Journal of Cellular Biochemistry 80:156–168 (2000)

Expression Screening of Factors Binding to the Osteocalcin Bone-Specific Promoter Element OC Box I: Isolation of a Novel Osteoblast Differentiation-Specific Factor

Heidi Hoffmann, Jack Green, André J. van Wijnen, Janet L. Stein, Gary S. Stein, and Jane B. Lian*

Department of Cell Biology, University of Massachusetts Medical Center, Worcester, Massachusetts 01655

Abstract Contributing to bone-specific expression of the osteocalcin gene is the promoter element OC Box I (−99 to −76), which binds both Hox proteins and another nonhomeodomain factor (designated OCBP for osteocalcin-box binding protein) (Hoffmann et al. [1996] J Cell Biochem 61:310–324). OCBP correlates with increased promoter activity and may, therefore, be important to development or maintenance of the osteoblast phenotype. To identify OCBP candidates, we used a multimerized OC Box I sequence to screen a γgt11 cDNA expression library, constructed from the rat osteosarcoma osteoblastic ROS 17/2.8 cell line, for cDNA clones encoding factors that recognize this element. Mutant OC Box I sequences that do not bind OCBP and/or homeodomain proteins were used to counterscreen the cDNA isolates. Clones showing binding specificity were sequenced and further characterized for patterns of expression in different tissues and cell lines. Among the characterized nonhomeodomain-related isolates, we identified a nucleolin, a clone encoding rCAP2 that is related to myogenic differentiation and more importantly, a cDNA clone containing a previously uncharacterized gene that has been designated as a cell differentiation-related factor (DRF). DRF mRNA is highly expressed in ROS 17/2.8 cells and in a developmentally regulated pattern during osteoblast differentiation, being upregulated at the postproliferative maturation transition and coinciding with the induction of osteocalcin gene expression. The 7.7-kb transcript encoded by clone 44 is abundantly expressed in osteoblasts, but the mRNA was not present at detectable levels in bone and soft tissues by Northern blot analysis. However, related expressed sequence tags were recently reported in cDNA libraries of rat lung and mouse sympathetic ganglion. The identification of DRF represents a novel osteoblast differentiation-specific marker related to osteocalcin expression. The identification of DRF may further facilitate definition of gene regulatory mechanisms mediating the final stages of bone cell differentiation J. Cell. Biochem. 80:156–168, 2000. © 2000 Wiley-Liss, Inc.

Key words: expression screening; osteocalcin; OC Box I; differentiation-related factor

Mesenchymal cells can differentiate into osteoblasts, chondrocytes, myocytes, and adipocytes [Taylor and Jones, 1979; Grigoriadis et al., 1988, 1990; Yamaguchi and Kahn, 1991]. The commitment of mesenchymal cells to the osteoblastic lineage is influenced by several factors including members of the transforming growth factor-β family, particularly, the bone morphogenic protein 2 (BMP-2), and the RUNX/CBFA/AML runt domain transcription factor family; most significantly, CBFA1/AML-3 [Merriman et al., 1995; Ducy et al., 1997; Geoffroy et al., 1995; Otto et al., 1997; Komori et al., 1997; Banerjee et al., 1996a, 1997]. However, control of cellular phenotype involves multiple factors that regulate the expression of tissue-specific genes throughout development. For example, Msx-2 and Dlx-5 homeodomain factors influence osteoblast differentiation and/or bone-specific gene expression [Hoffmann et al., 1994; Ryoo et al., 1997; Towler et al., 1994].

Grant sponsor: National Institutes of Health; Grant numbers: AR33920, DE12528, AR39588.

The contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health.

Heidi Hoffmann's current address is AlphaGene, 260 West Cummings Park, Woburn, MA 01801.

*Correspondence to: Jane B. Lian, Department of Cell Biology, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01655-0106.

Received 14 August 1998; Accepted 22 June 2000

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Osteoblast development in culture is characterized by progression through sequential stages of differentiation (i.e., cell growth, formation of the extracellular matrix, matrix maturation, and mineralization). Each stage is marked by the temporal expression of specific genes. During the onset of extracellular matrix formation, collagen type I (α1), fibronectin, and transforming growth factor β (TGF-β) are expressed during cell growth. The postproliferative matrix maturation period is characterized by expression of alkaline phosphatase, while during mineralization, expression of osteopontin and osteocalcin is upregulated (reviewed in [Lian et al., 1992; Stein and Lian, 1993]). This progression of gene expression is also observed in vivo during development [Weinreb et al., 1990; Nakase et al., 1994; Sommer et al., 1996]. Factors that bind to promoter elements involved in the bone-specific expression of the osteocalcin gene have proven to be candidates for the control of development of the osteoblast phenotype [Merriman et al., 1995; Banerjee et al., 1997; Ducy et al., 1997]. Therefore, further characterization of factors interacting with highly conserved OC promoter elements may yield novel insights into developmental control of the bone cell phenotype.

Several elements in the rat osteocalcin promoter have been shown to control tissue-specific expression of this gene. These elements include the three RUNX2/CBFA1/AML-3 recognition sites [Merriman et al., 1995] and the OC Box I, which is located in the proximal osteocalcin promoter and is part of a minimal promoter of 108 nucleotides that can regulate tissue-specific expression [Heinrichs et al., 1995; Hoffmann et al., 1996]. The OC Box I is a highly conserved 24 nucleotide sequence [Lian et al., 1989a] that has been shown to bind the homeodomain proteins Msx-1 and Msx-2 [Hoffmann et al., 1994; Towler et al., 1994] and Dlx-5 [Ryoo et al., 1997]. In addition, a nonhomeodomain protein in nuclear extracts from osteoblasts associates with this transcription element. This nonhomeodomain protein has been found only in nuclear extracts from bone-derived cell lines and was named the OCBP for *OC* Box *b*inding *p*rotein [Hoffmann et al., 1996]. Promoter elements similar to OC Box I are found in the collagen type I [Goldberg et al., 1995; Rossert et al., 1996; Dodig et al., 1996], bone sialoprotein [Li and Sodek, 1993; Yang and Gerstenfeld, 1997] promoters, and osteopontin [Yang and Gerstenfeld, 1997], further suggesting the importance of this element in regulation of bone-specific transcription.

