CCAAT/Enhancer-binding Proteins (C/EBP) β and δ Activate Osteocalcin Gene Transcription and Synergize with Runx2 at the C/EBP Element to Regulate Bone-specific Expression*

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CCAAT/enhancer-binding proteins (C/EBP) are critical determinants for cellular differentiation and cell type-specific gene expression. Their functional roles in osteoblast development have not been determined. We addressed a key component of the mechanisms by which C/EBP factors regulate transcription of a tissue-specific gene during osteoblast differentiation. Expression of both C/EBPβ and C/EBPδ increases from the growth to maturation developmental stages and, like the bone-specific osteocalcin (OC) gene, is also stimulated 3–6-fold by vitamin D3, a regulator of osteoblast differentiation. We characterized a C/EBP enhancer element in the proximal promoter of the rat osteocalcin gene, which resides in close proximity to a Runx2 (Cbfa1) element, essential for tissue-specific activation. We find that C/EBP and Runx2 factors interact together in a synergistic manner to enhance OC transcription (35–40-fold) in cell culture systems. We show by mutational analysis that this synergism is mediated through the C/EBP-responsive element in the OC promoter and by a direct interaction between Runx2 and C/EBPβ. Furthermore, we have mapped a domain in Runx2 necessary for this interaction by immunoprecipitation. A Runx2 mutant lacking this interaction domain does not exhibit functional synergism. We conclude that, in addition to Runx2 DNA binding functions, Runx2 can also form a protein complex at C/EBP sites to regulate transcription. Taken together, our findings indicate that C/EBP is a principal transactivator of the OC gene and the synergism with Runx2 suggests that a combinatorial interaction of these factors is a principal mechanism for regulating tissue-specific expression during osteoblast differentiation.

The CCAAT/enhancer-binding proteins (C/EBPs) comprise a family of transcription factors that are critical for normal cellular differentiation and metabolic functions in a variety of tissues. There are currently six members of the C/EBP family designated as C/EBPα, -β, -δ, -γ, -ε, and -ζ (1), most of which are expressed in liver, spleen, and adipocytic tissues. However, more selective expression in other tissues has been observed among the family members (2–7). Isoforms of the C/EBP proteins are known, and all function by homo- or heterodimerization with one another and interaction with other transcriptional activators or co-activators such as NF-κB, Stat3, c-Myc, PU.1, SP-1, ATF-2, PPARγ, and Runx-1 (8–14).

Very little is known about the role of C/EBP factors in osteogenesis. Targeted disruptions of C/EBP genes have been performed, but in none of the studies were gross abnormalities of the skeleton observed (6, 15–17). However, recent studies have identified C/EBP regulation of genes expressed in osteoblasts. The insulin-like growth factor 1 is a key regulator of osteoblast growth and differentiation (18). C/EBPδ enhances either basal or prostaglandin E2-activated transcription of the insulin-like growth factor 1 promoter in osteoblasts (19, 20). Expression of COX-2 and the α1 subunit of type I collagen is also regulated in osteoblasts by C/EBP factors (21, 22). The interaction of C/EBPα with a Runx1 factor (23) is also particularly relevant for postulating a role for C/EBP factors in osteoblast differentiation.

The Runx-related transcription factors (Runx/AML/Cbfα/PEBP2α) represent essential gene regulatory proteins that control lineage commitment for hematopoiesis (24–26) and osteogenesis (27, 28). Runx2 (AML3/Cbfa1/PEBP2a) is the most abundant Runx-related protein in osteogenic and chondrogenic cell lineages (29–31). Genetic ablation of the Runx2 gene causes developmental defects in osteogenesis (27), and hereditary mutations in the Runx2 gene are linked to specific ossification defects as observed in cleidocranial dysplasia (32). Runx2 is essential for osteoblast differentiation (27, 29, 30) and regulates expression of numerous bone-related genes (29, 31, 33–35). The importance of Runx2 in expression of the bone-specific osteocalcin (OC) gene is well documented (36, 37). Thus, Runx2 performs specialized functions during bone-tissue development and differentiation in vivo. However, it is noteworthy that osteoblast-specific transcription of osteocalcin occurs even in the absence of Runx sites in the rat OC promoter (38), suggesting a tissue-specific role for other regulatory factors in osteoblasts.

