 7·4), 20 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0·1% SDS and protease inhibitors). Lysates were sonicated for 20 min on ice and centrifuged at 10 000 ***g*** for 10 min to sediment particulate material. Protein concentrations of the supernatants were determined as described by Lowry *et al.* (1951). SDS-polyacrylamide gel electrophoresis was performed on 10% polyacrylamide gels and the resolved proteins were transferred onto nitrocellulose membranes. Membranes were blocked

with 0·1% Tween-20 TBS containing 2% BSA and 3% dry milk, at pH 7·4, for one hour. Polycloncal antibodies against C/EBPα, -β, or -δ were added and incubation was continued for another hour. After washing in 0·1% Tween-20 TBS, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse antibodies for one hour. After extensive washing, bands were visualized by chemiluminescence using an ECL kit (Amersham Biosciences), according to the manufacturer's instructions.

Immunoprecipitations

MC3T3-E1 cells were lysed in 50 mM Tris–HCl pH 7·5, 150 mM NaCl, 1% NP-40, and 0·5% sodium deoxycholate, containing a cocktail of protease inhibitors (Sigma). Lysates were then precleared for 3 h at 4 °C with protein G-Sepharose (Roche, Mannheim, Germany). For immunoprecipitation of endogenous Runx2 or C/EBPδ from MC3T3-E1 cells, we used goat anti-Runx2 or anti-C/EBPδ. An isotype-matched antibody (anti-thyroglobulin) was used as a control. The lysates were incubated for 3 h at 4 °C prior to being incubated with protein G-Sepharose. After extensive washing, the immunoprecipitates were electrophoresed in SDS–polyacrylamide gel and the expression levels of the proteins of interest were verified by western analyses using specific antibodies.

Transfections and reporter assays

Transient transfections were performed in triplicate, and transfection efficiencies were monitored using cytomegalovirus-β-galactosidase (pCMV-β-gal, Promega Corp.) vectors in parallel cultures. For these experiments, osteoblastic cells were plated at high density (3×10^5 cells/well) onto 12-well plates. Appropriate plasmids were transfected into each well using Lipofectamine Plus reagent (Invitrogen Corp.) according to the manufacturer's instructions. Cell lysates (0·25 ml/well) were prepared using the Promega Luciferase assay system and reporter activity was measured using a luminometer (Lumat LB 9507, Berthold, Bad Wildbad, Germany). All luciferase values were normalized against the β-galactosidase activity from the cotransfected pCMV-β-gal plasmid. All values, means, and standard deviations were expressed relative to basal promoter activity as fold inductions.

Glutathione S-transferase (GST) pull-down analyses

GST fusion proteins were induced in *Eschericia coli* BL21 for 3 h at 25 °C by adding isopropyl-1-thio-β-D-galactopyranoside (100 μM final concentration) to a 100-ml bacterial culture ($A_{600} \approx 0{\cdot}5$). After induction, bacteria were pelleted for 20 min at 3000 × ***g*** and resuspended in 20 ml ice-cold binding buffer (20 mM Tris–HCl, pH 7·5, 150 mM NaCl, 0·1% Nonidet P-40, 0·1 mM PMSF, 1 mM EDTA). The bacteria were then lysed using three freeze-thaw cycles (5 min in liquid nitrogen followed by thawing for 10 min at 37 °C). The lysed bacteria were then subjected to three 10-s rounds of sonication (7 μ amplitude) at 4 °C, and bacterial debris was pelleted by centrifugation at 15 000 × ***g*** for 30 min at 4 °C. Supernatants were stored frozen at –20 °C in 100-μl aliquots until needed. Free GST lysates were prepared in a similar manner from *E. coli* BL21 transformed with pGEX-3 vector. Free GST and GST fusion proteins were purified on a glutathione–Sepharose 4B column (Amersham Biosciences) according to the manufacturer's recommendations, and then dialyzed against binding buffer.

For the GST pull-down assays, equal amounts of purified recombinant GST or the GST fusion proteins were immobilized on glutathione–Sepharose beads and washed four times with 1 ml wash buffer (20 mM Tris–HCl, pH 7·5, 150 mM NaCl, 1 mM EDTA, 0·1% NP-40, 1 mM NaF, 2 μg/ml aprotinin, 0·1 mM PMSF) at 4 °C. ^{35}S-Labeled Runx2 was synthesized in rabbit reticulocyte lysate by coupled *in vitro* transcription and translation (TNT T7-coupled reticulocyte lysate system; Promega Corp.), and then added to immobilized GST or GST fusion proteins and incubated for 2 h. After binding, proteins bound to the beads were eluted with elution buffer (10 mM reduced glutathione, 20 mM Tris–HCl (pH 7·5), 0·1 mM PMSF, 0·1% NP-40, 2 μg/ml aprotinin). Samples were then separated in SDS-polyacrylamide gel and analyzed by autoradiography.

Electrophoretic mobility shift assay

Nuclear extracts were prepared as described by Dignam *et al.* (1983). Briefly, cells were washed with ice-cold PBS, and then resuspended in hypotonic lysis buffer containing 20 mM HEPES (pH 8·0), 25% glycerol, 1·5 mM $MgCl_2$, 0·2 mM EDTA, 0·3% Triton X-100, 0·6% ammonium sulfate, 1 mM dithiothreitol and the protease inhibitors. The protein concentrations of the nuclear extracts were determined by the Bradford assay (BioRad) using bovine serum albumin as a standard. *In vitro* translated C/EBPα, -β, or -δ was obtained by transcribing and translating pSV-SPORT-C/EBPα, -β, or -δ expression plasmids respectively, using the TNT SP6/T7-coupled reticulocyte lysate system. Protein concentration was measured using parallel [^{35}S]methionine-labeled reactions. Oligonucleotide probes corresponding to the OSE2 site in the mouse osteocalcin promoter (5′-GATCCGCTGCAATCACC AACCACAGCA-3′) (Ducy & Karsenty 1995) and the optimal consensus C/EBP element (5′-ATTTTTGCG

CAATTTTATTGCGCAATCAATATTGAATAA-3′; two adjacent C/EBP binding sites are underlined) (Elberg *et al.* 2000), and the putative C/EBP binding site in OG2 promoter (C1, 5′-TCCCCACCAACCACAAGAAATGCCCTACAACCGG-3′; C2, 5′- AGCTGCCCTGAACTGGGCAAATGAGGACATTACTG-3′; C3, 5′-CCTTGCCCAGGCAGCTGCAATCACCAACCACA-3′; the bases corresponding to consensus binding site are underlined) were generated using an oligonucleotide synthesizer (Bioneer Corp.). Complementary oligonucleotides were annealed and labeled with [α-^{32}P]dCTP. The binding reaction was performed by incubating 10 μg nuclear protein from cultured cells or *in vitro* translated proteins in 20 mM HEPES (pH 8·0), 25% glycerol, 1·5 mM $MgCl_2$, 300 mg bovine serum albumin, and 1 mg poly(dI-dC) in a final volume of 10 μl for 10 min at 25 °C. Labeled oligonucleotides were added to the reaction mixture and incubated for an additional 20 min on ice. To prove the involvement of C/EBPδ or Runx2 in the formation of a ternary complex, nuclear lysates were preincubated at 4 °C for 1 h with anti-C/EBPδ or anti-Runx2 antibody prior to the addition of poly(dI-dC) and radiolabeled probe DNA. Samples were electrophoresed in 4% nondenaturing polyacrylamide gels, which were then dried and autoradiographed.