We therefore used OC Box I sequences to isolate DNA binding proteins from a λgt11 expression library constructed from the cDNA of rat osteosarcoma cells, ROS 17/2.8. Northern analysis using the isolates to probe RNA from different cell and tissue types as well as from osteoblast cultures at various stages during development revealed that expression of one novel clone (clone 44) is continuously increased during osteoblast differentiation. This clone, designated differentiation-related factor (DRF), represents a novel marker to dissect gene regulatory mechanisms during bone cell phenotype development.

MATERIALS AND METHODS

Library Screening

One million clones of a λgt11 cDNA library representing mRNAs expressed in ROS 17/2.8 cells were induced to express proteins in *Escherichia coli* Y1090R$^-$ and assayed for association with OC Box I sequences (OC2; Table I) as described in Ausubel et al. [1989]. After blocking with 5 mM Tris/7.5, 0.1 mM EDTA, 0.1 mM dithiothreitol (DTT) for 1 h at room temperature, the filters were washed three times, 10 min per wash in binding buffer (1 mM Tris/pH 7.5, 5 mM NaCl, 5% glycerol, 5% sucrose, 0.02 mM EDTA, 0.75 mM $MgCl_2$, 0.1 mM DTT, and 0.1% NP-40) and then hybridized for 1 h at room temperature in binding buffer plus 1×10^6 cpm /ml probe (OC2 sequences, Table I), 5 μg/ml heat-denatured salmon sperm DNA and 0.5 mg/ml bovine serum albumin (BSA). The binding buffer was established [Hoffmann et al., 1996] to enhance protein–DNA interactions with the OC Box. The filters were washed in binding buffer four times (10 min per wash) at room temperature, blotted dry, and exposed to X-ray film overnight at -80°C with intensifying screens. Phage that showed association with probe on duplicate filters were isolated. Each of the isolated phage was further characterized and counterscreened using the methods described above with the oligonucleotides WTOC, OC8, and HOX as probes (sequences in Table I).

The probes were prepared by labeling the ends with ^{32}P and ligating the oligonucleotides together. The sense oligonucleotide (300 ng)

TABLE I. Summary of Characterization of Binding Specificities of λgt11 Isolates;[a] The 44 Isolates Are Classified into 5 Different Categories Based on Their Recognition of the Sequences Detailed in Lower Part of Table[b]

Probes[a]	
WTOC	ATGACCCCCAATTAGTCCTGGCAG
OC2	ATGACCCggAATTAGTCCTGGCAG
OC8	ATGACCCgactgctcTCCTGGCAG
HOX	GCCTCCAATTAGTGT

No. of isolates[b]	Phenotype	Probe WTOC	Probe OC8	Probe HOX
12	OCBP candidates[c]	Y	N	N
4	Hox-related OC factors[c]	Y	N	Y
6	Junction sequence specific	Y	Y	N
18	Nonspecific associations	Y	Y	Y
4	False positives	N	N	N

[a]Detail of the sequences used to probe plaque lifts of the λgt11 library and isolates. Lowercase letters indicate sequences that are mutated from the wild-type OC Box I osteocalcin promoter element. HOX indicates sequences of the homeodomain binding site that were used to determine which proteins might be homeodomain related.
[b]All of the isolates bind to the OC2 sequences that were used in the original isolation of the clones.
[c]Categories that are of interest for further study.

was labeled with ^{32}P-λ-ATP, whereas the complimentary oligonucleotide (600 ng) was phosphorylated with nonradioactive ATP. The two reactions were combined, boiled, and slow cooled. After cooling, ATP and T4 polynucleotide ligase were added, and the reaction was incubated at 16°C overnight. Excess nucleotides were removed by centrifugation of the sample through a G-25 Sephadex column.

RNA Analysis

Total cellular RNA was extracted with TriZol (GIBCO/BRL, Gaithersburg, MD) according to the manufacturer's instructions. Bone (calvaria and long bone) and soft tissues were harvested from 8-week-old mouse (C57/Bl) and rat (Sprague-Dawley). Frozen tissues were pulverized before resuspension in TriZol solution to extract the RNA. Extracted RNA was incubated for 30 min at 37°C with 20 U of RNasin (Promega, Madison, WI) and 20 U of RNase free DNase I (Promega) in 10 mM Tris-Cl, pH 8.3, 50 mM KCl, and 1.5 mM $MgCl_2$. Samples were phenol/chloroform extracted, chloroform extracted, and then ethanol precipitated. RNA was resuspended in diethylpyrocarbonate-treated water. RNA (10 μg) was separated by electrophoresis in 1% agarose/17.6% formaldehyde gels, and the integrity of the RNA was assessed after ethidium bromide staining by the ratio of 28S/18S ribosomal RNA. RNA was transferred from the denaturing gel onto Zeta-Probe membrane (Bio-Rad, Hercules, CA) overnight by capillary action using 20× SSC buffer (3 M NaCl, 0.3 M sodium citrate). RNA was cross-linked to filters by ultraviolet irradiation for 1 min and the blots were stored until use. The RNA blots were prehybridized with a solution of 50% formamide, 0.12 M Na_2HPO_4/pH 7.2, 0.25 M NaCl, 7% sodium dodecyl sulfate (SDS) and 1 mM EDTA for 10 min at 43°C. Next, 10^6 cpm/ml random primed (Prime It Kit; Stratagene, La Jolla, CA) ^{32}P-dCTP (3,000 Ci/mmol; NEN, Boston, MA) labeled cDNA probes specific for the genes specified on each figure were heat denatured and added to the prehybridization buffer, and the RNA blots were hybridized overnight at 42°C. After hybridization, blots were washed in buffer of progressive stringency starting at 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate), 0.1% SDS at room temperature, to a final stringency of 0.1× SSC, 0.1% SDS at 55°C.

