Journal of Cellular Biochemistry 74:596–605 (1999)

Cbfa1 Isoform Overexpression Upregulates Osteocalcin Gene Expression in Non-osteoblastic and Pre-osteoblastic Cells

Z.S. Xiao, T.K. Hinson, and L.D. Quarles*

Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710

Abstract The mouse *Cbfa1* gene potentially encodes several proteins that differ in their N-terminal sequences, including an osteoblast-specific transcription factor, *Cbfa1/Osf2*, a *Cbfa1* isoform (*Cbfa1/iso*), and the originally described *Cbfa1* gene product (*Cbfa1/org*). Uncertainty exists about the function of these potential isoforms of the *Cbfa1* gene. To examine the transactivation potential of different *Cbfa1* gene products, we compared the ability of *Cbfa1/Osf2*, *Cbfa1/iso*, and *Cbfa1/org* overexpression to activate an osteocalcin promoter/reporter construct in NIH3T3 fibroblasts, C3H10T1/2 pluripotent cells and MC3T3-E1 pre-osteoblasts. These three cell lines were transiently cotransfected with a 1.3-kb mouse osteocalcin promoter luciferase-fusion construct (p1.3OC-luc) and different amounts of expression vectors containing the respective full-length *Cbfa1* isoform cDNAs. Using transfection protocols with lower amounts of expression plasmid DNAs, we found that all three *Cbfa1* isoforms stimulated osteocalcin promoter activity in each of the cell types, consistent with the their ability to induce expression of an osteoblast-specific gene both in non-osteoblast cells and in osteoblast cell lines. However, using transfection protocols with higher amounts of expression plasmids containing *Cbfa1* cDNAs, we found that the *Cbfa1/Osf2* and *Cbfa1/org* had less transactivating potential compared with *Cbfa1/iso*. Our studies suggest that the 87-amino acid N-terminus of *Cbfa1/Osf2* is not crucial for optimal transactivation, whereas the 19-amino acid N-terminal sequence of *Cbfa1/iso* augments transcriptional activation only at high doses of the expression plasmid. The physiological significance of these in vitro findings remain to be determined. J. Cell. Biochem. 74:596–605, 1999.

Key words: transcription factors; *Cbfa1*; *Osf2*; osteoblasts

The *Cbfa1* (core-binding factor) gene, also referred to as PEBP2αA (polyomavirus enhancer-binding protein), and AML-3 (acute myelogenous leukemia) encodes a transcription factor that plays a critical role in osteoblastic differentiation [Komori et al., 1997]. *Cbfa1* gene products form complexes that bind to consensus *cis*-acting OSE2 elements in genes that are coordinately regulated during osteoblast maturation [Banerjee et al., 1997; Geoffroy et al., 1995; Merriman et al., 1995]. The essential role of *Cbfa1* in osteoblast differentiation is supported by the failure of endochondral and membranous bone formation after targeted disruption of the *runt* domain of the *Cbfa1* gene in mice [Komori et al., 1997; Otto et al., 1997]. In addition, inactivating mutations of human *CBFA1* are the cause of cleidocranial dysplasia (CCD), an autosomal-dominant condition characterized by abnormal skeletal genesis and the arrest of osteoblast development [Mundlos et al., 1997].

The *CBFA1/Cbfa1* gene comprises at least 9 exons [Mundlos et al., 1997; Ahn et al., 1996; Xiao et al., 1998a], referred here as exons 1–9 (Fig. 1A). The tissue-specific use of different translational start sites in the mouse gene potentially gives rise to at least three isoforms that differ in their N-terminal sequences and possible transactivation potentials. The originally described *Cbfa1* cDNA (*Cbfa1/org*, also referred to as *Cbfa1*/p56) uses a translation start site in exon 3 (previously referred to as exon 1) [Ogawa et al., 1993; Satake et al., 1995; Mundlos et al., 1997]. *Cbfa1/org*, which was originally thought to be a T-cell-specific gene, is

Grant sponsor: National Institutes of Health; Grant number: R01-AR37308; Grant number: R01-AR43468; Grant sponsor: National Institute of Arthritis and Musculoskeletal and Skin Diseases.

*Correspondence to: L.D. Quarles, Duke University Medical Center, P.O. Box 3036, Durham, NC 27710. E-mail: quarl001@mc.duke.edu

