MOLECULAR BASIS OF CELL AND DEVELOPMENTAL BIOLOGY:

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Runt Domain Factor (Runx)-dependent Effects on CCAAT/Enhancer-binding Protein δ Expression and Activity in Osteoblasts*

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Thomas L. McCarthy‡, Changhua Ji‡, Yun Chen‡, Kenneth K. Kim‡, Masayoshi Imagawa§, Yoshiaki Ito¶, and Michael Centrella‡‖

From the ‡Department of Surgery, Plastic Surgery Section, Yale University School of Medicine, New Haven, Connecticut 06520, the §Laboratory of Environmental Biochemistry, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-Oka, Suita, Osaka, Japan, and the ¶Department of Viral Oncology, Institute for Virus Research, Kyoto University, Shogoin, Sakyo-ku, Kyoto 606-01, Japan

Transcription factor CCAAT/enhancer-binding protein δ (C/EBPδ) is normally associated with acute-phase gene expression. However, it is expressed constitutively in primary osteoblast cultures where it increases insulin-like growth factor I synthesis in a cAMP-dependent way. Here we show that the 3′ proximal region of the C/EBPδ gene promoter contains a binding sequence for Runt domain factor Runx2, which is essential for osteogenesis. This region of the C/EBPδ promoter directed high reporter gene expression in osteoblasts, and specifically bound Runx2 in osteoblast-derived nuclear extract. C/EBPδ gene promoter activity was reduced by mutating the Runx binding sequence or by co-transfecting with Runx2 antisense expression plasmid, and was enhanced by overexpression of Runx-2. Exposure to prostaglandin E_2 increased Runx-dependent gene transactivation independently of Runx2 binding to DNA. Runx2 bound directly to the carboxyl-terminal region of C/EBPδ itself, and its ability to drive C/EBPδ expression was suppressed when C/EBPδ or its carboxyl-terminal fragment was increased by overexpression. Consistent effects also occurred on C/EBPδ-dependent increases in gene expression driven by synthetic or insulin-like growth factor I gene promoter fragments. These interactions between Runx2 and C/EBPδ, and their activation by prostaglandin E_2, provide new evidence for their importance during skeletal remodeling, inflammatory bone disease, or fracture repair.

CCAAT/enhancer-binding proteins (C/EBPs)[1] are classified as members of the basic leucine zipper (bZIP) transcription factor family, which includes several highly related C/EBP isoforms, the cAMP response element-binding protein, activating transcription factors, and the AP-1-binding proteins Fos and Jun. Each family member can homo- or heterodimerize, a necessary component for DNA binding and transcriptional activation. Similar to other basic leucine zipper-like factors, C/EBPs are associated with genes controlled by cAMP-dependent protein kinase A (PKA) in several tissues. Notably, activation of C/EBPs can initiate downstream gene cascades related to the acute-phase response, healing, and tissue growth and remodeling (1–6).

Various C/EBP family members are expressed by liver, fat, colon, and mammary cells, and by monocytes, macrophages, and osteoblasts. The relative levels of individual C/EBPs vary with cell differentiation and can be modulated by lipopolysaccharide, thermal injury, hypoxia, and inflammation (2, 4–13). For example, C/EBPβ and C/EBPδ are specifically induced during infection and inflammation, whereas C/EBPα is often down-regulated (5, 9). The *c/ebpβ* and *c/ebpδ* genes exhibit unusual structure in that they each lack intron sequences (14, 15). Promoter regions for both *c/ebp* genes were recently isolated (16, 17). The C/EBPβ gene promoter has two cAMP response element-binding protein sites near the TATA box that function in liver during regeneration or activation by PKA (17). In HepG2 hepatoma cells, stimulatory effects by interleukin 6 on C/EBPδ expression involve nuclear factors termed acute-phase response element/signal transducers and activators of transcription 3 (APRE/STAT3) and Sp1 (16, 18). Also, C/EBP-binding sites nearly 3000 base pairs downstream of coding DNA have been suggested to autoregulate the C/EBPδ gene (19).

We previously reported that C/EBPδ pre-exists in primary cultures of differentiated osteoblasts. When these cells are treated with prostaglandin E_2 (PGE_2), parathyroid hormone (PTH), or other agents that increase intracellular cAMP and activate PKA, cytoplasmic C/EBPδ rapidly translocates to the nucleus. It then binds to a single *cis*-acting element within exon I of the gene encoding insulin-like growth factor I (IGF-I) and enhances new IGF-I expression (11, 20). However, when osteoblasts are exposed to glucocorticoid or to estrogen at concentrations that have permissive or suppressive effects on the rate of bone formation, we noted rapid changes in C/EBP synthesis and activity that significantly alter PKA-dependent effects on IGF-I expression (11, 13). Differences in C/EBPδ and IGF-I expression that occur in this way parallel changes in matrix protein synthesis by bone cells, and may thereby help to determine bone integrity. Nevertheless, little is known about *cis*-acting elements that regulate C/EBPδ gene expression in osteoblasts.

Earlier studies revealed that expression of the osteoblast phenotype occurs in concert with a nuclear transcription factor

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‖ To whom correspondence should be addressed: Dept. of Surgery, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06520-8041. Tel.: 203-785-4927; Fax: 203-785-5714; E-mail: michael.centrella@yale.edu.

[1] The abbreviations used are: C/EBP, CCAAT enhancer-binding protein; PKA, protein kinase A; PGE_2, prostaglandin E_2; PTH, parathyroid hormone; IGF-I, insulin-like growth factor I; Runx, Runt domain factor; GAL4-DBD, GAL4 DNA-binding domain; PKAreg, PKA regulatory subunit; PKAregμ, mutant PKA regulatory subunit; EMSA, electrophoretic mobility shift assay; ETS, expressed tag sequence; STAT, signal transducers and activators of transcription.

TABLE I

Oligonucleotide probes and DNA sequences used to assess Runx-dependent effects on C/EBP δ gene expression

C/EBPδ probes were derived from the 5′ region of the rat C/EBPδ gene at the positions indicated. Probe −175/−147μ was designed to include a disruption (bold) in the Runx binding sequence. Probes Runx, HS3D, SP1, and RCP were designed to include consensus Runx, C/EBP, or Sp1 binding sequences, as indicated.