Chromatin immunoprecipitation (ChIP) assays

MC3T3-E1 cells were treated with 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate for 12 days to induce osteogenic differentiation. Chromatin-binding proteins were cross-linked by treating the cells with 1% formaldehyde in PBS for 15 min at room temperature. Cells were washed with ice-cold phosphate-buffered saline, scraped, and swollen on ice for 10 min in phosphate-buffered saline containing protease inhibitors (Roche Applied Science, Indianapolis, USA). Cell extracts were prepared in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl (pH 8·1) and protease inhibitors). Extracts were sonicated with a microtip on ice to obtain DNA fragments ranging from 200 to 1000 bp. Five microliters of supernatant were saved as input DNA, and the remaining soluble chromatin fragments were diluted 1:10 in ChIP dilution buffer (0·01% SDS, 1·1% Triton X-100, 1·2 mM EDTA, and 16·7 mM Tris–HCl) containing protease inhibitors.

The chromatin suspension was precleared using Protein-A beads (Amersham Biosciences), and then incubated with 1 μg of an antibody specific to Runx2 or C/EBPδ at 4 °C overnight. Normal rabbit IgG and irrelevant anti-thyrotropin (TSH) receptor antibody was used as a negative control. Protein-A beads were added to the samples and incubated for 15 min at 4 °C. The beads were collected by centrifugation at 10 000 × ***g*** for 3 min and washed once in low salt buffer (0·1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris–HCl, pH 8·0), four times in high salt buffer (0·1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris–HCl, pH 8·0), once in lithium chloride buffer (250 mM LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris–HCl, pH 8·0), and once in 1 × TE (10 mM Tris–HCl, pH 8·0, 10 mM EDTA) at room temperature for 10 min each. The protein–DNA complexes were then eluted from the beads using elution buffer (50 mM $NaHCO_3$, 1% SDS), and the cross-links were removed by heating at 67 °C for 4–5 h. DNA was purified from the complexes by phenol/chloroform extraction and ethanol precipitation, and analyzed by PCR using the following primer pairs encompassing the −285/−84 region of the OG2 promoter: P1, 5′-CCAGCTGAGGCTGAGAGAGA-3′ and P2, 5′-CTAATTGGGGGTCATGTGCT-3′ (see Fig. 7A). As a control, PCR using primer pairs encompassing the –1247/−1145 region, which does not contain either the C/EBP or the OSE2 site (P3, 5′-GCAGTTAAGAGCCACTGACT-3′ and P4, 5′-AAGAGGGCATCAGATTCCAC-3′), was performed in parallel.

Results

C/EBPβ and -δ are expressed in murine osteoblastic cell lines

We examined the expression of C/EBP factors in mouse calvarial cells and in a series of osteoblastic and non-osteoblastic cell lines. Western blotting analysis revealed that both C/EBPβ and -δ were expressed in murine calvarial cells, MC3T3-E1, ST2, C3H10T1/2, rat osteogenic sarcoma ROS 17/2·8 cells, and 3T3-L1 adipocytes (Fig. 1A). However, C/EBPα was not detected in calvarial cells and three osteoblastic cell lines, MC3T3-E1, ST2 and ROS 17/2·8. C3H10T1/2 cells, a mesenchymal cell line, expressed little C/EBPα compared with 3T3-L1 adipocytes, which were used as a positive control.

Northern blot analysis of RNA extracted from MC3T3-E1 cells showed that C/EBPβ mRNA expression level was high during the early phase of osteoblastic differentiation (days 0–3) and then gradually down-regulated during the culture period (days 7–19, Fig 1B). However, the mRNA expression of C/EBPδ showed a biphasic pattern, decreasing during the early phase of matrix maturation (days 0–7), followed by an increase with mineral deposition (days 12–19). Moreover, the later increase in C/EBPδ expression coincided with peak expression of osteocalcin, suggesting that C/EBPδ may participate in the regulation of osteocalcin expression. The peak expression of Runx2, the essential element for osteocalcin expression, seems to precede the expression of osteocalcin.

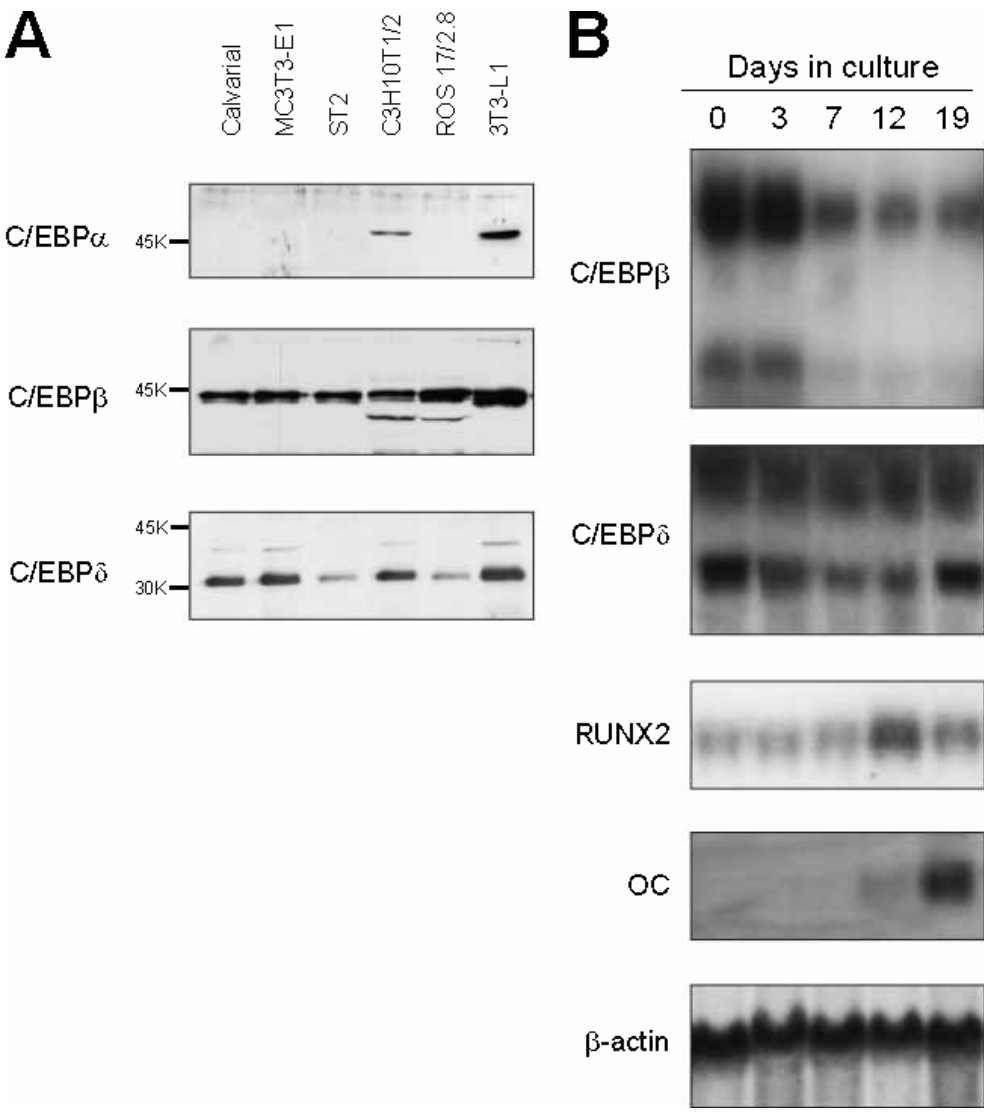

Figure 1 Expression of C/EBPs in osteoblastic and non-osteoblastic cells. (A) Cellular lysate proteins (20 μg/lane) from mouse calvarial cells, MC3T3-E1, ST2, C3H10T1/2, ROS 17/2·8, and 3T3-L1 cells were loaded into 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred to nitrocellulose. Immunoblots were probed with antibody for C/EBPα, -β, or -δ. (B) Changes in the level of C/EBP mRNA during the osteoblastic differentiation of MC3T3-E1 cells. MC3T3-E1 cells were cultured in DMEM/F12 containing 10% FBS, and harvested at confluence (day 0, lane 1) or after inducing osteoblastic differentiation with 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate for 19 days. Total cellular RNA was isolated and 20 μg RNA were added to each lane. Northern blots were probed for C/EBPβ, -δ, Runx2, osteocalcin (OC), and β-actin.