Received 28 December 1998; Accepted 5 March 1999

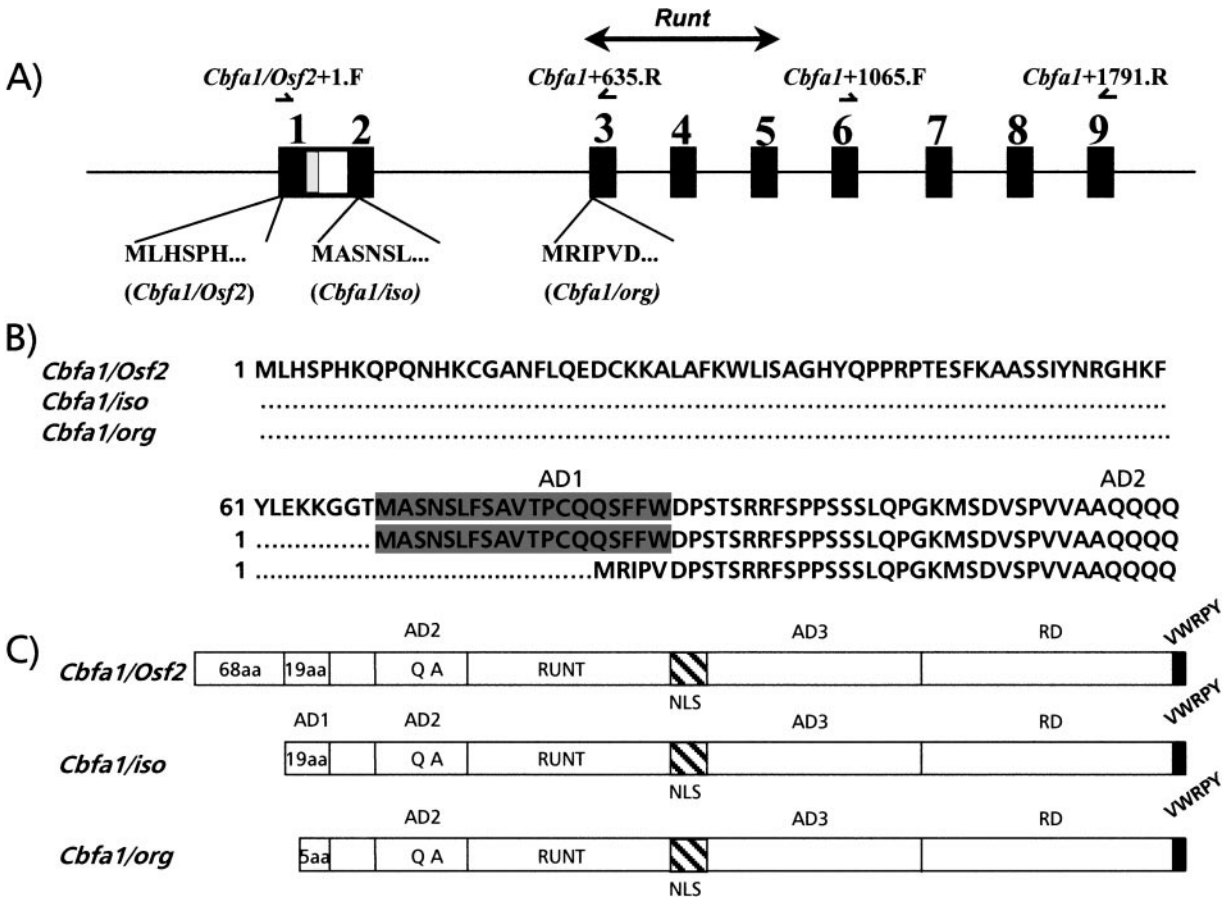

Fig. 1. Three potential N-terminal isoforms derived from the *Cbfa1* gene. **A:** Structure of the *Cbfa1* gene. Exons 1 and 2 are separated by a short intronic segment (mini-intron), whereas exon 2 is separated from exon 3 by a large intronic region [Xiao et al., 1998a; Geoffrey et al., 1998]. The unique *Cbfa1*/*Osf2* isoform utilizes the translation initiation site in exon 1. The *Cbfa1* isoform (*Cbfa1/iso*) is generated from an alternative translational start site located in exon 2. The start site for the original *Cbfa1* (*Cbfa1/org*) gene product that begins with the amino acid sequence MRIPVD is in exon 3 (formerly called exon 1). *Arrows*, locations of some of the primer pairs used to amplify *Cbfa1*. **B:** Comparison of the deduced amino acid sequences of the 5′ end of the *Cbfa1* gene products. The 513-amino acid *Cbfa1/org* protein starts with the 5-amino acid N-terminal sequence "MRIPV." The 528-amino acid *Cbfa1*/*Osf2* protein has a novel 87-amino acid N-terminus. The 528-amino acid *Cbfa1/iso* begins with a 19-amino acid N-terminus that is homologous to an overlapping region of *Cbfa1*/*Osf2*. Beginning with the sequence "DPSTSRR..." the 3′ ends of the respective *Cbfa1* gene products are identical. **C:** Schematic representation of the transactivation domains of the three *Cbfa1* expression constructs. *Cbaf1*/*Osf2* and *Cbfa1/iso* share a 19-amino acid activation domain (AD1), but *Cbfa1*/*Osf2* has an additional 68-amino acid N-terminal sequence [Thirunavukkarasu et al., 1998], whereas *Cbfa1/org* lacks AD1. The *runt* domain, AD2, AD3, and RD are conserved between the three isoforms. AD, activation domain; RD, repression domain; NLS, nuclear localization signal.

predicted to encode a 513-amino acid protein that begins with the amino acid sequence MRIPVD [Ogawa et al., 1993; Satake et al., 1995; Mundlos et al., 1997] (Fig.1A,B). Alternative translation start sites in exons 1 and 2 (previously referred to as exons −1 and 0) encode two additional isoforms with different N-terminal sequences [Xiao et al., 1998a] (Fig. 1A,B). The *Cbfa1* isoform (*Cbfa1/iso*, also referred to as *Cbfa1*/p57) is encoded by exon 2 and contains 19 additional amino acids at its N-terminus, beginning with the sequence MASNSL [Stewart et al., 1997; Mundlos et al., 1997]. A novel *Cbfa1*/*Osf2* isoform [Ducy et al., 1997] derived from exon 1 contains an additional 87 amino acids that begins with the N-terminal amino acid sequence MLHSPH (Fig. 1A,B). This *Cbfa1*/*Osf2* isoform was originally designated as the osteoblast-specific gene product responsible for *Cbfa1* gene function. The remaining exons 3–9 encode the putative ATP binding site, the glutamine/alanine-rich domain, the *runt* domain region, two proline, serine, threonine rich regions, and a nuclear ma-

trix target site that is common to all three isoforms [Zeng et al., 1997; Bae et al., 1994](Fig. 1C). The presence of multiple isoforms of the *Cbfa1* gene is consistent with other members of the CBFA/Cbfa transcription superfamily, including *Cbfa2* and *Cbfa3*, that exist as multiple isoforms with different transactivation potentials [Bae et al., 1994; Zeng et al., 1997].

Uncertainty exists regarding which of the *Cbfa1* gene products regulate osteoblast-specific gene expression. The originally described *Cbfa1/org* gene product beginning with the amino acid sequence MRIPVD is not expressed in osteoblasts [Xiao et al., 1998a; Geoffroy et al., 1998] and to date has only been identified in T cells [Ogawa et al., 1993; Satake et al., 1995]. Nevertheless, forced overexpression of *Cbfa1/org* paradoxically inhibits type I collagen expression in MC3T3- E1, C2C12 cells, C3H10T1/2, and osteocalcin gene expression ROS 17/2.8 [Tsuji et al., 1998]. In other studies, overexpression of *Cbfa1/org* stimulates osteopontin expression and transforming growth factor-β (TGF-β) receptor promoter activity in osteoblast cell lines [Tsuji et al., 1998; Ji et al., 1998]. Still, other studies suggest the novel *Cbfa1/Osf2* N-terminal sequence is essential for osteoblast-specific gene expression. In this regard, Ducy et al. [1997] found that forced expression of the unique *Cbfa1*/*Osf2* N-terminal sequence, encoding the additional 87-amino acid *Osf2* N-terminal sequence, upregulates osteoblast-specific genes in non-osteoblastic cells in vitro. Recent data showing the inefficient translation of the *Cbfa1*/*Osf2* isoform in osteoblasts [Thirunavukkarasu et al., 1998] and the absence of a conserved open reading frame (ORF) in the human *CBFA1* gene [Xiao et al., 1998a] raise questions about the relevance of this isoform. Indeed, the osteoinductive potential may not be limited to the unique *Cbfa1*/*Osf2* N-terminal sequence, as overexpression of the truncated *Cbfa1/iso* also stimulates osteoblast-specific gene expression [Thirunavukkarasu et al., 1998].

