Journal of Cellular Biochemistry 73:114–125 (1999)

# Transient Upregulation of CBFA1 in Response to Bone Morphogenetic Protein-2 and Transforming Growth Factor β1 in C2C12 Myogenic Cells Coincides With Suppression of the Myogenic Phenotype but is Not Sufficient for Osteoblast Differentiation

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**Abstract** The bone morphogenetic protein (BMP)-2 is a potent osteoinductive signal, inducing bone formation in vivo and osteoblast differentiation from non-osseous cells in vitro. The *runt* domain-related protein Cbfa1/PEBP2αA/AML-3 is a critical component of bone formation in vivo and transcriptional regulator of osteoblast differentiation. To investigate the relationship between the extracellular BMP-2 signal, Cbfa1, and osteogenesis, we examined expression of Cbfa1 and osteoblastic genes during the BMP-2 induced osteogenic transdifferentiation of the myoblastic cell line C2C12. BMP-2 treatment completely blocked myotube formation and transiently induced expression of Cbfa1 and the bone-related homeodomain protein Msx-2 concomitant with loss of the myoblast phenotype. While induction of collagen type I and alkaline phosphatase (AP) expression coincided with Cbfa1 expression, Cbfa1 mRNA was strikingly downregulated at the onset of expression of osteopontin (OPN) and osteocalcin (OCN) genes, reflecting the mature osteoblast phenotype. TGF-β1 treatment effectively suppressed myogenesis and induced Cbfa1 expression but was insufficient to support osteoblast differentiation reflected by the absence of ALP, OPN, and OCN. We addressed whether induction of Cbfa1 in response to BMP-2 results in the transcriptional activation of the OC promoter which contains three enhancer Cbfa1 elements. Transfection studies show BMP-2 suppresses OC promoter activity in C2C12, but not in osteoblastic ROS 17/2.8 cells. Maximal suppression of OC promoter activity in response to BMP-2 requires sequences in the proximal promoter (up to nt -365) and may occur independent of the three Cbfa sites. Taken together, our results demonstrate a dissociation of Cbfa1 expression from development of the osteoblast phenotype. Our findings suggest that Cbfa1 may function transiently to divert a committed myoblast to a potentially osteogenic cell. However, other factors induced by BMP-2 appear to be necessary for complete expression of the osteoblast phenotype. J. Cell. Biochem. 73:114–125, 1999. 

**Key words:** osteogenic commitment; Msx-2; Cbfa1; osteocalcin promoter

Numerous factors, including hormones, growth factor signaling proteins, and transcription factors, have been implicated in skeletal development and the control of osteogenesis [Hogan, 1996; Erlebacher et al., 1995]. Studies of expressed transgenes and null-mutations in mice, as well as observed effects of these factors on induced osteogenesis in in vitro model systems, have revealed important stages in regulation of commitment to the osteoblast phenotype, as well as the subsequent expression and maintenance of osteoblast differentiation. However, very few studies have addressed the interrelationship of factors fundamental to development of the osteoblast phenotype.

The discovery of the Cbfa1/AML-3/PEBP2αA as a bone restricted transcription factor was a significant milestone in osteoblast biology [Mer-

Contract grant sponsor: National Institutes of Health; Contract grant numbers: DE12528 and AR39588.

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Received 8 September 1998; Accepted 30 October 1998

riman et al., 1995; Ducy et al., 1997; Banerjee et al., 1997]. Deletion of the Cbfa1 gene by homologous recombination in mice results in absence of ossified tissue owing to a maturational arrest of osteoblast differentiation [Komori et al., 1997]. Simultaneously it was revealed that a heterozygous mutation of the gene caused cleidocranial dysplasia syndrome in human [Mundlos et al., 1997] and mouse [Otto et al., 1997]. Cbfa1 is a member of a large gene family of runt homology domain proteins. Cbfa proteins are derived from these distinct gene products, Cbfa1 (AML-3), Cbfa2 (AML-1B), and Cbfa3 (AML-2), which produce numerous mRNA splice variants that generate multiple Cbfa protein isoforms. Several Cbfa1 splice variants have been identified including OSF-2 [Ducy et al., 1997], *til*-1 [Stewart et al., 1997], and the isoform first identified by Ito and coworkers [Ogawa et al., 1993b]. The major translated mRNA in osteoblasts originates from MASN in exon 0 [Stewart et al., 1997; Thirunavukkarasu et al., 1998]. The importance of Cbfa1 proteins in regulation of osteoblast differentiation and bone specific gene expression of several osteoblast marker genes has been demonstrated including the bone-specific osteocalcin gene [Banerjee et al., 1997; Ducy et al., 1997] and the TGF-β1 receptor [Ji et al., 1998].

Bone morphogenetic proteins (BMPs) were originally identified in demineralized bone matrix as factors that induce ectopic bone formation when implanted into rat muscular tissue [Urist, 1965]. At least 12 BMPs have been identified which are highly conserved members of the transforming growth factor-β (TGF-β) superfamily [Centrella et al., 1994; Dube and Celeste, 1995; Celeste et al., 1995; Hu et al., 1998]. Among these genes, BMP-2, -4, and -7 have been shown to be potent bone inducing agents [Wang et al., 1990; Sampath et al., 1992; Katagiri et al., 1994]. Previous in vitro studies demonstrated that BMP-2 not only stimulated the maturation of osteoblastic progenitor cells [Yamaguchi et al., 1991], but also induced differentiation of pluripotent C3H10T1/2 fibroblastic [Katagiri et al., 1990] or W-20–17 stromal cells [Thies et al., 1992] into cells of the osteoblastic lineage.