C/EBPδ activates osteocalcin gene expression in osteoblasts

The possibility that C/EBPs activates osteocalcin expression was investigated. ROS 17/2·8 and MC3T3-E1 cells were stably transduced with C/EBPβ or -δ retrovirus and the expression of transduced genes was verified by Western blot analysis (Fig. 2A,B). ROS 17/2·8 cells were found to constitutively express osteocalcin and the level of its expression was robustly increased by stable transduction of C/EBPδ (Fig. 2C). Overexpression of C/EBPβ also increased osteocalcin mRNA levels, albeit less strongly than C/EBPδ (Fig. 2C). Expression of osteopontin and collagen type 1 exhibited a similar response. MC3T3-E1 cells expressed only low levels of osteocalcin in the basal condition, but stable transduction with C/EBPδ greatly enhanced its expression (Fig. 2D). In contrast, the expression level of

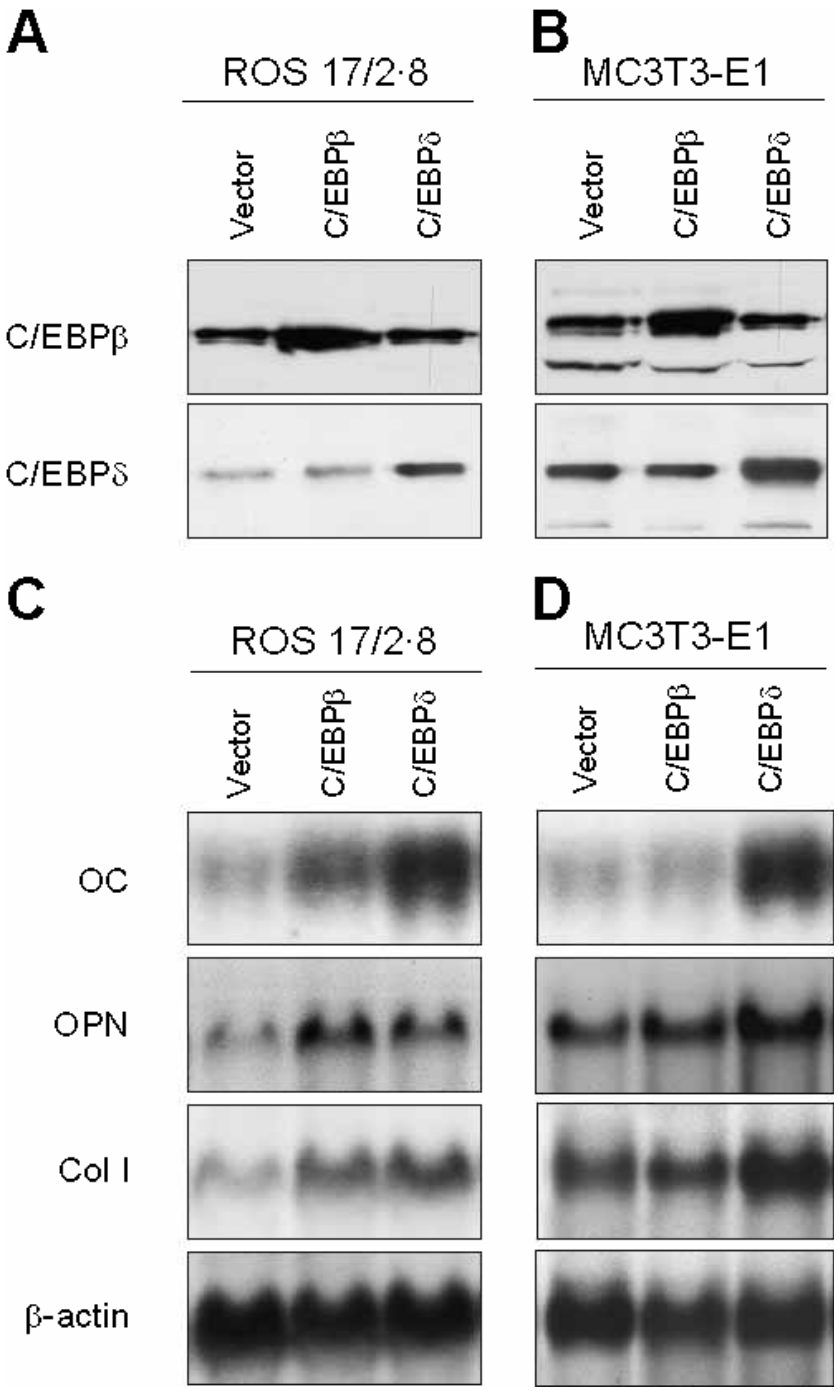

Figure 2 C/EBPs regulate osteocalcin gene expression. ROS 17/2·8 (A and C) or MC3T3-E1 cells (B and D) stably transduced with retrovirus encoding empty vector, C/EBPβ or -δ were cultured in DMEM/F12 containing 10% FBS, and harvested at confluence. Cellular lysate proteins (20 μg/lane) from these cells were subject to immunoblotting as in Fig. 1 to verify the expression of transduced genes in ROS 17/2·8 (A) and MC3T3-E1 (B) cells using antibodies for C/EBPβ or -δ. Osteoblast-specific gene expression in ROS 17/2·8 (C) or MC3T3-E1 (D) cells were analyzed by northern blotting. Total cellular RNA was isolated and 20 μg RNA were added to each lane. Northern blotting was performed as in Fig. 1 using cDNA probes for osteocalcin (OC), osteopontin (OPN), collagen type 1 (Col I), and β-actin.