To date, however, the function of the three isoforms of the *Cbfa1* gene have not been directly compared in the same experimental system. In the current study, we evaluated the effect of forced expression of the three *Cbfa1* N-terminal isoforms on osteocalcin promoter activity in pluripotent C3H10T1/2 cells, MC3T3-E1 pre-osteoblasts and NIH3T3 fibroblasts.

MATERIALS AND METHODS

Constructs

The full-length *Cbfa1/iso* cDNA (Fig. 1C) was generated by reverse transcription-polymerase chain reaction (RT-PCR) amplification, using the forward primer *Cbfa1*/*Osf2* +205.F (5′-ATGGCGTCAAACAGCCTCTTCAGCGCAG-3′) and reverse primer *Cbfa1* +1791.R (5′-TCAATATGGCCGCCAAACAGACTCATCC-3′) from total RNA derived from MC3T3-E1 osteoblasts. The resulting product was cloned into pCR2.1 vector (Invitrogen, Carlsbad, CA) and sequenced to confirm the amplification of the full-length *Cbfa1/iso* sequence. The full-length *Cbfa1/iso* cDNA was subcloned into the *Nhe*I-*Xho*I site of the pcDNA3.1 expression vector (Invitrogen).

The full-length *Cbfa1/org* that contains a different 5-amino acid N-terminal sequence (Fig. 1C) was generated by XL-PCR (Perkin-Elmer, Norwalk, CT), using the forward primer 5′-ATGCGTATTCCTGTAGATCCGAGCACCAG-3′ that contains the 15 nucleotides encoding these 5 amino acids and the reverse primer *Cbfa1* +1791.R. The PCR product was initially cloned into pCR2.1 vector, sequenced, and later subcloned into the *Nhe*I-*Xho*I site of the pcDNA3.1 expression vector. The full-length bicistronic *Cbfa1*/*Osf2* cDNA (Fig. 1C) was generated by ligating an N- terminal cDNA encoded by exons 1 and 2 with the remaining 3′ end of the *Cbfa1/iso* cDNA encoding exons 3–9. First, the 5′-end of the cDNA (encoded by exons 1, 2, and 3; see Fig. 1) was generated by RT-PCR amplification of total RNA derived from MC3T3-E1 osteoblasts using the forward primer *Cbfa1*/*Osf2* +1.F (5′-ATGCTTCATTCGCCTCACAAACAACCAC-3′) and the reverse primer *Cbfa1* + 647.R (5′-TTGAAGGCCACGGGCAGGGTCTTGTTGCAC-3′). The resulting product was cloned into pCR2.1 and sequenced. Next, to generate the pcDNA3.1-*Cbfa1*/*Osf2* expression construct, the 5′-end cDNA (exon 1, 2, and 3) of *Cbfa1*/*Osf2* was reamplified by XL-PCR using the primers (5′-AAGCTGGCTAGCATGCTTCATTCGCCTCAC-3′) and (5′-TTGAAGGCCACGGGCAGGGTCTTGTTGCAC-3′) that contained restriction enzyme sites at the respective 5′ and 3′ ends. The resulting XL-PCR product was double-digested with restriction enzyme *Nhe*I-*Bam*HI and ligated into *Nhe*I-*Bam*HI double-digested pcDNA3.1-*Cbfa1/iso* construct. The XL-PCR conditions used in all

amplifications were as follows: denaturation at 95°C for 30 s, annealing and extension at 68°C for 5 min for 30 cycles, and all templates were amplified in the presence of 1.1 M betaine or 10% dimethylsulfoxide (DMSO) [Kureishi et al., 1992]. All three pcDNA 3.1 *Cbfa1* constructs were confirmed by restriction enzyme digest and by sequencing of their 5′ ends.

Cell Culture

The C3H10T1/2 cell line was grown in basal medium Eagle (Gibco-BRL, Gaithersberg, MD) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), the MC3T3-E1 cell line was grown in α-MEM (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS), and the NIH3T3 cells were grow in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) supplemented with 10% FBS. All growth media contained 100 U/ml penicillin and streptomycin, and the cells were incubated in a humidified incubator with 5% CO_2 at a temperature of 37°C.

Transient Transfection

These cell lines were respectively cotransfected with three constructs: (1) pcDNA 3.1 expression vector containing the *Cbfa1* isoform of interest (0.1–2.0 μg), (2) 1.3-kb mouse osteocalcin promoter luciferase-fusion plasmid (p1.3OC-luc; 1.0 μg), and (3) pSV β-gal plasmid (0.5 μg), using a Lipofectamine (Gibco-BRL) protocol. Carrier DNA was added to achieve equivalent amounts of total added DNA in each transfection experiment. 2×10^5 cells were plated in a 60-mm dish and were incubated for 12 h at 37°C in a CO_2 incubator. The cells were then rinsed twice and were transfected with the DNA-liposome complexes for 5 h in serum-free medium combined with 6 μl of Lipofectamine and culture for an additional time periods for 19–67 h in complete growth medium. For the control, the cells were transfected with an empty expression vector, using Lipofectamine. Luciferase activity was measured using the luciferase assay kit (Promega, Madison, WI) according to the manufacturer's protocol, using a Optocom I luminometer (MGM Instruments, Cambridge, MA). β-Galactosidase activity was measured with a commercial kit (Promega, Madison, WI), using a colorimetric assay, and total protein content was determined with a Bio-Rad Protein assay kit (Bio-Rad, Hercules, CA) based on the Bradford method using bovine serum albumin (BSA) as the standard [Bradford, 1976]. The luciferase activity was normalized by the β-galactosidase activity and the amount of protein. To obtain the relative luciferase activity the normalized value was divided by that obtained with cotransfection of the pcDNA 3.1 empty expression vector and p1.3OC-luc.

Northern Blot

Total cellular RNA was isolated by a single-step RNA isolation method using TRIzol reagent (Life Technologies, Baltimore, MD). Northern blot analysis was carried out as previously described [Guo et al., 1997; Xiao et al., 1998b]. Briefly, 15 μg of total RNA was fractionated in a 1.2% formaldehyde agarose gel, transferred to Nytran Plus membrane and the membrane was exposed in an ultraviolet (UV) crosslinker for 2 × 1,200 μJ. Hybridization was performed according to the northern hybridization protocol (5 Prime → 3 Prime Inc, Boulder, CO). The membrane was incubated for 4 h at 42°C in a pre-hybridization working solution at a ratio of 1 ml 2× pre-hybridization buffer: 1 ml of deionized formamide: 10 μl of 10 mg/ml denatured salmon sperm DNA : 5 μl of 20 mg/ml denatured yeast RNA. Labeled *Cbfa1*/*Osf2*-specific probe (exons 1 and 2, 2×10^6 cpm/ml) was added in a hybridization working solution at a ratio of 2 ml 2× hybridization buffer: 2 ml of deionized formamide : 20 μl of 10 mg/ml denatured salmon sperm DNA: 10 μl of 20 mg/ml denatured yeast RNA : 1 ml 50% dextran sulfate. The membrane was hybridized overnight at 42°C. The blot was washed in 2× SSC/0.1% sodium dodecyl sulfate (SDS) at room temperature for 30 min, followed by a wash with 0.1× SSC/0.1% SDS at 50°C for 30 min. The membrane was then exposed with an intensifying screen to X-ray Biomax film (Kodak, Rochester, NY) for 3 days at −80°C.