BMP-2 is not only a potent inducer of osteogenesis, but can block the differentiation pathway of C2C12 myoblasts into mature muscular cells by suppressing the master control genes for myoblast differentiation [Katagiri et al., 1994]. Subsequently, expression of typical osteoblast phenotypic markers are induced, such as alkaline phosphatase activity, competency for parathyroid hormone responsiveness, and osteocalcin production, in continued BMP-2 treated C2C12 cells [Katagiri et al., 1994]. More recently, it has been shown that inhibition of myoblastic differentiation and induction by BMP-2 of osteoblast differentiation involves the BMP receptor-1A and the transducers Smad1 and Smad5 [Namiki et al., 1997; Yamamoto et al., 1997; Nishimura et al., 1998]. Subclonal cell lines of C2C12 that stably express a kinase domain-truncated BMPR-1A, differentiate into myoblasts and not osteoblasts in the presence of BMP-2. In addition, transfection of Smad1 or Smad5 into C2C12 cells decreased myogenic promoter activity in the absence of BMP-2 [Yamamoto et al., 1997], while C-terminal truncated Smad1 and Smad5 proteins increased myogenic promoter activity and blocked the BMP-2 signal. Thus, BMP-2 induction of the transdifferentiation of C2C12 myoblasts to osteoblasts provides an excellent model to investigate mechanisms regulating commitment to the osteoblast phenotype.

In this study, we examined the temporal expression of the Cbfa1 gene during BMP-2 induced osteogenesis using this model and the functional activity of BMP-2 in relation to Cbfa activation of the osteocalcin promoter. Our results contribute to an understanding of the molecular mechanism by which BMP-2 and TGF-β1 plays a central role in initial commitment towards the osteoblast lineage by activation of Cbfa1. However, BMP-2 provides additional signals for complete expression of the osteoblast phenotype. Furthermore, BMP-2 appears to antagonize Cbfa1 enhancer activity on the osteocalcin promoter in C2C12 cells, consistent with the transitory effects of BMP-2 on Cbfa1 induction during osteogenesis.

## MATERIALS AND METHODS

### Cell Culture Systems

Mouse myoblast cell line, C2C12, was purchased from the ATCC. C2C12 cells were maintained in DMEM (Gibco, Grand Island, NY) containing 5% FBS (Gibco) and 100 U/ml of penicillin G and 100 μg/ml of streptomycin at 37°C in a humidified atmosphere of 5% $CO_2$ in air. Bioactive recombinant human BMP-2 was produced and purified from the conditioned medium of CHO cells and the purity and bioactiv-

ity was checked as described previously [Wang et al., 1990]. Recombinant human TGF-β1 was purchased from R&D Systems Inc. (Minneapolis, MN). The cells were inoculated at $1 \times 10^6$ cells/100 mm culture dishes. To examine the effects of BMP-2 on the cellular differentiation, the growth medium was replaced with DMEM containing 5% FBS after 24 h of inoculation. The cells were cultured for 1, 3, and 7 days with or without treatment of 300 ng/ml of BMP-2 or 5 ng/ml of TGF-β1. Statistical significance between control and treated groups was observed only in stripped serum and after 48–72 h in independent experiments and assays. Student unpaired *t*-tests were applied for n = 3 samples per group and in at least two independent experiments.

### Constructs and Stable Cell Lines

A panel of OC promoter-CAT reporter gene constructs with deletions in the OC promoter region have been described previously [Aslam et al., 1995]. Each of these constructs (-1097, WT/CAT; -365, Bgl II/CAT; -108, Aos/CAT) was stably integrated into ROS 17/2.8 cells as documented previously [Frenkel et al., 1996]. In addition, we prepared an OC promoter-CAT construct in which three Cbfa1 sites (nt -604 to -599, Site A; nt -440 to -435, Site B; nt -136 and -130, Site C) [Merriman et al., 1995] were mutated within the context of the 1.1 kb rat OC promoter. For each site, two substitution mutations were incorporated into the Cbfa recognition motif (5'-YGTGGYY). Substitution mutations in the OC promoter were generated by a PCR based approach using synthetic oligonucleotides (Integrated DNA Technologies, Inc.). These oligonucleotides are shown with mutations in lower case and the Cbfa recognition motif underlined:

Site A-5′CCATCAAAA**ag**ACTAAAT

AAGAAATGCC3′;

SiteB-5′CATTACTGA**tt**GCTCTTCCTGGGG3′;

SiteC-5′GTCACCAA**ga**ACAGCATCCTTTG3′.

Using a pUC/M13 reverse primer (Promega, Madison, WI) and the oligos bearing the mutated sequence, three independent PCR reactions were performed, and all three products digested in parallel with Hind III. The region upstream of each Cbfa site was generated by PCR, digested with SphI and a three-way ligation performed with the Hind III-SphI pGEM-7zf(+) vector (Promega). To generate the triple site mutant (mABC), the plasmids containing the mutation in Site A (mA) and in Site B (mB) were digested with MfeI and ligated to generate an intermediate construct with mutated Sites A and B (mAB). In the final step, the mAB plasmid was digested with Bgl II-Hind III to release the wild-type Site C which was replaced with the mutant Site C plasmid. Incorporation of the mutation in each site was confirmed by dideoxy sequencing.

To generate cells with genomically integrated OC-CAT reporter gene constructs, wild-type and Cbfa mutant constructs were stably transfected into ROS 17/2.8 cells by the calcium phosphate method as previously described [Frenkel et al., 1996]. For each construct, four 100 mm plates were transfected with 15 μg of OC-CAT plasmid and 5 μg of pCEP 4 (Invitrogen, San Diego, CA) encoding the Hygromycin B-Phosphotransferase gene. Cells were harvested at 95% confluency and replated for selection in media containing 55 U/ml Hygromycin B (Calbiochem, La Jolla, CA). Resistant colonies (between 60 and 75) from each plate were pooled and propagated as polyclonal cell lines. Each pool was expanded until $\sim 20 \times 10^8$ cells were available for preparation of frozen stocks. Cells were routinely maintained in media containing Hygromycin B for measuring CAT activity and growth factor responsiveness.