Cell Culture

Rat osteosarcoma cells (ROS 17/2.8 [Majeska et al., 1980]) were grown in F-12 (GIBCO, Gaithersburg, MD) medium supplemented with 5% fetal calf serum. Rat UMR-106 [Partridge et al., 1980] were cultured in minimal essential medium (MEM: GIBCO) supplemented with 10% fetal calf serum. HeLa S3 cells [Puck et al., 1956] were grown in Joklik-modified minimal essential medium (GIBCO) supplemented with 5% fetal calf serum and 5% horse serum. Mouse MC3T3-E1 cells [Kodama et al., 1981] were grown in α-MEM (GIBCO) plus 10% fetal calf serum. Human osteosarcoma cell line SAOS-2 (obtained from American Type Culture Collection [ATCC], Rockville, MD) was grown in Dulbecco-modified Eagle medium (DME: GIBCO) supplemented with 10% fetal calf serum. H4 rat hepatoma cells (obtained from ATCC) were grown in MEM (GIBCO) 5% fetal calf serum and 5% horse

serum. FRTL-5 cells [Vitti et al., 1982] were grown in Coon's modified F12 medium supplemented with 5% calf serum and 1.7% 6H [Ambesi-Impiombato and Perrild, 1989]. Mouse embryo cell line 3T3-L1 was obtained from ATCC and grown in DME plus 5% fetal calf serum. Mouse mammary tumor cells (C127, obtained from ATCC) were grown in DME plus 5% fetal calf serum and 5% horse serum. Normal human fetal lung fibroblasts (IMR-90 [Nichols et al., 1977]) were grown in DME plus 10% fetal calf serum.

Mouse C2C12 myoblasts were plated in DME with 15% fetal calf serum. The media was replaced 24 h after plating with DME and 5% fetal calf serum also containing 400 ng/ml of BMP-2 (a gift from Drs. John Wozney and Vicki Rosen, Genetics Institute, Cambridge, MA). Cells were harvested 24 h after plating (0 days), 24 h after the addition of BMP-2 (1 day), and 24 h after each feeding on days 3 and 6 of BMP-2 treatment.

Normal osteoblasts (ROB cells) obtained from calvariae of fetal rats of 21-day gestation were isolated and subjected to sequential digestion of 20, 40, and 90 min at 37°C in 2 mg/ml collagenase P (Boehringer-Mannheim, Indianapolis, IN) with 0.25% trypsin (Gibco, Grand Island, NY). Cells released in initial digests were discarded, and those released from the third digestion were plated at a density of 4×10^5 cells/100-mm dish. Cells were fed every 2 days with MEM (Gibco) supplemented with 25 μg/ml ascorbic acid. All subsequent feedings contained medium supplemented with 10% fetal calf serum, 50 μg/ml ascorbic acid, and 10 mM β-glycerol phosphate. Culture conditions for differentiation and mineralization were used as detailed in Aronow et al. [1990] and Bellows et al. [1986]. ROB cells were also treated for 24 h before harvest at three stages of maturation with 10^{-7} M dexamethasone, 10^{-8} M $1,25(OH)_2D_3$ (a gift from Dr. M. Uskokovic, Hoffmann-La Roche Inc., Nutley, NJ) or 2 ng/ml TGF-β (R&D Systems, Minneapolis, MN) to assess hormonal and growth factor regulation.

Plasmids and Constructs

The λgt11 inserts were amplified using PCR primers which hybridized to λgt11 sequences. The amplification primers contained a Bgl II site and the products were digested with Bgl II and cloned into the Bam H1 site of the PQE vector (Qiagen, Chatsworth, CA) or digested with EcoRI and cloned into the EcoRI site of pGEX-5X-1 or 5X-2 (Pharmacia, Piscataway, NJ).

Production of GST/Nucleolin Fusion Protein

λgt11 clone number 3 (nucleolin) was subcloned into pGEX-5X-1 (Pharmacia Biotech) and transformed into in *E. coli* BL21. Expression of a GST/nucleolin fusion protein was induced by growth in 0.1 mM IPTG for 1 h. Cells were pelleted and resuspended in ice-cold buffer containing 20 mM Tris-Cl/pH 8.0, 0.2 mM EDTA, 1 M NaCl, 1 mM phenylmethylsulphonyl fluoride, 1 mM DTT, and 1 mg/ml lysozyme and sonicated three times for 15 s. Triton X-100 was added to a final concentration of 1%, and the mix was incubated for 30 min at 4°C. The supernatant was either used at this stage (bacterial lysate) or purified by binding to a glutathione Sepharose 4B column (Pharmacia Biotech) and releasing the fusion protein by the addition of excess glutathione (10 mM reduced glutathione in 50 mM Tris HCl/pH 8.0).

Gel Mobility Shift Assay

Probe was prepared by 5′ end labeling, using ^{32}P-γ-ATP and T4 polynucleotide kinase. For each binding reaction, 40 fmol probe and 5 μl purified protein were used in a reaction containing 5 μg BSA (fraction V, Sigma), 0.1% NP40, 10 mM DTT, 1 μg poly(dI-dC), 25% glycerol, 25% sucrose, 37 mM $MgCl_2$, 50 mM Tris-HCl/pH 7.5, 250 mM NaCl, and 1 mM EDTA. The suspension was incubated for 10 min at room temperature. Protein–DNA complexes were separated on a 6.5% (30:0.8) nondenaturing polyacrylamide gel. The gel was electrophoresed in 0.5× TBE buffer for 3 h at 250 V. Gels were dried and autoradiographed on Kodak XAR film, using an intensifying screen, at −70°C. Competitions were performed in reactions as described above with the addition of 50× molar excess of unlabeled oligonucleotide competitors.

RESULTS AND DISCUSSION

Isolation of cDNAs Encoding Proteins with Recognition for the OC Box I Transcriptional Regulatory Element of the Osteocalcin Gene

A rat osteosarcoma cDNA library in λgt11 was plated and induced to express proteins using IPTG. Duplicate filter lifts were made of

each library plate. The membranes were hybridized with a multimerized, radiolabeled probe (OC2; Table I, top) encoding the tissue specific 24 nucleotide osteocalcin transcriptional element, OC Box I (nt −99 to −76, relative to the ATG codon) [Lian et al., 1989b] with a two-nucleotide mutation. OC2 was chosen for the original screening because the two-nucleotide mutation was previously shown to increase the affinity of all the factors (homeodomain related and a bone-specific factor OCBP) that associate with OC Box I [Hoffmann et al., 1996]. Positive clones were replated and screened two more times with OC2 to isolate individual phage encoding a protein with affinity for the altered OC Box I sequences. We purified 44 isolates in this manner.