Reverse Transcription-Polymerase Chain Reaction

RT-PCR was done using the Titanô One Tube RT-PCR kit purchased from Boehringer-Mannheim (Indianapolis, IN). The reverse transcription reaction using 1.0 μg of total RNA treated with DNase I (Stratagene, La Jolla, CA) was incubated at 50°C for 30 min. PCR was performed with thermal cycling parameters of 94°C for 30 s, 60°C for 30 s, and 68°C for 45 s for the indicated number of cycles, followed by a final extension at 68°C for 7 min. To amplify the

Cbfa1/org [Ogawa et al., 1993] containing the original *Cbfa1* 5′ untranslated sequence, we designed forward primer *Cbfa1*/5′UTR.F (5′CACTTTGCAAAGAGCAGGAG3′) and reverse primer *Cbfa1*/5′UTR.R (5′AGCCACAAGTTAGCGAAGTG3′). To amplify the *Cbfa1*/*Osf2* containing the unique *Cbfa1*/*Osf2* 5′-end sequence, we used forward primer *Cbfa1*/*Osf2* +1.F (5′ATGCTTCATTCGCCTCACAAAC3′) in combination with the reverse primer *Cbfa1*+ 655.R (5′CTACAACCTTGAAGGCCACG3′) (Fig. 1A). To amplify the 3′ end of the *Cbfa1* transcript we used primers *Cbfa1*+1065.F (5′AGGACAGAGTCAGATTACAG3′) and *Cbfa1*+1791.R (5′TCAATATGGCCGCCAAACAG3′) (Fig. 1A). Primers for mouse β-actin were used to amplify a 245-bp β-actin fragment to control for the amount and integrity of RNA in the RT-PCR reactions [Guo et al., 1997; Xiao et al., 1998b].

Statistics

One-way analysis of variance (ANOVA) was performed with each isoform as a function of plasmid dose. Values sharing a superscript within an isoform group are not different at $P < 0.05$. Values represent the mean ±SEM of a minimum of three separate experiments.

RESULTS

Endogenous Expression of *Cbfa1* Transcripts and Basal Osteocalcin Promoter Activity in NIH3T3, C3H10T1/2, and MC3T3-E1 Cells

Initially, we performed Northern blot and RT-PCR analysis to characterize the expression of the 5′ ends of the various *Cbfa1* gene transcripts in NIH3T3 fibroblasts, pluripotent C3H10T1/2, and MC3T3-E1 pre-osteoblasts. We failed to detect any *Cbfa1* messenger RNA (mRNA) transcripts in NIH3T3 fibroblasts by either Northern or RT-PCR analysis under the study conditions (Fig. 2). By contrast, we observed high expression of the predicted size mRNA transcript containing the 5′ end of the *Cbfa1* gene that encodes the unique *Cbfa1*/*Osf2* and *Cbfa1/iso* sequences in MC3T3-E1 pre-osteoblasts. This same transcript was also identified in C3H10T1/2 pluripotent cells, but at lower levels. Consistent with prior studies [Xiao et al., 1998a], we were unable to detect a mRNA transcript corresponding to the 5′ end of *Cbfa1/org* by RT-PCR in either NIH3T3, C3H10T1/2, or MC3T3-E1 osteoblasts (data not shown).

We also assessed basal osteocalcin promoter activity in these three cell lines. Consistent with their non-osteoblast phenotype, NIH3T3 fibroblasts had lower promoter activity (12, 502 ± 551 light U/μg protein/β-galactosidase) compared with osteocalcin promoter activity in C3H10T1/2 cells (41,966 ± 1,399 light U/μg protein/β-galactosidase), which have the capacity to differentiate into osteoblasts [Tsuji et al., 1998], and in pre-osteoblastic MC3T3-E1 (30,448 ± 1,871 light U/μg protein/β-galactosidase), which have low levels of osteocalcin transcripts [Quarles et al., 1997].

Effect of Transient Overexpression of *Cbfa1* Isoforms on Osteocalcin Promoter Activity in C3H10T1/2 Cells

We measured osteocalcin promoter activity as a specific and sensitive way to monitor osteoblast-specific gene expression in cells transfected with expression plasmids containing the various *Cbfa1* isoforms [Frendo et al., 1998]. The magnitude of the osteocalcin reporter gene response to the three transfected *Cbfa1* N-terminal isoforms in pluripotent C3H10T1/2 cells depended on the amounts of transfected mammalian expression vectors, the particular isoform, and the time after transfection (Fig. 3A). Optimal transactivation was observed after 48 h and was sustained for the 72-h culture period (Fig. 3B). *Cbfa1*/*Osf2* and *Cbfa1/org* acted as partial transactivators, achieving maximal induction at a plasmid dose of 0.5 μg, but a paradoxical relative suppression at higher plasmid amounts (Figs. 3, 4A). By contrast, transfection with *Cbfa1/iso* resulted in a dose-dependent induction of the osteocalcin promoter, achieving maximal stimulation only at a plasmid dose of 2.0 μg (Figs. 3, 4A). At the higher amounts of the respective transfected constructs, the inductive effects of the *Cbfa1/iso* construct was more pronounced in C3HT101/2, due both to its greater stimulation of the osteocalcin promoter and to a relative repression in the stimulation observed with the high doses of the other isoforms lacking AD1 (Fig. 4A). The amount of plasmid used in the transfection experiments resulted in corresponding differences in *Cbfa1* transcripts measured by RT-PCR. In this regard, increment in the expression of the 3′ end of *Cbfa1* transcripts was small after transfection with 0.5 μg of the expression plasmid, but a more pronounced increment in *Cbfa1* transcripts was observed with cells trans-