### Northern Blot Analysis

Total cellular RNA was extracted from C2C12 cells at the above mentioned time points according to an acid guanidinium thiocyanate-phenol-chloroform method [Chomczynski and Sacchi, 1987]. Ten microgram of total cellular RNA was separated on a 1% agarose/5.5% formaldehyde gel and transferred to Hybond-N membranes (Amersham, Amersham, UK) using 20× sodium chloride sodium citrate buffer. RNA was cross-linked to the filter by UV irradiation for 1 min. DNA probes, rat osteocalcin [Lian et al., 1989], rat alkaline phosphatase [Noda and Rodan, 1986], human type I collagen [Chu et al., 1982], rat osteopontin [Oldberg et al., 1986], human fibronectin [Kornblihtt et al., 1983], mouse matrix Gla protein [Luo et al., 1995], mouse Msx-2 [Catron et al., 1996], rat Dlx-5 [Ryoo et al., 1997], and mouse PEBPα2A/Cbfa1 [Ogawa et al., 1993b], were labeled with α-$^{32}$P dCTP (3,000 Ci/mmol;

NEN, Boston, MA) using the random primer technique [Feinberg and Vogelstein, 1983]. The blot was prehybridized in 50% formamide, 5 × SSPE, 5 × Denhardt's solution, 0.1% SDS and 100 μg/ml salmon sperm DNA at 42°C for 3 h. For hybridization, $1 \times 10^6$ cpm/ml of heat denatured radioactive DNA probe was added and incubated at 42–65°C overnight. Following hybridization, the blot underwent several different stringent washes and exposed to Kodak XAR film at −70°C with intensifying screens.

### Transfection Assays

Cells were plated at a density of 4–5 × $10^5$ cells/100 mm plate for use in transient transfection experiments. C2C12 cells, MC3T3-E1 cells, ROS 17/2.8 cells, and primary cultured rat osteoblast cells were transfected by the DEAE-dextran method [Ryoo et al., 1997]. The total amount of exogenous DNA was maintained at 20 μg/100 mm culture plate consisting of 2 μg of luciferase construct, 8 μg of CAT construct and 10 μg of Salmon sperm DNA. All plasmid DNAs were prepared using the Qiagen Plasmid Kit (Germany) and checked for supercoiled structure and compared with expression of plasmids of similar quality. Cells were transfected with DNA in the presence of 200 μg/ml DEAE-dextran (Pharmacia, Gaithersburg, MD) and 50 μg/ml chloroquine (Sigma, St. Louis, MO) for 2 h, shocked briefly with 10% glycerol in serum free F12 media, then incubated in their maintenance medium. All cells were harvested with phosphate buffered saline (PBS, pH 7.4) 48–72 h after transfection with or without treatment of 300 ng/ml of BMP-2 for final 48 h. The cell pellet was treated with 1x lysis buffer (0.25 M Tris-HCl, pH 8.0, 0.1% Triton X-100; Promega). Luciferase activity was determined with luciferase assay system (Promega). Luminescence was determined by MonoliteTM 2010 (Analytical Luminescence Laboratory, San Diego, CA). Chloramphenicol acetyltransferase (CAT) activity was determined as described previously [Ryoo et al., 1997]. The samples were incubated with 0.25 μCi (1 Ci = 37 Gbq) of $^{14}$C chloramphenicol (Dupont, Boston, MA) for 4–12 h. After ethyl acetate extraction, the samples were separated by chromatography on TLC plates (Whatman, Clifton, NJ). Radioactivity on the TLC plates was quantitated using a β-scope 603 blot analyzer from Betagen (Mountain View, CA).

## RESULTS

### Selective Expression of Bone Phenotypic Markers in Response to BMP-2 Induced Osteogenic Differentiation of C2C12 Cells

C2C12 myoblasts normally differentiate along the myogenic lineage. However, these myoblastic cells can be induced by BMP-2 to transdifferentiate into osteoblasts. Numerous multinucleated myotubes are generated when C2C12 cells are cultured in low mitogen medium (Fig. 1a, day 7). Consistent with previous observations, BMP-2 or TGF-β1 each completely inhibit the formation of multinucleated myotubes (Fig. 1b,c), but only BMP-2 leads to the expression of osteogenic markers of the mature osteoblast phenotype, e.g., osteocalcin. BMP-2, but not TGF-β1, induced the expression of alkaline phosphatase (ALP) and osteocalcin (OCN) within 3 and 7 days, respectively (Fig. 2). The untreated cells (0 time and control group undergoing myotube formation) do not express mRNAs for the osteogenic markers, ALP and OCN during the entire experimental period. Osteopontin (OPN), which is expressed in many tissues but abundantly in bone, was induced by the change to low serum for myotube formation in the control group. In the control group during myoblastic differentiation and the TGF-β1 group, OPN mRNAs are detected only at day 1 at the early proliferation stage. In contrast, BMP-2 treatment suppressed osteopontin expression on day 1, but significantly induced OPN expression after day 3 on day 6 (Fig. 2). Another osteoblast marker gene, α1(I) collagen (Col I), was minimally expressed and increased in the differentiated myoblasts (day 7). TGF-β1 treatment enhanced α1(I) collagen from day 1. Collagen mRNA remained at the increased level through the experimental period (Fig. 2). However, in response to BMP-2, Col I expression was significantly induced and continuously increased during osteoblastic differentiation. Matrix gla protein (MGP) and fibronectin (FN) did not show a significantly different expression pattern either by BMP-2 or TGF-β1 (Fig. 2) when compared to control.