C/EBPδ probes	Sequence	Possible sites[a]
−175/−127wt	AGTAGGTCCAAACCGCACAAACAGGAAGGAGGGAAGGCCAAGAGGTGCGG	Runx/ETS
−175/−147wt	AGTAGGTCCAAACCGCACAAACAGGAAGG	Runx
−175/−147μ	AGTAGGTCCAAAC**AT**CACAAACAGGAAGG	Runx mutation
−131/−103	TGCGGGCAGAGGGCGGGTCGTTCCCAGCA	Sp1
Consensus probes		
Runx	CGTATTAACCACAATACTCG	Runx
HS3D	GAGCAGATAGAGCCTGCGCAATCGAAATA	C/EBP
SP1	GTACATTCGATCGGGCGGGGCGAGCGATC	Sp1
RCP	TAGCTGCAGCCAGAGGCCGTTGATTGCCCCATCCTGACCACAGCCCTACTTCCTCCCCTGGGCTG	C/EBP, Runx

[a] Regions containing possible nuclear factor binding sites were determined by MatInspector, version 2.2 (51), and are underlined.

which was variously termed polyoma enhancer-binding protein 2-αA1, acute myelogenous leukemia factor (AML)-3, or core binding factor (CBF)a1 (21–23), one of three closely related gene homologues. These factors share a *Runt* homology domain sequence first defined by analogy to the Runt nuclear factor involved in body segmentation, sex determination, and neurogenesis in *Drosophila.* The Runt domain directs DNA binding and heterodimer formation with several other nuclear factors, including C/EBPβ, whereas the carboxyl-terminal region contains a transactivating domain that drives gene expression (24–32). The "Runx" nomenclature has recently been developed to incorporate cross-genus similarities among these factors, and in this system the homologue especially enriched in differentiated osteoblasts is designated as Runx2.[2] Runx-dependent gene expression increases in parallel with osteoblast differentiation (21–23), and loss of Runx2 by homozygous gene deletion in mice arrests skeletal tissue development (33, 34). Moreover, suppression of nuclear Runx2 protein by persistent exposure to, or high levels of glucocorticoid limits Runx-dependent gene expression in fetal rat osteoblasts (35).

Studies in several cell culture models suggest a synergistic interaction between members of the C/EBP and Runt domain factor gene families. This requires proximal *cis*-acting elements that occur in a small number of genes, and on a physical interaction between the *trans*-acting nuclear factors themselves (27, 30). However, based on the expression of both Runx2 and C/EBPδ in osteoblast-enriched cultures and on a detailed examination of the C/EBPδ gene promoter sequence (16, 36), we predicted an additional, upstream regulatory event. In this study we describe the presence of a functional Runx binding element in the C/EBPδ gene promoter and its ability to regulate C/EBPδ gene expression. We further report effects by PGE_2 on Runx2 activation, direct interactions between the isoforms Runx2 and C/EBPδ, and how these events may allow discriminate control of Runx- and C/EBP-dependent gene expression in isolated osteoblasts.

EXPERIMENTAL PROCEDURES

Cell Cultures—Osteoblast-enriched cell cultures were prepared from parietal bones of 22-day-old Harlan Sprague-Dawley rat fetuses (Charles River Breeding Laboratories, Raleigh, NC) by methods approved by Yale Animal Care and Use Committee. Sutures were eliminated by dissection and cells were released by five sequential digestions with collagenase (37). Cells from the last three digestions exhibit high levels of PTH receptors and type I collagen synthesis, an increase in osteocalcin expression in response to vitamin D_3, and high alkaline phosphatase specific activity (37–39). By these criteria, differential sensitivity to transforming growth factor-β, bone morphogenic proteins, various prostaglandins, and the ability to express nuclear factor Runx2 and form mineralized nodules *in vitro*, osteoblast-enriched cultures are well distinguished from less differentiated periosteal cells (23, 40–42). Cells were plated at 4,000/cm^2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Life Technologies).

Transfection Constructs—Reporter plasmid constructs containing rat C/EBPδ promoter fragments spanning nucleotides −2700 to +42, −175 to +42, and −79 to +42 were produced as described previously (16). The nucleotide fragment −535 to +42 was subcloned by restriction enzyme digestion and ligated into empty vector. The Runx-binding site (underlined) (5′- . . . CCAAACCGCACAA . . . -3′) in the −175 to +42 C/EBPδ promoter fragment was modified with a mutation (boldface) (5′- . . . CCAAAC**AT**CACAA . . . -3′) by polymerase chain reaction. Parental plasmids and reporter constructs pSXN1C, p1711b/Luc, and 4XHS3D were described previously (11, 13, 20, 23, 35, 43). Plasmid pRCP/L was created by inserting probe RCP (Table I) into the Rous sarcoma virus minimal promoter plasmid construct used to prepare 4XHS3D. The 5xGAL4 reporter construct and expression vectors encoding the GAL4 DNA binding sequence and the VP16 gene transactivation domain were generously provided by Dr. I. Sadowski, University of British Columbia, Vancouver (44, 45). The expression construct encoding murine Runx2 (initially designated as polyoma enhancer binding protein P2-αA1) was previously reported (25). An antisense Runx2 expression construct was produced by ligating a 2.25-kilobase *Bgl*II/*Eco*RI restriction fragment of the murine gene in reversed orientation into vector pSV7d (46). Expression constructs encoding rat C/EBPδ or murine Runx2 (lacking 18 amino-terminal amino acids) fused to the GAL4 DNA-binding domain (GAL4-DBD), or various fragments of C/EBP fused to VP16 transactivation domains δ (see Fig. 4), were produced by restriction enzyme cleavage and re-ligation into the vectors obtained from Dr. Sadowski (44, 45). Expression constructs encoding native PKA regulatory subunit (PKAreg) or a mutant PKAreg subunit unresponsive to cAMP (PKAregμ) were obtained from Dr. G. S. McKnight (University of Washington, Seattle, WA) (47, 48). An expression construct encoding full-length murine Runx2 with *myc*-His epitope tags was generated by subcloning into vector pcDNA3 (Invitrogen).

Transfections—Promoter/reporter, expression, or empty vector plasmid constructs, pretitrated for optimal expression efficiency, were transfected using LT1 (Mirus Corp.). Briefly, cultures at 50–70% confluent density were exposed to plasmids for 16 h in serum-depleted medium, and then supplemented to obtain a final concentration of 5% fetal bovine serum. For reporter gene assays, cultures were expanded for 48 h, treated as indicated in the figures in serum-free medium, rinsed, and lysed. Nuclear-free supernatants were analyzed for reporter gene activity and corrected for protein content. To account for interference by competition among plasmids for limiting transcriptional components, control cultures were transfected with equivalent amounts of empty expression vectors. Transfection efficiency was assessed in parallel in cells transfected with positive and negative reporter plasmids, as described previously (11, 13, 20, 23, 35, 43, 49).