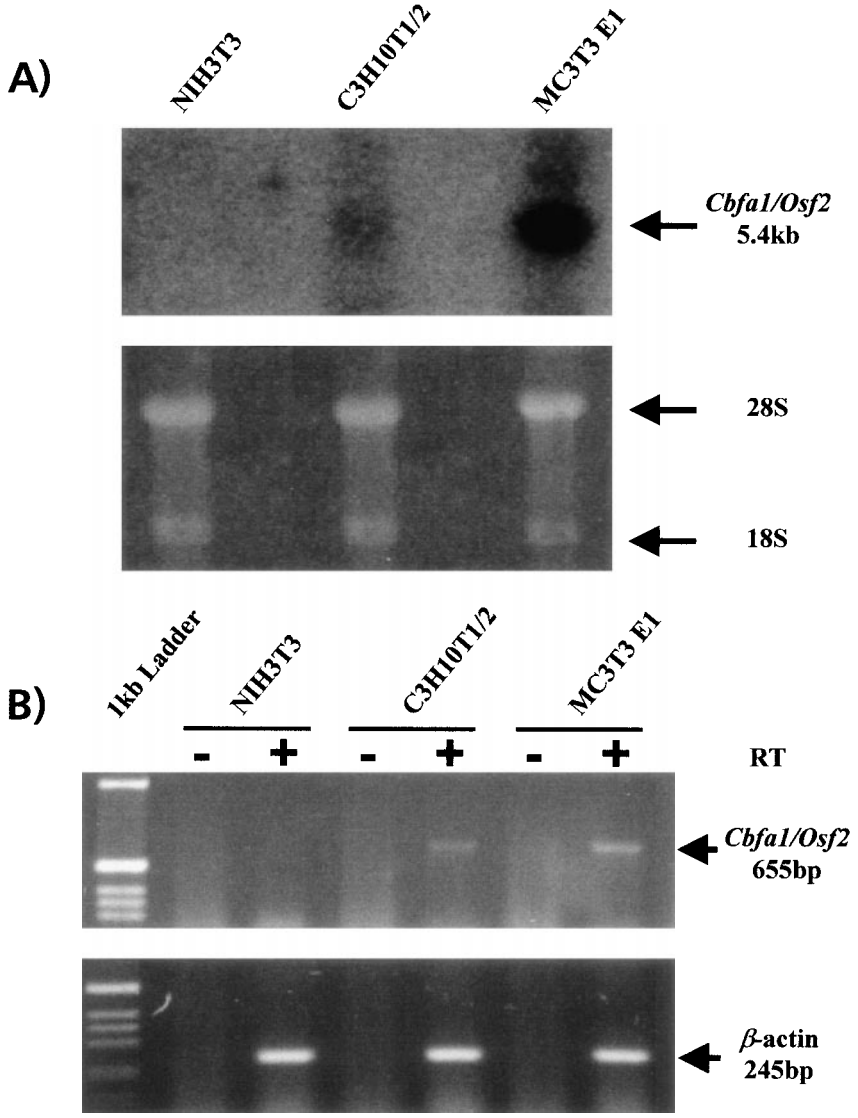

Fig. 2. Endogenous expression of mRNA transcripts derived from exons 1 and 2 of the *Cbfa1* gene. **A:** Northern blot analysis of *Cbfa1/Osf2* transcript expression in various cell lines using a probe to the sequences encoded by exons 1 and 2. High levels of the predicted 5.4-kb transcript containing the unique *Cbfa1/Osf2* sequence was present in MC3T3-E1 pre-osteoblasts and pluripotent C3HT101/2 cells, but not in NIH3T3 fibroblasts (top). Ethidium bromide staining demonstrated equal loading of total RNA as evidenced by the equivalent 28S and 18S ribosomal bands (bottom). **B:** Reverse transcription-polymerase chain reaction (RT-PCR) amplification of the 5′ region of the *Cbfa1/Osf2* transcripts. RT-PCR analysis using primers designed to amplify the sequences transcribed by exons 1, 2, and 3 resulted in the predicted 655 bp (38 cycles) RT-dependent product in C3H10T12 cells and MC3T3-E1 pre-osteoblasts, but no product was identified in NIH3T3 fibroblasts (top). Amplification of β-actin was similar in the different cell lines. ±, the respective presence and absence of reverse transcriptase.

fected with 2.0 μg of plasmid DNAs (data not shown).

Transfection of *Cbfa1* Gene Products in Other Cell Lines

To more fully characterize the transactivation potential of the *Cbfa1* gene products, we compared the ability of all three isoforms to stimulate osteocalcin promoter activity in other cell lines as a function of plasmid dose. In NIH3T3 fibroblasts (Fig. 4B) and MC3T3-E1 pre-osteoblasts (Fig. 4C), cotransfection of the smaller amount of the three different *Cbfa1* isoforms with 1 μg of p1.3OC-luciferase resulted in significant increments in osteocalcin promoter activity, similar to those in C3H10T1/2 transfection studies (Fig. 4A). There were no differences in the magnitude of the response between the various isoforms at these low doses in either cell line. By contrast, after NIH3T3 fibroblasts or MC3T3-E1 pre-osteoblasts were transiently transfected with 2 μg of the vector containing the respective isoforms, a significantly greater induction of osteocalcin promoter activity occurred with the *Cbfa1/iso* containing AD1 compared with either the *Cbfa1/Osf2* or the original *Cbfa1* construct, similar to what we observed in C3H10T1/2 cells (Fig. 3).

DISCUSSION

Our study is the first to compare directly the transactivation potential of three different N-terminal *Cbfa1* isoforms derived from the *Cbfa1* gene (Fig. 1). Our approach permits the func-