### Transient Expression of CBFA1 in Response to BMP-2 and TGF-β1 Induced Transdifferentiation of Myoblastic C2C12 Cells

To determine the mechanism of the stimulatory effect of BMP-2 on osteoblastic differentiation, we investigated mRNA expression of tran-

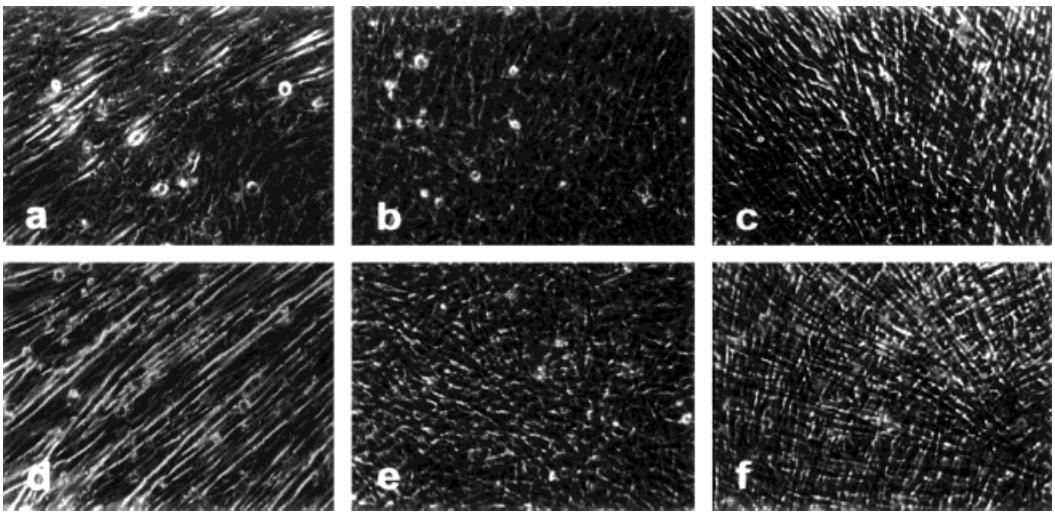


**Fig. 1.** The effects of BMP-2 and TGF-β1 on C2C12 cell differentiation. The cells were cultured for 3 days (**a–c**) or 7 days (**d–f**) under low mitogenic medium (DMEM with 5% fetal bovine serum) in the absence (a,d) or presence of 300 ng/ml of BMP-2 (b,e) or 5 ng/ml of TGF-β1 (c,f) as described in Materials and Methods (× 100, inverted phase contrast microscope).

scription factors involved in osteogenesis including the runt domain factor, Cbfa1, and the homeodomain factors, Msx-2 and Dlx-5. Cbfa1 mRNA was not expressed at any time in the control cultures. Notably, Cbfa1 was not immediately induced by BMP-2 at day 1, but detected transiently, expressed 3 days after BMP-2 treatment, and then strikingly diminished by day 7 (Fig. 3). This expression pattern of Cbfa1 occurred similarly following TGF-β1 treatment (Fig. 4). These results suggest that Cbfa1 expression is related to commitment to differentiation along a non-myogenic pathway. However, expressing Cbfa1 does not necessarily lead to induction of osteogenic related genes as reflected by the results in response to TGF-β1 and the delay in induction of OCN and OPN until day 7 in BMP-2-treated cells.

Msx-2 expression was maintained at a constitutive level in control cultures, while BMP-2 transiently induced Msx-2 mRNA level at 3 d. These expression patterns suggest that the up-regulation of these two bone related transcription factors (Cbfa1 and Msx-2) is a key event in the early stages (day 3) of osteogenic C2C12 cells (Fig. 4). Dlx-5 is another homeotic gene, but in contrast to Msx-2, is expressed at later stages of osteoblast differentiation during mineralization [Ryoo et al., 1997]. Dlx-5 mRNA is not detected in control cultures or in response to BMP-2 or TGF-β1 at any time. Taken together, these results indicate that Cbfa1 induction by BMP-2 or TGF-β1 is a transitional event

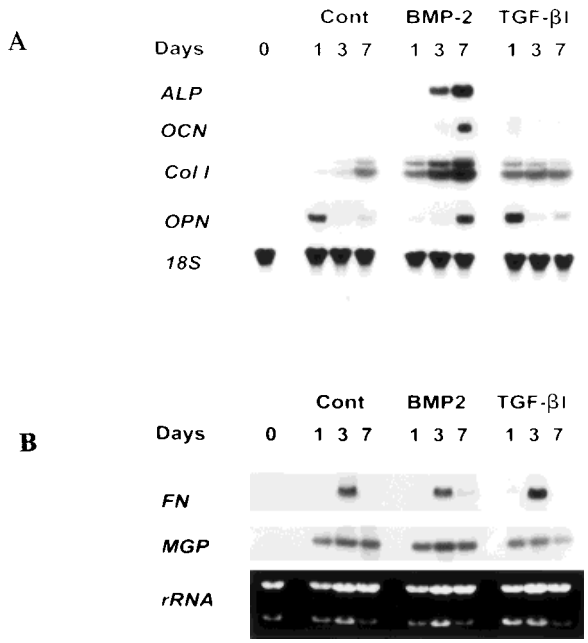


**Fig. 2.** Northern blot analysis of the expression patterns of osteoblast phenotypes in C2C12 cells after treatment of BMP-2 and TGF-β1. Cells were plated at a density of $6.5 \times 10^5$ cells/100 mm culture plate and allowed their attachment for 24 h (day 0). Cells were further cultured with or without BMP-2 (300 ng/ml) and TGF-β1 (5 ng/ml) for the indicated period. Ten microgram of total cellular RNA was loaded for each lane. **A:** Expression pattern of osteoblast marker genes ALP, alkaline phosphatase; OCN, osteocalcin; Col I, α1(I) collagen; OPN, osteopontin. **B:** MGP, matrix gla protein; FN, fibronectin expressions were demonstrated. Consistent RNA loading was represented by 18S ribosomal RNA or etidium bromide staining of RNA.

