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C/EBP β binds the P1 promoter of the Runx2 gene and up-regulates Runx2 transcription in osteoblastic cells

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Abstract

The Runx2 factor is an essential component of the regulatory mechanisms that control transcription during skeletogenesis. Runx2/p57 expression in osteoblastic cells is controlled by the P1 promoter, which is recognized by key regulators of osteoblast differentiation including homeodomain factors and Wnt- and BMP-signaling mediators. Here, we report that the transcription factor C/EBP β up-regulates Runx2/p57 expression by directly binding to the Runx2 P1 promoter in mesenchymal, pre-osteoblastic and osteoblastic cells. This C/EBP β -mediated up-regulation is principally dependent on C/EBP site II that is located within the first 180 bp of the proximal P1 promoter region and is highly conserved among mouse, rat, and human Runx2 genes. Our studies reveal how the C/EBP β factor, known to have a key role during osteogenesis, contributes to regulating the expression of Runx2, the master regulator of osteoblast differentiation.

Keywords

Runx2; Bone-specific transcription; C/EBP β

Introduction

The Runx2 transcription factor is an essential regulatory component during bone formation in mammals as it controls the expression of several key osteoblast-related genes (Lian et al. 2004; Karsenty et al. 2008; Marie, 2008). Runx2 null mice exhibit dramatic defects in osteogenesis and hereditary mutations in the human RUNX2 gene can be linked to ossification defects such as those reported in Cleidocranial Dysplasia (Otto et al. 1997; Otto et al. 2002).

Runx2 expression in osteoblastic cells is controlled by several regulatory elements distributed within the P1 promoter (Drissi et al. 2000; Zambotti et al. 2002; Lengner et al.

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2005; Gaur et al. 2005; Lee et al. 2005; Hassan et al. 2006, 2007), which are recognized by cognate transcription factors during osteogenesis. Among these factors the homeodomain proteins *Dlx3*, *Dlx5*, *Msx2*, *CDP/Cut* (Lee et al. 2005; Hassan et al. 2006), and *Hoxa10* (Hassan et al. 2007), as well as transcriptional regulators like AP-1 (Zambotti et al. 2002), β -catenin/TCF (Gaur et al. 2005), *Nkx3.2* (Lengner et al. 2005), NF-1 (Zambotti et al. 2002), Sp1 and Ets (Zhang et al. 2009). We have recently shown that in osteoblastic cells *Runx2* expression involves a specific chromatin remodeling process within the first 400 bp of the *Runx2* P1 promoter region (Cruzat et al. 2009). The changes in chromatin structure occur with elevated levels of histone H3 and H4 acetylation and are independent of SWI/SNF complex activity (Cruzat et al. 2009). However, the precise mechanism(s) involved in this remodeling process and the specific chromatin remodelers responsible remain undefined.

CCAAT/Enhancer Binding-Proteins (C/EBPs) are members of the CREB/ATF family of bzip (Basic Leucine Zipper) transcription factors (Nerlov, 2007). Most of these factors are broadly expressed in mammalian cell types, where they significantly contribute to control of tissue-specific genes (Nerlov, 2007). C/EBP β exists as three protein isoforms generated by alternative translation initiation: p38/C/EBP β -1 (also known as Liver Activating Protein* or LAP*), p33/C/EBP β -2 (LAP), and p20/C/EBP β -3 (Liver Inhibitory Protein or LIP). All three isoforms contain the bzip DNA-binding domain and retain all (C/EBP β -LAP* and C/EBP β -LAP) or only part (C/EBP β -LIP) of the N-terminal transcription regulatory domain (Calkhoven et al. 2000; Xiong et al. 2001). C/EBP β -LIP has been shown to have dominant-negative activity at C/EBP β -LAP*- and C/EBP β -LAP-target genes (Ramji and Foka, 2002).

Several reports support an important role for C/EBP β during osteogenesis (Marie, 2008). C/EBP β knock out mice exhibit delayed bone formation and decreased expression of osteoblast marker genes such as alkaline phosphatase and osteocalcin (Tominaga et al. 2008; Smink et al. 2009). Also, bone-targeted overexpression of C/EBP β -LIP in mice results in osteopenia from reduced bone formation (Harrison et al. 2005). C/EBP β can up-regulate osteoblast-related genes during osteogenesis by synergizing with *Runx2* (Gutierrez et al. 2002; Hata et al. 2005; Shirakawa et al. 2006) and ATF4 (Tominaga et al. 2008) transcription factors as well as by recruiting SWI/SNF chromatin-remodeling activity to target promoters (Villagra et al. 2006). It has been also demonstrated that C/EBP β can interact with key components of the Mediator complex (Mo et al. 2004; Li et al. 2008) as well as with transcriptional co-regulators like CBP/p300 (Mink et al. 1997), G9A (Pless et al. 2008), and PRMT4/CARM1 (Kowenz-Leutz et al. 2010) which contain histone-modifying activities. Therefore, this factor may be regulating target genes in osteoblastic cells through several different pathways.

Sequence analysis of the first 1 kb of the *Runx2* P1 promoter predicts four high-affinity C/EBP β -binding sites, which are conserved among mouse, rat and human genomes (see Figure 1A). Because C/EBP β (C/EBP β -LAP* and C/EBP β -LAP) is expressed in osteoblastic cells and its levels increase during osteogenesis (Gutierrez et al. 2002 and data not shown), it is important to define the role of this transcription factor in the P1-driven transcription of the *Runx2* gene in osteoblasts. Here, we report that C/EBP β up-regulates the *Runx2/p57* expression by directly interacting with the *Runx2* P1 proximal promoter in mesenchymal, pre-osteoblastic and osteoblastic cells.

Materials and Methods

Cell Culture

Rat-derived ROS17/2.8 osteoblastic cells and mouse C2C12 cells were cultured as described previously (Majeska et al. 1980; Cruzat et al. 2009). Mouse MC3T3-E1 and *Runx2*-null

TERT-immortalized osteoprogenitor cells (Bae et al. 2007) were grown in α -minimal essential medium (MEM) supplemented with 10% fetal bovine serum.

Expression constructs

Luciferase-reporter constructs driven by the Runx2 P1 promoter (p2.8P1mRunx2-Luc, p600P1rRunx2-Luc, p490P1rRunx2-Luc, p288P1rRunx2 and p108P1rRunx2) were described previously (Drissi et al. 2000; Zhang et al. 2009). All mutant versions of the p288P1rRunx2-Luc vector (MUT1, MUT2 and MUT1+2) were generated with the Quick-Change Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. For oligonucleotides utilized see supplementary figure S3. Expression vectors pC/EBP β -LAP*, pC/EBP β -LAP and pC/EBP β -LIP were kindly provided by Dr. Jose L. Gutierrez (University of Concepcion, Concepcion, Chile). The pC/EBP β plasmid containing the mouse C/EBP β cDNA was generated from MSV/C/EBP β (Gutierrez et al., 2002). mC/EBP β cDNA was cleaved and then inserted into the *EcoRI/XhoI* sites of pcDNA3.1/His (Invitrogen, Carlsbad, CA, USA), which contains both 6xHis and Xpress tags. The bacterial vector coding for His-C/EBP β was generated from MSV/C/EBP β (Gutierrez et al. 2002) by digestion with *SphI/HindIII* and subsequent insertion into the pQE-80L plasmid (QIAGEN Inc., Valencia, CA, USA). The bacterial expression vector coding for GST-Runx2 was described previously (Paredes et al. 2002; Sierra et al. 2003).