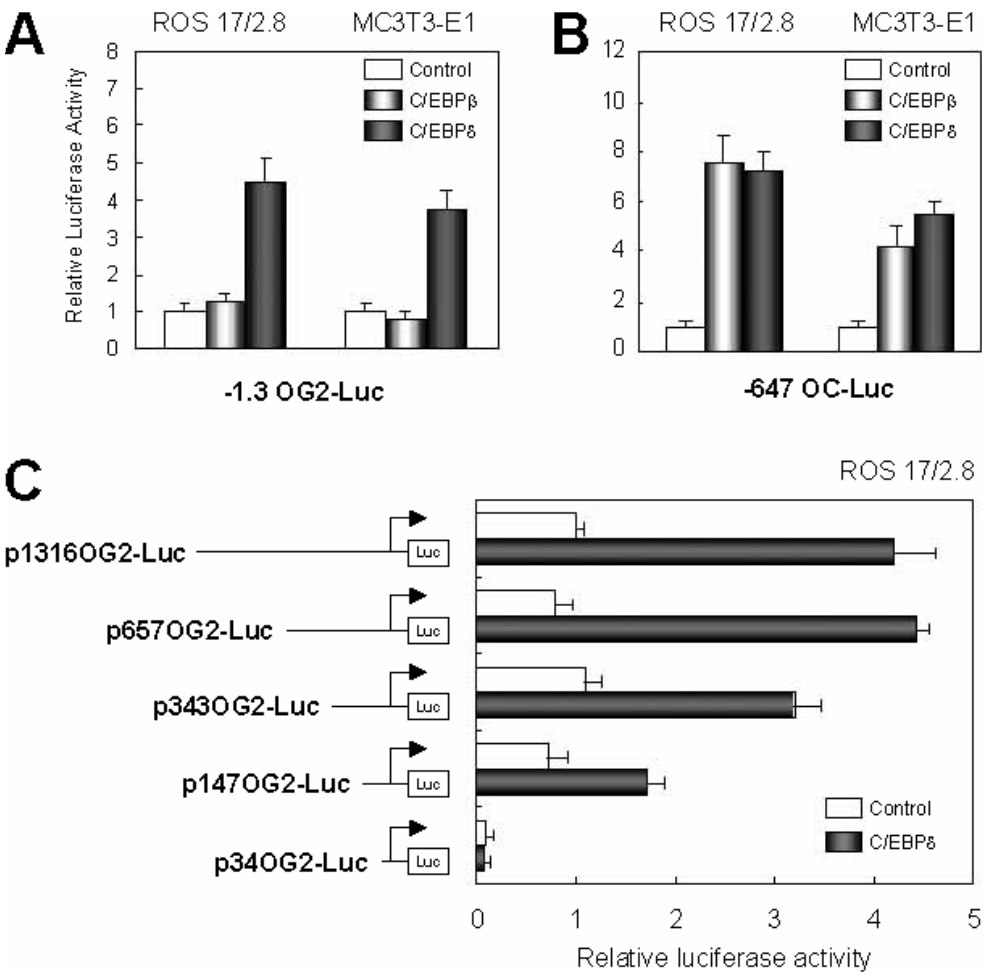

Figure 3 C/EBPs regulate mouse and rat osteocalcin promoter activity. (A, B) Mouse osteocalcin (−1·3 OG2-Luc (A)) or rat osteocalcin (−647 OC-Luc (B)) plasmids were cotransfected with C/EBPβ or -δ expression plasmids, or with empty vector into ROS 17/2·8 or MC3T3-E1 cells. (C) ROS 17/2·8 cells were cotransfected with deletion mutants of the OG2-Luc plasmid and C/EBPδ expression plasmid, or empty vector. Forty-eight hours after transfection, cells were harvested and luciferase activities were measured. Luciferase activity values were normalized for transfection efficiency against the β-galactosidase activity from the cotransfected pCMV-β-gal plasmid. All values, means, and standard deviations are expressed relative to basal promoter activity as fold inductions. Values are presented as means±S.D., and results are representative of three experiments each performed in triplicate.

osteocalcin was unaffected by the overexpression of C/EBPβ (Fig. 2D). The expression of other osteoblast-specific genes, including osteopontin and collagen type 1, showed a similar pattern. These results suggest that C/EBPδ increased the expression of osteocalcin and of other osteoblast-specific genes in both mouse and rat osteoblastic cell lines, whereas C/EBPβ has species-dependent effects on osteoblasts.

C/EBPδ activates osteocalcin gene transcription from mouse osteocalcin OG2 promoter

To further confirm the role of C/EBPs on osteocalcin expression, we analyzed luciferase activity in cells transfected with the mouse osteocalcin OG2 promoter–luciferase construct, −1·3 OG2-Luc. The transient transfection of −1·3 OG2-Luc into ROS 17/2·8 cells resulted in constitutive reporter activity, and this activity increased four-fold in cells cotransfected with the C/EBPδ construct, whereas the reporter activity was unchanged by cotransfection with C/EBPβ (Fig. 3A). The same pattern of C/EBPδ-mediated induction of reporter activity was noted in MC3T3-E1 cells. Again, transient transfection of C/EBPβ had no effect on OG2 promoter activity. These results are consistent with the findings of northern blotting and suggest that mouse osteocalcin OG2 promoter responds to C/EBPδ but not to C/EBPβ. In contrast, transient transfection of

C/EBPβ or -δ into ROS 17/2·8 cells enhanced the rat osteocalcin promoter activity by 7·5- and 7-fold respectively (Fig. 3B), which is consistent with a previous study by Gutierrez *et al.* (2002). In MC3T3-E1 cells, overexpression of C/EBPβ or -δ stimulated the reporter activity by 4- and 5·5-fold respectively (Fig. 3B).

In order to identify the promoter region that facilitates the transactivation by C/EBPδ, we studied a series of luciferase reporter constructs with serial deletions of the OG2 promoter. In DNA transfection experiments, the basal promoter activity in ROS 17/2·8 cells was not significantly altered until the region –147 to –34 was deleted. Cotransfection of C/EBPδ in this setting consistently increased promoter activity by 2- to 4·5-fold. However, the removal of the sequence between –657 to –343 and − 343 to –147 resulted in a decreased C/EBPδ-mediated stimulation. Furthermore, the deletion of the sequence between –147 and –34 completely abolished both the basal promoter activity and the C/EBPδ-mediated activation (Fig. 3C). These results suggest that there may be more than one C/EBP site in this OG2 promoter segment that could mediate the C/EBPδ-mediated transactivation.

C/EBPδ does not directly bind to the OG2 promoter

Given the results of the deletion mutant analysis, we next systemically analyzed the –657 to +13 region of the OG2 promoter for its contribution to the C/EBP-mediated activation. Sequence analysis of this region using a computer program (Alibaba 2·1; http://www.gene-regulation.com/pub/programs/alibaba2) reveals the presence of three putative C/EBP motifs (C1, − 607 to –598; C2, − 466 to –456; and C3, − 141 to –150, Fig. 4A).

To determine the possibility that these putative sites mediate the enhancement of the promoter activity by C/EBPs, we generated a series of deletion mutants that lack each of the putative C/EBP sites. For this study, we used mouse mesenchymal C3H10T1/2 cells, which lack Runx2 or Runx2 binding activity (Ducy *et al.* 1997). As shown in Fig. 4B, the reporter activity from the wild-type p657OG2-Luc, which contains Runx2 and three putative C/EBP binding sites, was increased by 1·8- or 2·4-fold by the transient transfection of C/EBPδ or Runx2 expression vector respectively into C3H10T1/2 cells. Notably, cotransfection of Runx2 and C/EBPδ results in a robust activation of the p657 OG2-Luc reporter activity, demonstrating a functional synergism between these two factors. However, deletion of each of three putative C/EBP sites (p657ΔC1OG2-Luc, p657ΔC2OG2-Luc, and p657ΔC3OG2-Luc) did not result in a significant change in the slightly increased promoter activity by C/EBPδ. In contrast, deletion of the Runx2 site (p657ΔOSE2OG2-Luc) completely abolished both the Runx2-mediated activation and synergism between C/EBPδ and Runx2. To further confirm the role of putative C/EBP sites, gel mobility shift analysis was performed using oligonucleotides containing three putative elements (Fig. 4C). However, none of these sites were able to bind to *in vitro* translated C/EBPs (Fig. 4D, data not shown for C1- and C2-C/EBP). As a control, we observed binding activity between the C/EBP consensus element and *in vitro* translated C/EBPs (Fig. 4D, right panel). Although we cannot exclude the possibility that C/EBP proteins may bind to the noncanonical C/EBP site, these results suggest that three putative C/EBP sites do not have binding activity to the C/EBP proteins nor do they contribute to the activation of the OG2 promoter. Based on the results in Fig. 4B, it may be deduced that the synergistic activation of OG2 promoter by Runx2 and C/EBPδ may be mediated by the Runx2-binding element, OSE2 of the OG2 promoter.