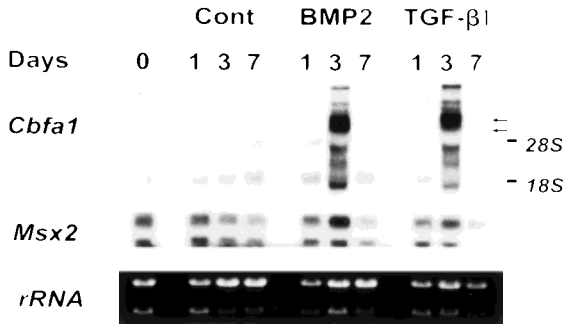


**Fig. 3.** Northern blot analysis of the expression patterns of osteoblast specific transcription factors, Cbfa1(a) and Msx-2(b), in C2C12 cells after treatment of BMP-2 and TGF-β1. Cells were plated at a density of $6.5 \times 10^5$ cells/100 mm culture plate and allowed their attachment for 24 h (day 0). Cells were further cultured with or without BMP-2 (300 ng/ml) and TGF-β1 (5 ng/ml) for the indicated period. Ten microgram of total cellular RNA was loaded for each lane. Consistent RNA loading was represented by etidium bromide staining of rRNA. Arrows indicated the position of two major transcripts of Cbfa1 gene, 18S and 28S ribosomal RNA.

early in the osteoblast differentiation process. Furthermore, expression of Cbfa1 by TGF-β1 is not sufficient for the completion of osteoblastic differentiation. These findings suggest other factors that either promote or inhibit osteogenesis are produced in response to BMP-2 or TGF-β1, respectively.

### The OC Promoter Contains a Negative BMP-2 Responsive Gene Regulatory Element

The levels of Cbfa1 mRNAs are dramatically increased 3 days following BMP-2 induced differentiation of C2C12 cells into the osteoblast lineage. Cbfa1 is a well documented enhancer of OC promoter activity. However, osteocalcin expression is not detected until day 7. We therefore assessed whether the elevated levels of Cbfa1 mRNA induced by BMP-2 reflect increased transcriptional activity of the prototypical Cbfa1 responsive promoter, osteocalcin. Previously, we have shown that the osteocalcin promoter contains three Cbfa1 binding sites [Merriman et al., 1995] and any one of these sites is sufficient for bone-specific activation of the OC gene in osseous cells [Banerjee et al., 1997].

We experimentally addressed the effect of BMP-2 on osteocalcin promoter activity in C2C12 cells and osteoblasts initially by performing transient transfection assays using a series of OC promoter/CAT reporter gene constructs. One of these (-108 nt/OCCAT) spans the proximal promoter of the OC gene and is required for transcription [Hoffmann et al., 1996]. The proximal OC promoter encompasses the tissue-specific OC Box I which interacts with the bone-related homeodomain proteins Msx-2 and Dlx-5 [Hoffmann et al., 1994; Ryoo et al., 1997]. However, the -108/OC promoter lacks Cbfa1 binding sites. The second construct (nt -365/OCCAT) includes a TGFβ responsive element which interacts with an AP-1 heterodimer composed of fra-2/jun-D, and the contiguous proximal Cbfa1 recognition motif (Site C) [Merriman et al., 1995; Ducy et al., 1997]. The third construct (-1.1 nt/OCZCAT) includes the VDRE domain flanked by two distal Cbfa1 sites (Site A, -605 to -599, and Site B, -441 to -435). This set of OC promoter constructs was transfected in parallel into C2C12 cells in the presence and absence of BMP-2 treatment.

The results show that BMP-2 has no significant effects on the transcriptional activity of the -1100 nt OC promoter (Fig. 4). However, deletion of sequences between -1100 to -365 reveals that OC transcription is responsive to BMP-2, as reflected by a dramatic four- to five-fold reduction of -365 OC promoter activity upon BMP-2. The suppressive activity of this BMP-2 responsive element is severely diminished by deletion of sequences between nt -365 to -108. The -108 OC promoter displays a statistically significant but numerically modest BMP-2 response (1.4-fold). The biological relevance of this result remains uncertain. Our findings suggest that sequences in the OC promoter segment from -365 to -108 contain a potent BMP-2 responsive element that down-regulates OC gene transcription.

To assess whether similar BMP-2 repression of OC promoter activity occurs within the context of a mature osteoblast environment, we performed transfection assays in ROS 17/2.8 cells having stably integrated wild type sequences and a mutated OC promoter. To evaluate the direct contributions of Cbfa interactions with the OC promoter in relation to BMP-2 regulation and the possibility that Cbfa interactions may mask BMP-2 responsiveness, we analyzed a stably integrated transgene with site-specific mutations of all three Cbfa1 sites in -1100 OC promoter in ROS 17/2.8 cells. Only the -365 OC sequence appeared responsive to BMP-2 when stably integrated in ROS 17/2.8, similar to the transient transfection observed in C2C12 cells. However, when the fold suppres-