Transient transfections and reporter assays

C2C12 and MC3T3-E1 cells were plated in 24- and 12-well plates (21 and 15 mm diameter wells, respectively) and transiently transfected with the FuGENE 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA). ROS17/2.8 cells were plated in 24-well plates and transfected with Lipofectamine 2000 reagent (Invitrogen). Mouse Runx2-null TERT-immortalized cells were plated in 24-well plates and transfected with the FuGENE 6 Transfection Reagent (Roche Diagnostics). The cells were transfected with 250 or 500 ng of the firefly luciferase reporter plasmids and 12.5 or 25 ng, respectively, of the *Renilla* luciferase (pRL-SV40) plasmid as internal control (Promega Corp. Madison, WI, USA). The total amount of DNA was maintained at a constant level (1.3 μ g for 12-well and 650 ng for 24-well) by adding an appropriate amount of pBluescript (pBS) plasmid (Agilent Technologies Inc.). After 24 h, the cells were harvested using 50 μ L of passive lysis buffer (Promega) per well. Cell lysates (20 μ l) were evaluated for firefly luciferase activity using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions and normalized to values of *Renilla* luciferase activity.

Chromatin immunoprecipitation (ChIP)

ChIP studies were performed as described earlier (Soutoglou et al. 2002; Villagra et al. 2006; Cruzat et al. 2009) with modifications. ROS17/2.8 cell cultures (100 mm diameter plates) were washed with phosphate buffered saline (PBS 1X) and incubated for 10 min with 1% formaldehyde and gentle agitation at room temperature. The cross-linking was stopped by the addition of 0.125 M glycine for 5 min. The following experimental steps were performed on ice or at 4°C. The cells were washed with 5 ml of PBS, scraped off in the same volume of PBS, and collected by centrifugation at 1,000 x *g* for 5 min. The cell pellet was resuspended in 1 ml of lysis buffer (50 mM Hepes, pH 7.8, 20 mM KCl, 3 mM MgCl₂, 0.1% Nonidet P40, and a mixture of proteinase inhibitors), incubated for 10 min on ice and homogenized by Dounce homogenizer. The cell extract was collected by centrifugation at 1,500 x *g* for 5 min and resuspended in 0.3 ml of sonication buffer (50 mM Hepes, pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate acid, 0.1% SDS, and a mixture of proteinase inhibitors) for each 100 mm plate. To reduce the length of the chromatin fragments to 500 bp or smaller (confirmed by electrophoretic analysis and PCR

amplification), the extract was sonicated with a Misonix sonicator (model 3000), using 15-s pulses at 30% power. After centrifugation at 16,000 x *g* for 10 min, the supernatant was collected, frozen in liquid nitrogen, and kept at -80 °C. An aliquot was used for A_{260} measurements. Cross-linked extracts (8 A_{260} units) were resuspended in sonication buffer to a final volume of 500 μ l. The samples were precleared by incubation with 50 μ l of protein A/G-agarose beads (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 1 h at 4 °C with agitation. After centrifugation at 1,000 x *g* for 5 min, the supernatant was collected and immunoprecipitated with anti-C/EBP β polyclonal antibody (C-19, Santa Cruz Biotechnology) and anti-Runx2 (M-70, Santa Cruz Biotechnology) for 12h at 4°C. The immunocomplexes were recovered with the addition of 50 μ l of protein A-agarose beads and subsequent incubation for 1 h at 4°C with agitation. The complexes were washed once with: sonication buffer, sonication buffer plus 500mM NaCl, LiCl buffer (100 mM Tris-HCl, pH 8.0, 500 mM LiCl, 0.1% Nonidet P40, and 0.1% deoxycholic acid), and with Tris-EDTA (TE) buffer pH 8.0 (2mM EDTA and 50 mM Tris-HCl, pH 8.0), washing each time for 5 min at 4°C. The protein-DNA complexes were then eluted by incubation with 100 μ l of elution buffer (50 mM NaHCO₃ and 1% SDS) for 15 min at 65°C. After centrifugation at 10,000 x *g* for 5 min, the supernatant was collected and NaCl added to a final concentration of 200 mM. This mixture was incubated with 20 μ g/ml of RNase A for 12–16h at 65°C to reverse the cross-linking. The proteins were digested with 100 μ g/ml of proteinase K for 2 h at 50°C and the DNA recovered by phenol/chloroform extraction and ethanol precipitation using glycogen (20 μ g/ml) as a precipitation carrier. The PCR primers used to evaluate the rat Runx2 P1 promoter region were: forward -179, 5'-GGAGGGGAGAAGGAAAAAGA-3'; reverse +147, 5'-AGCACTCACTGACTCGGTTG-3'.

Western Blotting

Nuclear extracts were prepared as reported previously (Paredes et al. 2004) from ROS17/2.8, C2C12, MC3T3-E1 and Runx2-null TERT-immortalized osteoprogenitor cells, transfected with the appropriate expression vector as described in each figure legend. Proteins were detected by Western blot using specific antibodies: C/EBP β (C-19), TFIIB (C-18) (Santa Cruz Biotechnology) and Xpress (Invitrogen).

Protein Expression and Protein-DNA interaction analyses

The recombinant protein GST-Runx2 was obtained by expression in *Escherichia coli* (*E.coli*) BL21 and purified in a glutathione-Sepharose column (GE Healthcare, Pittsburgh, PA, USA) as previously reported (Paredes et al. 2002; Sierra et al. 2003; Paredes et al. 2004). His-C/EBP β was obtained following transformation of *E.coli* BL21 and subsequent purification using a Ni-NTA (QIAGEN Inc.) column, according to the manufacturer's instructions and as described previously (Paredes et al. 2004). The purity and integrity of these proteins were evaluated by SDS/PAGE and by Western blot analysis using specific antibodies (Santa Cruz Biotechnology). The specific DNA binding activity of the bacterially expressed proteins was analyzed as described previously (Paredes et al. 2002) by electrophoretic mobility shift assay (EMSA), using oligonucleotides end-labeled with [γ -³²P]ATP and T4 polynucleotide kinase (For oligonucleotide sequences, see supplementary figure S3). Binding reactions contained 50 ng of purified proteins, 50 fmol of each labeled oligonucleotide, and were performed in 20 μ l of 10 mM Tris/HCl pH 7.4, 100 mM KCl, 1 mM EDTA, 0.05 % Nonidet P40, 0.5 mM PMSF, 1 mM DTT, 10% (v/v) glycerol, 50 μ g/ μ l BSA, 1 μ g/ μ l poly [d(I-C)]. Protein-DNA complexes were fractionated in 3.5% (w/v) polyacrylamide gels (acrylamide:bis-acrylamide ratio 60:1) and 0.5X TBE (1x TBE is 45mM Tris/borate/1mM EDTA), dried and visualized by autoradiography.

Results

Several reports support a critical role for the transcription factor C/EBP β during osteogenesis (Marie, 2008) by controlling the expression of various osteoblast-phenotypic genes (Tominaga et al. 2008; Smink et al. 2009; Gutierrez et al. 2002; Villagra et al. 2006). Sequence analyses of the first 1 kb of the Runx2 gene P1 promoter, reveals four high affinity C/EBP binding sites that are conserved among the mouse, rat, and human genomes (Figure 1A, B). Hence, we first addressed whether these sites contribute to transcription of this osteoblast master regulator in mesenchymal or osteoblast-lineage-committed cells. We performed transient transfection experiments using a 2.8 Kb segment of the Runx2 P1 promoter driving the expression of the Luciferase (Luc) reporter gene and found that forced expression of C/EBP β /LAP* in both mouse mesenchymal C2C12 and pre-osteoblastic MC3T3 cells (Figure 2A, upper and lower graphs, respectively) stimulate Runx2 P1 activity in a dose-dependent manner. These results indicate that C/EBP β /LAP* functions as a positive regulator of the Runx2 P1 promoter in both uncommitted C2C12 cells and in MC3T3 cells that are committed to the osteoblast lineage.