RNA Preparation and Assay—Total RNA was extracted with acid guanidine-monothiocyanate, precipitated with isopropyl alcohol, dissolved in sterile water, and hybridized with ^{32}P-labeled antisense RNA fragments encoding rat C/EBPδ or 18 S rRNA. Unhybridized RNA was digested with RNase A and T1 and protected fragments of C/EBPδ (190 nucleotides), or 18 S (80 nucleotides) were collected with isopropyl alcohol and resolved on a 5% denaturing polyacrylamide gel. Bands

[2] Runt Domain Factor Nomenclature Committee, unpublished report.

were visualized by fluorography and quantified by densitometry, as described previously (13, 20).

Nuclear Protein Extracts—Cells were rinsed, harvested by scraping and centrifugation, and lysed in hypotonic buffer supplemented with phosphatase and protease inhibitors and 1% Triton X-100. Nuclei were collected by centrifugation and resuspended in hypertonic buffer with glycerol, phosphatase, and protease inhibitors. Released nuclear proteins were separated from insoluble material by centrifugation and stored at −75 °C (11, 20, 49).

Electrophoretic Mobility Shift Assay (EMSA)—Commercial double-stranded probes (Table I) were radiolabeled by annealing complementary oligonucleotides, and overhangs were filled with dNTPs and [α-^{32}P]dATP using the Klenow fragment of DNA polymerase I. 5–10 μg of nuclear extract protein was preincubated on ice without or with unlabeled specific or nonspecific competitor DNA, supplemented with ^{32}P-labeled probe (0.1–0.2 ng at 5×10^4 cpm). In some samples, nuclear extract was preincubated with antiserum for 30 min before adding ^{32}P-labeled probe. Radioactive complexes were resolved on a 5% non-denaturing polyacrylamide gel and visualized by autoradiography.

Protein Interactions—Fragments of C/EBPδ and Runx2 were cloned into expression plasmids that fused them to DNA encoding either DNA binding or gene transactivation domains, which, if brought together by interactions between C/EBPδ and Runx2, highly enhance gene expression through a heterologous gene promoter (44, 45). Alternately, osteoblast-enriched cultures were transfected with *myc*-His epitope-tagged Runx2 and GAL4-DBD epitope-tagged C/EBPδ. Nuclear extracts were combined with ProBond nickel chelating resin to collect transfected, histidine-tagged Runx2, and the resin was eluted with imidazole. Eluates were fractionated by electrophoresis on a 12% SDS-polyacrylamide gel, blotted onto ImmobilonP membranes (Millipore), probed with antibody to the epitope tags on C/EBPδ or Runx2, and visualized with ECL (Amersham Pharmacia Biotech) reagents and chemiluminescence (13, 23).

Reagents—PGE_2 was obtained from Sigma. Oligonucleotide probes were obtained from Life Technologies. Antiserum to human Runx2 (initially designated as AML-3) was generously provided by Dr. Scott W. Hiebert (Vanderbilt University) (50). Nonimmune rabbit serum and antibody to C/EBPδ and GAL4-DBD were obtained from Santa Cruz Biotechnologies. Anti-*myc* antibody was obtained from Invitrogen.

Statistical Analysis—Statistical differences were assessed by one-way analysis of variance and the Kruskal-Wallis method for post-hoc analysis, with SigmaStat software.

RESULTS

Runx-binding Sequence in the C/EBPδ Gene Promoter—C/EBPδ gene promoter fragments of 2700, 535, and 175 nucleotides directed similarly high levels of reporter gene expression in osteoblasts, whereas the 3′ terminal 79-nucleotide promoter fragment supported less than 5% of maximal activity (Fig. 1*A*). The sequence between nucleotides −175 and −79 contains consensus binding sites for nuclear transcription factors of the Runx, ETS, Sp1, STAT, and AP4 gene families (51) (Fig. 1*B*). By gel shift analysis, strong nuclear factor binding to DNA occurred with ^{32}P-labeled probes that spanned nucleotides −175 to −147 (−175/−147wt), and −175 to −127 (−175/−127wt), which included the full Runx or Runx and ETS binding sequences. Radiolabeled complex formation was completely reduced by mutation of the Runx binding sequence in ^{32}P-labeled probe −175/−147 μ. Competition with unlabeled homologous oligonucleotides, or with oligonucleotide encoding only a consensus Runx binding sequence also suppressed complex formation by both ^{32}P-labeled probes. In contrast, an unlabeled probe homologous except for a mutated Runx binding sequence (−175/−147 μ), or one containing a nonspecific C/EBP binding sequence (HS3D) (20) had no suppressive effect. Complex formation was also significantly modified by anti-Runx2 specific antibody (α-Runx2), but not by anti-C/EBPδ (α-C/EBP) or non-immune rabbit IgG (Fig. 1*C*). Consistent with the abundance of Sp1 in osteoblast nuclear extract (49), a ^{32}P-labeled probe defining the Sp1 binding sequence formed a specific complex by EMSA (see Fig. 3*B*, below). Not surprisingly, mutation of the Sp1 binding sequence suppressed basal C/EBPδ gene promoter activity (not shown), predicting its importance for basal C/EBPδ gene expression. However, the ^{32}P-labeled probe −175/−127wt, which contains Runx- and ETS-binding sites, was suppressed to a similar extent by either the consensus Runx oligonucleotide or the homologous C/EBPδ promoter-derived oligonucleotide. Therefore, little or no ETS-related factors appear to exist in osteoblasts or bind to this region of the C/EBPδ gene promoter under basal conditions.