C/EBPδ enhances the transcriptional activity of Runx2 through physical interaction

We next investigated the possibility that C/EBPδ interacts with Runx2 thereby enhancing the transcription activity of Runx2 at the OSE2 element. Towards this end, transient transfection was performed on C3H10T1/2 cells. As shown in Fig. 5A, transfection of p6OSE2-Luc, in which luciferase expression is controlled by six tandem copies of the OSE2 element (Ducy & Karsenty 1995), resulted in only a low level of luciferase activity in the absence of Runx2. However, cotransfection of the Runx2 expression vector (pcDNA3-til) enhanced transcription in a dose-dependent manner (Fig. 5A). The transfection of these cells with C/EBPδ in this setting enhanced Runx2-dependent transcription threefold (Fig. 5B). Mutation of the OSE2 sites in the p6 OSE2 m-Luc plasmid abolished the induction of transcription by Runx2 as well as C/EBPδ-mediated activation (Fig. 5B). These results suggest that C/EBPδ-mediated enhancement of osteocalcin promoter activity depends on Runx2 expression as well as on the presence of an intact OSE2 site, the Runx2-binding sequence in osteocalcin promoter.

We next examined the possibility that Runx2 physically interacts with C/EBPδ to synergistically activate osteocalcin promoter. First, we directly assayed protein interactions *in vitro*, using GST-C/EBP and *in vitro* translated Runx2 labeled with [^{35}S]methionine. As shown in Fig. 5C, ^{35}S-labeled Runx2 associated with GST-C/EBPδ but not with GST alone. To further evaluate the ability of endogenous C/EBPδ to interact with endogenous Runx2 *in vivo*, we performed a coimmunoprecipitation experiment followed by Western blot analyses. As shown in Fig 5D, immunoprecipitation of MC3T3-E1 lysates either with anti-Runx2 or

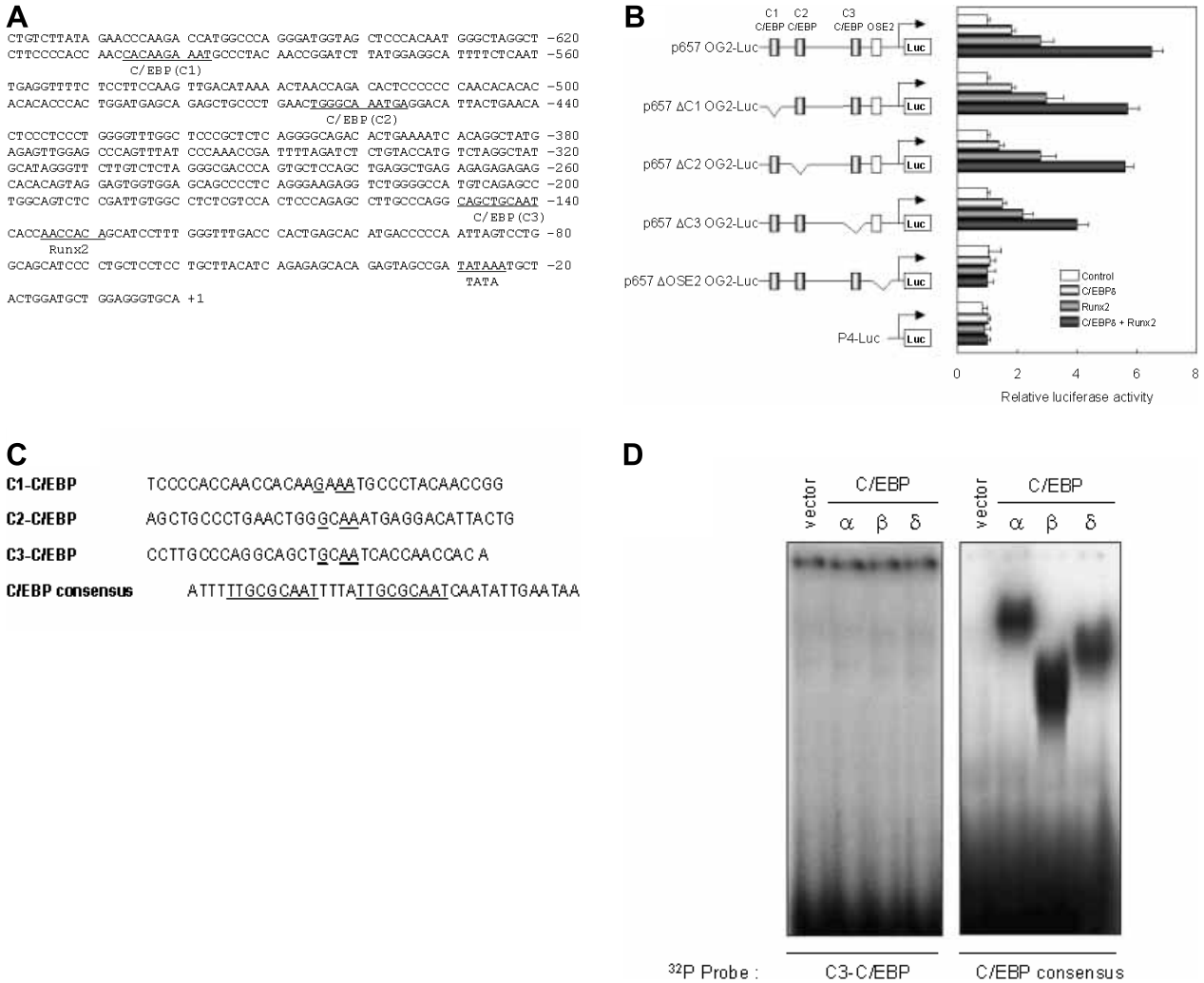

Figure 4 Lack of binding between C/EBPs and the putative C/EBP element in OG2 promoter. (A) Sequence of the mouse osteocalcin OG2 promoter –680 to +1relative to the transcription start site. The putative C/EBP sites (C1, C2, and C3), Runx2 site, and TATA box are underlined and labeled. (B) C3H10T1/2 cells were transiently transfected with deletion mutants of the OG2-Luc plasmid and 0·5 μg C/EBP and/or Runx2 expression constructs as indicated. Luciferase activity values were normalized for transfection efficiency against the β-galactosidase activity from the cotransfected pCMV-β-gal plasmid. All values, means, and standard deviations are expressed relative to basal promoter activity as fold inductions. Values are presented as means±S.D., and results are representative of three experiments each performed in triplicate. (C) The nucleotide sequences of the synthetic oligonucleotides corresponding to the three putative (C1-, C2-, and C3-C/EBP) and the consensus C/EBP site (two adjacent C/EBP binding sites are underlined) used for gel mobility shift assays are shown. (D) An electrophoretic mobility shift assay was performed on the ^{32}P-labeled oligonucleotide containing the putative C/EBP element in the OG2 promoter (C3-C/EBP) or on the consensus C/EBP oligonucleotides in the presence of *in vitro* translated C/EBPα, -β, or -δ. C/EBPα, -β, or -δ proteins were obtained by transcribing and translating the pSV-SPORT-C/EBPα, -β, or -δ expression plasmids respectively, using the TNT SP6/T7-coupled reticulocyte lysate system. No binding activity was demonstrated between *in vitro* translated C/EBP proteins and the putative oligonucleotide probe (C3-C/EBP).

anti-C/EBPδ antibody could pull down Runx2 (Fig. 5D, left panel). We also found that immunoprecipitation of Runx2 or C/EBPδ coprecipitated C/EBPδ (Fig 5D, right panel). Collectively, these findings indicate that C/EBPδ physically interacts with Runx2 and enhances its transcriptional activity at the OSE2 element, the Runx2-binding sequence in the osteocalcin promoter.