Activation of tissue-specific genes is controlled by combinatorial mechanisms that rely on local features of the promoters, including organization of control elements in the target genes and/or the interplay between DNA-binding proteins and various transcriptional co-regulators (39). Both C/EBPβ and Runx factors have been shown to cooperate with chromatin remodeling factors (p300, SWI/SNF) and other enhancer-binding proteins (40, 41). For example, Ets-1, c-Myc, Sp1, and C/EBP,
together with Runx factors, stimulate the transcription of hematopoietic and osteogenic genes (13, 14, 23, 42–44), whereas PPARγ and Stat3 interactions with C/EBPa are driving forces for adipocyte differentiation (45). Given these observations, i.e. the presence of both C/EBPβ and -δ and Runx2 in osteoblasts (29, 30, 46) and C/EBPα-Runx1 protein-protein interactions in regulation of a hematopoietic specific gene (13), we addressed the possible role of C/EBP factors in osteoblasts and in the regulation of a bone-specific gene, osteocalcin.

Here we report that C/EBPβ and -δ, but not -α, are developmentally expressed during osteoblast differentiation and are up-regulated in response to 1,25(OH)2D3, a hormone that promotes osteoblast differentiation. We have identified a C/EBP-responsive regulatory element in the proximal promoter of the bone-specific osteocalcin gene. Deletion or mutation of this motif abrogates transcriptional enhancement by C/EBP factors. Furthermore, we provide the first demonstration that Runx2 and C/EBP factors. Furthermore, we provide the first demonstration that Runx2 and C/EBP factors. Consequently, we have identified a C/EBP-responsive regulatory element in the proximal promoter of the bone-specific osteocalcin gene. Deletion or mutation of this motif abrogates transcriptional enhancement by C/EBP factors. Furthermore, we provide the first demonstration that Runx2 and C/EBP factors. Consequently, we have identified a C/EBP-responsive regulatory element in the proximal promoter of the bone-specific osteocalcin gene. Deletion or mutation of this motif abrogates transcriptional enhancement by C/EBP factors. Furthermore, we provide the first demonstration that Runx2 and C/EBP factors.

**MATERIALS AND METHODS**

Site-directed Mutagenesis and Expression Constructs— Constructs containing the rat OC (-1907/+23 or -208/+23) promoter fused to the chloramphenicol acetyltransferase (CAT) gene have been described previously (47). The -208 OC- CAT/C/EBP mt plasmid, containing mutation of the C/EBP binding site in the -208 OC promoter (shown in lowercase) was generated by a PCR-based approach (48) with the following synthetic oligonucleotides: 5′-GGTTTGACCTACATGACTATGCAGCTATGAC-3′, pUC/M13 reverse primer: 5′-TCACACAGGAAAAGGCATATGATGAC-3′, pUC/M13 forward primer: 5′-CGAC 3′, pUC/M13 reverse primer: 5′-TCACACAGGAAAAGGCATATGATGAC-3′, pUC/M13 forward primer: 5′-CGACGGCTTCTTCCGAGTCA GCAG 3′ (PCR 2), and -208 OC-CAT as template; this mutation introduced a unique site for the SpeI restriction enzyme. The PCR products were digested with BamHI-SpeI (PCR1) and ApaI-SpeI (PCR2).

A three-way ligation reaction was set using BamHI-digested -208 OC-CATH as backbone.

The -208 OC-CAT Runx mt plasmid was generated by digestion of the mC CAT plasmid (36) with SphI and XhoI. The PCR fragment was digested with MluI and cloned into the pCMV-5CAT plasmid (37) to obtain the C/EBP-responsive regulatory element in the proximal promoter.

