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FAST TRACK

Runt Homology Domain Proteins in Osteoblast Differentiation: AML3/CBFA1 Is a Major Component of a Bone-Specific Complex

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Abstract The AML/CBFA family of runt homology domain (rhd) transcription factors regulates expression of mammalian genes of the hematopoietic lineage. *AML1, AML2,* and *AML3* are the three AML genes identified to date which influence myeloid cell growth and differentiation. Recently, AML-related proteins were identified in an osteoblast-specific promoter binding complex that functionally modulates bone-restricted transcription of the osteocalcin gene. In the present study we demonstrate that in primary rat osteoblasts AML-3 is the AML family member present in the osteoblast-specific complex. Antibody specific for AML-3 completely supershifts this complex, in contrast to antibodies with specificity for AML-1 or AML-2. AML-3 is present as a single 5.4 kb transcript in bone tissues. To establish the functional involvement of AML factors in osteoblast differentiation, we pursued antisense strategies to alter expression of rhd genes. Treatment of osteoblast cultures with rhd antisense oligonucleotides significantly decreased three parameters which are linked to differentiation of normal diploid osteoblasts: the representation of alkaline phosphatase–positive cells, osteocalcin production, and the formation of mineralized nodules. Our findings indicate that AML-3 is a key transcription factor in bone cells and that the activity of rhd proteins is required for completion of osteoblast differentiation. J. Cell. Biochem. 66:1–8, 1997.

Key words: AML/CBF/PEBP2; regulatory element; AML-3; osteoblasts; differentiation

Transcriptional regulation of hematopoiesis-specific genes (T-cell–, B-cell–, and myeloid-specific) has been attributed to members of the AML/CBF family of proteins. The DNA-recognition component of these heteromeric proteins directly binds to a sequence-specific enhancer core motif TGTGGT that is frequently found in a wide variety of viral as well as eukaryotic genes [Meyers and Hiebert, 1995; Speck and Stacy, 1995]. Recently, AML-1 binding sequences were identified in the bone-specific osteocalcin (OC) gene [Merriman et al., 1995], and the transactivating form of AML-1 (AML-1B) was shown to enhance OC gene expression [Geoffroy et al., 1995; Banerjee et al., 1996].

Three related but distinct genes, *AML1, AML2,* and *AML3,* which are localized on different chromosomes, have been identified [Levanon et al., 1994]; all contain a highly conserved runt homology domain (rhd) [Kagoshima et al., 1993]. Genes encoding both subunits (AML-1 and CBFβ) of human core binding factor (CBF) exhibit chromosomal translocations that result in fusion proteins which are frequently associated with human leukemias [Nucifora and Rowley, 1995; Ohki, 1993; Meyers and Hiebert, 1995; Meyers et al., 1995]. The association of these proteins with leukemia as well as transcriptional regulation of target genes has prompted extensive analyses of these wild-type and chimeric regulatory factors [Meyers and Hiebert, 1995; Speck and Stacy, 1995]. To date, most studies have focused on AML1. AML-1 is abundant in T-cells and exhibits close sequence identity to AML-3. AML-3, though T-cell–specific, is

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present in low levels in hematopoietic cell lines tested [Meyers et al., 1996]. Studies also reveal that AML-2, which is expressed predominantly in B-cells and in myeloid cells, is functionally similar to AML-1B [Meyers et al., 1996]. Similarly, PEBP2αA1 (murine homologue of AML-3) is known to transcriptionally activate T-cell–specific genes [Ogawa et al., 1993].

Osteoblast differentiation is a multistep process proceeding through defined stages of maturation from a committed progenitor cell capable of proliferation to a postproliferative osteoblast expressing bone phenotypic markers. The final stage of osteoblast differentiation to an osteocyte involves mineralization of its surrounding extracellular matrix. The postproliferative expression of alkaline phosphatase and later of osteocalcin during mineralization provides markers to assess development of the osteoblast phenotype [Owen et al., 1990].