C/EBPδ forms a ternary complex with Runx2 in the context of OSE2

To further clarify the role of the cooperative protein interactions in the context of the OSE2 site, we performed a gel mobility shift assay using an oligonucleotide probe representing the OSE2 motif of

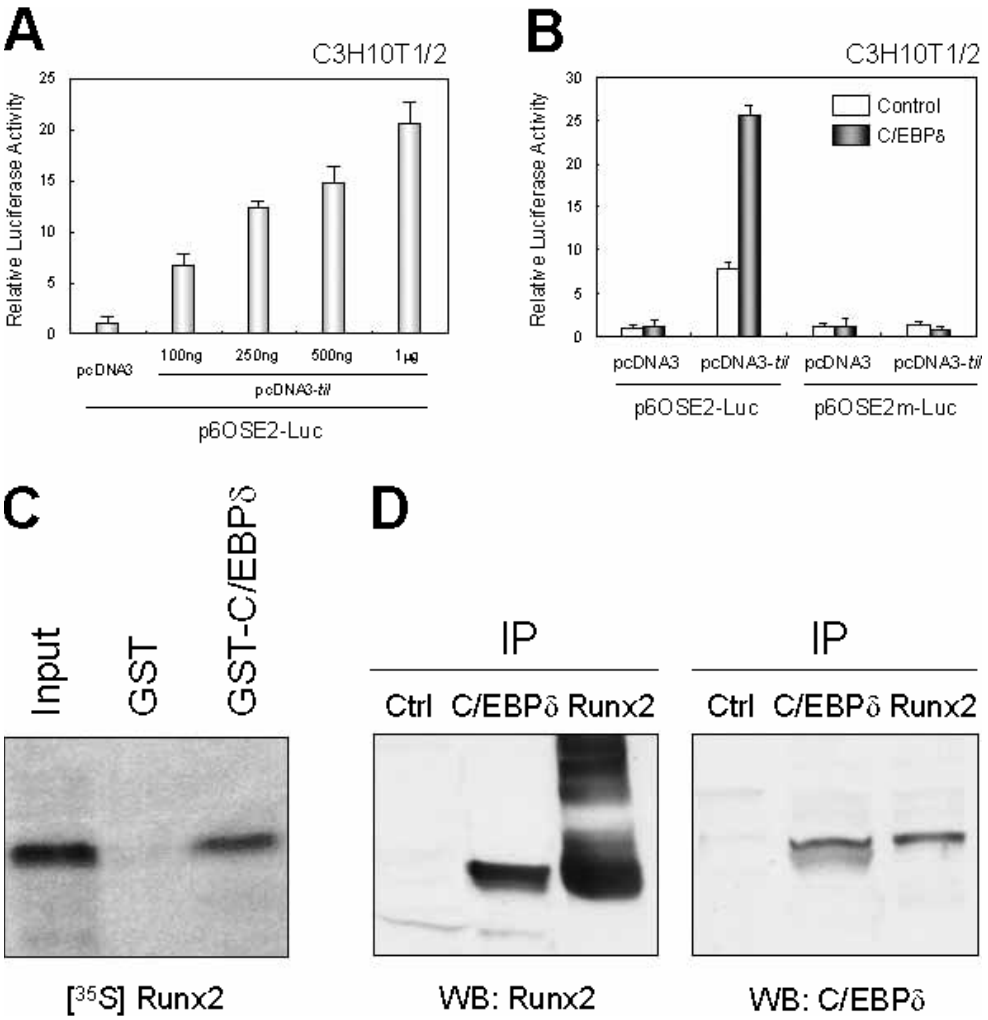

Figure 5 C/EBPδ enhances the transcriptional activity of Runx2 through physical interaction. (A) C3H10T1/2 cells were cotransfected with increasing amounts of pcDNA3-til and p6 OSE2-Luc plasmids and assayed for luciferase activity. (B) C3H10T1/2 cells were cotransfected with p6 OSE2-Luc and expression plasmids for C/EBPδ, Runx2 (pcDNA3-til), or the respective controls as indicated. Forty-eight hours after transfection, cells were harvested and luciferase activity was assayed as in Fig. 3. Values are presented as means±S.D., and results are representative of three experiments each performed in triplicate. (C) Pull-down experiments were performed to examine the *in vitro* binding of C/EBPδ and Runx2 using GST-C/EBPδ and *in vitro* translated Runx2 labeled with [^{35}S]methionine. Interacting protein was visualized following gel electrophoresis and autoradiography. (D) The *in vivo* association of endogenous C/EBPs and endogenous Runx2 in MC3T3-E1 cells was assessed by coimmunoprecipitation. Nuclear extracts from MC3T3-E1 cells were incubated with anti-Runx2, anti-C/EBPδ, or control (Ctrl) antibody. Protein A Sepharose beads were added to all reactions. Proteins bound to the beads were resolved by SDS-PAGE and immunoblotted with anti-Runx2 (left panel) or anti-C/EBPδ (right panel) antibodies. IP, immunoprecipitation; WB, Western blotting.

mouse OG2 promoter. As shown in Fig. 6A, incubation of nuclear extracts from MC3T3-E1 cells overexpressing C/EBPδ with radiolabeled OSE2 probe gave rise to a shifted band (*) that may represent the Runx2–DNA complex. In addition, we observed a slowly migrating complex (**), which increased in intensity in a dose-dependent manner on transfecting increasing amounts of C/EBPδ expression plasmid (Fig. 6A, lanes 1–4). Furthermore, this higher complex was competed by nonlabeled C/EBP consensus (lanes 6–8) and OSE2 oligonucleotides (lanes 12–14) but not by the mutant C/EBP (lanes 9–11) or mutant OSE2 oligonucleotides (lanes 16–18). The formation of this complex was abolished when an antiserum specific for C/EBPδ was