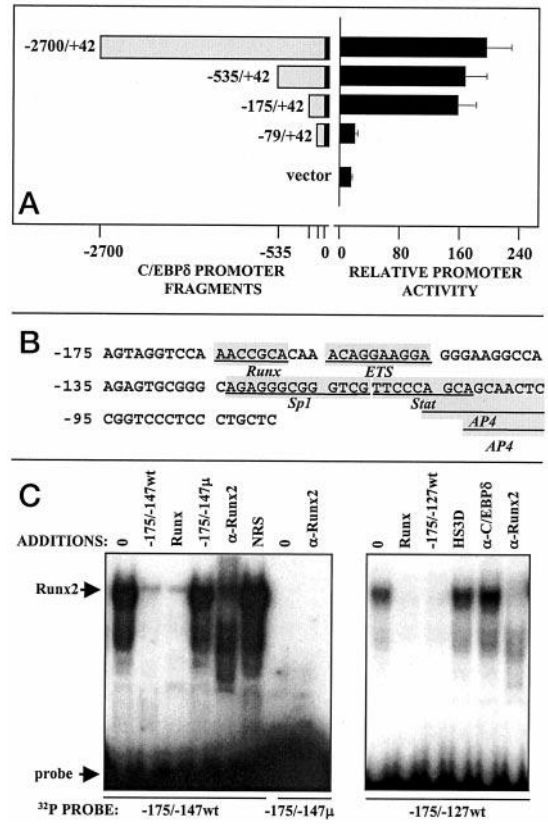

FIG. 1. **Runx-binding site in the proximal promoter region of the C/EBPδ gene.** In *A*, osteoblast-enriched cultures were transfected with 150 ng/500 μl of empty vector or plasmids containing fragments of the rat C/EBPδ promoter as shown. Reporter gene activity was measured after 48 h. Data are mean ± S.E. from 18 replicate cultures per condition, and five to six experiments. *B* shows the sequence between nucleotides −175 and −80 from the rat C/EBPδ gene promoter, defining the region that differs between plasmid constructs −175/+42 and −79/+42. Sequences known to bind various nuclear factors are designated by *lines* and *shaded boxes*, and labeled below. In *C*, oligonucleotide probes containing native or mutated sequences (Table I) were examined by EMSA. ^{32}P-Labeled oligonucleotide probes are indicated *below*, and additions of unlabeled oligonucleotides at 50-fold molar excess, antisera (α-Runx2, α-C/EBPδ), or normal rabbit serum (*NRS*) at 0.5 μl per reaction are indicated *above*.

Runx-dependent Effects on C/EBPδ Gene Promoter Activity—Mutation of the Runx-binding site in the C/EBPδ gene promoter reduced reporter gene expression by approximately 50% (Fig. 2*A*). In addition, forced overexpression of Runx2 above the level already present in osteoblasts (21–23) further enhanced C/EBPδ gene promoter activity by 80% (Fig. 2*B*). Co-transfection with an expression construct encoding an antisense sequence to Runx2, which potently suppressed the Runx-dependent promoter/reporter construct SXN1C (23, 35) (Fig. 2*C*), also reduced C/EBPδ gene promoter activity by 50% (Fig. 2*D*). Consistent with its stimulatory effect on C/EBPδ gene promoter activity, forced overexpression of Runx2 increased the steady state levels of C/EBPδ mRNA by 100–150% (Fig. 2*E*). Thus, approximately half of basal C/EBPδ gene promoter activity appears sensitive to endogenous Runx protein in osteoblasts, and an increase in Runx expression has an appropriate stimulatory effect on C/EBPδ expression.

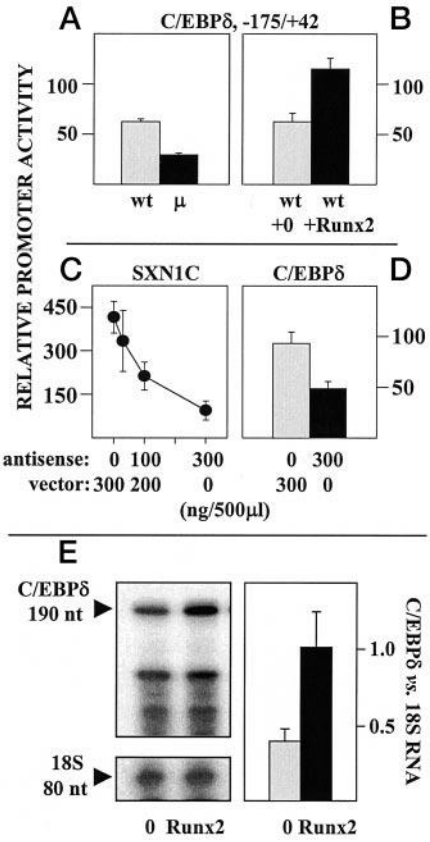

FIG. 2. **Runx regulates C/EBPδ gene promoter activity in osteoblasts.** In *A*, osteoblast-enriched cultures were transfected with 150 ng/500 μl of native C/EBPδ gene promoter/reporter plasmid construct −175/+42 (wt), or one containing the mutated (μ) Runx binding sequence shown in Table I. In *B*, cells were co-transfected with native C/EBPδ promoter/reporter plasmid −175/+42 and 50 ng/500 μl of empty vector (0) or an expression construct encoding murine Runx2. In *C*, cells were co-transfected with 100 ng/500 μl of plasmid SXN1C, containing two Runx DNA-binding sites inserted into plasmid pGL3-Promoter, and with empty vector or a Runx antisense expression construct at the concentrations shown. In *D*, cells were co-transfected with native C/EBPδ promoter/reporter plasmid −175/+42 and empty vector or the Runx antisense expression construct, as indicated. Data are mean ± S.E. from nine or more replicate cultures per condition, and three or more experiments. In *E*, total RNA prepared from cultures transfected with empty vector (0) or the Runx2 expression construct was examined by RNase protection assay with specific antisense probes for rat C/EBPδ and 18 S rRNA, as indicated on the *left*. *Numbers* show protected fragment size in nucleotides (*nt*), relative to a sizing ladder. The stimulatory effect of Runx2 overexpression assessed in six separate studies is shown on the *right*.