Several regulatory elements in the 5′ proximal promoter of the OC gene contribute to control of bone tissue–specific expression. These promoter elements include the highly conserved OC Box I (nt −99 to −76) [Towler et al., 1994; Hoffmann et al., 1994, 1996; Heinrichs et al., 1995] as well as OC Box II (−138 to −130) [Banerjee et al., 1996], which contains one of the three AML binding sites in the rat OC promoter. The osteoblast-specific complex (OBSC) [Banerjee et al., 1996] that interacts with OC Box II in the OC gene promoter includes an AML-related protein and its heterodimer partner CBFβ [Banerjee et al., 1996]. Using antisera that recognize the amino terminus of AML-1B, we observed an incomplete supershift in ROS 17/2.8 nuclear extracts, suggesting only partial identity of the factor to AML-1B. Furthermore, several transcripts were detected in both normal diploid rat osteoblasts and rat osteosarcoma cells [Merriman et al., 1995], raising the possibility of the presence of a potentially osteoblast-specific AML family member.

Here we have identified AML-3 as the AML member involved in the osteoblast-specific complex and have addressed the importance of the rhd class of proteins as regulators of osteoblast phenotype development.

MATERIALS AND METHODS

Rat Tissues and Cells

Normal diploid osteoblasts obtained from fetal rat calvariae were isolated and maintained as described [McCabe et al., 1996]. ROS 17/2.8 and ROS 25/1 rat osteosarcoma [Majeska et al., 1980] and Saos-2 human osteosarcoma (American Type Culture Collection, Rockville, MD) cell lines were maintained as described. Jurkat, a T-cell line, was maintained in RPMI 1640 (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum. Organs, soft tissues, and bones were obtained from female Sprague Dawley rats (Charles River, Cambridge, MA).

Plasmids

Osteocalcin promoter–CAT (chloramphenicol acetyltransferase) constructs containing wild-type (WT-CAT) and mutant (CM1-CAT or MUT-CAT) AML-1 binding sequences as well as insertless control (−108-CAT) [Banerjee et al., 1996] were used for transient transfection assays. CMV expression plasmids containing cDNAs of AML-1B, the transactivating form of AML-1 [Meyers et al., 1995], AML-2 [Meyers et al., 1996], and PEBP2αA1 (murine homologue of AML-3; obtained from Dr. S.-C. Bae, Chungbuk National University, Cheongu, Korea) were used for overexpression studies. PEBP2αA1 (murine) and AML-3 (human) are identical at the amino acid level [Levanon et al., 1994].

Analysis of Protein-DNA Interactions

Nuclear extracts were prepared from proliferating (day 3), differentiated (day 14), and mineralized (day 23) primary rat osteoblasts as described [Hiebert et al., 1991; Hoffmann et al., 1994] using 0.45 M KCl for extraction. Electrophoretic mobility shift assays were performed using conditions previously described [Ausubel et al., 1989; McCabe et al., 1996]. DNA binding reactions were performed as described previously [McCabe et al., 1996; Meyers et al., 1996]. Nuclear extracts (4 μg) were incubated with 10 fmol of the ^{32}P end-labelled double-stranded AML binding site consensus oligonucleotide (5′-CGAGTATTGTGGTTAATACG-3′) as the probe and nonspecific competitor DNA. Protein-DNA complexes were resolved in 4% nondenaturing polyacrylamide gels. Antisera supershift experiments contained polyclonal antiserum directed to an AML-1 peptide containing 17 N-terminal amino acids of AML-1 [Meyers et al., 1995], AML-2 antiserum directed to an AML-2–specific peptide [Meyers et al., 1996], AML-3 antiserum directed to an AML-3–specific peptide [Meyers et al., 1996], or preimmune serum (control). For peptide competition, the specific antigenic peptide of AML-3 was used. AML-3

peptide (8 μg) was incubated for 30 min with nuclear extract prior to addition of the antibody and the probe to the reaction. Gels were dried and exposed to Kodak films (Eastman Kodak, New Haven, CT) at −70°C for 6–12 h.