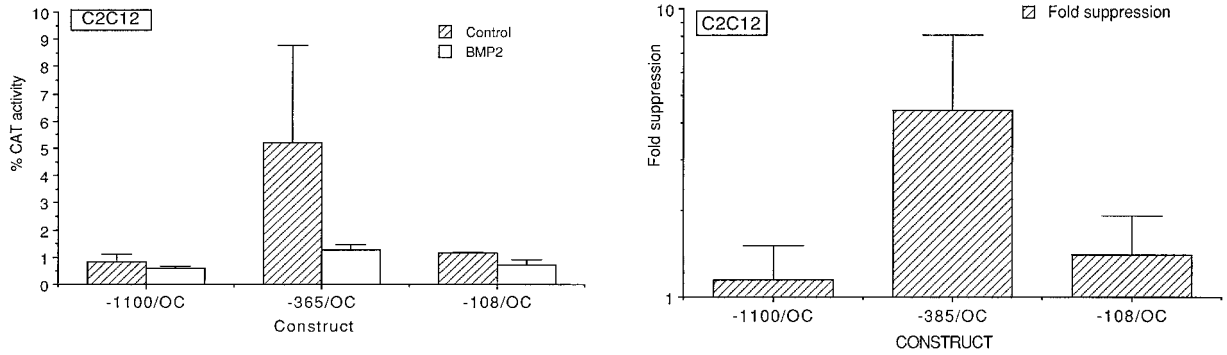


**Fig. 4.** BMP-2 repression of OC promoter activity during the early stages of osteogenic differentiation in C2C12 cells requires the nt -365/-108 promoter segment. **A:** BMP-2 responsiveness of the OC promoter in C2C12 cells. The bar-graph shows transcriptional activity (expressed as % CAT activity) of a 5' deletion series of OC prmoter/CAT reporter gene constructs upon transfection in C2C12 cells in the absence (control) and presence (300 g/ml) of the BMP-2. The data plotted represent the average CAT activity (error bars indicate standard deviations) observed for each sample from a typical experiment performed in triplicate. The results show that CAT values in transfected cells treated with BMP-2 are consistently lower than those in control cells. **B:** Delineation of a BMP-2 dependent repression domain in the nt -365/-108 promoter segment. The bar-graph shows a summary of four independent transfection experiments as described in A (up to 14 replicates) in which OC promoter activity observed for control and BMP-2 treated cells was expressed as the average "fold repression" ([% CAT activity in control cells]/[% CAT activity in the presence of BMP-2]; error bars indicate standard deviations). Statistical analysis was performed using the unpaired Student's *t*-test (Statview, MacIntosh Program). The BMP-2 response of the -365/CAT construct is significantly different from that of the -108/CAT construct (with $P < 0.01$) and the -1100/CAT construct (with $P < 0.005$). Values greater than 1 observed for the -108/CAT and -365/CAT constructs represent statistically signifcant differences ($P < 0.05$) for CAT activity observed in the absence and presence of BMP-2. We note that the -1100/CAT construct is not BMP-2 responsive ($P > 0.4$) suggesting the presence of a regulatory domain between nt -1100/-365 which appears to mask the BMP-2 response.

sion was compared to the BMP-2 effect in the pGEM empty vector stable cell lines (Fig. 5), no statistically significant differences among the promoter segments, mutant constructs, and pGEM control are observed.

These findings demonstrate that BMP-2 can suppress osteocalcin promoter activity in the non-osseous C2C12 cell line and the effect may be independent of Cbfa interactions. In mature osteoblast-like cells, as the ROS cell line, BMP-2 responsiveness may be masked by endogenous levels of enhancer factor that contribute to constitutive expression of the OC gene.

## DISCUSSION

Bone morphogenetic proteins represent a family of cytokines, many of which reside in demineralized bone matrix, and induce ectopic bone formation when implanted into muscle tissue [Urist, 1965]. The molecular mechanism of the ectopic bone formation induced by BMP was partly explained by in vitro conversion of the differentiation pathway of C2C12 myoblasts into the osteoblastic lineage by recombinant human BMP-2 [Katagiri et al., 1994]. The dedifferentiation of C2C12 myoblasts followed by commitment to the osteogenic phenotype and expression of osteoblast differentiation markers in response to BMP-2 provides an excellent model to investigate molecular mechanisms for osteogenesis, particularly when contrasted to the effects of TGF-β1. Under the culture conditions for myogenic differentiation, the presence of 300 ng/ml of BMP-2 or 5 ng/ml TGF-β1 almost completely inhibits the formation of myotubes. However, only BMP-2 confers competency for the expression of osteoblastic marker genes, type I collagen, alkaline phosphatase, osteopontin, and osteocalcin, in a time-dependent manner.

In order to elucidate the molecular mechanism underlying the arrest of C2C12 myoblast differentiation by BMP-2 and TGF-β1 and the consequent induction of osteogenesis only by BMP-2, we examined expression of the bone restricted transcription factor, Cbfa1. In our studies, Cbfa1 expression was induced in C2C12 cells at day 3 of BMP-2 treatment, but subsequently only trace levels detected at day 7 of BMP-2 treatment. When these findings are compared to the result that Cbfa1 was induced by BMP-7 in C3H10T1/2 from day 2 and that Cbfa1 expression was gradually increased until day 6 [Tsuji et al., 1998], it is apparent that cell types exhibiting distinct phenotypic properties can respond somewhat differently to a BMP signal.