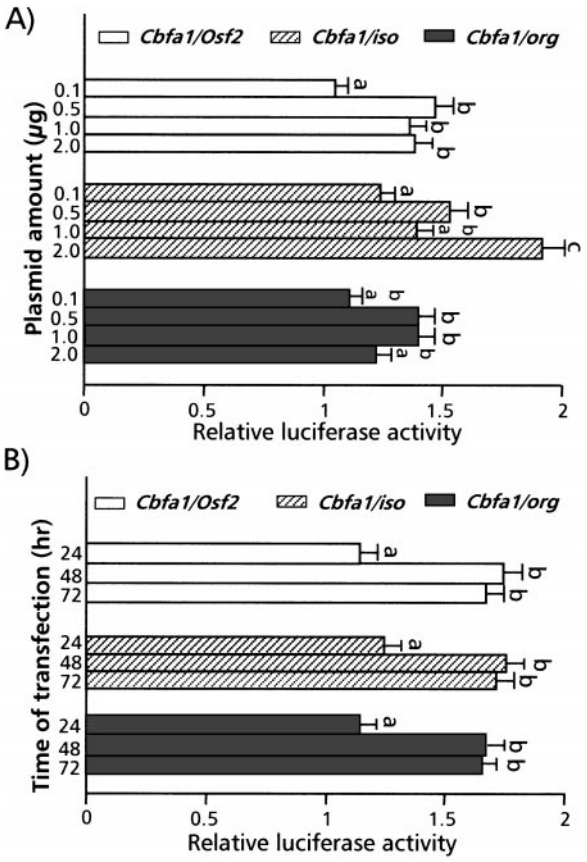

Fig. 3. Dose- and time-dependent effects of transient overexpression of *Cbfa1* isoforms on osteocalcin promoter activity in C3H10T1/2 cells. **A:** Transfections with increasing doses of expression plasmid. C3H10T1/2 cells were transiently transfected with 1.0 μg of p1.3OC-luciferase and the indicated amounts of pcDNA3.1 expression vector containing either full-length *Cbfa1*/*Osf2* that contains both ATG codons (Met^1 and Met^{69}), *Cbfa1/iso* containing only the second ATG codon (Met^{69}) and encoding the AD1 domain, or *Cbfa1/org* that lacks the AD1 domain as described in Fig. 1. **B:** Time course of *Cbfa1* isoform-stimulated osteocalcin promoter activity. C3H10T1/2 cells were transiently transfected with 1.0 μg of p1.3OC-luciferase and 0.5 μg of pcDNA3.1 vector expressing the various *Cbfa1* isoforms. Luciferase assays were performed after various culture periods as described under Materials and Methods. Values sharing the same letter superscript are not significantly different at $P < 0.05$. Values represent the mean ± SEM of three separate experiments. Relative luciferase activity was determined by dividing the corrected luciferase activity by the luciferase activity of cells transfected with the empty expression vector as described under Materials and Methods.

tional assessment of the different N-terminal amino acids in the context of other domains present in the remainder of the *Cbfa1* protein (Fig. 1C). To accomplish this, we transfected cDNAs encoding the original 513-amino acid *Cbfa1* gene product derived from the ATG in exon one, the full-length 597-amino acid *Cbfa1*/*Osf2* protein derived from the ATG in exon 1, and the truncated 528-amino acid *Cbfa1/iso* protein derived from the ATG in exon 2. We found that the amount of transfected *Cbfa1* cDNAs influenced osteocalcin gene transcription in an isoform-specific manner. At lower amounts of expression plasmid DNAs, all three *Cbfa1* isoforms resulted in equivalent stimulation of osteocalcin promoter activity in fibroblasts, C3H10T1/2 cells and MC3T3-E1 pre-osteoblasts (Figs. 3, 4). By contrast, using transfection protocols with higher amounts of plasmid DNAs, we found that the *Cbfa1*/*Osf2* and *Cbfa1/org* had less transactivating potential compared with *Cbfa1/iso*. At these higher amounts of expression plasmid DNAs, the 528-amino acid Cbfa/*iso* containing AD1 was the most potent inducer of osteocalcin activity in these cells (Figs. 3, 4). *Cbfa1/org* and *Cbfa1*/*Osf2* failed to fully activate the osteocalcin promoter at high expression plasmid concentrations (Fig. 4).

Our findings indicate that all three isoforms have the potential to activate the osteocalcin promoter when the amount of the expression plasmid is less than the reporter plasmid. The similar transactivating potential of all three *Cbfa1* isoforms which share the AD2, the *runt* domain, and AD3 [Thirunavukkarasu et al., 1998] suggest that these domains are sufficient to activate the osteocalcin promoter (Fig. 3). The presence of similar activating domains in the related *Cbfa2* and *Cbfa3* genes, including the *runt* domain that is responsible for the DNA binding, potentially explains why these gene products also stimulate osteoblast gene transcription in vitro [Banjeree et al., 1997; Ji et al., 1998]. Moreover, studies of protein-DNA interactions using *Cbfa1* constructs with different N-termini show equal binding to the OSE2 element [Thirunavukkarasu et al., 1998]. Because all *Cbfa1* isoforms, as well as other *runt* domain proteins, are capable of stimulating the osteocalcin promoter reporter construct to some degree [Changhua et al., 1998], the cell-type-specific expression of a particular Cbfa protein/isoform (Fig. 2), rather than the transactivating potential of different N-terminal sequences, may be a major determinant of cell-specific function.

Regardless, our studies suggest that the unique 87-amino acid *Osf2* N-terminal sequence potentially encoded by exon 1 is not needed for the induction of osteoblast-related gene expression. The nonessential role of the N-terminal *Osf2* sequence in the transcrip-