Western Blot Analyses

Nuclear extracts (30 μg per lane) were resolved in 12% SDS-PAGE and electroblotted (using a Semidry Electroblotter; Owl Scientific Plastics, Cambridge, MA) onto nitrocellulose membrane (Protran 0.2 μm; Schleicher and Schuell, Keene, NH) according to the manufacturer's specifications. Western blot analyses were performed as described [McCabe et al., 1996]. Membranes were incubated in a 1:100–150 dilution of antibody in Tris-buffered saline containing 1% bovine serum albumin. Affinity-purified antibodies specific for AML-3 [Meyers et al., 1996] were used in these studies. Peptide competition was performed with an AML-3–specific antigenic peptide [Meyers et al., 1996]. Membranes were incubated with secondary antibody for 45 min followed by chemiluminescent detection using an ECL (Amersham Life Sciences, Arlington Heights, IL) kit according to the manufacturer's specifications. Membranes were exposed to 10 s to 1 min to Amersham Hyperfilm for detection of signals.

RNA Analysis

Total RNA was isolated from soft tissues and bones of 8-week-old rats (2-week-old rats for calvariae samples). Frozen specimens were pulverized and total RNA isolated using Trizol® (GIBCO-BRL) according to the manufacturer's specifications. RNA (10 μg per lane) was separated in an 1% agarose-formaldehyde gel, transferred onto Zetaprobe membrane (Bio-Rad Labs, Hercules, CA), and hybridized to probes specific for PEBP2αA1 (murine homologue of AML-3). A 287 bp N-terminal fragment (BamHI-Ncol) of mouse PEBP2αA1 cDNA (obtained from Dr. S.-C. Bae) was used as the AML-3–specific probe. Hybridization was performed at 68°C and the blots washed extensively in buffer containing 0.1% SSC and 0.1% SDS at 55°C. Data were analyzed using a Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Antisense Studies

Phosphorothioate-modified antisense oligonucleotides directed against a segment of the runt homology domain were used in these studies. All AML-related sequences in GenBank were aligned using the Multiple Sequence Analysis program by GCG (Wisconsin Package Version 8.0; Genetics Computer Group, Madison, WI), and rhd regions that were 100% conserved amongst all species were chosen. All potential antisense and scrambled (control) sequences were checked for homology with unrelated genes using BLAST (National Center for Biotechnology Information, NIH, Bethesda, MD) and FastA (GCG). The sequences 5′ AGCACGGAGCAGAGGAAGTT 3′ and 5′ CGGACAGGTGAAGGACGT 3′ demonstrating negligible homology to unrelated genes were utilized for antisense and scrambled (control) oligonucleotides, respectively. Starting on day 1, primary rat osteoblast cultures plated at a density of 0.01×10^6 per well on 24-well plates were fed every 24 h with fresh media supplemented with 2 μM, 10 μM, or 20 μM oligonucleotides per well. Cell viability and cell growth were not affected due to treatments with 20 μM oligonucleotides as determined by cell counts at different points during the time course. Media were collected at the end of every 24 h to determine levels of secreted osteocalcin/day by a radioimmunoassay using reagents characterized in these laboratories [Owen et al., 1990]. At day 23, cultures were fixed and histochemically stained for alkaline phosphatase activity by incubating cell layers with 20 mg/ml naphthol AS-MX phosphate disodium salt and 40 mg/ml Fast Red TR salt (Sigma, St. Louis, MO) in Tris buffer, pH 8.4, at 37°C for 30 min. The number of mineralized nodules per well was also assessed. Statistical analyses were done using Statview (Abacus Concepts, Inc., Berkeley, CA).