The 44 isolates were then probed with three additional sequences (detailed in Table I, top) to determine the binding specificity of each isolate with respect to the individual regulatory elements within the OC Box. The probes contained the wild-type OC Box I sequences (WTOC), an eight nucleotide mutation of OC Box I that eliminates homeodomain and OCBP binding (OC8), or a homeodomain binding consensus sequence (HOX). Binding with the WTOC probe but not the OC8 mutation was used to indicate specificity of the protein–DNA interactions. Association with the HOX probe was used to determine which clones encoded proteins belonging to the homeodomain family of transcription factors that associate with OC Box I. The binding characteristics of the 44 isolates are summarized in Table 1B. Of the 44 isolates, twelve encoded proteins that bound to the WTOC probe but not OC8 or HOX sequences and were considered candidates for the OCBP. Four isolates, interacting with the WTOC and HOX probes but not OC8, were considered candidates for homeodomain proteins that bind OC Box I. Six isolates bound the WTOC and OC8 probes but not the HOX probe, indicating interaction with the junction sequences created by ligating multiple OC Box oligos together. Eighteen isolates bound all of the probes and represent nonspecific DNA binding proteins. Thus, our expression screen for OC Box I binding proteins has yielded two classes of cDNAs that appear to bind to the homeodomain motif (four clones) or the overlapping recognition motif for a nonhomeodomain protein OCBP (12 clones).

Expression of the Isolates During Growth and Differentiation of the Myoblast and Osteoblast Phenotype

The 16 isolates that were classified as potential bone-specific osteocalcin-box binding proteins (OCBP) and homeodomain factor candidates were further screened for tissue specificity of expression. RNA from 3-week-old mouse muscle, liver, and calvaria and from rat osteosarcoma (ROS 17/2.8), mouse myoblast (C2C12), and human lung fibroblast (IMR-90) cell lines were hybridized with radiolabeled sequences from different λGT11 isolates. Four representative isolates, clones 3, 19, 35, and 44, illustrate the different patterns of expression observed (Fig. 1). Clone 3 was a potential OCBP candidate by virtue of its probe-binding association (Table I), but it is expressed in all of the cell and cell lines tested, and therefore does not exhibit the tissue specificity of the OCBP.

Based on the expression screening, clone 35, which bound HOX and WTOC probes but not OC8 in the plaque screening experiment, is a potential homeodomain clone. The Northern blot results (Fig. 1) reveal that clone 35 is expressed more prevalently in muscle than in bone tissue and cell lines. To assess expression of clone 35 during muscle cell differentiation, cultures of the premyogenic line C2C12 were grown to confluency (controls) and were chronically treated after plating with BMP-2 (which blocks muscle and induces osteoblastic differentiation) or with TGF-β (which blocks myotube formation but does not mediate osteogenic differentiation) to alter expression of the muscle cell phenotype. Control and treated cells were harvested 24 h after feeding on days 1, 3, and 6 of culture, and RNA was isolated from each group. Morphologically, the myoblast phenotype was clearly repressed by treatment with BMP-2, as previously reported ([Katagiri et al., 1994] and our data not shown). The RNA was probed with radiolabeled osteocalcin cDNA or clone 35 sequences (Fig. 2). Expression of clone 35 is clearly downregulated by treatment with BMP-2. Osteocalcin, a bone marker, is induced by the BMP-2 treatment, demonstrating induction of the osteoblast phenotype. These data demonstrate an inverse relationship between the expression of clone 35 and osteocalcin. Clone 35 is a potential candidate for repression of the bone cell phenotype.

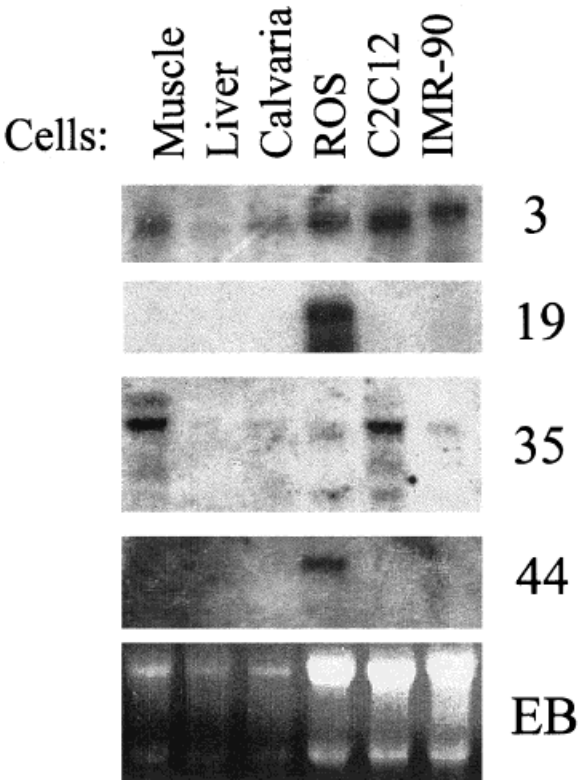


Fig. 1. Preliminary analysis of tissue specificity of the lambda isolates. RNA was isolated from muscle, liver, and calvaria isolated from 3-week-old mice and from rat osteosarcoma (ROS), mouse myoblast (C2C12), and human lung fibroblast (IMR-90) cell cultures. Ten micrograms of RNA from each sample was separated on a 1% agarose/formaldehyde denaturing gel and transferred overnight by capillary action onto Zetaprobe membrane. The membranes were hybridized with random-prime labeled probes generated from the sequences of the indicated lambda isolates. The EB samples show the ethidium bromide staining of the ribosomal RNA to indicate relative loading of the RNA species.