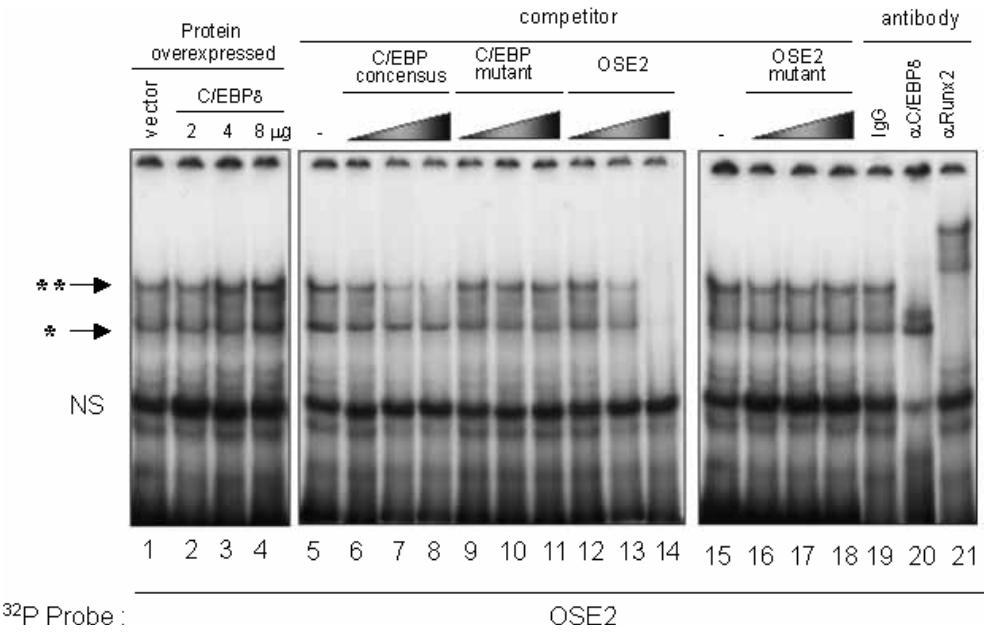

Figure 6 Runx2 and C/EBPδ form a ternary complex at the OSE2 site. (A) Electrophoretic mobility shift assays showed a Runx2-C/EBPδ interaction at the OSE2 element. ^{32}P-Labeled oligonucleotide containing the OSE2 element in the OG2 promoter was incubated with 10 μg nuclear extracts from MC3T3-E1 cells transfected with increasing amounts of C/EBPδ expression plasmids (lanes 1–4). Two specific DNA–protein complexes (* and **) were identified. Competition experiments were performed in the absence (lane 5 and 15) or presence of 12·5-, 25-, and 50-fold molar excess of nonlabeled C/EBP consensus (lanes 6–8), C/EBP mutant (lanes 9–11), OSE2 (lanes 12–14), or OSE2 mutant (lanes 16–18) oligonucleotides. The formation of the higher complex (**) was abolished when an antiserum specific for C/EBPδ was added to the reaction mixture (lane 20) and it was supershifted by the addition of anti-Runx2 antibody (lane 21). NS, non-specific band.

added to the reaction mixture (compare lanes 19 and 20) and was supershifted by the addition of anti-Runx2 antibody (lane 21), suggesting that C/EBPδ is implicated in the formation of the ternary complex (**).

To further validate the *in vivo* occupancy of the C/EBP or OSE2 site in the OG2 promoter by Runx2 and C/EBPδ, we performed ChIP assays. For this experiment, MC3T3-E1 cells were treated with ascorbic acid and β-glycerophosphate for 3, 12, or 19 days to induce osteoblastic differentiation, and cross-linked with formaldehyde. The primers used in the PCR analysis amplify a 202-bp fragment of the OG2 promoter region encompassing the C3-OSE2 element. Since the C3 and OSE2 elements were too close, the primers (P1 and P2) were designed to encompass both sites together. The position of this fragment relative to OSE2 and the C/EBP site is shown in Fig. 7A. As shown in Fig. 7B, antibodies against C/EBPδ failed to precipitate the OG2 promoter containing the C3-OSE2 element on day 3 of osteoblastic differentiation (lane 1), whereas Runx2 was weakly but clearly associated with chromatin in this stage of differentiation (lane 2). We were able to demonstrate more robust PCR bands from chromatins immunoprecipitated with both C/EBP and Runx2 antibodies in cells harvested on days 12 (lanes 3, 4) and 19 (lanes 5, 6) of culture. In control experiments, an irrelevant TSH receptor antibody (lane 10) or normal rabbit IgG (lane 11) could not immunoprecipitate this region of the OG2 promoter. In addition, primer pairs that do not contain the OSE2 element (P3-P4) were also used as a negative control (lanes 7, 8). Although these ChIP analysis results *per se* cannot exclude the possibility that C/EBPδ may directly bind to the C3 site of the promoter, the lack of positive PCR bands on day 3 with subsequent increases on days 12 and 19 further support the notion that the cooperative interaction between Runx2 and C/EBPδ at the OSE2 element may contribute to the C/EBPδ-mediated activation of the mouse osteocalcin OG2 promoter.

Discussion

This study demonstrates that C/EBPδ in conjunction with Runx2 cooperatively activate mouse osteocalcin promoter, and suggests that they may play an important regulatory role in the differentiation of osteoblasts. Mouse calvarial cells and the osteoblastic cell line