Transient Transfection Assays and Overexpression Studies

ROS 17/2.8 cells were transiently transfected by the DEAE-Dextran method [Hoffmann et al., 1994]. For overexpression studies, 5 μg of the reporter plasmid was cotransfected with 500 ng of the expression plasmid and 500 ng of pRSV-luc (internal control). The amount of total DNA was maintained at 15 μg/100 mm plate with salmon sperm DNA (Sigma, molecular biology grade). All experiments were done three times in triplicate using at least two different DNA preparations.

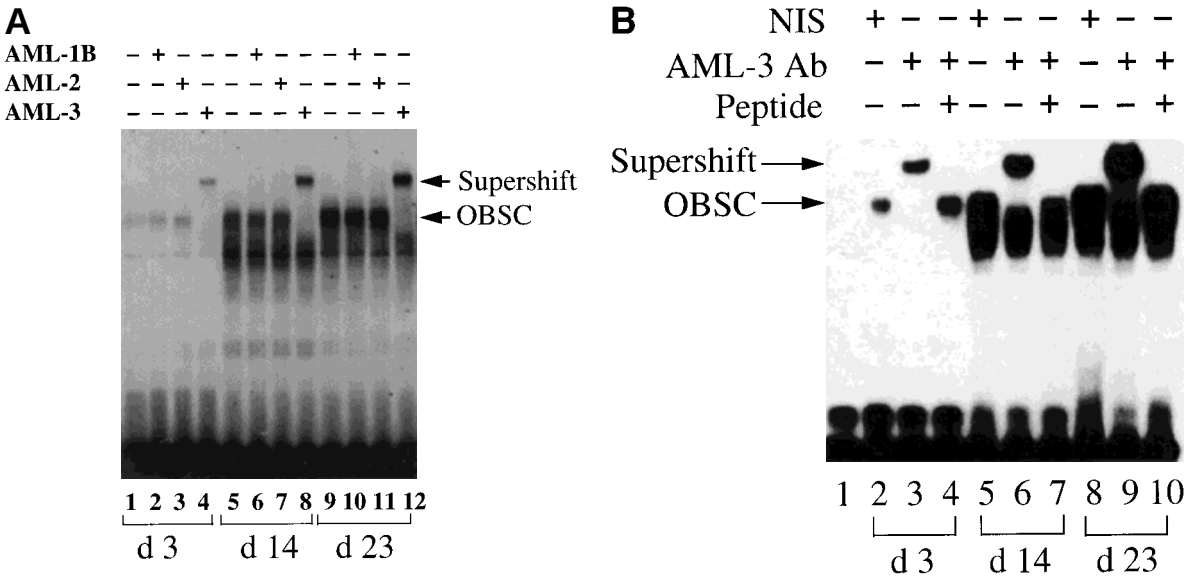


Fig. 1. Supershift mobility assays of the AML-related osteoblast-specific complex (OBSC). **A:** Rat osteoblast nuclear extracts (day 3: **lanes 1–4;** day 14: **lanes 5–8;** day 23: **lanes 9–12**) were incubated with anti–AML-1 (lanes 2,6,10), anti–AML-2 (lanes 3,7,11), or anti–AML-3 (lanes 4,8,12) antibodies in binding reactions using a probe containing the consensus AML-1 binding site. Assays were done as described [Meyers et al., 1996]. Lanes 1,5,9 contain nuclear extracts alone. **B:** Rat osteoblast nuclear extracts (day 3: *lanes 2–4;* day 14: *lanes 5–7;* day 23: *lanes 8–10*) were incubated with anti-AML-3 antibody in binding reactions using a probe containing the consensus AML-1 binding site. Assays were done as described [McCabe et al., 1996]. Lane 1: Probe alone. Lanes 2,5,8: Nuclear extracts incubated with preimmune serum (NIS). Lanes 3,6,9: Nuclear extracts incubated with 2 µl of AML-3 antibody. Lanes 4,7,10: Eight micrograms of the specific antigenic peptide (Pep) to AML-3 were incubated in the presence of the antibody. Dried gels were exposed for 6 h. OBSC and the supershifted bands are indicated.