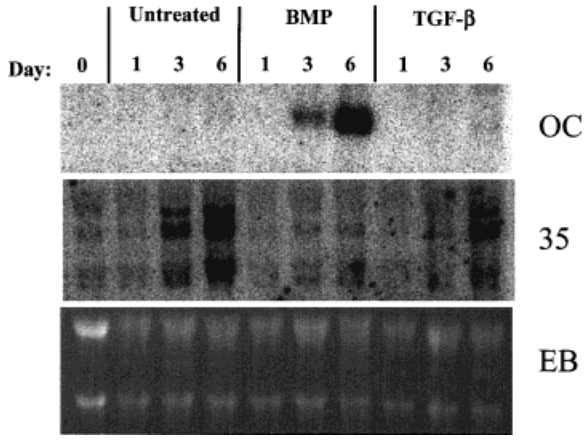


Fig. 2. Clone 35 expression parallels development of the myoblast phenotype. C2C12 cells were grown in culture for 1, 3, or 6 days with chronic treatment of bone morphogenic protein (BMP)-2, transforming growth factor β (TGF-β) or untreated. Cells harvested on the day of plating (0) were used as a control to indicate initial expression of genes before development of the myoblast phenotype. RNA was extracted from each time point and treatment condition, separated on a 1% agarose/formaldehyde denaturing gel, and transferred by capillary action onto Zetaprobe membrane. Ethidium bromide staining (EB) of the ribosomal RNA separated on the agarose gel is shown to indicate relative loading consistency. The northern was probed with sequences from osteocalcin cDNA (OC) or clone 35 (35).

Clones 19 and 44 are classified as OCBP candidates (Table I) because they bind to the WTOC probe but not OC8 and HOX. Clones 19 and 44 are expressed strongly in ROS 17/2.8 cells (Fig. 1) and were therefore tested for expression during development of the bone cell phenotype in cultured primary rat calvarial osteoblasts (ROB). Osteocalcin expression increases during mineralization, reflecting the extent of differentiation in osteoblasts. Expression of clone 19 is upregulated as ROB cells differentiate during mineralization (Fig. 3). However, it does not parallel the expression of osteocalcin but peaks at day 23, after osteocalcin reaches maximal levels. Thus, clone 19 appears to represent a marker characteristic for the most mature osteoblast embedded in a mineralized extracellular matrix.

In contrast, clone 44 expression patterns are similar to those of osteocalcin during development of the osteoblast phenotype (Fig. 3). Interestingly, clone 44 was also expressed early in culture on day 3. This early developmental expression of clone 44 was observed only in primary cultures, and not in secondary cultures that were passaged on day 5 (data not shown). Thus, the expression of clone 44 in the day 3 cells isolated directly from the fetal calvaria may reflect residual mRNA from the bone in vivo. The upregulation of clone 44 at day 10 reflects modifications in gene expression required for the progression of osteoblast differentiation during this critical transition period in bone cell phenotype development. Consequently, clone 44 represents a novel marker for studying bone-cell-specific gene regulatory mechanisms during osteoblast maturation.

The detection of clone 44 in freshly isolated osteoblasts from rat calvaria for the primary cultures appeared inconsistent with the lack of expression in calvaria tissue of the mouse (Fig. 1, lane 3). To determine whether species specificity was a factor in tissue detection of clone

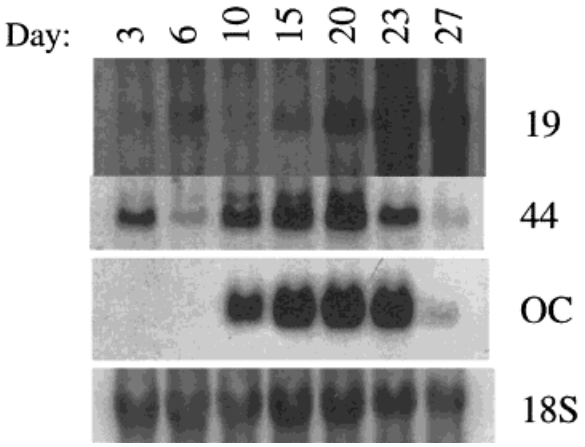


Fig. 3. Expression of clones 19 and 44 increases as the bone cell phenotype develops. Rat calvaria osteoblasts were grown in culture from 3 to 27 days and harvested at various time points as indicated above each lane. RNA was extracted from the cultured cells and separated on a 1% agarose/formaldehyde denaturing gel and transferred onto Zetaprobe membrane for northern analysis. The RNA blot was probed with labeled sequences from clone 19, clone 44, osteocalcin cDNA (OC), or 18S ribosomal RNA (18s).

44, the representation of clone 44 mRNA in rat tissues was examined. ROS 17/2.8 cells and a time course of primary rat calvarial osteoblasts were included as positive controls (Fig. 4). Clone 44 could not be detected in whole tissues by Northern analyses of 15 μg total cellular RNA per lane (Fig. 4a). Thus, although clone 44 is undetectable in whole tissues, a significant mRNA level is expressed in relation to osteoblast differentiation and its levels increase with maximum osteocalcin transcription in primary cultures (Fig. 4b, representing a different experiment than shown in Fig. 3). The transcript is also represented in Poly A+ RNA (Fig. 4c) from the bonelike ROS 17/2.8 cells. Thus, these findings indicate that postnatal tissue levels of clone 44 are not readily detectable, but the results confirm the abundance of clone 44 in osteoblasts during their differentiation. Therefore, tissue specificity of clone 44 could not be established by these studies.

Sequence Analysis and Characterization of the cDNA Isolates

Clones 3, 19, 35, and 44 were sequenced and analyzed for identity or similarity with genes reported in GenBank. The alignment of the sequences isolated in the lambda clones is detailed in Figure 5.