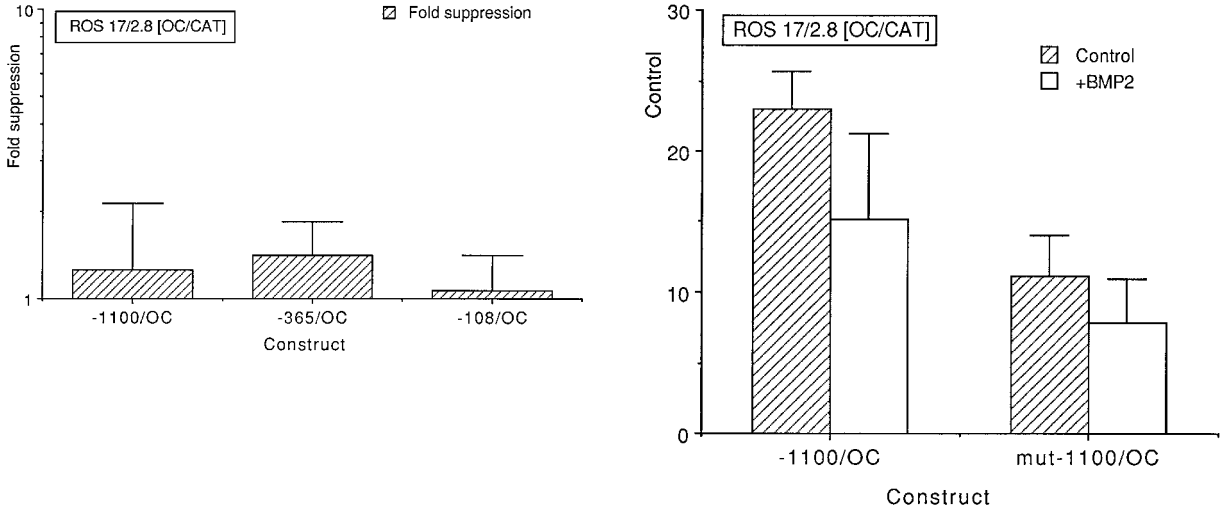


**Fig. 5.** Activity of the OC promoter in response to BMP-2 in ROS 17/2.8 osteoblastic cells. **A:** Transcriptional activity of a series of 5' osteocalcin promoter-CAT reporter constructs stably integrated into ROS 17/2.8 cells was examined in response to 300 ng/ml BMP-2. The data plotted represent the average CAT activity observed for n=9 to n=12 per group and assayed in independent experiments. OC promoter activity observed for control in BMP-2 treated cells is expressed as the mean "fold repression" (% CAT activity in control cells divided by % CAT activity in the presence of BMP-2) and corrected for background BMP-2 activity on pGEM empty vector. **B:** BMP-2 activity on the native OC promoter (-1100 nt) is compared to mutant OC promoter stably integrated into ROS 17/2.8 cells. Standard deviations are indicated by the error bars. No group exhibited statistically significant differences including the -1100 OC promoter in which the three Cbfa motifs, sites A, B, and C [Merriman et al., 1995], are mutated.

Alternatively, BMP-2 and BMP-7 may induce a different cascade of factors regulating Cbfa1 expression. As previously reported, myoblastic differentiation of MyoD-overexpressed C3H10T1/2 and NIH3T3 cell lines can be blocked by BMP-2 treatment [Katagiri et al., 1997]. However, osteoblast differentiation was induced only in C3H10T1/2 cells, but not in NIH3T3 cells, by Smad1 and Smad5 [Yamamoto et al., 1997]. These findings suggest that response to the same signal is cell type dependent, possibly due to differences in the composition of preexisting signal transduction machinery.

BMPs are extracellular signalling molecules that can initiate the cascade of events leading to osteoblast differentiation [Heldin, 1997]. The propagation of a BMP-2-mediated signalling pathway in the C2C12 cell line can be summarized as follows. Accommodation of BMP-2 by BMPR-II stimulates subsequent dimerization of the receptor ligand complex to BMPR-IA, which activates receptor serine/threonine kinase (RS/TK) activity in the cytoplasmic domain of BMPR-1A [Namiki et al., 1997]. The activated RS/TK stimulates Smad 1 or Smad 5 by phosphorylation of their C-terminal ends. The phosphorylation of these proteins enhance hetero-oligomerization of Smad1 or Smad5 with Smad4 and other proteins [Chen et al., 1996]. Consequently, the complexes translocate from the cytoplasm to the nucleus to regulate gene expression [Kretzschmar et al., 1997a,b]. Currently, several proteins other than Smads are suspected to be involved in hetero-oligomerization of the Smad proteins [Chen et al., 1996]. Notably, TGF-β1 binds its own receptor system, initially type II receptors which bind to type I receptors through phosphorylation of Smad2 and Smad 3 by RS/TK activity of TGFR-I [Lagna et al., 1996; Liu et al., 1996; Nakao et al., 1997; Hu et al., 1998]. Thus, both BMP-2 and TGF-β1 signalling share Smad4 to enhance DNA binding activity of other Smad proteins. However, they differ with respect to their ability to phosphorylate serine residues in Smads; TGF-β1 cannot phosphorylate Smad5 and Smad1 [Nishimura et al., 1998]. Formation of the Smad4-Smad5 heterodimer complex appears to be a key step in the BMP signalling pathway which mediates BMP-2 induced osteoblast differentiation of C2C12 mesenchymal cells [Nishimura et al., 1998].

As demonstrated in Figure 3, Cbfa1 was induced not only by BMP-2 but also by TGF-β1 treatment. However, the expression of Cbfa1 in TGF-β1 treated cells did not influence expression of the osteoblast marker genes, alkaline phosphatase, and osteocalcin. Moreover, the ex-

pression pattern of α1(I) collagen and osteopontin in response to TGF-β1 differed from that of BMP-2 treatment, further supporting independent pathways for the effects of TGF-β1 and BMP-2 in C2C12 cells. Our results reveal that Cbfa1 is expressed in response to both BMP-2 and TGF-β1 coinciding with arrest of the myoblast phenotype. Therefore, these factors may mediate initial stages of transdifferentiation through Cbfa1. Cbfa1 is a necessary transcription factor for commitment toward the osteogenic lineage reflected by the suppression of myoblast differentiation. However, based on the results in TGF-β1 treated cells, Cbfa1 is not a sufficient transcription factor for the induction of genes representing the mature osteoblast phenotype. Our conclusions are strongly supported by the results that prolonged treatment of Cbfa1$^{-/-}$ with BMP-2 cells induced alkaline phosphatase and osteocalcin expression despite the absence of Cbfa1 [Komori et al., 1997].