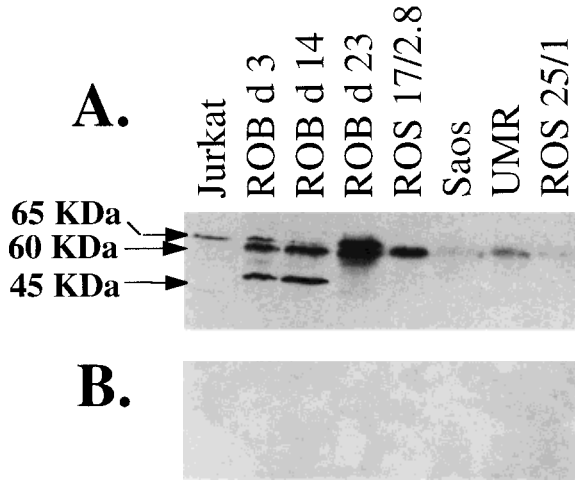
RESULTS

AML-3 Is a Major Component of the Osteoblast-Specific Complex in Primary Rat Osteoblasts

The AML recognition sequence TGTGGT binds an osteoblast-specific complex [Banerjee et al., 1996; Merriman et al., 1995] in ROS 17/2.8 cells which have characteristics of the mature osteoblast phenotype. To investigate which AML family members are involved in the formation of this complex, we performed immunoshift assays using a panel of specific antibodies and the AML binding site consensus oligonucleotide (which is homologous to the OC Box II sequence) as the probe (Fig. 1A). Our results indicate that a major component of this DNA binding activity in nuclear extracts from primary rat osteoblasts is AML-3, as indicated by a complete supershift of the AML-related complex by the AML-3–specific antibody (Fig. 1A, lanes 4,8,12) at all three stages of development (day 3, day 14, and day 23). Also, an increasing representation of the AML-3–related DNA binding activity is observed as the cells progress toward mineralization. AML-1 (Fig. 1A, lanes 2,6,10) and AML-2 (Fig. 1A, lanes 3,7,11) antibodies showed little or no ability to supershift the osteoblast-specific complex (OBSC). Specific antigenic peptide to AML-3 completely prevented formation of the supershifts (Fig. 1B, lanes 4,7,10). Thus, the major component of the osteoblast-specific complex of primary rat osteoblasts is AML-3 or an AML-3–related protein.

Expression of AML-3 During Osteoblast Growth and Differentiation

We also carried out Western analyses of nuclear extracts using antibodies specific for AML-3 (Fig. 2). We examined primary rat osteoblasts at three stages of maturation and several osteoblast cell lines exhibiting different phenotypic properties [Rodan and Noda, 1990]. AML-3 was detected in early stage osteoblasts (day 3 and day 14) as two immunoreactive species of 45 kDa and 60 kDa, whereas two species of 60 kDa and 65 kDa were present in mature day 23 osteoblasts. The smaller species is not present in mature differentiated osteoblasts (day 23) or in the ROS 17/2.8 cell line. The specificity of antibody-protein interactions was confirmed by