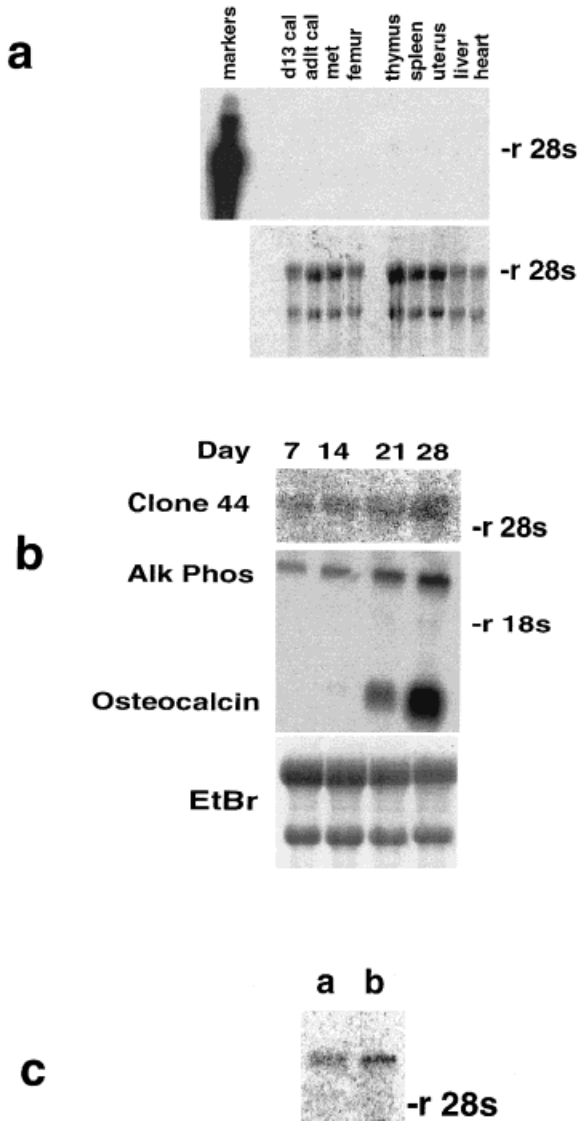


Fig. 4. Tissue and osteoblast representation of clone 44 mRNAs. **a:** Northern blot analysis of total cellular RNA (15-μg lane) from postnatal rat tissues. **Left to right lanes:** day 13 calvaria (d13 cal); 8-week-old tissues: calvaria (adlt cal), femur metaphysis (met), femur diaphysis (femur), thymus, spleen, uterus, liver, and heart. Ribosomal RNA (r28S) is indicated. The lower panel shows the ethidium bromide stained gel. **b:** Transcripts (7.7 kb) of clone 44 detected by Northern blot analysis in osteoblasts from cultured fetal rat calvaria cells (days 7, 14, 21, and 28) is shown in the top panel using the same labeled probe as in (a). Middle panel demonstrates alkaline phosphate (Alk Phos) and osteocalcin mRNA expression in the same blot. The ribosomal 28S and 18S RNA markers are indicated. The lower panel shows the ethidium bromide (EtBr) staining of the same gel. **c:** Ten microgram total cellular RNA (a) or 2 μg polyA+ RNA (b) from ROS 17/2.8 cells harvested at confluency.

Clone 19 is homologous with a central region of the VL30 gene, a repetitive genetic element. Although the VL30 gene is known to produce an RNA product that can interact with other nucleic acid molecules, it does not have a known protein product [Torrent et al., 1994]. VL30 gene expression is upregulated during malignant growth and anoxia [Firulli et al., 1993], and VL30 RNA/RNA duplexes assist in virion packaging. However, it remains unclear why this clone was identified during our screen as a candidate for a DNA binding protein. In our osteoblast cultures, VL30 expression peaked after osteocalcin in the late mineralization period, when apoptosis occurs [Lynch et al., 1998]. Interestingly, VL30 expression has been observed in relation to modification in intracellular calcium [Magun and Rodland, 1995] and with apoptosis induced by glucocorticoids [Gruol and Altschmied, 1993].

Clone 35 (muscle related) is homologous with the C-terminus of rCAP2 (adenylyl cyclase-associated protein-2) and contains an additional 170 nucleotides 3′ of the sequences reported in GenBank. The protein contains two distinct functional regions that are separated by a proline hinge [Swiston et al., 1995]. The C-terminus has been demonstrated to bind actin [Hubberstey et al., 1996] and is associated with morphological and nutritional defects in yeast [Gerst et al., 1991]. The messenger for rCAP2 is expressed as the myoblast phenotype develops. It is possible that rCAP2 may repress the osteoblast phenotype in mesenchymal cells.

Clone 3 is homologous with the central region of rat nucleolin. This region of the gene contains a nucleic acid binding motif and has previously been reported to influence transcriptional activity [Yang et al., 1994]. Nucleolin has high affinity for G-G paired DNA [Hanakahi et al., 1999]. Nucleolin also recognizes matrix attachment regions (MARs) [Dickinson and Kohwi-Shigematsu, 1995]. MARs are known to influence transcriptional activity (reviewed in Bode et al. [1995] and Stein et al. [1991]). The various DNA sequences that have been published (MARs [Dickinson and Kohwi-Shigematsu, 1995] and promoter sequences [Yang et al., 1994]) and the OC Box I sequences used in this study are clearly distinct and do not define a consensus sequence. Thus, we investigated the specific association of nucleolin with the OC Box.

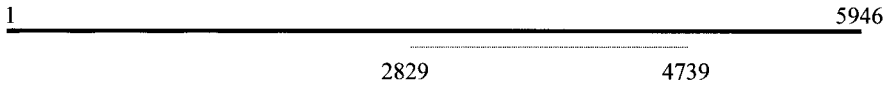
Clone 3 (nucleolin) was subcloned into a GST fusion expression vector, and the chimeric GST/nucleolin protein was expressed in *E. coli* and tested for binding activity either in bacterial cell lysates or after partial purification. The chimeric protein was assayed for binding activity and specificity to OC Box I sequences using a gel mobility shift assay (Fig. 6). The data demonstrate that nucleolin binds to OC Box I and has varying affinity for sequences containing different mutations of OC Box I that were used as competitors. These studies demonstrate that the pattern of competition does not reflect the expected properties of the OCBP. For example, the CC mutant that binds OCBP [Hoffmann et al., 1996] does not compete for the nucleolin/DNA interaction. In addition, the interactions with the AA mutant and the Hox consensus rule out the possibility that nucleolin is interacting with the homeodomain motif. Nucleolin may therefore represent a third class of factors associating with these sequences. Nucleolin is a multifunctional phosphoprotein whose levels are related to cell proliferation. Nucleolin has recently been shown to bind to G-rich oligonucleotides that exhibit antiproliferative activity [Bates et al., 1999]. Nucleolin binding to the OC Box may relate to the stringently regulated postproliferative activation of osteocalcin gene transcription.