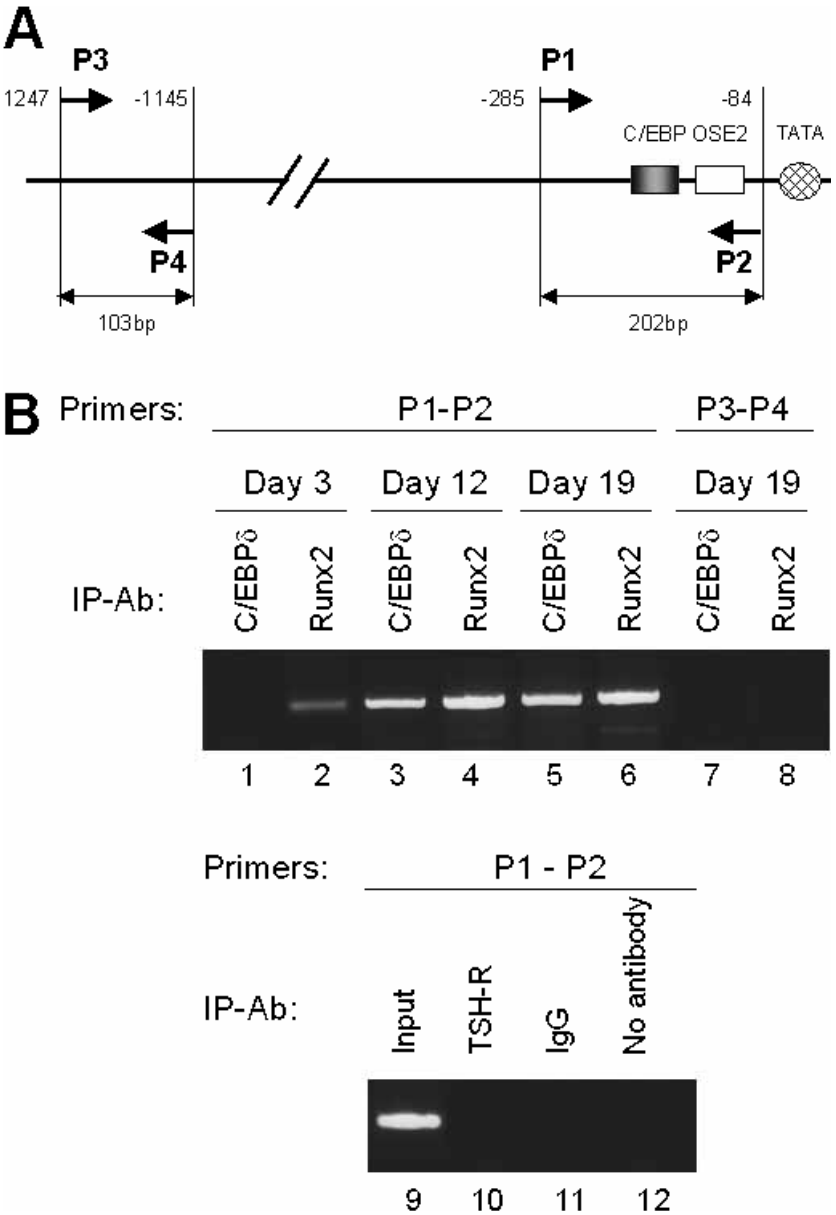

Figure 7 *In vivo* occupancy of the proximal osteocalcin promoter by Runx2 and C/EBPδ. (A) Illustration of relative positions of putative C/EBP and OSE2 elements and primers (P1 to P4) used in ChIP analysis. (B) MC3T3-E1 cells were treated with β-glycerophosphate and ascorbic acid for 3, 12, or 19 days to induce differentiation. Cells were then crosslinked with formaldehyde for ChIP analysis using C/EBPδ (lanes 1, 3, 5, 7), Runx2 (lanes 2, 4, 6, 8), TSH receptor antibody (TSH-R, lane 10), rabbit IgG (lane 11) or without antibody (lane 12). The gels are ethidium bromide-stained for the PCR products obtained with ChIP DNAs using the OG2 promoter primers. Input-chromatin is also shown (lane 9). IP-Ab, antibodies used for immunoprecipitation.

MC3T3-E1 express C/EBPβ and -δ but not C/EBPα, and we found that the overexpression of C/EBPδ in osteoblasts upregulates osteocalcin gene expression. This activation seems to be a consequence of the physical interaction between C/EBPδ and Runx2 at the OSE2 site, resulting in the synergistic activation of osteocalcin promoter. These results provide a mechanistic basis for the regulation of Runx2 transcriptional activity by C/EBPδ through functional cooperation between these two factors.

We found that mouse calvarial cells and osteoblastic MC3T3-E1 cells express C/EBPβ and -δ but not C/EBPα, confirming the earlier reports in rat osteoblasts (Gutierrez *et al.* 2002). In contrast, pluripotent mesenchymal C3H10T1/2 cells express all three repertoires of C/EBPs, including C/EBPα. The expression of C/EBPδ showed a biphasic pattern, i.e. downregulation after reaching confluency with a subsequent increase during the later stage of maturation, when osteocalcin expression is maximal in mouse osteoblastic cell lines. Our data is consistent with findings in primary rat osteoblasts, and suggest that C/EBPδ is linked to the expression of osteocalcin (Gutierrez *et al.* 2002). It is worth noting that although Runx2 is an essential transcription factor for osteoblast differentiation and the synthesis of osteoblast-specific proteins, its abundance is downregulated during osteoblast maturation when osteocalcin expression is upregulated. Moreover, it has been shown that C/EBPδ is positively regulated by Runx2 (McCarthy *et al.* 2000*b*). This dynamic pattern of C/EBPδ and Runx2 expression suggests that cooperative activation of osteocalcin promoter by C/EBPδ may confer a tissue-specific, temporal form of regulation in amplifying Runx2-mediated transcription and osteoblastic differentiation. Indeed, we found that the retroviral overexpression of C/EBPδ resulted in the upregulation of the expression of osteocalcin and of other osteoblast-specific genes.

In contrast to C/EBPδ, the expression of C/EBPβ in MC3T3-E1 cells gradually reduced during culture, and the overexpression of C/EBPβ did not enhance the expression of osteocalcin or its transcriptional activity. Our findings in this context are analogous to those of Iyer *et al.* (2004) who reported that the overexpression of C/EBPβ in MC3T3-E1 cells suppressed osteogenic differentiation, but promoted proliferation. In contrast, both C/EBPβ and -δ have been shown to enhance osteocalcin transcription from rat osteocalcin promoter (Gutierrez *et al.* 2002), and C/EBPβ was found to promote osteoblastic differentiation of mesenchymal cells (Hata *et al.* 2002). The reason for this seeming discrepancy concerning the role of C/EBPβ on osteoblast phenotype is unclear. The discordant response of the osteocalcin promoter may be attributed to a different set of *cis* acting elements located in the mouse and rat osteocalcin promoter. More importantly, these results may suggest that species-specific expressions of different isoforms of C/EBP proteins play different roles during osteoblast differentiation, from the stage of lineage commitment from mesenchymal progenitors to the later stage of mineralization, to ensure the fine regulation of osteoblastic differentiation. Further investigations to identify the coactivators or corepressors involved in this process will increase our understanding of the detailed underlying molecular mechanisms.

Although our findings that C/EBPδ enhances Runx2-mediated osteocalcin promoter activity concurs with rat osteocalcin promoter results, the mechanism whereby they exert cooperative activation is different. In rat osteocalcin promoter, Gutierrez and coworkers have shown that both C/EBPβ and -δ bind to a C/EBP motif located distal to the Runx2 site (Gutierrez *et al.* 2002). However, the C/EBP site identified in rat osteocalcin promoter is not conserved in mouse or human osteocalcin promoter and we were unable to identify the corresponding C/EBP element in mouse osteocalcin OG2 promoter. Instead, three putative C/EBP sites were identified in the −657/+34 region of the OG2 promoter. However, we found that none of these sites were able to bind to *in vitro* translated C/EBPs. Indeed, the sequences of all the three putative sites diverge from the consensus C/EBP domain (RTTGCGYAAY), suggesting that these sites are not good candidates for the actual binding for C/EBPs. Although we cannot exclude the possibility that a C/EBP responsive element is located further upstream or downstream in the osteocalcin locus, we provide evidence that C/EBPδ upregulates osteocalcin expression indirectly by affecting Runx2 function. First, C/EBPδ was found to activate osteocalcin promoter activity only in cells that express Runx2. Secondly, the presence of both Runx2 and an intact OSE2 site was found to be necessary for the C/EBP-mediated enhancement of osteocalcin promoter. Thirdly, we demonstrated an interaction between C/EBPδ and Runx2 in MC3T3-E1 cells, in which C/EBPδ-mediated activation was observed. Finally, gel mobility shift assays and ChIP assays demonstrated that the interaction of C/EBPδ and Runx2 occurs in the context of OSE2. Therefore, we conclude that the enhancement of Runx2-mediated transcription by C/EBPδ results from the protein interaction between Runx2 and C/EBPδ at the OSE2 sequence.

The remaining issue to be answered is the possibility that C/EBPs activate Runx2-mediated osteocalcin promoter activation by indirect mechanisms. A recent study identified C/EBPδ as a principal cAMP-activated regulator of the IGF-I gene transcription in osteoblasts, and that activated C/EBPβ binds specifically to HS3D to activate IGF-I gene transcription through promoter 1 (Umayahara *et al.* 1997). As IGF-I, an essential growth factor that regulates the growth and remodeling of skeletal tissue, is also involved in the activation of osteocalcin promoter activity through both protein kinase (PK) A and PKC signaling cascades (Boguslawski *et al.* 2000), C/EBPβ or -δ might indirectly affect osteocalcin promoter activity in this way.

To summarize, we found evidence that C/EBPδ regulates mouse osteocalcin OG2 promoter via its cooperative interaction with Runx2 transcription factor. Further investigations of the physiologic role of C/EBPs

on osteoblast differentiation will contribute to our understanding of bone development and remodeling.

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