PGE_2-dependent Activation of Runx2—PGE_2 rapidly increases translocation of pre-existing C/EBPδ from the cytoplasm to the nucleus in osteoblasts, where it drives new IGF-I mRNA expression in a cAMP-dependent way (20). The protein synthesis inhibitor cycloheximide in part reduces the amount of C/EBP in nuclear extracts, suggesting that PGE_2 may also increase new C/EBPδ expression in these cells (13, 20). In agreement with this, PGE_2 increased C/EBPδ gene promoter activity by approximately 60%. In combination with forced overexpression of Runx2, PGE_2 enhanced C/EBPδ gene promoter activity to 300% of control, whereas forced expression of Runx2 antisense severely reduced basal and PGE_2-induced promoter activity (Fig. 3*A*). Nonetheless, PGE_2 treatment had no effect on nuclear factor binding to DNA probes defined by regions of the C/EBPδ gene promoter encompassing the Runx-, ETS-, or Sp1-binding sites (Fig. 3*B*). However, other evidence indicated that PGE_2 enhanced the biochemical activity of Runx2 in osteoblasts. First, PGE_2 significantly increased the

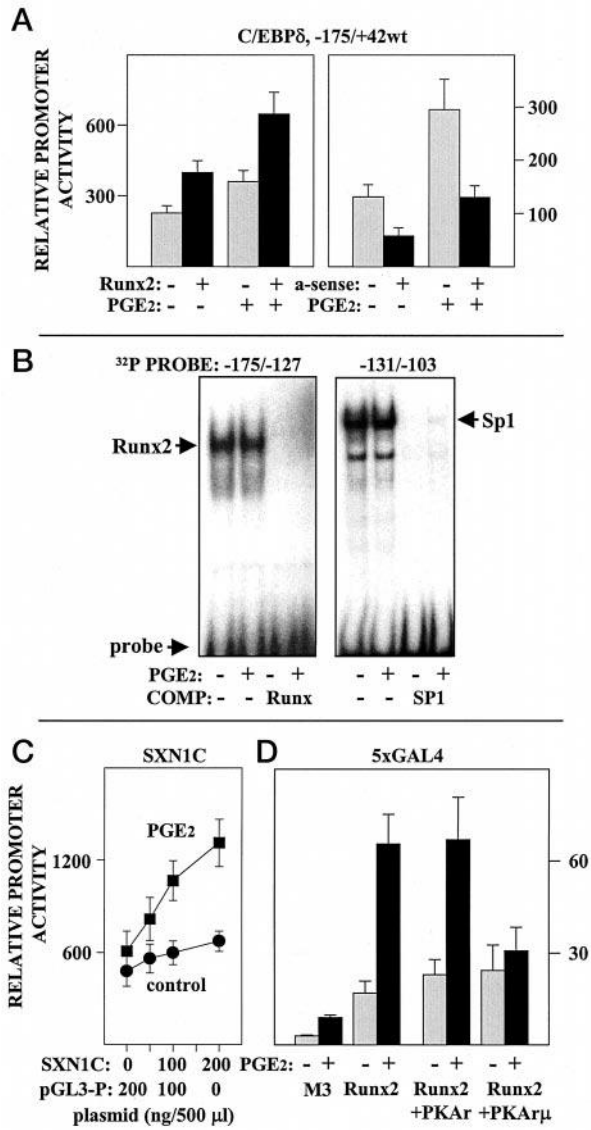

FIG. 3. **PGE_2 increases C/EBPδ gene promoter activity and enhances transactivation by Runx2.** In *A*, osteoblast-enriched cultures were co-transfected for 48 h with native C/EBPδ promoter/reporter plasmid −175/+42wt and empty vector or expression constructs encoding murine Runx2 or Runx2 antisense (a-sense) as described in the legend to Fig. 2. In *B*, nuclear protein extract from control (−) or PGE_2 (+) treated cells was examined by EMSA with ^{32}P-labeled probes −175/−127wt or −131/−103 without or with the unlabeled consensus competitors Runx or SP1 (Table I), as described in the legend to Fig. 1. In *C*, cells were co-transfected for 48 h with parental plasmid pGL3-Promoter (pGL3-P) or SXN1C at the concentrations shown. In *D*, cells were co-transfected for 48 h with 200 ng/500 μl 5xGAL4 reporter plasmid and either 50 ng/500 μl of parental vector encoding the GAL4-DBD (M3), or M3 supplemented with cDNA encoding all but the 18 carboxyl-terminal amino acids of native Runx2 (Runx2). Some cells were co-transfected with 5xGAL4, the M3/Runx2 expression construct, and 100 ng/500 μl of expression constructs encoding either native PKAreg subunit (PKAr), or mutated PKAregμ (PKArμ) that fails to bind cAMP and release the catalytic subunit of PKA. To assess downstream effects on C/EBPδ expression, cells were treated for 24 h with vehicle or 10^{-6} M PGE_2. To assess more immediate effects on Runx2 activation, cells were treated for 6 h with vehicle or 10^{-6} M PGE_2. Data from reporter assays are mean ± S.E. from nine or more replicate cultures per condition, and three experiments. Analogous results occurred in at least two separate EMSA studies.

ability of endogenous Runx2 to drive gene expression by the synthetic promoter/reporter construct SXN1C, which contains two Runx-binding sites, without effecting gene expression driven by the parental construct pGL3-Promoter (pGL3-P) that

lacks these sequences (Fig. 3*C*). Second, in cells co-transfected with a Runx2 expression construct fused to the GAL4-DBD (M3/Runx2) and the promoter/reporter gene construct 5xGAL4, PGE_2 increased ectopic Runx2-dependent gene expression by 4-fold. Finally, forced expression of mutated PKA regulatory subunit PKAregμ, which fails to bind cAMP and retains the PKA catalytic subunit in an inactive complex (47), suppressed the stimulatory effect of PGE_2, whereas native PKAreg did not (Fig. 3*D*). Together, these results show that PGE_2 enhanced Runx2 activity in a PKA-dependent way independently of its ability to bind DNA promoter elements.