Of significance, two other nuclear matrix–associated transcription factors, CBF/AML and YY1, regulate expression of the osteocalcin gene [Merriman et al., 1995; Guo et al., 1995]. The CBFA/AML *runt* homology factors, including the bone-specific CBFA1/AML-3 factor that exclusively associates with the nuclear matrix [Merriman et al., 1995], bind to three sites in the osteocalcin promoter and contribute to bone tissue-specific expression. YY1 is only partially associated with the nuclear matrix and attenuates vitamin D enhancement of osteocalcin transcription through binding to the vitamin D response element (−460 to −446) and competition with TFIIB [Guo et al., 1996]. Nucleolin may facilitate additional interactions of the OC gene with the nuclear matrix by tethering the proximal promoter via OC Box I, which can both promote and repress transcriptional activation [Hoffmann et al., 1994; Ryoo et al., 1997].

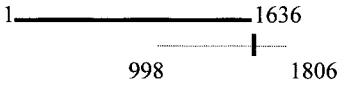
The largest open reading frame of clone 44 is only 114 amino acids and is unlikely to encode a sequence-specific transcription factor. Clone

Clone 3; accession number M55015, Nucleolin

Clone 19; accession number L06433, VL30



Clone 35; accession number U31935, rCAP2



Clone 44

```
1
CCAATTAAGC AGTTTTTATT TTTGCAATAT AGGATGAGAG GTCTTTGTTA
51
CTGGAGGAAA ATGACACAAG TTCAATAAAG CGTAACACGT CGGTGGTGAA
101
GGTCCCTTGA CAGTTACACT GCCACGGCCT TGGGATGGCC TTGACTAAGG
151
AACTAAATGT CATGTCCTCT CTAGGAACGA TGGTAAAGGA AAGACTTTTT
201
TCTCACCTAG TACGGAAGAA AATCATTACC AATATAGAAA GGCAACAGTC
251
ATTAAATAAA TTAAAAGCAT CTTTAAACTT TTTCCATATA CATATAATAT
301
ATATTCCAAT TGCCATTGAC TGTACAAGCA TTAAATTTCC AACTAATACA
351
TTTACAGAAA CTTGTGTGAA AAGCTGAAGG GAAAGTTTTG TCTTTAGAAA
401
GGGGAATCAT TTTATCACTC AAAAGCTACA TTAAAATACT GAGAAATCAG
451
ATTTGCCCCG GATTTCTTAC TTAGTTGTTT ATTCATCCTA TGCGTCAGGA
501
ATGCATAGCT ATTTTCTAGA CCAACCTTGA TTCATCATGT TTAGACGTTT
551
GCTACTCGGT AGCACAGAGA AAATGTAAAG CTTCAAGATT AACTGAATAG
601
TGTAGTTACA TAAACATTGT ATAAGTCAGG CTGGTGACAT TGTTACTCAT
651
GATTTTTTAT GAAATATTAA ATGGTAATAC AATTTATACA GAGATAGTAA
701
AACATTACCT TAGTGATAAA TGACCATCGG CTAATACGTA GCTAGGTAGG
751
GTAGGATTTA ACCTGGAATA CTGCAATCCT GAGGTCCAGG CATTCAATCT
801
TCCAGTTTCG TTCTACAACA TTAAGTTCCT TAAATTATAT ATTATTCACA
851
CAGGTTACAA TGAA
```

Fig. 5. Alignment of clones 3, 19, and 35 with previously known sequences and details of clone 44 sequences. GenBank sequences are represented graphically to show the alignment of the lambda isolates. The GenBank accession number and the name of the protein that is homologous with the lambda isolates are given. The upper line represents the GenBank clone and the numbers indicate the number of nucleotides reported. The lower, dashed lines represent the lambda isolates and are aligned with the region of the GenBank clone that is found in the isolate. The numbering on the isolates indicates the nucleotides in the GenBank sequence that are present in the lambda clone.