The temporal sequence of events we have observed suggests the possibility of an interaction between Cbfa1 and transcriptional machineries downstream of BMP-2 or TGF-β1 signal transduction. Cbfa1 induction by BMP-2 requires more than 24 h, which suggests that Cbfa1 is not an immediate target for the BMP-2 signalling pathway. Therefore the difference in expression of osteoblast marker genes in response to BMP-2 compared with TGF-β1 treated C2C12 cells might be accounted for by protein-protein interaction of Smads, as well as Cbfa. Other protein-protein interactions may be between Smads and Cbfas or Cbfas and cell-type specific partner proteins. Cbfa factors must dimerize with Cbfβ to initiate transactivation [Ogawa et al., 1993a]. However, Cbfa1 also can heterodimerize with proteins that suppress activity. Recently, proteins related to the drosophila Groucho (human homologue designated TLE [Stifani et al., 1992; Aronson et al., 1997] and rat homologue designated R-esp [Schmidt and Sladek, 1993]) which are expressed in many tissues, have been characterized as such Cbfa partner proteins. Provided that the Cbfa1 isoforms upregulated in response to BMP-2 are transcriptionally active, the possibility arises that OC gene transcription should be highly enhanced by BMP-2 as an initial step towards osteoblast phenotype commitment in C2C12 mesenchymal progenitor cells. It has been well established that Cbfa1 activates osteocalcin expression. Overexpression of Cbfa1 dramatically enhances osteocalcin gene expression and in transient transfection of OC-reporter constructs, several studies have demonstrated that the Cbfa sequences confer tissue specific expression [Banerjee et al., 1996, 1997; Ducy et al., 1997]. However, in C2C12 cells, BMP-2 and TGF-β1 induce Cbfa1 mRNA, but a DNA binding protein competent to activate transcription of osteocalcin is not apparently produced in response to TGF-β1.

The spectrum of protein-protein interactions required to regulate activity of Cbfa1 is appreciated in our studies based on the observations that BMP-2 inhibition of promoter activity overrides the Cbfa enhancer activity on the osteocalcin promoter. This finding appears to be consistent with the necessity for transient expression of Cbfa1 during C2C12 transdifferentiation to committed osteoblasts. The Cbfa class of runt domain proteins has demonstrated specific DNA binding activity with Cbfa/AML consensus sequences in the osteocalcin promoter that correlates with enhancer activity [Banerjee et al., 1997]. In ROS 17/2.8, BMP-2 suppressor effects on the OC promoter are not observed. Here, perhaps, the high endogenous levels of Cbfa1 and competency for formation of the osteoblast-specific DNA binding complex [Banerjee et al., 1997] that contributes to enhanced promoter activity is not modified by BMP-2. In one early study, the rat osteocalcin promoter was observed to be resistant to the effects of BMP-2 during a 72 h transfection of mouse stromal cell line MLB13MYC clone 17 [Goto et al., 1996]. This result suggests Cbfa1 may form a complex in response to BMP-2 that is not capable of transactivating promoters like osteocalcin. Notably, alkaline phosphatase, which is not regulated by Cbfa1, is expressed first in BMP-2 induction of osteogenesis. In C2C12 cells, osteocalcin finally becomes expressed when Cbfa1 mRNA levels are downregulated on day 7. Perhaps the induced Cbfa levels in response to BMP-2 have autocrine effects which leads to the downregulation of its own mRNA. Thus, numerous signalling pathways may converge to regulate Cbfa mRNA expression and DNA binding activity. Cbfa1 activity in response to BMP-2 in C2C12 cells clearly differs from Cbfa activity in mature osteoblasts.

We compared the expression of Cbfa1 to the expression of two homeodomain proteins Msx-2 and Dlx-5, which are important for early limb development in vivo [Ferrari et al., 1995] and

involved in osteogenesis. Msx-2 is abundantly expressed in early osteoprogenitors and must be downregulated for the final stages of osteoblast differentiation in vitro [Ryoo et al., 1997; Hoffmann et al., 1996], while Dlx-5, which we demonstrated exhibits bone tissue specific expression, is expressed at the mineralization stage of rat osteoblast differentiation in vitro [Ryoo et al., 1997]. Msx-2 was transiently induced at day 3 in BMP-2 treated cells, but not in response to TGFβ, suggesting that its induction also reflects the commitment to differentiation of C2C12 to osteoblasts. In contrast, Dlx-5 was not detected in BMP-2 treated C2C12 cells. This finding may reflect the lack of mineral deposition and final stages of differentiation to osteocytes after 7 days of BMP-2 treatment.

The delay in expression of Cbfa responsive genes, osteocalcin, and osteopontin, in BMP-2 treated cells could be accounted for by Msx-2. Msx-2, a characterized suppressor of osteocalcin transcription, is also upregulated on day 3 when Cbfa1 is induced. Transcriptional regulation occurs directly by protein binding to homeodomain DNA recognition motifs [Hoffmann et al., 1994; Towler et al., 1994] and through heterodimeric combinations with selected proteins. Abate-Shen and co-workers [Zhang et al., 1997] demonstrated that the N terminal of Msx factors interacts with Dlx family members; the formation of a Dlx-5/Msx-1 heterodimer results in de-repression of Msx-1-mediated inhibition of the myoD enhancer element. Thus, in the absence of detectable Dlx-5 in BMP-2 differentiated C2C12 cells, the suppressor effects of Msx-2 on osjteoblast expressed genes may remain until Msx-2 is downregulated.

Taken together, our findings demonstrate that Cbfa1 plays a central role in BMP-2 and TGF-β1 induced transdifferentiation of C2C12 cell at an early restriction point to divert the cells from the myogenic pathway. However, commitment and progression of osteogenes requires interactions with BMP-2 signaling machinery, but not with TGF-β1. Cbfa1 appears necessary but to sufficient for osteoblast differentiation and further studies would be required to elucidate mechanisms for the final stages of osteoblast maturation. Our observations reveal that multiple factors and signalling pathways may synergize/converge or function independently to regulate osteogenesis.

## ACKNOWLEDGMENTS

This work was supported in part by Basic Medical Research Fund of Ministry of Education, Republic of Korea, 1997, and by the National Institutes of Health. The contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health.

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