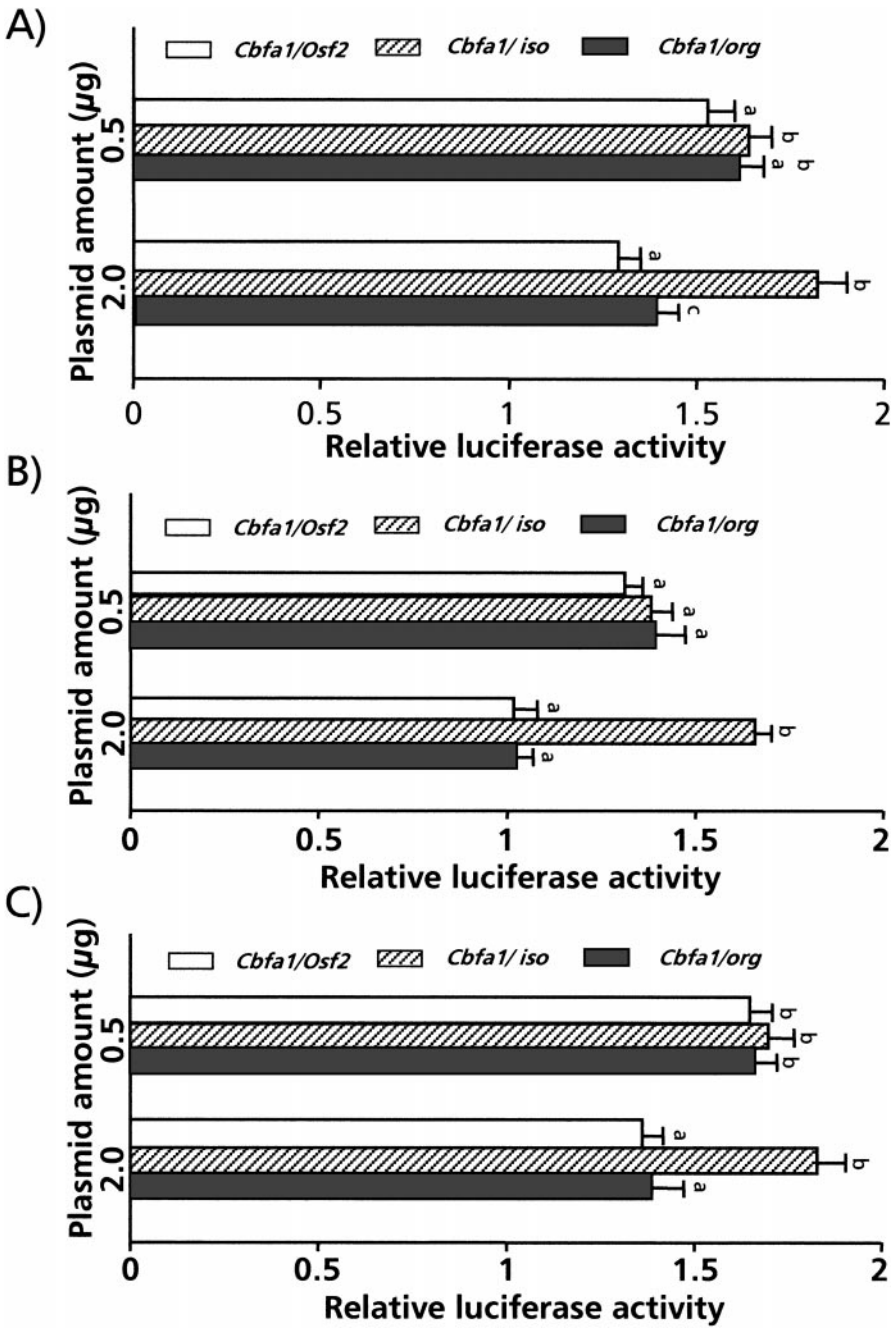

Fig. 4. Comparison of transactivating role of the 19-amino acid N-terminal domain of the *Cbfa1* isoform in C3H10T1/2 Pluripotent cells, NIH3T3 fibroblasts, MC3T3-E1 pre-osteoblasts. **A:** C3H10T1/2 cells, (**B**) NIH3T3 fibroblasts, or (**C**) MC3T3-E1 pre-osteoblasts were transiently transfected with 1.0 μg of p1.3OC-luciferase and either 0.5 μg or 2.0 μg of the pcDNA3.1 expression vector containing either full-length *Cbfa1*/*Osf2*, *Cbfa1/iso*, or *Cbfa1/org*. Values sharing the same letter superscript are not significantly different at $P < 0.05$. One-way ANOVA was performed within a cell type as a function of the transfected *Cbfa1* isoform. Values represent the mean ±SEM of a minimum of seven separate experiments. Relative luciferase activity was determined by dividing the corrected luciferase activity by the luciferase activity of cells transfected with the empty expression vector as described under Materials and Methods.

tional activation of osteoblast differentiation is consistent with the absence of a homologous N-terminus corresponding to the *OSF2* coding sequence in the human *CBFA1* cDNA [Lee et al., 1997; Xiao et al., 1998a].

We observed a greater transcriptional effect of the *Cbfa1/iso* under experimental conditions in which the amount of expression plasmid exceeds that of the reporter plasmid. Consequently, it remains possible that different N-terminal domains contribute to the variable transactivation potential of the three *Cbfa1* isoforms observed in our dose-dependent studies. In support of this possibility, other investigators have demonstrated that the 19-amino acid N-terminal sequence of *Cbfa1/iso* represents an activation domain (AD1) [Thirunavukkarasu et al., 1998]. The fact that AD1 enhances transactivation, albeit only at high plasmid amounts, is consistent with the additive effects of its additional N-terminal transactivation domain. This enhancement might be

attributable to unidentified coactivators that interact with the putative 19-amino acid N-terminus of *Cbfa1/iso* under certain experimental conditions [Yamaoka et al., 1998]. Regardless, it is likely that a balance between the number of transactivation and repression domains present in the various *Cbfa1* gene products controls the transactivation potential of this transcription factor in osteoblasts [Thirunavukkarasu et al., 1998].

Potential nonspecific secondary effects of transfection protocols that use higher amounts of plasmid DNAs, however, limit the conclusions that we can make regarding the role of the 19-amino acid N-terminal AD1. In general, experimental conditions characterized by the lower ratio of overexpression plasmid to reporter plasmid are more likely to be representative of the normal physiologic state. Most studies that identify optimal responses to various Cbfa proteins use expression to promoter/reporter constructs ratios of <1 [Ji et al., 998; Geoffroy et al., 1995]. We could demonstrate greater activity of the *Cbfa1/iso* containing AD1 only when the amount of the expression plasmid exceeded the amount of the promoter/reporter plasmid. Such potential excess of *Cbfa1* proteins may bind to suboptimal and incorrect *cis*-acting sites on the promoter and lead to erroneous regulation of gene transcription. Indeed, others have shown that excessive expression plasmid can paradoxically inhibit the transactivation potential of the related *Cbfa2* transcription factor [Bae et al., 1994].

In spite of significant increases in osteocalcin promoter activity in response to transient transfection with *Cbfa1* cDNAs, the magnitude of the response was small. The increase in osteocalcin activity after transient transfection with *Cbfa1* isoforms was less than the 5-fold increase in osteocalcin promoter activity that typically occurs during differentiation of MC3T3-E1 [Quarles et al., 1997]. The relative inefficiency of transient transfections might contribute to the limited response. The number of *cis*-acting OSE2/*Cbfa1* binding domains, which numbered two in the osteocalcin promoter, might also limit the response [Frendo et al., 1998]. Indeed, similar studies performed with a reporter construct containing tandem repeats of multiple OSE2 *cis*-acting elements resulted in greater stimulation by transfected *Cbfa1* cDNAs [Thirunavukkarasu et al., 1998]. The inability of *Cbfa1/iso* to maximally stimulate osteocalcin promoter activity could also represent the need for factors other than *Cbfa1* gene products. Recent studies demonstrating that *Cbfa1* is necessary but not sufficient for osteoblast differentiation support this possibility [Ryoo et al., 1998; van den Bos 1998].

In conclusion, forced overexpression of three *Cbfa1* isoforms in vitro demonstrates variable induction of osteocalcin promoter activity that depends on the amount of plasmid DNA in the transfection protocol and possibly the N-terminal sequence. All isoforms, including *Cbfa1/iso*, *Cbfa1/org*, and *Cbfa1/Osf2*, display similar transactivation potential at optimal ratios of expression plasmid to reporter constructs. The *Cbfa1* isoform that contains the 19-amino acid AD1 N-terminal sequence augments activation of the osteocalcin promoter/reporter in vitro only under limited experimental conditions. Moreover, the unique N-terminal *Osf2* sequence, previously thought to represent the osteoblast-specific transactivator, appears to lack a distinct regulatory function.

ACKNOWLEDGMENTS

The authors thank Lauren L. Green for secretarial assistance in the preparation of this manuscript.

REFERENCES

Ahn MY, Bae SC, Maruyama M, Ito Y. 1996. Comparison of the human genomic structure of the Runt domain-encoding PEB2/CBFα gene family. Gene 168:279–280.