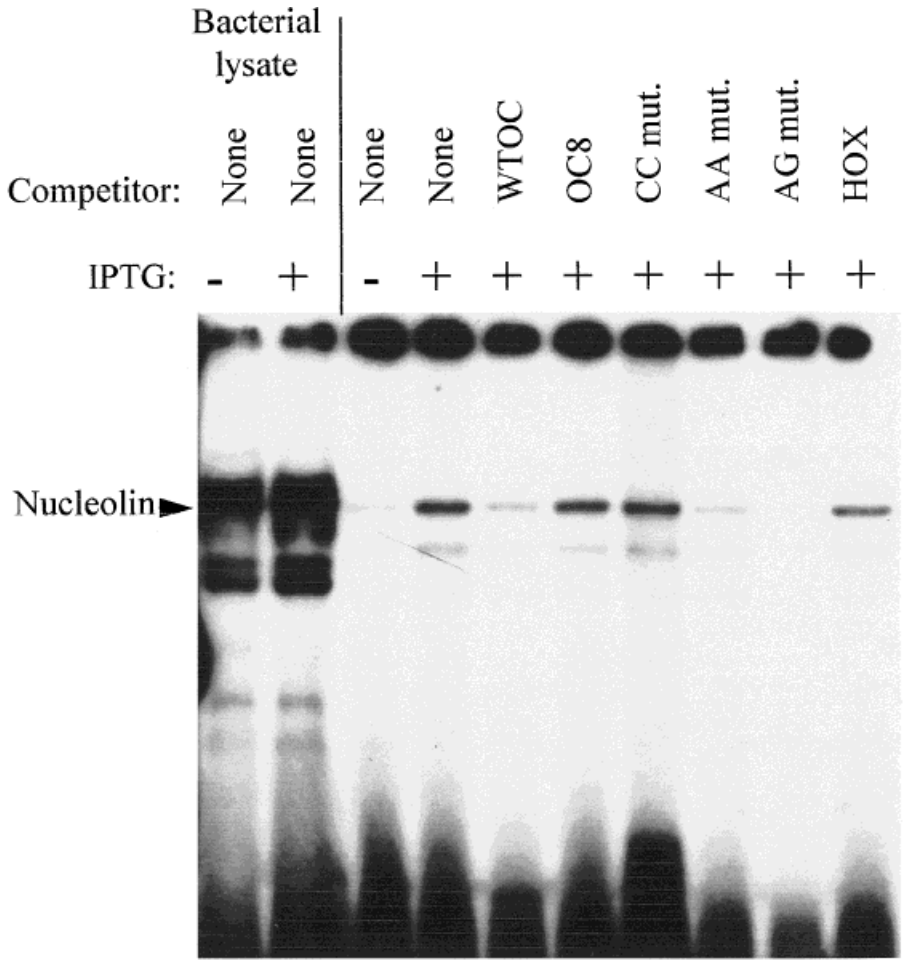


Fig. 6. Isolated nucleolin protein binds OC Box I sequences with specificity. Clone 3 was subcloned into a pGEX-5X vector and transformed into *Escherichia coli* JM105 cells. The bacteria were grown in the absence or presence of IPTG at a 1-mM final concentration as indicated above each lane. Protein was purified from the cultures and used as is (bacterial lysate) or further isolated using glutathione-Sepharose beads. The lysate or purified protein was then incubated with a radiolabeled 24 nucleotide OC Box I oligo (WTOC) in the presence or absence of a nonradiolabeled competitor as indicated above each lane. The binding reactions were separated on a 6.5% 30:0.8 polyacrylamide gel in 0.5× TBE and autoradiographed. The sequences of the oligonucleotide competitors are detailed at the bottom of the figure.

```
Competitors:
WTOC      ATGACCCCCAATTAGTCCTGGCAG
OC8       ATGACCCgactgctcTCCTGGCAG
CC mut    ATGACCCggAATTAGTCCTGGCAG
AA mut.   ATGACCCCCggTTAGTCCTGGCAG
AG mut.   ATGACCCCCAATTtcTCCTGGCAG
HOX          GCCTCCAATTAGTGT
```

44 has homology with rat (GenBank accession number AA 800581), human (number AA 376739), and mouse (number AA 354375) expressed sequence tags reported in GenBank. Full-length cDNAs were isolated from mouse neonate sympathetic ganglia and a rat lung library (unpublished data). Thus, clone 44 does not represent the osteocalcin box binding protein (OCBP), which is observed in nuclear extracts from only osteoblastic cell lines [Hoffmann et al., 1996]. The functional significance of clone 44 and related cDNAs remains to be established.

Further Characterization of Expression of Clone 44 and Significance

We examined whether expression of clone 44 is regulated by hormones and growth factors

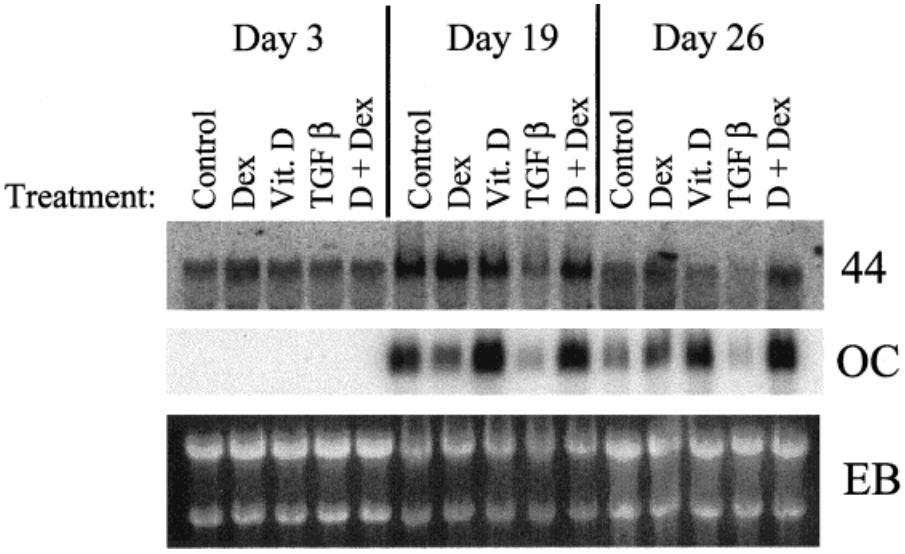


Fig. 7. Expression of clone 44 is modulated by treatment with dexamethasone (Dex), vitamin D (Vit. D), and transforming growth factor β (TGF-β). Primary rat osteoblast cultures were chronically treated with dexamethasone, vitamin D, TGF-β, or vitamin D and dexamethasone together and harvested on day 3, 19, or 26 of culture. RNA from these cultures were separated on a denaturing agarose gel and transferred to Zetaprobe membrane for northern analysis. The RNA was probed with clone 44 (44) or osteocalcin (OC) sequences that were random prime labeled. Ethidium bromide (EB) staining of the ribosomal RNA is shown to indicate the relative loading of each RNA sample.

that modulate osteocalcin expression (reviewed in Stein et al. [1996]). Analysis of clone 44 expression in ROB cells treated with dexamethasone, vitamin D, and TGF-β and harvested during proliferation (day 3), a period of active deposition of mineral (day 19), and heavily mineralized mature cultures (day 26), demonstrates that expression of clone 44 is influenced by these hormones as a function of the stage of maturation during osteoblast differentiation (Fig. 7). Expression of clone 44 is evident on day 3 during the early growth stage (see also Fig. 3). However, clone 44 expression is not affected by various hormonal treatments at day 3 in culture but is affected at later stages of culture, indicating again that this may represent residual message from the isolated calvaria cells and not active transcription. At day 19, expression levels of both 44 and osteocalcin are elevated. Both transcripts are repressed by TGF-β. However, unlike osteocalcin, clone 44 expression is upregulated by dexamethasone and is not stimulated by vitamin D. On day 26, after mineralization, both osteocalcin and clone 44 basal levels are decreased relative to day 19. Again, the only significant regulation of clone 44 observed is the decrease by TGF-β, which parallels the TGF-β1 effect on osteocalcin [Banerjee et al., 1996b]. Taken together, our findings indicate clone 44 represents a novel differentiation-specific marker that is developmentally regulated and uniquely responsive to the physiologic mediator TGFβ. This novel gene represented in clone 44 and designated DRF for differentiation-related factor may be a useful marker for probing gene regulatory mechanisms during the postproliferative transition period of osteoblast differentiation.

ACKNOWLEDGMENTS

We thank Judy Rask for manuscript preparation, Dr. William Devito, Department of Endocrinology, for the FRTL-5 cells and media. We also thank Liz Buffone for assistance in cell culture.

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