Interactions between Runx2 and C/EBPδ—Because PGE_2 can increase new C/EBPδ expression, the higher levels of C/EBPδ that accumulate in this way might more readily interact with Runx2 (27, 30) and thereby alter its ability to regulate gene expression. To address this, we first used a mammalian two-hybrid gene expression assay that requires a physical interaction between Runx2 fused to the GAL4-DBD (M3/Runx2) and C/EBPδ fused to the VP16 gene transactivation domain (C/EBPδ/VP16) in order to enhance gene expression. Co-expression of intact C/EBPδ/VP16 (amino acids 1–268) increased the effectiveness of M3/Runx2 by 6-fold. C/EBPδ fragments lacking the carboxyl-terminal region (fragments containing amino acids 1–92 or 1–151) had no effect. However, co-transfection with a C/EBPδ/VP16 expression construct retaining the carboxyl-terminal dimerization and DNA-binding domains of C/EBPδ (21Δ151) produced results analogous to those by intact C/EBPδ (Fig. 4*A*). Consistent with this, Runx2 formed intranuclear protein-protein complexes with either the native or the 21Δ151 forms of C/EBPδ (Fig. 4*B*). These findings agreed with previous results that demonstrated physical interactions between the homologues Runx1 and C/EBPα (27, 30), and localized them to the Runt domain common to all Runx proteins (52). Our results extend this to show that this interaction also requires the DNA-binding and/or dimerization domain of C/EBPδ. Nonetheless, forced expression of either native C/EBPδ or the 21Δ151 C/EBPδ deletion fragment potently suppressed C/EBPδ gene promoter activity in cells with endogenous Runx protein, as well as the stimulatory effect of Runx2 overexpression (Fig. 4*C*). Because of this, we wished to determine if gene promoter activity could occur in osteoblasts within the context of proximal Runx and C/EBP *cis*-acting elements. Cells were transfected with a synthetic promoter/reporter construct termed pRCP/L that we designed for this purpose. In untreated osteoblasts, where Runx associates with the nucleus and C/EBPδ remains in the cytoplasm, pRCP/L-dependent gene expression was 60% higher than that directed by the parental vector devoid of Runx- and C/EBP-binding sites. PGE_2 had no significant effect on promoter activity in vector transfected cells, but synergistically increased reporter gene expression by 340% in osteoblasts transfected with pRCP/L (Fig. 5*A*). Similar to results with a C/EBPδ gene promoter fragment (Fig. 3*B*), the Runx binding element in pRCP/L was utilized by factor from control and PGE_2-treated cells, whereas binding to the C/EBP element only occurred with factor from the treated cell nuclear extract (Fig. 5*B*). Therefore, osteoblasts appear to utilize and respond to Runx and C/EBP in different ways, depending in part on the intracellular location of these *trans*-acting factors, and on the presence of Runx, C/EBP, or composite Runx/C/EBP *cis*-acting elements.

Effects on C/EBP-dependent Gene Promoter Activity—Changes in C/EBPδ expression by way of Runx2 or its activation should produce appropriate downstream effects on C/EBP-dependent gene expression. This was first examined with the synthetic reporter construct HS3D, which contains 4 tandem C/EBP binding sequences. Basal and PGE_2-induced reporter

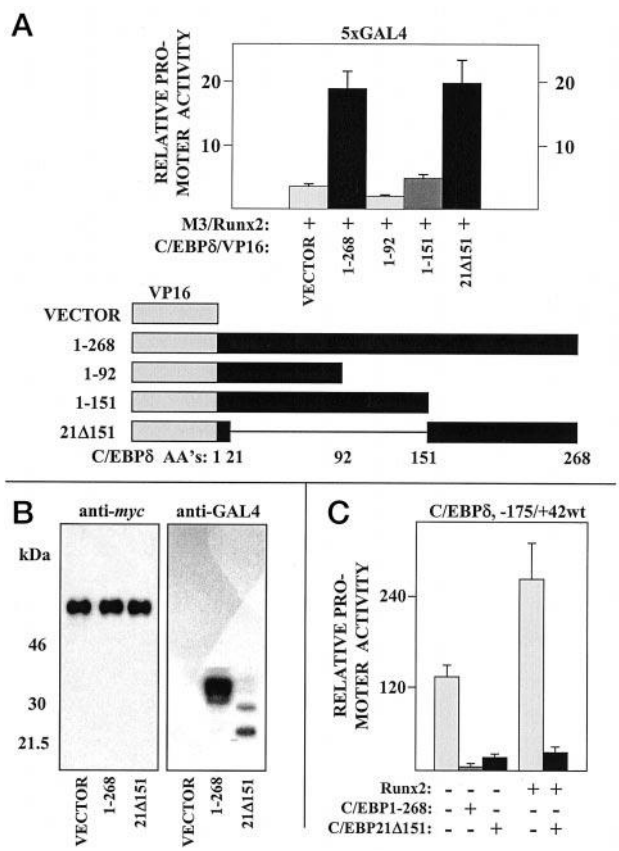

FIG. 4. **Interactions between Runx2 and C/EBPδ, and downstream effects on C/EBPδ gene promoter activity.** In *A*, osteoblast-enriched cultures were co-transfected with 200 ng/500 μl 5xGAL4 reporter plasmid, 50 ng/500 μl M3/Runx2, and 50 ng/500 μl of either the VP16 vector encoding a strong gene transactivation domain, or VP16 supplemented with cDNA encoding native (fragment 1–268), truncated (fragments 1–92 and 1–151), or internally deleted C/EBPδ (fragment 21Δ151), as indicated. *AA's* denotes amino acids. No significant activity occurred in cells transfected with VP16 constructs in the absence of M3/Runx2 (not shown). In *B*, cells were transfected with expression constructs encoding *myc*-His tagged Runx2, and empty GAL4-DBD vector, full-length (1–268) or deleted (21Δ151) GAL4-DBD-tagged C/EBPδ. Equal amounts of nuclear protein collected with affinity resin that binds histidine-tagged Runx2 were fractionated by SDS-polyacrylamide gel electrophoresis, blotted, and probed with antibody to c-*myc* to visualize Runx2, or with antibody to GAL4-DBD to visualize C/EBP. Similar results were found in 2 studies. In *C*, cells were co-transfected with 150 ng/500 μl native C/EBPδ promoter/reporter plasmid −175/+42, and 75 ng/500 μl of empty vector (−), native C/EBPδ 1–268, or the 21Δ151 deletion fragment, without or with 50 ng/500 μl of parental vector (−) or Runx2 expression construct, as indicated. In *A* and *C*, data are mean ± S.E. from nine or more replicate cultures per condition, and three or more experiments.

gene expression driven by HS3D was potently suppressed by co-transfection with the Runx2 antisense construct. PGE_2 further increased promoter activity that was induced by Runx2 overexpression, and both effects were reduced by the C/EBPδ fragment 21Δ151 (Fig. 6*A*). Entirely consistent effects occurred in cells expressing the native IGF-I gene promoter/reporter construct 1711b, which contains 1711 base pairs of IGF-I gene promoter DNA and a portion of exon 1 where the HS3D-binding site was first defined (Fig. 6*B*). Therefore, even within the context of maximal IGF-I gene promoter where no Runx-binding site exists, Runx2 appears to have an important indirect influence that is mediated by its ability to control C/EBPδ expression in osteoblasts.