We next determined whether the four C/EBP elements within the first 600 bp of the Runx2 P1 promoter were contributing to the C/EBP β -mediated effect. Using a series of deletion constructs of the rat Runx2 P1 promoter (see Figure 1C), we determined the requirement of the four C/EBP sites for the C/EBP β response. As shown in Figure 1C, these constructs contain either all four C/EBP sites I, II, III and IV (p600P1rRunx2-Luc), sites I, II and III (p490P1rRunx2-Luc), sites I and II (p288P1rRunx2-Luc), or only site I (p108P1rRunx2-Luc). We found that increased expression of C/EBP β in both C2C12 and MC3T3 cells enhances the activity of the P1 promoter deletion constructs that retain the putative C/EBP site II (Figures 3A, 3B and 3C). The shorter construct retaining only the most proximal C/EBP site I responded poorly to the increasing levels of the C/EBP β factor in both cell lines (Figure 3D). The C/EBP β -mediated up-regulation of the Runx2 P1 promoter was also determined in ROS17/2.8 osteoblastic cells (supplementary Figure S1A) although in this case the stimulatory effect was less marked. This result is likely due to the high concentrations of endogenous C/EBP β proteins already present in these cells (supplementary Figure S1A, lower panel and Shin et al. 2006). Together these results indicate that the putative C/EBP site II is a key contributor to the C/EBP β -dependent stimulation of Runx2 gene P1 promoter activity, and most prominently in non-osseous and early progenitor cells.

C/EBP β exists as three protein isoforms which are generated by alternative translation initiation (Nerlov, 2007). The two larger isoforms, C/EBP β -LAP* and C/EBP β -LAP (Figure 4A), have been shown to function principally as activators of gene transcription, while the third shorter C/EBP β -LIP isoform (Figure 4A) can function as a negative regulator (Nerlov, 2007). Hence, we determined whether a specific C/EBP β protein isoform is regulating the Runx2 P1 promoter activity. As shown in Figure 4B, forced expression of either C/EBP β -LAP* or C/EBP β -LAP proteins (see also supplementary Figure S2) equally upregulated Runx2 P1 promoter activity in MC3T3-E1 cells in a dose-dependent manner. This result indicates that the first 21 aminoacid residues at the N-terminus of C/EBP β -LAP* do not significantly contribute to the C/EBP β -mediated stimulation of the Runx2 P1 promoter. Interestingly, co-expression of C/EBP β -LIP (see Figure 4D) inhibited in a dose-dependent manner both C/EBP β -LAP*- and C/EBP β -LAP-enhanced Runx2 P1 promoter activity (Figure 4C). These results are in agreement with the previously described role of C/EBP β -LIP as a negative regulator of transcription (Nerlov, 2007; Ramji and Foka, 2002) and suggest that binding of C/EBP β -LAP* or C/EBP β -LAP to the Runx2 promoter is required during C/EBP β -mediated transcriptional up-regulation of Runx2. Similar results were obtained when these analyses were performed in C2C12 cells (not shown) indicating that C/

EBP β -LAP* or C/EBP β -LAP can stimulate Runx2 P1 promoter activity in both mesenchymal and osteoblast-committed cell lineages.

We next determined whether an intact C/EBP site II was required for C/EBP β -mediated up-regulation of the Runx2 P1 promoter activity. For this purpose, the p288P1rRunx2-Luc reporter construct, containing the C/EBP sites I and II (see Figure 5A), was used as a template to mutate both elements, either independently or in combination, by site-directed mutagenesis (see Methods). Changes in two consensus nucleotides of the C/EBP sites (see Figure 5A), which abolish C/EBP β -binding, were selected based on previous reports (Gutierrez et al. 2002; Chen et al. 2005) and confirmed by protein-DNA interaction analyses (see below). As shown in Figure 5B, mutation of the C/EBP site II (MUT2) significantly, although not completely, inhibits the ability of the Runx2 P1 promoter construct to respond to C/EBP β over-expression, both in C2C12 (Figure 5B, upper graph) and in MC3T3 (Figure 5B, lower graph) cells. In contrast, mutation of C/EBP site I (MUT1) does not affect the ability of C/EBP β factors to stimulate this Runx2 P1 promoter construct (Figure 5B, upper and lower graphs). Similarly, a double mutation of C/EBP sites I and II (MUT1+2) reduces the C/EBP β -mediated up-regulation of the Runx2 P1 promoter to a level equivalent to that obtained when only C/EBP site II is mutated. Together, these results confirm that C/EBP site II is a principal contributor during C/EBP β -mediated activation of the Runx2 P1 promoter, both in mesenchymal and osteoblast-committed lineages.

As shown in Figure 5B, even in the absence of a fully functional C/EBP site II, the p288P1rRunx2-Luc construct exhibits enhanced promoter activity during C/EBP β over-expression. This result may be related to an indirect effect through a different transcription factor that is induced by the forced expression of C/EBP β in the cells. Alternatively, it may be the result of C/EBP β functioning at this Runx2 promoter region through a different regulatory element. Previous reports indicate that C/EBP β can form functional complexes with the transcription factor Runx2 to up-regulate the expression of bone-related genes (Gutierrez et al. 2002; Villagra et al. 2006; Tominaga et al. 2008; Shin et al. 2006). Moreover, these Runx2-C/EBP β -containing complexes can still bind to target promoters in the presence of an intact consensus element for only one of these two factors (Gutierrez et al. 2002).

The p288P1rRunx2-Luc promoter construct contains 3 conserved Runx binding sites (-119/-114; -85/80; and -74/-69, see supplementary Figure S3), which have been shown to be recognized by Runx2 (Drissi et al. 2000 and see Figure 7). We determined whether the presence of the transcription factor Runx2 in the cells is important during C/EBP β -mediated up-regulation of Runx2 P1 promoter activity. The plasmid coding for C/EBP β -LAP* and the p288P1rRunx2-Luc construct (wild-type and with the C/EBP sites mutated) were co-transfected into calvarial-derived osteoprogenitor cells from Runx2 null mice (Bae et al. 2007). Interestingly, we observed that the overall basic activity of these Runx2 P1 promoter constructs is higher in these Runx2-null cells than in both C2C12 and MC3T3 cells. This result suggests that the lack of Runx2 generates a cellular background that is more suitable for Runx2 gene transcription (see Discussion). As shown in Figure 6A, mutation of C/EBP site II (MUT2) results in 50 percent reduction of the p288P1rRunx2-Luc construct basal activity, thus confirming the relevant role of C/EBP site II in Runx2 P1 promoter activity. In contrast, mutation of C/EBP site I (MUT1) produces only a minor effect on the Runx2 P1 promoter activity (~ 25 percent reduction) indicating that in the absence of Runx2, this site contributes partially to maintenance of the overall promoter activity. Importantly, we determined that over-expression of C/EBP β -LAP* stimulates the wild-type basal activity of the p288P1rRunx2-Luc construct as well as the activity of the P1 promoter sequence carrying a mutated C/EBP site I (Figure 6). However, high concentrations of C/EBP β are unable to enhance the activity of the reporter constructs with mutations in the C/EBP site II

(MUT2 and MUT1+2) (Figure 6 and data not shown). Therefore, our results demonstrate that in Runx2-null cells Runx2 P1 promoter activity can also be stimulated by C/EBP β . In addition, we conclude that in the absence of an intact C/EBP site II, the C/EBP β factor is unable to up-regulate Runx2 P1 promoter activity when the transcription factor Runx2 is not present.