**RESULTS**

C/EBP-mediated Transcriptional Control

To test the role of C/EBP factors in osteoblast differentiation, we used a C/EBP-responsive reporter construct (p3×C/EBP-CAT) that contains three copies of the C/EBP binding site from the rat osteocalcin promoter (-137/68) and was driven by the human histone H4 promoter (-14, and 20) were prepared by a modified Dignam method (52) with 0.42 mM KCl for extraction. ROS 17/2.8 and HeLa cells were plated in 100-mm plates at a density of 0.5 × 106 cells. Cells were collected at 95% confluence by scraping with ice-cold phosphate-buffered saline (PBS) solution. Whole-cell isolation procedure was carried out on ice. Cells from five plates were pooled into a 50-ml polypropylene tube and pelleted by centrifugation at 165 × g for 5 min at 4 °C. Cells were gently resuspended in 5-10 volumes of Nonidet P-40 lysis buffer (10 mM Tris, pH 7.4, 3 mM MgCl2, 10 mM NaCl, 0.5% Nonidet P-40) supplemented with 1× Complete protease inhibitor mixture (Roche Molecular Biochemicals), 25 μM MG132 (Sigma-Aldrich) and extracted at 4 °C for 15 min, followed by centrifugation at 16,000 × g for 15 min. The supernatant was transferred to a clean microcentrifuge tube and precleared with 20 μl of protein A/G Plus-agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), at 4 °C for 30 min. The beads were collected by centrifugation at 1000 × g for 5 min at 4 °C. Xpress antibody (3 μg, Invitrogen Corp., Carlsbad, CA) was added to the precleared cell lysate followed by incubation at 4 °C for 1 h. To precipitate the immunocomplexes, 50 μl of protein A/G Plus-agarose beads were added and further incubated at 4 °C with agitation for 1 h. The beads were washed twice with 1 ml of washing buffer (20 mM Tris, pH 8.0, 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 50 mM NaCl, 2 mM EDTA, 1× Complete; 25 μM MG 132), and suspended in 1× SDS sample buffer, and analyzed by Western blotting.

Western Blot Analysis—Transfected or untransfected HeLa and ROS 17/2.8 cells cultured on 100-mm dishes were lysed on the plate by adding 300 μl of SDS lysis buffer (2% SDS, 10 mM dithiothreitol, 10% glycerol, 2 mM urea, 1.0 mM phenylmethylsulfonyl fluoride, 10 mM Tris-HCl, pH 8.0, 0.002% bromphenol blue, 1× protease inhibitor mixture). Proteins (30–40 μg) were resolved in 10% SDS-PAGE and transferred to Trans-Blot membrane (Bio-Rad). Antibodies against C/EBPβ, C/EBPδ, and lamin B were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Epitope-tagged Runx proteins were detected by mouse monoclonal horseradish peroxidase-conjugated Xpress antibody (Invitrogen Corp., Carlsbad, CA). Monoclonal antisera for tubulin was purchased from Sigma-Aldrich.

**RESULTS**

C/EBP Family Members Are Expressed during Osteoblast Differentiation and Are Regulated by Vitamin D3.—We initially assessed the expression of C/EBP factors in various bone tissues and during development of the osteoblast phenotype. Fig. 1A shows that C/EBPβ and -δ mRNAs are present in calvarial tissue (lane 2) and in cortical and trabecular bone (data not shown) at levels similar to those in a representative soft tissue, muscle (lane 1). C/EBPα is not detected in bone tissue, consistent...
ent with its pivotal role for adipogenesis. Osteoblast markers (OC, Runx), which are not present in soft tissues, are shown for comparison. The abundance of C/EBPβ and -δ in bone prompted examination of expression of the C/EBP family members from growth to differentiation stages of osteoblasts in vitro, representing proliferation (days 3–5), matrix maturation (days 7–12), and the mineralization stage (days 19–22), reflected by peak levels of histone H4, alkaline phosphatase (ALP), and OC, respectively (Fig. 1B).