DISCUSSION

Osteoblast differentiation and activity require the expression of specific nuclear transcription factors that drive the

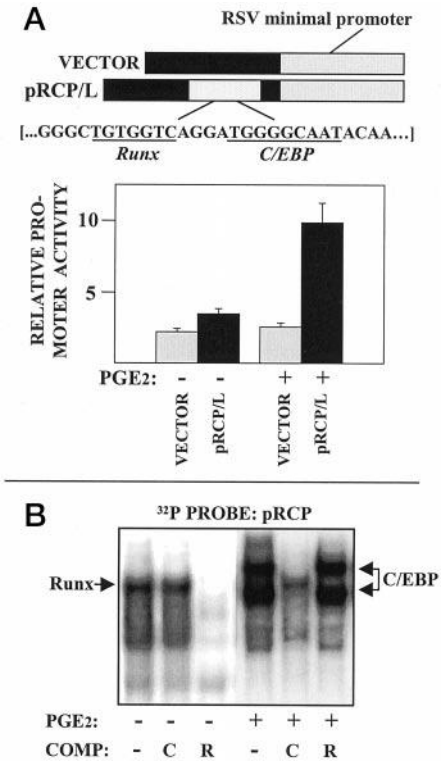

FIG. 5. **Interactions between Runx2 and C/EBPδ on the activity of a composite gene promoter in osteoblasts.** In *A*, cells were transfected with 750 ng/500 μl of empty vector or pRCP/L for 48 h, and treated for 24 h with vehicle (−) or 10^{-6} M PGE_2. Data are mean ± S.E. from nine or more replicate cultures per condition, and three or more experiments. In *B*, nuclear protein extract from cells treated for 6 h with vehicle (−) or PGE_2 (+) was examined by EMSA with ^{32}P-labeled probe pRCP without or with the unlabeled consensus competitors C/EBP (*C*) or Runx (*R*) (Table I), as described in the legend to Figs. 1 and 3. Analogous effects by PGE_2 on Runx and C/EBP binding to consensus elements were observed in three or more studies.

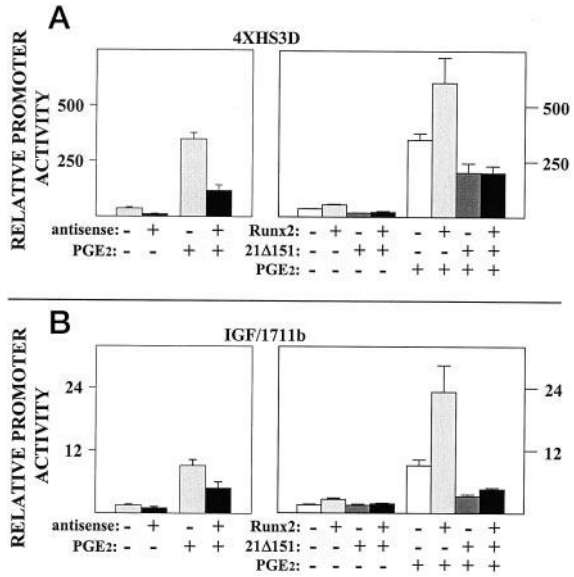

FIG. 6. **Effects by Runx2 on downstream, C/EBP-dependent reporter gene, and IGF-I expression.** In *A*, osteoblast-enriched cultures were co-transfected for 48 h with 300 ng/500 μl of promoter/reporter plasmid 4XHS3D, containing 4 tandem CEBP/δ DNA-binding sites, empty vectors (−), and 300 ng/500 μl of Runx2 antisense (antisense), 50 ng/500 μl of native Runx2 (Runx2), or 75 ng/500 μl of C/EBPδ deletion (21Δ151) expression constructs, as indicated. In *B*, cells were co-transfected with 300 ng/500 μl of promoter/reporter plasmid IGF/1711bLuc (IGF/1711b) containing the IGF-I gene promoter and its 3′ C/EBP-sensitive domain, and the same combination of empty vectors or expression constructs described in *A*. Cells were then treated for 24 h with vehicle or 10^{-6} M PGE_2. Data are mean ± S.E. from nine or more replicate cultures per condition, and three or more experiments.

synthesis of pertinent downstream genes. These complex events are modified by and also integrate signals generated by extracellular growth regulators and circulating hormones (53). In this study we report regulatory effects by transcription factor Runx2 on transcription factor C/EBPδ gene expression, and on C/EBPδ-dependent gene promoter activity. We show that Runx2-dependent gene transactivation can be enhanced by PGE_2 at least in part by effects on PKA. Moreover, in cells where high levels of C/EBPδ are achieved, Runx-dependent gene expression can be suppressed in a negative feedback-like way by direct interactions with the carboxyl-terminal region of C/EBPδ itself. These events may help to control the expression of IGF-I, which itself is regulated by C/EBPδ and influences bone formation and remodeling.

Loss of Runx2 by homozygous gene deletion in mice severely limits osteogenesis during fetal development (33, 34), and suppression of Runx activity in postnatal animals significantly reduces bone matrix protein expression and engenders osteopenia (54). Several genes expressed by osteoblasts contain Runx binding sequences, and others are reduced when functional Runx protein levels decline (35, 54–56), indicating important direct and indirect effects on osteoblast activity. Nonetheless, mice lacking C/EBPδ exhibit no readily obvious skeletal defect (4).[3] This suggests that under basal conditions other C/EBP isoforms may have compensatory effects, and that the influence of Runx2 is more pervasive. Even so, effects that may occur with aging, stress, fracture, or variations in steroid hormone status, where changes in local growth factor expression have a large impact on skeletal tissue integrity or repair, have not been resolved in C/EBPδ-deficient animals.

By various criteria, at least half of basal C/EBPδ gene promoter in osteoblasts relied on a single Runx binding sequence. Furthermore, treatment with PGE_2 enhanced Runx-dependent gene expression in part through effects on Runx2 activation. Runx-dependent changes in C/EBPδ expression were verified by appropriate effects on a synthetic C/EBP-sensitive reporter gene construct, and on the native IGF-I gene promoter. IGF-I is critical during longitudinal bone growth, and may contribute to bone formation during the coupled skeletal remodeling cycle that helps to maintain serum calcium homeostasis (13, 57, 58). Because PGE_2 and PTH, normally associated with bone resorption, also activate new IGF-I expression by osteoblasts through cAMP-dependent effects on C/EBPδ activation and synthesis (11–13, 20), we now consider C/EBPδ as a nuclear coupling factor for this process.