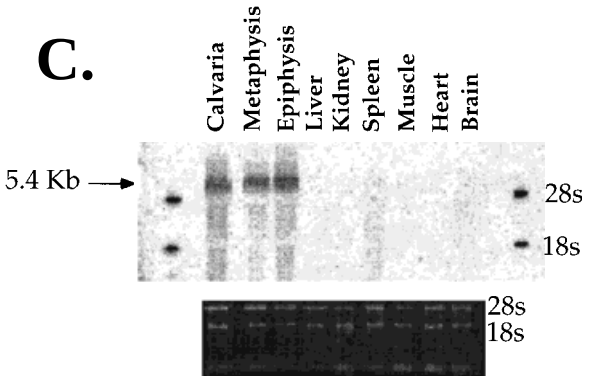


Fig. 2. Representation of AML-3 in osteoblasts and bone. **A,B:** The presence of immunoreactive proteins was determined by Western blot analyses performed on rat osteoblast (ROB) nuclear extracts obtained from different stages of growth and differentiation (proliferating day 3, differentiating day 14, and mineralizing day 23), Jurkat (T-cell line) and osteosarcoma lines (ROS 17/2.8, Saos-2, UMR, and ROS 25/1) as indicated. Samples (30 µg per lane) were resolved in 12% polyacrylamide gels and blotted onto nitrocellulose membranes. Blots were incubated with affinity-purified antibody specific for AML-3 and signal detected by chemiluminescence, and bands of interest are indicated (A). Blots were incubated with affinity-purified antibody in the presence of competitor antigenic AML-3 peptide (B). **C:** Tissue-specific expression of AML-3 was examined by Northern analysis. Total RNA (10 µg) isolated from bones and soft tissues of rats was resolved in a 1% agarose/formaldehyde gel and blotted onto a nylon membrane. RNA was hybridized with a probe specific for PEBP2αA1. The blot was analyzed in a PhosphorImager after 30 h exposure. Samples of trabecular and cortical bone of metaphysis, trabecular bone of epiphysis, liver, kidney, spleen, muscle, heart, and brain were obtained from 8-week-old rats, and calvariae was obtained from 2-week-old rats. Transcripts, 28s and 18s are indicated. The amount of RNA in each lane presented after ethidium bromide staining is indicated at the bottom.

competition with the specific antigenic peptide of AML-3 (Fig. 2B).

We next examined the tissue distribution of AML-3 using Northern hybridization. The representation of the respective mRNAs was compared in soft tissues and bones from rat (Fig. 2C). A major AML-3 transcript of 5.4 kb was observed in bone tissues only (calvaria, metaphysis, and epiphysis) but not in liver, spleen, and other soft tissues.

AML Family Members Transactivate Expression of Osteocalcin in Osseous Cells

The transactivation potential of AML-1B in ROS 17/2.8 osteosarcoma cells has been reported previously [Geoffroy et al., 1995; Banerjee et al., 1996]. Here we used ROS 17/2.8 cells to study the transactivation potential of the three AML members, all of which contain the runt homology domain. All three AML members were expressed in the presence of wild-type or mutant OC promoter–CAT constructs WT-CAT, MUT-CAT, and −108-CAT. Results indicate that overexpression of AML-1B, AML-2, and AML-3 each induces a five- to ninefold increase in the levels of OC promoter activity

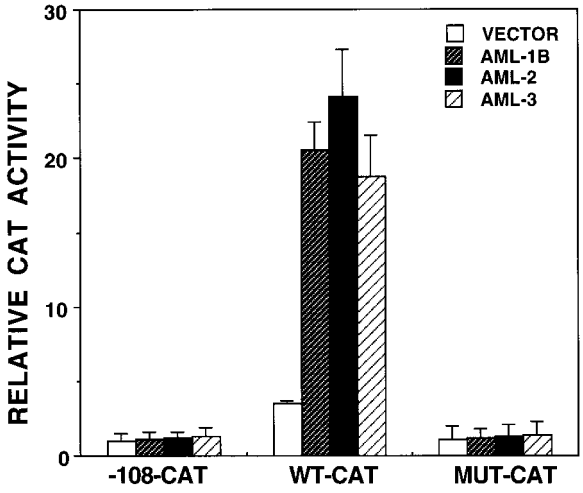


Fig. 3. Overexpression of AML members transactivate the rat osteocalcin promoter in osteoblast cells. ROS 17/2.8 cells were transiently transfected with 500 ng of either AML-1B, AML-2, or PEBP2αA1 (AML-3) overexpression plasmids or the CMV vector control and 5 µg of promoter-CAT construct containing wild-type (WT-CAT) or mutated OC Box II (MUT-CAT). CAT activity relative to −108-CAT (insertless control) cotransfected with CMV (vector) is shown. Data represent mean ± S.D. of three experiments, each done in triplicate.