Both C/EBPβ and -δ mRNAs are detected from the growth to maturation stages and are expressed in a biphasic pattern. In contrast, C/EBPα expression is not detected at any stage of osteoblast differentiation, consistent with its absence in bone tissue. Two sizes of C/EBPβ mRNA are present with the larger species appearing more constitutive, whereas the smaller transcript is expressed during the growth period (days 3–5). The latter transcript is decreased markedly during the matrix maturation stage (days 7–12, when alkaline phosphatase-positive cells are forming nodules), followed by a 4–5-fold increase in expression concomitant with mineral deposition and peak levels of osteocalcin and Runx2 expression (Fig. 1B). C/EBPδ mRNA is expressed in a manner similar to that for C/EBPβ, but the larger transcript is detected at very low levels. A significant (5-fold) temporal increase in C/EBPδ mRNA expression is observed during osteoblast differentiation from confluence (day 7) to the mature osteoblasts (day 22). Several Runx2 isoforms that result from utilization of alternative promoters and differential splicing are expressed (Fig. 1B). The increase in expression of the major Runx2 transcript during later stages of differentiation is consistent with increased Runx2 DNA binding activity in mature osteoblasts (29). Thus, the increases in C/EBPβ and C/EBPδ in the late stages of osteoblast differentiation appear to parallel peak expression levels for the osteoblast-related osteocalcin gene and the Runx2 transcription factor.

We further assessed the relationship of C/EBP factors to osteoblast differentiation. Vitamin D₃, a hormone that promotes osteoblast differentiation, is a known enhancer of many osteoblast-related genes (53). Expression of C/EBPβ is stimulated by vitamin D₃ at each stage of osteoblast maturation, 6-fold during growth (day 7) and nodule development (day 12) and 3-fold during mineralization (day 19). Vitamin D₃-dependent enhancement of C/EBPβ is similar to that of C/EBPβ except the -fold stimulation is lower (3-fold at all stages). Treatment of cells with vitamin D₃ has no effect on the expression of C/EBPα at any stage of differentiation (data not shown). The differentiation-promoting properties of vitamin D₃ are reflected by the decrease in histone H4 and the increase in OC expression (Fig. 1C). Thus, expression of both C/EBPβ and C/EBPδ is strongly enhanced upon treatment with vitamin D₃ in relation to osteoblast differentiation. Taken together, these data demonstrate that C/EBP transcription factors are expressed at significant levels in bone tissue, they increase during osteoblast differentiation in vitro, and their expression is up-regulated by 1,25(OH)₂D₃.

C/EBP Proteins Activate the Osteocalcin Gene through a C/EBP-responsive Element in the Proximal Promoter—The enhanced expression of C/EBPβ and -δ during mineralization relative to the onset of OC transcription and in response to vitamin D₃ suggests that these C/EBP transcription factors may contribute to osteoblast-specific expression of the OC gene. Previous studies using promoter deletion constructs of the rat OC gene have shown that the initial 200 bp of the promoter can confer tissue-specific expression (38). This region contains a Runx-responsive motif and a homeodomain box that also binds an osteoblast-specific complex (54). Sequence analysis of this
region reveals the presence of a C/EBP motif (Fig. 2A). To determine whether the C/EBP protein(s) can interact with this element, gel mobility shift analyses were performed using oligonucleotides (see "Materials and Methods") containing either wild type or mutated C/EBP binding sequences (Fig. 2A). Using nuclear extracts from day 20 primary rat osteoblast cultures, C, the specificity of the complexes is further demonstrated by competition assays. Oligonucleotides carrying the wild type C/EBP site from the OC gene (see "Materials and Methods") were incubated with 6 μg of nuclear proteins. The concentration ranges of wild type (WT) or mutant (mt) cold competitors are indicated at the top of each lane.

The presence of C/EBP in the protein complex binding to the OC promoter element suggests transcriptional regulation of the OC gene by C/EBP factors. This possibility was experimentally addressed by assessing the effect of forced expression of C/EBPβ and -δ on activity of full-length (~1.1 kb) and proximal (~208 bp) OC promoter-CAT reporter gene constructs in the osteoblastic ROS 17/2.8 cell line (Fig. 3). The results indicate that C/EBPβ and -δ significantly enhance OC promoter activity, 4–5-fold on the full-length promoter (Fig. 3A, left panel) and 8-fold on the proximal promoter segments (Fig. 3A, right panel). Western blot analysis shows that C/EBPβ and C/EBPδ proteins of the expected sizes are expressed (Fig. 3B). A representative CAT autoradiogram showing basal activity of the ~1.1 kb and ~208 OC-CAT and the extent to which these promoters are activated by C/EBPβ and -δ is presented in Fig. 3C. Thus, both C/EBPβ and -δ are potent activators of osteocalcin gene transcription.