Critical breaks in the hormone-C/EBPδ-IGF-I axis can occur at several levels. Earlier, we reported variations in C/EBPδ-dependent IGF-I expression in response to hydrocortisone and 17β-estradiol. This may result from changes in C/EBPδ activation, from its binding to other nuclear proteins, or from changes in C/EBPδ expression itself (1, 11, 13), predicting the complexity of this axis. Our current study focuses on a small DNA region upstream of the C/EBPδ gene transcription start site. This region comprises virtually all of the elements thought to be essential for basal C/EBPδ transcription in osteoblasts, as in liver or vascular smooth muscle cells (16, 36), but may not include other sequences necessary for hormone- or growth factor-dependent effects. Nevertheless, the presence of Runx2 in osteoblasts (21–23) and a critical Runx binding sequence that we now describe in the C/EBPδ gene promoter, provides a novel, tissue-restricted link between these nuclear factors, and ex-

[3] P. F. Johnson, personal communication.

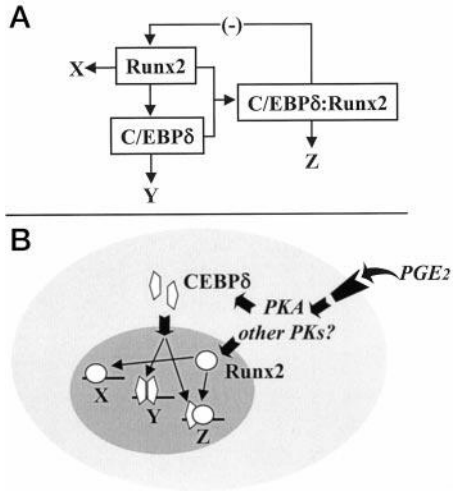

FIG. 7. **Model of independent and co-dependent effects by Runx and C/EBP gene family members on gene expression.** As shown in *A*, Runx2 can activate the promoters of X genes that contain Runx DNA binding sequences. Evidence from this report now includes C/EBPδ within this group. Similarly, C/EBPδ can activate Y gene promoters that contain C/EBP binding sequences. This may occur more readily in cells that express Runx protein and therefore contain a pre-existing pool of cytoplasmic C/EBPδ. When C/EBPδ levels rise significantly or become activated by hormones that increase cAMP and activate PKA, as shown in *B*, C/EBPδ accumulates in the nucleus. In this context it may associate with Runx protein and enhance Z gene promoters with proximal Runx and C/EBP binding sequences. Alternately, when nuclear C/EBP levels become exceedingly high, this interaction may eventually feedback and repress the expression of X genes, and thereby limit new C/EBPδ synthesis and its activity. Therefore, in the native state, Runx-dependent gene expression would persist until osteoblasts are appropriately activated, at which point different panels of genes would increase or decrease in controlled ways.

plains in part the pre-existing C/EBPδ found in osteoblasts.

Consistent with our results, previous studies indicate that Runx1-dependent gene expression may increase independently of total Runx1 binding to DNA. This effect occurred by activation of the extracellular signal-regulated kinase system, and related to specific phosphoamino acids within a protein subdomain that Runx1 shares with Runx2 (59). Here we show that expression of a mutated PKA regulatory subunit potently suppresses Runx2 activation by PGE_2, suggesting that it may also occur through a PKA-dependent event. After our studies were complete, similar observations were reported to explain the stimulatory effect of PTH on collagenase 3 gene expression in rat osteosarcoma cells, where other protein kinase systems did not appear to be involved (60). However, in preliminary studies, we noted that protein kinase C activators also can increase Runx2-dependent gene promoter activity in primary osteoblast cultures, and that Runx2 can be phosphorylated by osteoblast-derived extracts in an extracellular signal-regulated kinase-dependent way.[4] Moreover, long term exposure to agents that increase cAMP may target Runx2 to a proteolytic pathway in clonal murine MC3T3-E1 preosteoblasts (61). Therefore, intricate, and perhaps hormone- and context-specific control of Runx2 activity may occur in bone cells in multiple ways.

Runx1 and C/EBPα, which are both found in hematopoietic cells, physically interact by way of the Runt domain that occurs within all Runx isoforms (27, 30, 62). We also found a physical association between Runx2 and C/EBPδ within osteoblasts and that it requires the carboxyl-terminal region of C/EBPδ where the DNA binding and dimerization domains conserved among other C/EBP isoforms occurs. High levels of native C/EBPδ, or the 21Δ151 deletion fragment which retains these domains, reduced promoter activity by endogenous Runx2 as well by transfected Runx2 protein. Therefore, when C/EBPδ levels become sufficiently high, the same interaction may cause an eventual suppressive effect by C/EBPδ on its own expression in cells where Runx is involved. We recently reported that glucocorticoid increases new C/EBP expression by osteoblasts (13), suggesting that the inhibitory effect of this hormone on Runx2-dependent gene expression in bone (35) may be multifaceted and perhaps further dissociate the expression of genes controlled by these transcription factors.

In total, our results predict that gene expression through these two transcription factors can occur in multiple ways. As modeled in Fig. 7*A*, some genes (denoted as X), including C/EBPδ, could be regulated directly by Runx proteins, and other genes (denoted as Y) could be regulated directly by C/EBP gene family members. The presence of Runx could increase the synthesis of C/EBPδ, and therefore further enhance the expression of C/EBPδ-sensitive genes. Other genes (denoted as Z), controlled by promoters that contain contiguous C/EBP and Runx binding sequences, may be enhanced by heterodimer complexes of C/EBP and Runx. Sequence analysis of the Runx-sensitive region of the C/EBPδ gene promoter shows no proximal C/EBP binding sequence. Thus, formation of the same heterodimers that can enhance genes with contiguous C/EBP and Runx promoter elements could also limit gene expression that depends on individual Runx or C/EBP *cis*-acting elements. The majority of Runx pre-exists associated with the nucleus (63) and, at least in osteoblasts, the majority of C/EBPδ sequesters in the cytoplasm (20), limiting their ability to interact before PKA-dependent translocation of C/EBPδ (Fig. 7*B*). However, in transfected osteoblasts, interactions between these nuclear factors were observed in the absence of PGE_2. This is thought to occur because the high levels of C/EBPδ achieved by forced overexpression can overcome factors or events that normally sequester it in untransfected cells (12, 13). PGE_2 activates C/EBPδ- and Runx2-dependent gene expression at least in part by PKA-dependent events. Therefore, gene regulation and counter-regulation by these factors must be balanced in intricate ways. Until recently we knew little about C/EBPδ in osteoblasts, and even less about regulation of C/EBPδ synthesis. Our current studies provide novel evidence for important interactions between C/EBPδ and the essential osteoblast nuclear factor Runx2. Further studies are required to understand how these interactions may contribute to differential control of gene expression during osteoblast differentiation, and how these cells respond to other extracellular agents or events.

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