To show that C/EBP sites I and II in the Runx2 P1 promoter region are *bona fide* binding sites which can be recognized *in vitro* by C/EBP β , we performed electromobility shift assays (EMSA) using recombinant C/EBP β -LAP (His-tagged C/EBP β -LAP) and radio-labeled oligonucleotide probes including either C/EBP site I (probe site I) or C/EBP site II (probe site II). As shown in Figures 7A and 7B, both probes can be bound *in vitro* by C/EBP β -LAP. This interaction is specific as no retarded band is detected when mutant C/EBP site I or mutant C/EBP site II probes are utilized or when an excess (100 fold) of unlabeled specific oligonucleotide is included in the binding reaction. Similarly, formation of the specific protein-DNA complexes is inhibited when an excess (100 fold) of a unlabeled C/EBP consensus oligonucleotide (Osada et al., 1996; Ramji and Foka, 2002) is included, but not when unlabeled oligonucleotides carrying mutated C/EBP sites I or II are used as competitors.

We next evaluated *in vitro* whether the C/EBP β -LAP and Runx2 factors can recognize simultaneously, their cognate sites located within the most proximal 200 bp of the Runx2 P1 promoter region. For this purpose, we performed EMSA using recombinant his-tagged C/EBP β -LAP and GST-tagged Runx2/p57 produced in bacteria (Paredes et al. 2004) and PCR-generated radio-labeled DNA probes spanning the Runx2 P1 promoter sequences -179/-98 (82 bp probe) and -111/+15 (126 bp probe) (see details in supplementary Figure S3). As shown in Figure 7C, C/EBP β -LAP and Runx2 factors bind both independently and simultaneously to the Runx2 P1 promoter segments including the C/EBP sites I and II and the Runx elements. These results demonstrate that *in vitro*, these C/EBP sites are *bona fide* C/EBP β -binding elements, although site II shows more relevant functional regulatory activity. Our results also confirm previous reports indicating that Runx2 binds *in vitro* to its own P1 promoter (Drissi et al. 2000).

It was important to demonstrate that these two transcription factors interact with the Runx2 gene P1 promoter in the *in vivo* cellular environment of osteoblastic cells expressing Runx2. Hence, we performed chromatin immunoprecipitation (ChIP) analyses and found that both Runx2 and C/EBP β bind to the proximal Runx2 P1 promoter region in ROS17/2.8 osteosarcoma cells (Figure 7D), that actively transcribe the Runx2 gene (Paredes et al. 2004; Sierra et al. 2003; Cruzat et al. 2009). Taken together, these results indicate that C/EBP β can up-regulate transcription of the Runx2/p57 gene by directly binding to the proximal region of the P1 promoter.

Discussion

The C/EBP β transcription factor has been shown to be a key regulator of early and late osteogenesis (Marie, 2008). Here, we find that C/EBP β up-regulates Runx2/p57 expression by direct association with *bona-fide* C/EBP sites at the Runx2 gene P1 promoter sequence in mesenchymal, osteoprogenitor, pre-osteoblastic, and osteoblastic cells. The C/EBP β -mediated enhancement of Runx2 P1 promoter activity is principally, although not exclusively (see below), dependent on C/EBP site II, located within the proximal 180 bp promoter region immediately up-stream of the transcriptional start site. This C/EBP site II is highly conserved among the mouse, rat, and human Runx2 P1 promoter sequences, likely indicating that is also a conserved mechanism to regulate Runx2 transcription during bone cell formation.

In a recent report it was proposed that C/EBP β functions through the most distal C/EBP site IV of the Runx2 P1 promoter to down-regulate Runx2 transcription in uncommitted mesenchymal cells, expressing low levels of C/EBP β factor (Wiper-Bergeron et al. 2007). Although our results appear to contradict these findings, there are a number of reasons that may explain this discrepancy. In their study Wiper-Bergeron and colleagues did not consistently observe the inhibitory effect of C/EBP β on the Runx2 P1 promoter activity. This was the case when mesenchymal cell lines committed to the osteoblastic lineage and expressing C/EBP β were analyzed (Wiper-Bergeron et al. 2007). Several groups, including ours, have shown that osteoblastic cells co-express the Runx2/p57 and C/EBP β genes at high levels (Gutierrez et al. 2002; Shen and Christakos, 2005; Dhawan et al. 2005; Villagra et al. 2006), thus providing support for the idea that both factors function cooperatively during osteoblast differentiation to promote the expression of other key bone-related genes. Additionally, in their report these investigators did not determine whether direct binding of C/EBP β to the Runx2 P1 promoter was occurring under their experimental conditions. This opens the possibility of an indirect repressive mechanism activated by the forced expression of C/EBP β in the cells analyzed in their study. Also, these investigators neither evaluated the contribution of C/EBP sites II and III nor assessed the binding of C/EBP β to these *bona-fide* C/EBP sites. Despite these issues, it is still possible that in the study of Wiper-Bergeron et al. (2007) the arrival at different conclusions about the role of C/EBP β in Runx2 gene transcription may be related to the dissimilar cell backgrounds utilized in their over-expression experiments. Further studies that clarify this point will be required in the future.

TERT-immortalized calvarial cells from Runx2-null mice represent an osteoprogenitor cell model (Bae et al. 2007). We find that these cells not only express high levels of C/EBP β , but also exhibit high Runx2 P1 promoter activity when transiently transfected, further indicating that C/EBP β can function as an activator and not a repressor, of Runx2/p57 gene transcription. Interestingly, our results show that the absence of a functional C/EBP site II reduces proximal P1 promoter activity and abrogates the enhancement in this promoter activity that follows C/EBP β over-expression. This site represents a *bona-fide* C/EBP binding element as shown in our EMSA experiments using both short oligonucleotides carrying wild-type and mutated versions of this sequence and longer segments of the P1 proximal promoter region spanning C/EBP site II. In contrast, although mutation of C/EBP site I (also shown to be recognized *in vitro* by C/EBP β) slightly reduces Runx2 P1 promoter activity, it does not prevent C/EBP β -mediated up-regulation. Therefore, our results support a model in which C/EBP β -enhanced Runx2 transcription occurs principally through C/EBP site II. The fact that a Runx2-null cellular environment results in an apparently higher Runx2 P1 promoter activity may indicate that the transcriptional mechanisms operating through C/EBP β (and perhaps through other transcriptional regulators too) are over-stimulated in these cells, as part of a compensatory response seeking to elevate Runx2 expression. In agreement with this suggestion, it has been reported that this Runx2-null osteoprogenitor cell model responds to forced expression of Runx2 by rapidly becoming engaged in osteoblast differentiation and expressing key bone phenotypic markers (Bae et al. 2007).