To establish that the C/EBP element in the proximal region directly mediates the enhancement of promoter activity by C/EBPs, site-directed mutagenesis was performed. The C/EBP element in ~208 OC-CAT was mutated (see Fig. 2A) using the same oligonucleotides used in the gel shift assays. The results show that mutation of the C/EBP element blocks the C/EBP-mediated stimulation (6–8-fold) observed with the wild type promoter (Fig. 4). Therefore, responsiveness of the OC promoter to C/EBP transcription factors is dependent on the integrity of the C/EBP element in the basal promoter region.

**Functional Synergism between Runx and C/EBP Proteins Is Mediated through the C/EBP Element**—Runx2 is a well-characterized regulator of OC gene transcription and a Runx-responsive motif is located in close proximity to the C/EBP element in the proximal OC promoter. Therefore, functional interaction between these proteins in transcriptional regulation of the OC gene was tested. For these studies we selected HeLa cells, which lack Runx2 (Fig. 5A) and have no Runx DNA binding activity (Ref. 55 and data not shown). Consistent with these observations, we find low basal activity of the ~208 OC promoter in HeLa cells compared with osteoblastic ROS 17/2.8 cells, which express both C/EBPβ and Runx2 (Fig. 5). Interestingly, HeLa cells have significant levels of C/EBPβ (Fig. 5A). Based upon preliminary examination of dose-dependent effects of Runx2 and C/EBPβ individually on activity of the ~208 OC promoter (data not shown), we used a suboptimal concentration of each expression plasmid (0.4 μg) for these studies. Expression of either C/EBPβ or Runx2 in HeLa cells stimulates OC promoter activity 2–4-fold. However, co-expression of Runx2 and C/EBPβ results in a massive activation (30–40-fold) of the OC promoter (Fig. 6), demonstrating a functional synergism between these two proteins. We also observed a synergistic
interaction of Runx2 and C/EBPβ on the OC promoter in osteoblastic ROS 17/2.8 cells (20–25-fold stimulation compared with 3–5-fold for each protein alone; data not shown). This functional synergy between two positive regulators of OC transcription is consistent with increased expression of C/EBPβ and -δ, Runx2, and OC during late stages of osteoblast differentiation.

To investigate the specific contribution of the Runx and C/EBP elements and their cognate factors to synergistic activation of the OC promoter, a series of promoter constructs bearing mutations in either Runx or C/EBP binding sites were generated (Fig. 7A). Each of these constructs was tested for responsiveness to C/EBP and/or Runx2. Data pooled from four independent experiments show a consistent 30–40-fold synergistic enhancement of the wild type OC promoter by C/EBPβ and Runx2 (Fig. 7B). Mutation of the Runx site did not affect the synergistic response (23–26-fold enhancement). In contrast, we observed a loss of this functional synergism upon mutation of the C/EBP motif. A similar loss of synergistic activity was observed when both C/EBP and Runx sites were mutated (Fig. 7B). Co-expression of C/EBPβ and Runx2 resulted in a similar pattern of activation of these OC promoter constructs (data not shown). These findings suggest that the C/EBP regulatory element is required for the synergistic enhancer activity involving Runx2.

For further insight into the mechanisms involved in Runx2-C/EBP functional synergism, we tested a series of carboxy-terminal deletion mutants of Runx2 (Fig. 8A). Both mutant Runx2 proteins (1–361 and 1–230) are expressed (Fig. 8B), enter the nucleus and retain DNA binding activity (data not shown). Fig. 8C shows that both mutants also retain functional activity on the OC promoter but at a lower level than wild type Runx2. When the mutant Runx2-(1–361) was co-expressed with either C/EBPβ or -δ, synergistic activation (40–80-fold) of the OC promoter was observed. However, this synergism did not occur with the mutant Runx2-(1–230). These Runx mutation studies clearly demonstrate that a Runx2-C/EBP interaction is required to support synergistic activation of the OC promoter.