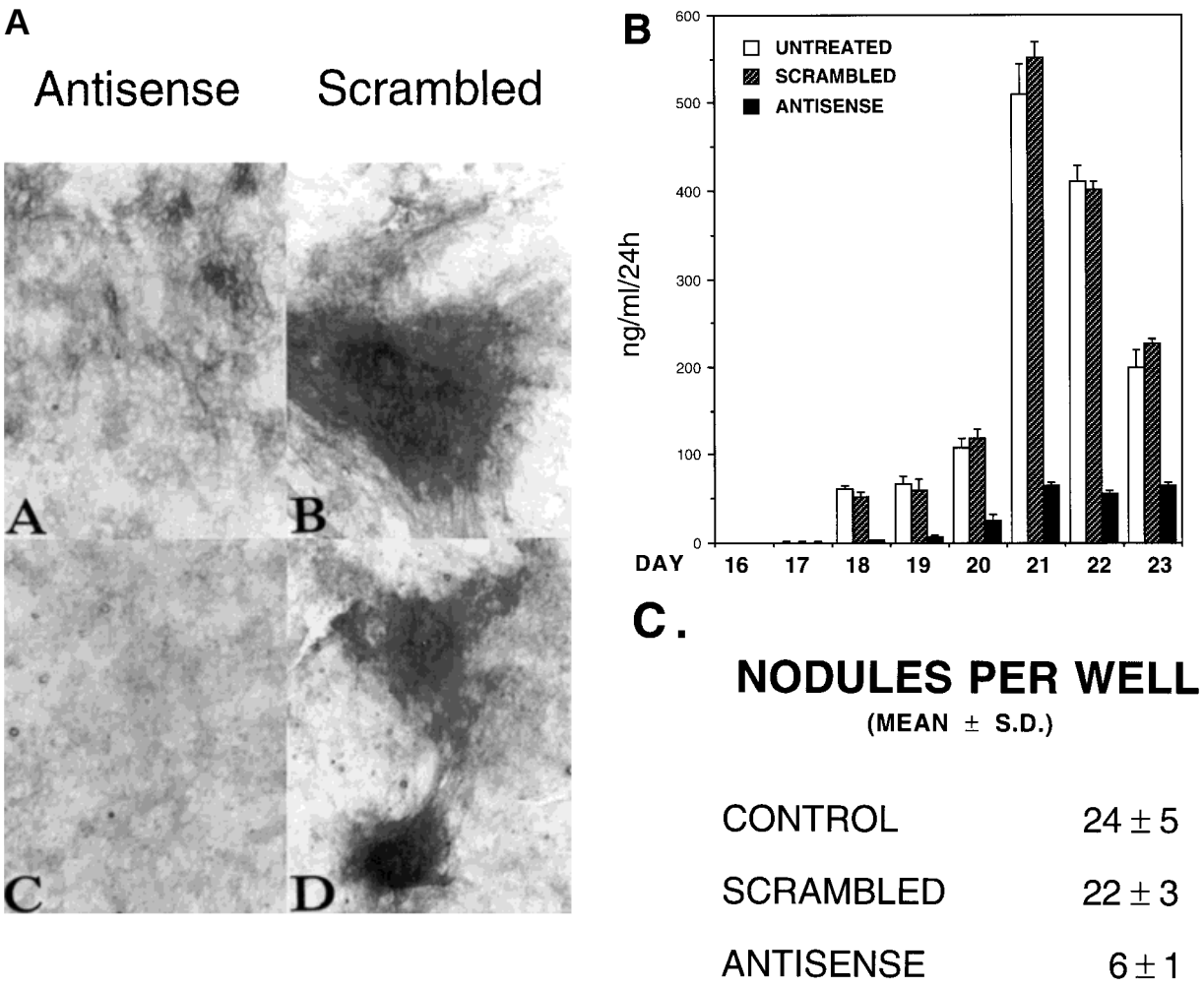


Fig. 4. Inhibition of the osteoblast phenotype by rhd antisense oligonucleotides. Rat osteoblast cultures were treated with 20-nt antisense and scrambled oligonucleotides every 24 h at 20 μM concentration from days 1–23. **A:** Alkaline phosphatase–stained day 23 osteoblast cultures treated with antisense (A,C) or scrambled (B,D) oligonucleotides. **B:** Levels of secreted osteocalcin (days 16–23) in untreated control and cells treated with antisense or scrambled oligonucleotides. Osteocalcin levels were analyzed by radioimmunoassay. Results represent mean ± S.D. of three independent experiments (n = 3). **C:** Table showing total number of nodules per 16 mm well in untreated, antisense, and scrambled oligonucleotide-treated cultures. Data represent mean ± S.D. of three experiments (n = 3) and are statistically significant ($P < 0.05$).

from the WT-CAT construct which contains a functional AML binding site (Fig. 3). Mutation of the AML binding element (MUT-CAT) results in almost complete loss of AML-stimulated promoter activity. Thus, AML family members have a transactivation function in osteoblasts. These results support the importance of rhd proteins in regulating expression of a bone-specific gene.

AML Proteins Are Involved in the Control of Osteoblast Differentiation

To determine whether AML proteins regulate osteoblast differentiation, we designed antisense oligonucleotides directed against the runt homology domain for translational arrest of all AML members present in osteoblasts. A dose-response curve of 2 μM, 10 μM, and 20 μM antisense oligonucleotide treatment of primary rat osteoblasts indicated that 10 μM is sufficient to elicit a response (data not shown). Figure 4A shows reduced staining of alkaline phosphatase, a marker of early osteoblast differentiation, following treatment with 20 μM antisense. Secreted osteocalcin levels indicate that osteocalcin production is substantially decreased as a consequence of antisense treatment (Fig. 4B). The number of mineralized nodules is reduced at day 23 in treated cul-