Our results show that when cells expressing low (C2C12 and MC3T3) or high (ROS17/2.8 cells) levels of Runx2 protein, are transfected with Runx2 P1 promoter constructs carrying mutated C/EBP sites (e. g. sites I and II in the 288 bp Runx2 P1 promoter construct), these reporter plasmids still retain the ability to at least partially respond to C/EBP β over-expression. Nevertheless, transfection of these same reporter constructs in the osteoprogenitor cell line lacking Runx2 (TERT-immortalized Runx2-null cells), does not result in C/EBP β -mediated enhancement. These results indicate that C/EBP β -mediated transcriptional up-regulation may also partially operate through Runx2 factors bound to the Runx2 P1 promoter. Alternatively, these findings may suggest that C/EBP β -mediated activation of the P1 promoter includes an indirect, yet undetermined component, which is

absent in cells lacking Runx2. In support of the first possibility and in agreement with previous reports (Drissi et al. 2001), we demonstrate here that the Runx2 protein binds to the proximal P1 promoter region both *in vitro* and in intact osteoblastic cells expressing Runx2. Importantly, both C/EBP β and Runx2 factors can simultaneously bind to this P1 promoter region *in vitro*. In addition, we and others have previously shown that C/EBP β can up-regulate osteoblast-related genes during osteogenesis by synergizing with Runx2 (Gutierrez et al. 2002; Hata et al. 2005; Shirakawa et al. 2006; Villagra et al. 2006). Moreover, these Runx2-C/EBP β -containing complexes can still bind target promoters in the presence of an intact consensus element for only one of these two factors (Gutierrez et al. 2002). A potential cooperative mechanism between C/EBP β and Runx2 to up-regulate Runx2 gene expression in osteoblasts is currently being investigated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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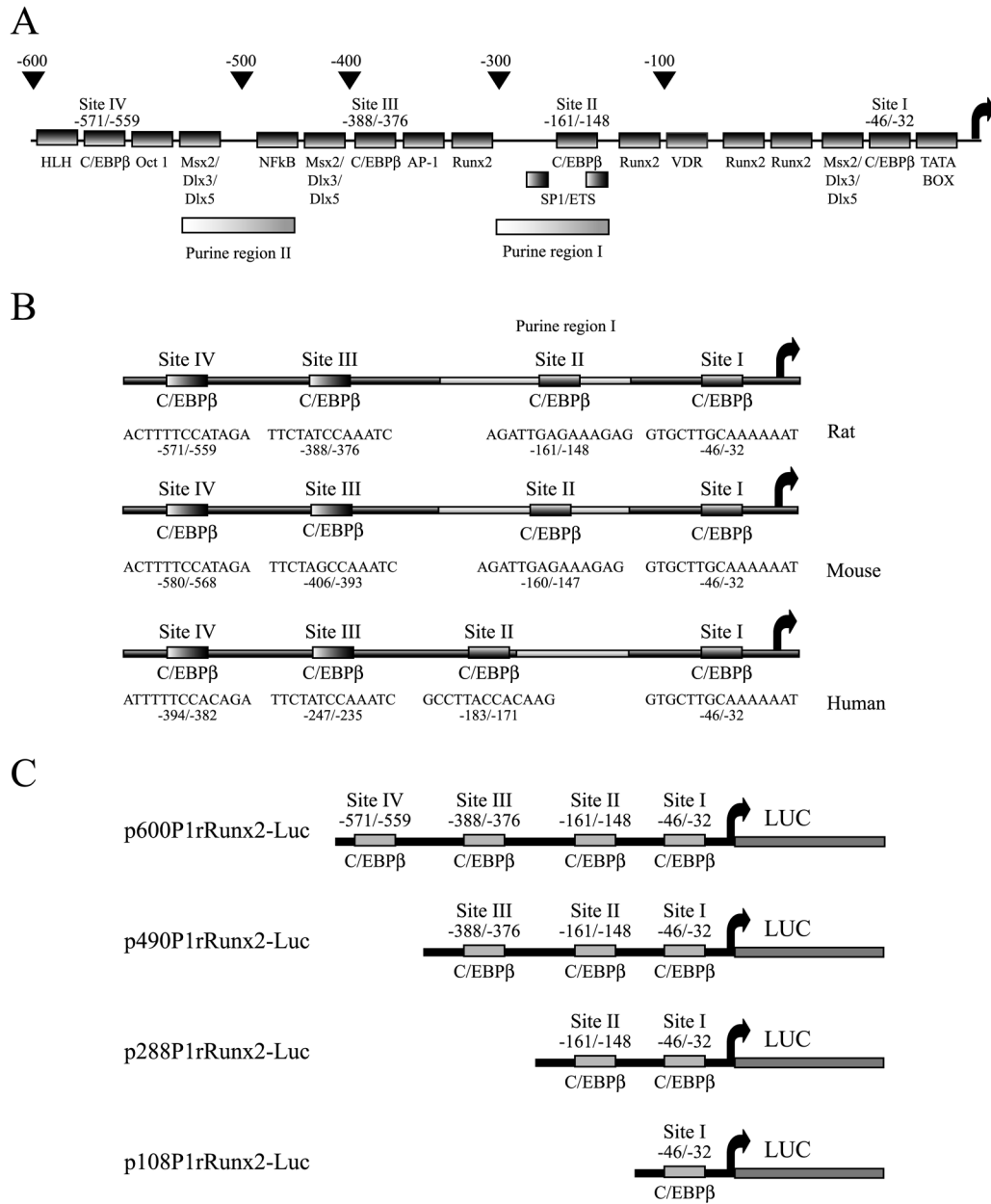


Figure 1. Regulatory sites in the Runx2 P1 promoter

A) Schematic representation of the regulatory elements present in the P1 promoter (first 600 bp) of the Runx2 gene. The diagram shows the positions of the different binding sites for transcription factors and the putative C/EBP sites. B) Comparison of the rat, mouse and Human Runx2 P1 promoters, showing the sequence of the four putative C/EBPβ-interaction sites, which are conserved among all three species. C) Schematic representation of the constructs carrying deletions of the rat Runx2 P1 promoter controlling the expression of the luciferase reporter gene. The presence of the putative C/EBP sites in the different constructs is indicated.