Bae SC, Ogawa E, Maruyama M, Oka H, Satake M, Shigesada K, Jenkins NA, Gilbert DJ, Copeland NG, Ito Y. 1994. PEBP2αB/mouse AML1 consists of multiple isoforms that possess differential transactivation potentials. Mol Cell Biol 14:3242–3252.

Banerjee C, McCabe LR, Choi JY, Hiebert SW, Stein JL, Stein GS, Lian JB. 1997. Runt homology domain proteins in osteoblast differentiation: AML3/CBFA1 is a major component of a bone-specific complex. J Cell Biochem 66:1–8.

Baskaran N, Kandpal RP, Bhargava AK, Glynn MW, Bale A, Weissman SM. 1996. Uniform amplification of mixture of deoxyribonucleic acids with varying GC content. Genome Res 6:633–638.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Ducy P, Zhang R, Geoffroy V, Ridall L, Karsenty G. 1997. *Osf2/Cbfa1*: a transcriptional activator of osteoblast differentiation. Cell 89:747–754.

Frendo J-L, Xiao G, Fuchs S, Franceschi RT, Karsenty G, Ducy P. 1998. Functional hierarchy between two OSE2 elements in the control of osteocalcin gene expression in vivo. J Biol Chem 273:30509–30516.

Geoffroy V, Corral DA, Zhou L, Lee B, Karsenty G. 1998. Genomic organization, expression of the human *CBFA1* gene, and evidence for an alternative splicing event affecting protein function. Mamm Genome 9:54–57.

Guo R, Quarles LD. 1997. Cloning and sequencing of Human PEX from a bone cDNA library: evidence for its developmental stage-specific regulation in osteoblasts. J Bone Miner Res 12:1009–1017.

Ji C, Casinghino S, Chang DJ, Chen Y, Javed A, Ito Y, Hiebert SW, Lian JB, Stein GS, McCarthy TL, Centrella M. 1998. CBFa (AML/PEBP2)-related elements in the TGF-β type 1 receptor promoter and expression with osteoblast differentiation. J Cell Biochem 69:353–363.

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. 1997. Targeted disruption of *Cbfa1* results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89:755–764.

Lee B, Thirunavukkarasu K, Zhou L, Pastore L, Baldini A, Hecht J, Geoffroy V, Ducy P, Karsenty G. 1997. Missense mutations abolishing DNA binding of the osteoblast-specific transcription factor OSF2/CBFA1 in cleidocranial dysplasia. Nature Genet 16:307–310.

Merriman HL, Van Wijnen AJ, Hiebert S, Bidwell JP, Fey E, Lian J, Stein J, Stein GS. 1995. The tissue-specific nuclear matrix protein, NMP-2, is a member of the AML/CBF/PEB2/Runt Domain transcription factor family: interactions with the osteocalcin gene promoter. Biochemistry 34:13125–13132.

Mundlos S, Otto F, Mundlos C, Mulliken JB, Aylsworth AS, Albright S, Lindhout D, Cole WG, Henn W, Knoll JH, Owen MJ, Mertelsmann R, Zabel BU, Olsen BR. 1997. Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. Cell 89:773–779.

Ogawa E, Maruyama M, Kagoshima H, Inuzuka M, Lu J, Satake M, Shigesada , ItoY. 1993. PEBP2/PEA2 represents a family of transcription factors homologous to the products of the *Drosophila runt* gene and the human AML1 gene. Proc Natl Acad Sci USA 90:6859–6863.

Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, Selby PB, Owen MJ. 1997. *Cbfa1*, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89:765–771.

Quarles LD, Siddhanti SR, Medda S. 1997. Developmental regulation of osteocalcin expression in MC3T3-E1 osteoblasts: minimal role of the proximal E-box *cis*-acting promoter elements. J Cell Biochem 65:11–24.

Ryoo HM, Lee MH, Javed A, Kim HJ, Shin HI, Choi JY, Rosen V, Stein JL, van Wijnen AJ, Stein GS, Lian JB. 1998. Transient expression of *Cbfa1* in response to bone morphogenetic protein-2 and transforming growth factor-beta 1 in C2C12 myogenic cells: a necessary milestone but not sufficient for osteoblast differentiation. Bone 23(suppl):S173.

Satake M, Nomura S, Yamaguchi-Iwai Y, Takahama Y, Hashimoto Y, Niki M, Kitamura Y, Ito Y. 1995. Expression of the runt domain-encoding PEBP2α genes in T cells during thymic development. Mol Cell Biol 15:1662–1670.

Stewart M, Terry A, Hu M, O'Hara M, Blyth K, Baxter E, Cameron E, Onions DE, Neil JC. 1997. Proviral insertions induce the expression of bone-specific isoforms of PEBP2alphaA (CBFA1): evidence for a new myc collaborating oncogene. Proc Natl Acad Sci USA 94:8646–8651.

Thirunavukkarasu K, Mahajan M, McLarren KW, Stifani S, Karsenty G. 1998. Two domains unique to osteoblast-specific transcription factor *Osf2*/*Cbfa1* contribute to its transactivation function and its inability to heterodimerize with Cbfb. Mol Cell Biol 18:4197–4208.

Tsuji K, Ito Y, Noda M. 1998. Expression of the PEBP2αA/AML3/*CBFA1* gene is regulated by BMP4/7 heterodimer and its overexpression suppresses type 1 collagen and osteocalcin gene expression in osteoblastic and nonosteoblastic mesenchymal cells. Bone 22:87–92.

van den Bos C. 1998. Human bone marrow-derived mesenchymal stem cells constitutively express *CBFA1*: lack of correlation of mRNA or protein levels with acquisition of osteoblastic phenotype. Bone 23(suppl):S150.

Xiao ZS, Thomas R, Hinson TK, Quarles LD. 1998. Genomic structure and isoform expression of the mouse, rat and human *Cbfa1*/*Osf2* transcription factor. Gene 214: 187–197.

Xiao ZS, Crenshaw M, Guo R, Nesbitt T, Drezener MK, Quarles LD. 1998. Intrinsic mineralization defect in *Hyp* mouse osteoblasts. Am J Physiol 275:E700–E708.

Yamaoka S, Courtois G, Bessia C, Whiteside ST, Weil R, Agou F, Kirk HE, Kay RJ, Isreal A. 1998. Complementation cloning of NEMO, a component of the IκB kinase complex essential for NF-κB activation. Cell 93:1231–1240.

Zeng C, Van Wijnen A, Stein J, Meyers S, Sun W, Shopland L, Lawrence J, Penman S, Lian J, Stein G, Hiebert S. 1997. Identification of a nuclear matrix targeting signal in the leukemia and bone-related AML/CBF-α transcription factors. Proc Natl Acad Sci USA 94:6746–6751.