To determine whether functional synergism between Runx2 and C/EBP requires a physical interaction, we performed co-immunoprecipitation studies. Wild type Runx2 and Runx2-(1–361), which lacks the carboxyl terminus, each form a complex with C/EBPβ, whereas Runx2-(1–230) fails to interact (Fig. 8D). The absence of C/EBPβ in the immunoprecipitated Runx2-(1–230) complex indicates that amino acids 230–361 of Runx2 are required for this interaction, consistent with the functional activity data (Fig. 8C). It was shown previously that the runt homology domain (RHD) of Runx1 supports interaction with C/EBPβ (13, 23). However, our Runx2 deletion analysis reveals that the RHD of Runx2 is not sufficient for C/EBPβ interaction (Fig. 8D). Our findings are consistent with the recent co-crystal structure of the RHD with C/EBPβ (bZIP), which demonstrates a lack of interaction of these two domains (56). Taken together our results demonstrate that the synergism observed on the OC promoter requires an interaction between Runx2 and C/EBP that involves a region of Runx2 outside the DNA binding domain.

To establish that the Runx2-C/EBP protein-protein interaction can occur when C/EBP proteins are bound to its regulatory element, we performed gel mobility shift assays with the C/EBP motif of the OC promoter. We compared nuclear extracts from HeLa cells lacking Runx2 and mature osteoblasts, which contain both Runx and C/EBP factors (see Fig. 5A). We find similar C/EBP complexes formed with both nuclear extracts; however, addition of antibody against Runx2 resulted in a supershift only with the bone cell extracts. These results...
provide evidence for a Runx2-C/EBP interaction that is independent of a Runx DNA binding site.

DISCUSSION

Our studies demonstrate that the C/EBP transcription factors support osteoblast-specific gene expression and may play an important regulatory role during osteoblast differentiation. C/EBPβ and α, but not C/EBPa, are expressed in skeletal tissues and are developmentally regulated during osteoblast maturation. Vitamin D3, a positive regulator of osteoblast differentiation and of the bone-specific osteocalcin gene, also increases expression of C/EBP factors. We find that osteocalcin is a downstream C/EBP target gene, strongly up-regulated in response to forced expression of C/EBP family members. The level of enhancement is equivalent to that observed for the bone-related Runx2 transcription factor. More importantly, we also demonstrate that Runx2 and C/EBPβ and α functionally cooperate for positive regulation of the OC gene and that the synergism is mediated through a physical interaction between Runx and C/EBP at the C/EBP element. We propose that C/EBP activity may be physiologically relevant to the spatio-temporal regulation of Runx2-dependent genes in mature osteoblasts.

Transcription of the OC gene is stringently regulated during osteoblast differentiation (12), but the mechanisms involved have not been completely elucidated. OC gene induction is
coupled to a post-proliferative increase in Runx2 DNA binding activity (29, 30). However, Runx proteins are present in skeletal progenitor cells and immature proliferating osteoblasts, in which OC gene expression is not activated (29). Thus, other factors are contributing to strong suppression of OC transcription in such cells, as well as to maximal levels of OC expression in mature osteoblasts. Our studies indicate that the activities of Runx proteins and transcriptional activation of Runx-responsive genes during skeletal development may be regulated in part by controlling cellular levels of Runx or C/EBP proteins and/or functional cooperation between Runx and C/EBP transcription factors. In support of this mechanism are the relative expression levels of each factor during osteoblast differentiation. Although C/EBP and Runx, SWI/SNF, and/or other components of the nuclear architecture.

among C/EBP, Runx, SWI/SNF, and/or other components of the poral modifications in the chromatin-related interactions (57, 61). Recent studies from our laborato-

64). Hence, C/EBP-dependent Runx synergism during (36, 62 necessary for bone-specific activation of the osteocalcin gene (41). Numerous studies have established that chromatin remodeling and modifications in nucleosomal organization are necessary for bone-specific activation of the osteocalcin gene (36, 62–64). Hence, C/EBP-dependent Runx synergism during development of the osteoblast phenotype may result from temporal modifications in the chromatin-related interactions among C/EBP, Runx, SWI/SNF, and/or other components of the nuclear architecture.

In summary, the data presented here address the mechanisms critical for the robust activation of the osteocalcin gene during osteoblast differentiation. Our studies indicate that these mechanisms require a C/EBP response element and syn-
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