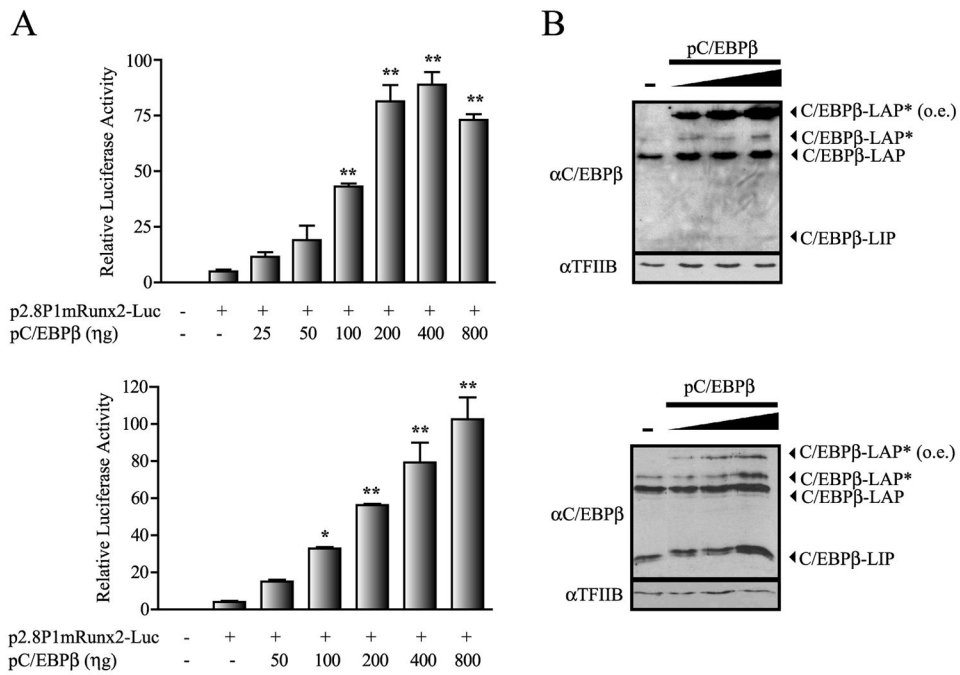


Figure 2. C/EBPβ up-regulates the Runx2 P1 promoter activity

A) C2C12 and MC3T3 cells (upper and lower graphs, respectively) were transiently co-transfected during 24 h with the 2.8 kb mouse Runx2 P1 promoter construct controlling the luciferase reporter gene (500 ηg) and increasing amounts of the pC/EBPβ construct. The presence (+) or absence (-) of each construct is indicated below the bars. The results represent at least three independent experiments, each assayed in triplicate. Each bar represents the mean \pm standard error. Statistical significance was determined by the ANOVA test (*: $p < 0.05$; **: $p < 0.01$). B) Analysis by Western blot of the pC/EBPβ over-expression. Nuclear extracts (10μg) isolated from transiently transfected C2C12 and MC3T3 cells (upper and lower blots, respectively) were analyzed by Western blot using polyclonal antibodies against C/EBPβ. The figure shows the presence of the different endogenous C/EBPβ isoforms and the ectopically over-expressed C/EBPβ (o.e.). Re-blotting against TFIIB was used to control for equal protein loading.

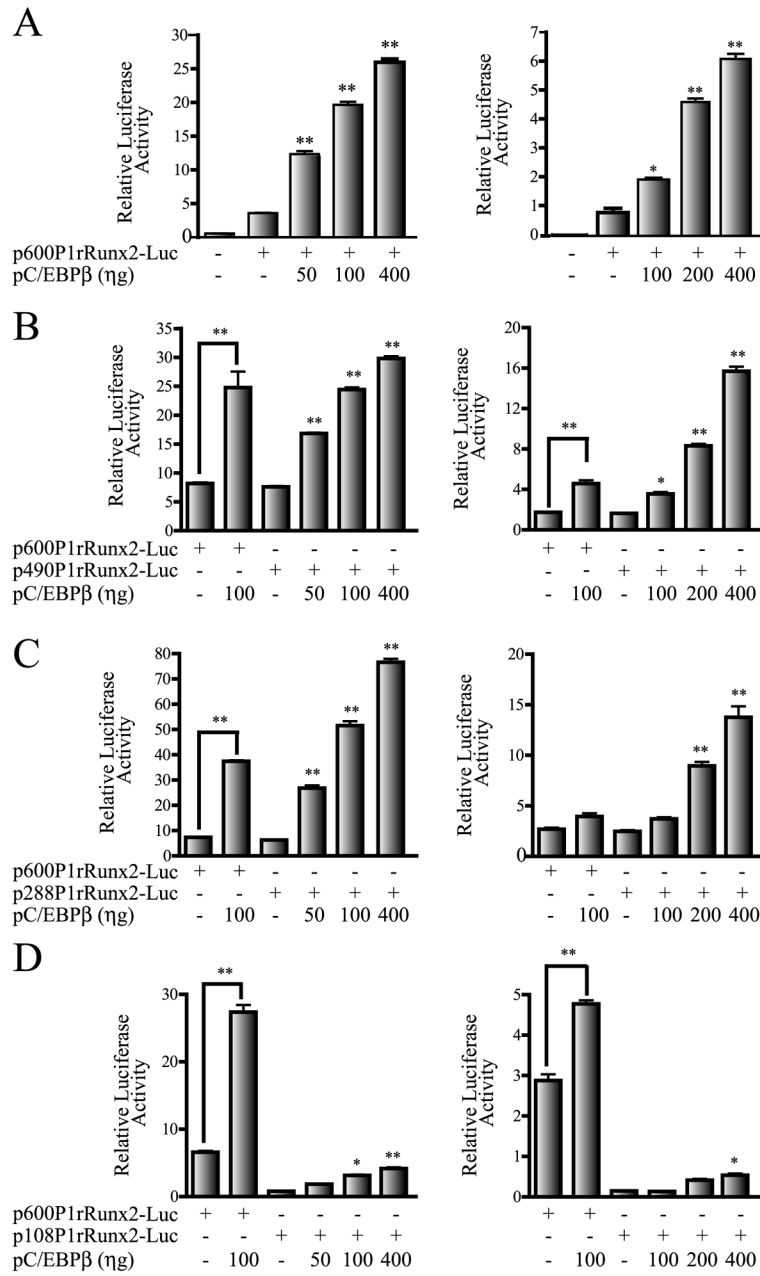


Figure 3. Putative C/EBP site II is a key component in the C/EBPβ-enhanced Runx2 P1 promoter activity

Transient co-transfection experiments were performed in C2C12 and MC3T3 cells (left and right panels, respectively), using luciferase reporter constructs carrying different deletions of the rat Runx2 P1 promoter (500 ng) and increasing amounts of the pC/EBPβ expression plasmid. A) 600 bp rat Runx2 P1 promoter construct, B) 490 bp Runx2 P1 promoter construct, C) 288 bp Runx2 P1 promoter construct, and D) 108 bp Runx2 P1 promoter construct. The presence (+) or absence (-) of each construct in the transfection mix is indicated below the bars. These results represent at least three independent experiments, each assayed in triplicate. Each bar represents the mean +/- standard error. Statistical significance was determined by the ANOVA test (*: p<0.05; **: p<0.01).