tures, reflecting a block in osteoblast maturation (Fig. 4C). Thus, translational arrest of AML proteins inhibits three principal markers of osteoblast differentiation. These findings support the functional role of rhd transcription factors in regulating development of the osteoblast phenotype.

DISCUSSION

The AML/CBFA–related transcription factors have been linked to hematopoiesis [Okuda et al., 1996; Wang et al., 1996] and to human leukemia where these genes are frequent targets of chromosomal translocations [Sakakura et al., 1994; Meyers and Hiebert, 1995; Speck and Stacy, 1995]. Therefore, past studies focused primarily on the influences of these factors on expression of genes in cells of myeloid lineage. It is evident, however, from recent studies as well as results presented here, that transcriptional activation mediated by AML proteins is not restricted to cells of the hematopoietic lineage but extends to cells of mesenchymal origin [Simeone et al., 1995; Geoffroy et al., 1995; Merriman et al., 1995; Banerjee et al., 1996].

In this report we demonstrate a functional requirement of AML transcription factors for the completion of osteoblast differentiation. However, the specific mechanisms and the genes that are targeted by inhibition of AML expression remain to be established. We show that AML-3 is expressed in bone tissue and that its expression correlates with active osteoblasts engaged in new bone formation. AML-3 transcripts were detected in calvariae from growing day 13 rat but not in adult calvariae (data not shown) which is devoid of new bone formation. Furthermore, the cancellous bone of the metaphysis and epiphysis, which is remodeled throughout adult life in the rat, expresses AML-3. Our studies also indicate that AML-3 is a major component of the osteoblast-specific complex that interacts with the AML transcription factor binding site. Although the three AML members can transactivate the OC promoter in transfection assays, the unique properties of the AML-3 protein may specify its inclusion in transcription factor complexes that mediate expression of osteoblast-specific genes. Inhibited development of the osteoblast phenotype by antisense-mediated suppression of the rhd transcription factor family further supports a major developmental role for these factors in bone formation.

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