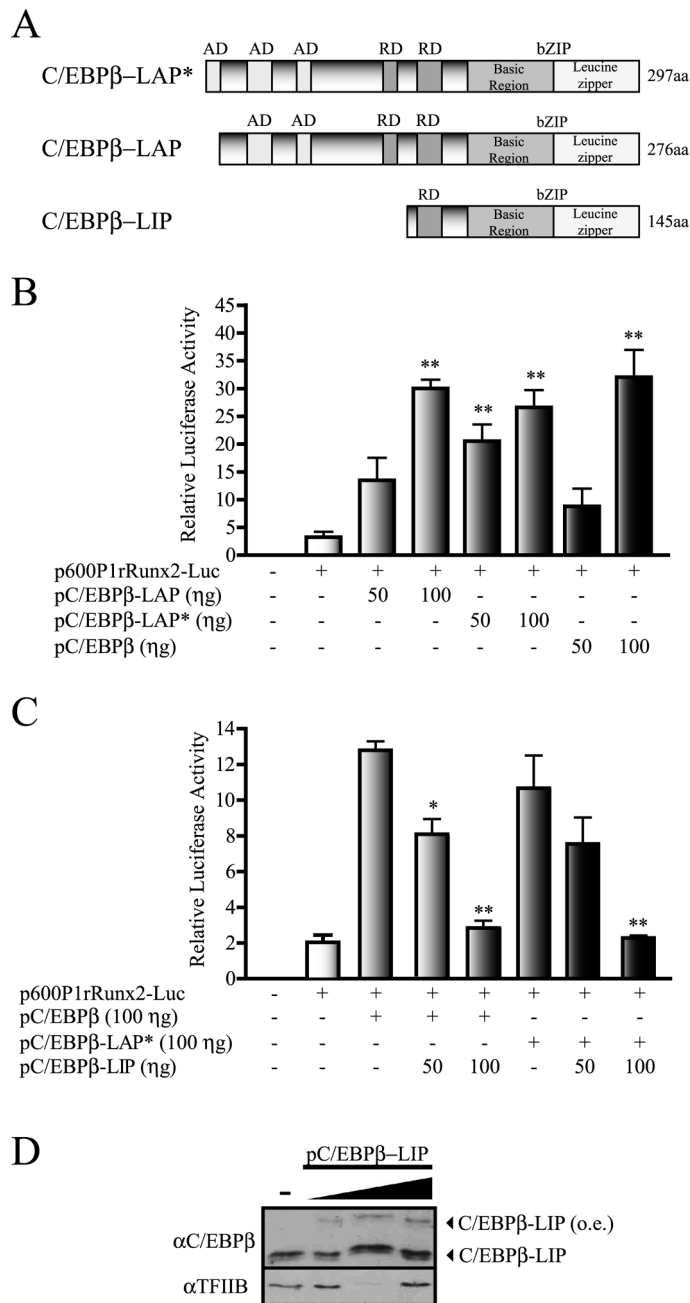


Figure 4. C/EBPβ isoforms LAP* and LAP can up-regulate the Runx2 P1 promoter activity
 A) Schematic representation of the three rat C/EBPβ isoforms (C/EBPβ-LAP*, C/EBPβ-LAP, and C/EBPβ-LIP). The diagram shows the presence of activation domains (AD), repression domains (RD) and the leucine zipper basic domains (bZIP), which support both DNA-binding (basic region) and dimerization (leucine zipper). B) MC3T3 cells were transiently transfected with 250 ng of the 600 bp rat Runx2 P1 promoter construct controlling the luciferase reporter gene and co-transfected with vectors that selectively express the C/EBPβ isoforms LAP*, LAP, or all three LAP*, LAP, and LIP. C) The effect of the selective over-expression of C/EBPβ-LIP on the C/EBPβ-LAP-enhanced Runx2 P1 promoter activity was also determined by transient transfection in MC3T3 cells. These results represent at least three independent experiments, each assayed in triplicate. Each bar

represents the mean \pm standard error. Statistical significance was determined by the ANOVA test (*: $p < 0.05$; **: $p < 0.01$). D) Western blot analysis showing C/EBP β -LIP over-expression in MC3T3 cells. Nuclear extracts (10 μ g) were resolved by SDS-PAGE (12% acrylamide) and revealed using specific antibodies against C/EBP β to detect the over-expressed (o.e.) xpress-tagged C/EBP β -LIP protein. Detection of TFIIIB was used to control for equal protein loading.

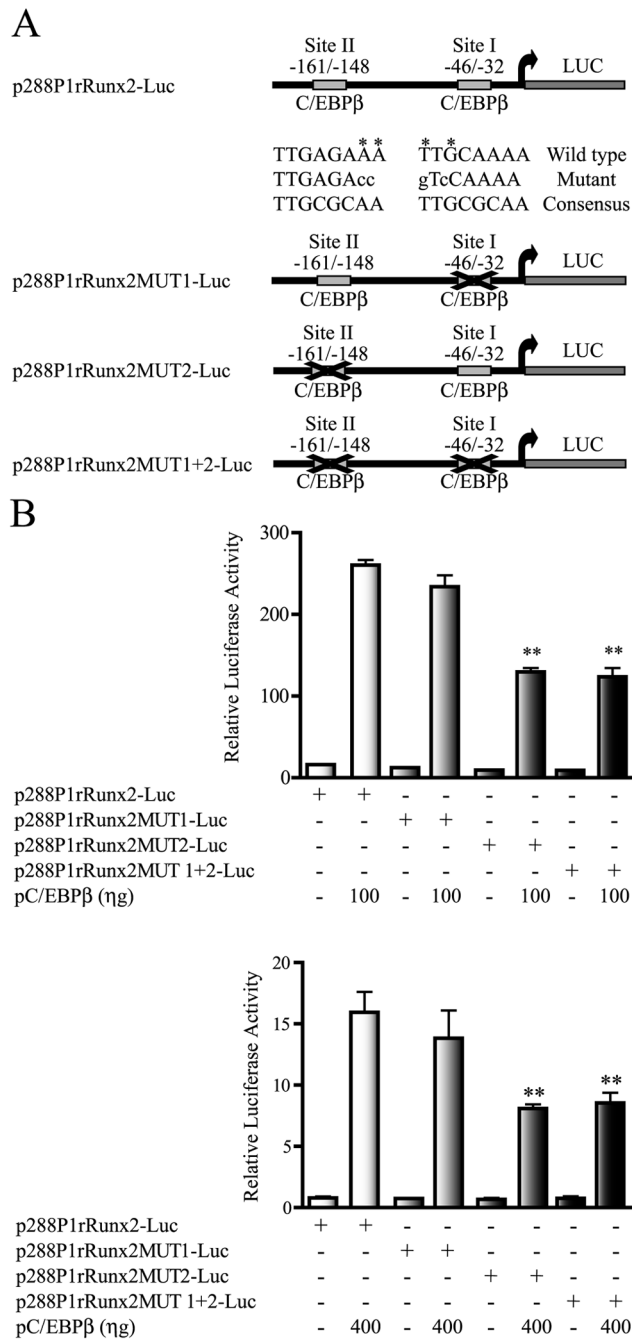


Figure 5. C/EBP site II is a principal component in the C/EBPβ-mediated transcriptional up-regulation of the Runx2 P1 promoter

A) Schematic representation of the wild type and mutated versions of the 288P1rRunx2-Luc reporter constructs. The figure shows the sequences of the putative C/EBP sites I and II present in these reporter constructs. Nucleotides marked with an asterisk were replaced by the nucleotides showed in lowercase letters. B) C2C12 and MC3T3 cells (upper and lower graphs, respectively) were transiently transfected with 250 ng of the reporter constructs containing intact putative C/EBP sites I and II (p288P1rRunx2-Luc), a mutated C/EBP site I (p288P1rRunx2MUT1-Luc), a mutated C/EBP site II (p288P1rRunx2MUT2-Luc), or both C/EBP sites I and II mutated (p288P1rRunx2MUT1+2-Luc). Cells were also co-transfected

with a vector for C/EBP β over-expression (pC/EBP β) in the amounts indicated under each graph. These results represent at least three independent experiments, each assayed in triplicate. Each bar represents the mean \pm standard error. Statistical significance was determined by the ANOVA test (*: $p < 0.05$; **: $p < 0.01$).

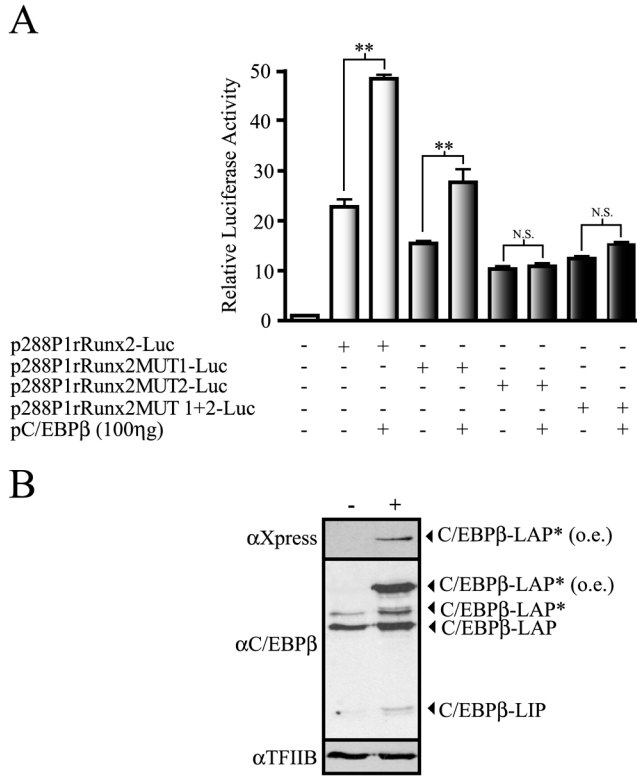


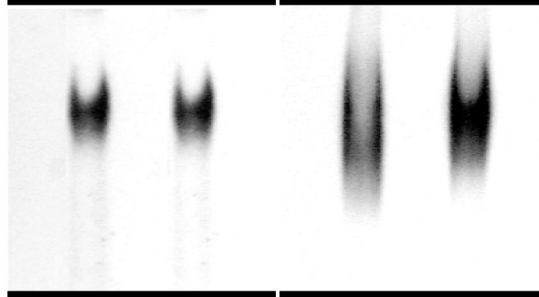
Figure 6. C/EBPβ requires the putative C/EBP site II to up-regulate the Runx2 P1 promoter activity in Runx2-null mice osteoprogenitor cells

A) TERT-immortalized mouse Runx2-null osteoprogenitor cells were co-transfected with 250 ng of the wild type or mutant versions of the p288P1rRunx2-Luc reporter construct and increasing amounts of the C/EBPβ expression vector (pC/EBPβ). Each bar represents the mean \pm standard error. Statistical significance was determined by the ANOVA test (*: $p < 0.05$; **: $p < 0.01$; N.S. not statistically significant). B) Western blot analysis of the mouse Runx2-null cells transfected with the pC/EBPβ vector. Nuclear extracts (10 μ g) were fractionated by SDS-PAGE (12% acrylamide) and revealed by Western blot using specific antibodies against C/EBPβ or the Xpress-tag to detect the over-expressed Xpress-tagged C/EBPβ/LAP* (o.e.). Detection of TFIIB was used to control for equal protein loading.

A

Competitor MUT2	-	-	-	-	-	-	-	-	+	-
Competitor MUT1	-	-	-	+	-	-	-	-	-	-
Competitor II	-	-	-	-	-	-	-	+	-	-
Competitor I	-	-	+	-	-	-	-	-	-	-
Probe MUT2	-	-	-	-	-	-	-	-	-	+
Probe II	-	-	-	-	-	+	+	+	+	-
Probe MUT1	-	-	-	-	+	-	-	-	-	-
Probe I	+	+	+	+	-	-	-	-	-	-
His-C/EBPβ	-	+	+	+	+	-	+	+	+	+

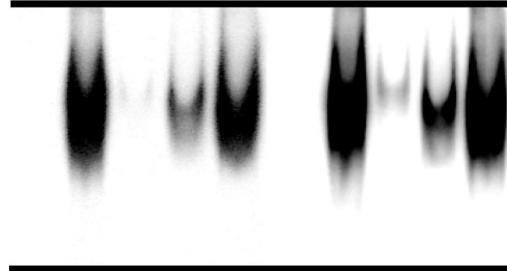
DNA-C/EBPβ ▶



B

Competitor MUT2	-	-	-	-	-	-	-	-	-	+
Competitor II	-	-	-	-	-	-	-	-	+	-
Competitor MUT1	-	-	-	-	+	-	-	-	-	-
Competitor I	-	-	-	+	-	-	-	-	-	-
Competitor Consensus	-	-	+	-	-	-	-	+	+	-
His-C/EBPβ	-	+	+	+	+	-	+	+	+	+
Consensus Probe	+	+	+	+	+	+	+	+	+	+

DNA-C/EBPβ ▶



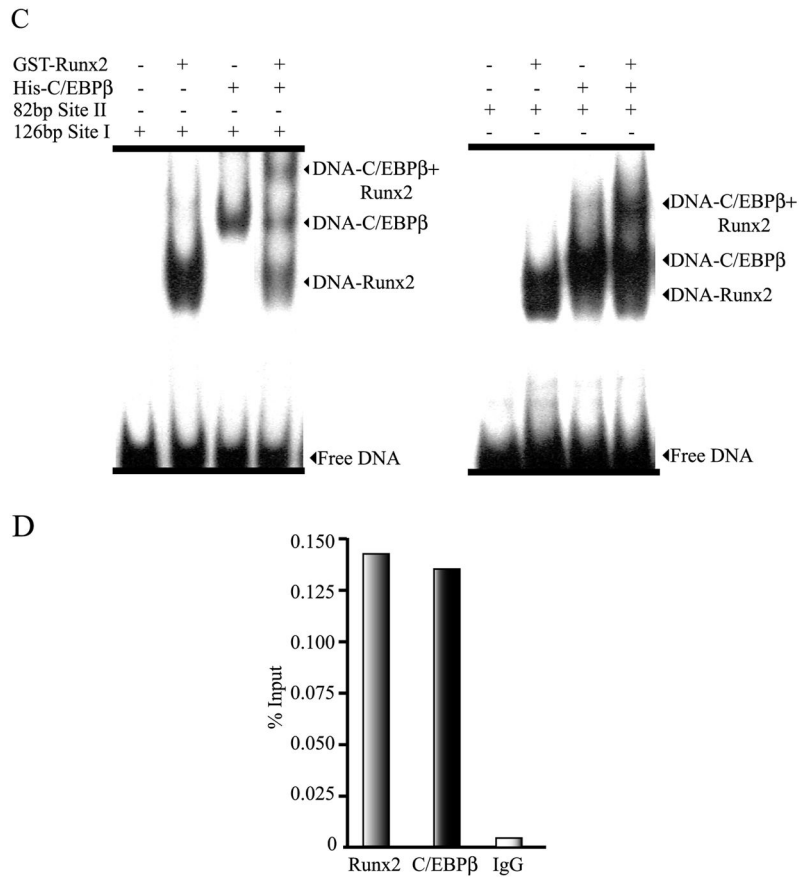


Figure 7. C/EBPβ and Runx2 factors interact independently and simultaneously with the Runx2 P1 promoter *in vitro* and in intact osteoblastic cells

The ability of C/EBPβ and Runx2 recombinant proteins to interact specifically with their cognate binding sequences in the P1 promoter was evaluated by EMSA as described in materials and methods. The combinations used in each binding reaction are indicated at the top of the gels. The positions of the specific protein-DNA complexes are also marked. A) Binding of purified His-C/EBPβ protein to the radio-labeled oligonucleotides (Probe) containing the C/EBP site I (left panel) or C/EBP site II (right panel). Competition assays were performed by adding an excess (100 fold) of the cold-specific oligonucleotide (competitor). B) Binding of His-C/EBPβ protein to a radio-labeled consensus C/EBP element (consensus probe) and competition assays using an excess of cold-oligonucleotides (competitor) containing either the wild-type sequence for C/EBP site I, C/EBP site II, or their mutated versions (MUT 1 and MUT2, respectively). C) Binding of purified His-C/EBPβ and GST-Runx2 proteins to radio-labeled Runx2 P1 promoter fragments containing the C/EBP site I (left panel) or C/EBP site II (right panel). D) Binding of endogenous C/EBPβ and Runx2 factors to the proximal Runx2 gene P1 promoter sequences in ROS17/2.8 cells, assessed by chromatin immunoprecipitation (ChIP). The enrichment of the Runx2 P1 promoter sequences in the precipitated DNA was determined by PCR amplification using specific primers and normalized to input material. IgG: non-specific immunoglobulin G.