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Expression and Regulation of Runx2/Cbfa1 and Osteoblast Phenotypic Markers During the Growth and Differentiation of Human Osteoblasts

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Abstract The runt family transcription factor (AML-3/PEBP2αA1/Cbfa1/RUNX2) plays a crucial role in formation of the mineralized skeleton during embryogenesis and regulates maturation of the osteoblast phenotype. Because steroid hormones and growth factors significantly influence growth and differentiation properties of osteoblasts, we addressed Cbfa1 as a target gene for regulation by dexamethasone (Dex), 1,25(OH)D_3 (vitamin D_3), 17β-estradiol, and transforming growth factor-β1 (TGF-β1). The representation of functional protein levels by Western blot analyses and gel mobility shift assays was examined during the growth and mineralization of several conditionally immortalized human osteoblast cell lines HOB 04-T8, 03-CE6, and 03-CE10, each representing different stages of maturation. In situ immunofluorescence demonstrates Cbfa1 is associated with nuclear matrix in punctate domains, some of which are transcriptionally active, colocalizing with phosphorylated RNA polymerase II. Although each of the cell lines exhibited different responses to the steroid hormones and to TGF-β1, all cell lines showed a similar increase in Cbfa1 protein and DNA binding activity induced only by Dex. On the other hand, Cbfa1 mRNA levels were not altered by Dex treatment. This regulation of Cbfa1 by steroid hormones in human osteoblasts contrasts to modifications in Cbfa1 expression in primary rat calvarial osteoblasts and the mouse MC3T3-E1 osteoblast cell line. Thus, these results reveal multiple levels of regulation of Cbfa1 expression and activity in osteoblasts. Moreover, the data suggest that in committed human osteoblasts, constitutive expression of Cbfa1 may be required to sustain the osteoblast phenotype. J. Cell. Biochem. 80:424–440, 2001. © 2001 Wiley-Liss, Inc.

Key words: osteoblast maturation; dexamethasone; vitamin D_3; 17β-estradiol; transforming growth factor-β1

The *runt* homology domain-related transcription factors (Cbfa/PEBP2αA/AML) are encoded by three related genes that have recently been shown to play key roles in regulating the development of hematopoietic [Okuda et al., 1996; Wang et al., 1996] and osseous tissues [Komori et al., 1997; Otto et al., 1997]. Cbfa2 (AML-1/PEBP-2αB) is critical for T and B cell differentiation [Okuda et al., 1996; Wang et al., 1996], and Cbfa3 (AML-2/PEBP-2αC) exhibits ubiquitous expression [Speck and Stacy, 1995]. Cbfa1/AML-3/PEBP-2αA was first cloned from T cells [Ogawa et al., 1993; Levanon et al., 1994; Satake et al., 1995], and later a splice variant was shown to be expressed abundantly in skeletal tissues [Banerjee et al., 1997; Ducy et al., 1997; Inada et al., 1999; Kim et al., 1999]. It was demonstrated to be a critical factor for embryonic bone development as demonstrated by the inhibition of bone tissue formation in the Cbfa1$^{-/-}$ null mutation mouse model [Komori et al., 1997]. In the Cbfa1 null mouse, although the skeleton develops through the early chondrogenic stages, mineralization in the hypertrophic zone of the growth plate and subsequent bone

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formation is blocked. Heterozygous loss of this gene in humans characterizes the cleidocranial dysplasia (CCD) syndromes [Mundlos et al., 1997; Otto et al., 1997].

Osteoblast differentiation requires a multistep series of events modulated by an integrated cascade of gene expression that supports proliferation and sequential expression of genes associated with each component of the bone-formation process [Stein et al., 1996]. Morphogenetic proteins and transcription factors, including homeodomain proteins, are required for stem cell commitment to the osteoprogenitor cell [reviewed in Aubin and Liu, 1996; Lian and Stein, 1999]. Osteoblast lineage cells are responsive to growth factors and hormones that stimulate genes to support development of the bone extracellular matrix (ECM). Mineralization of the matrix results in maturation of a surface osteoblast to a differentiated osteocyte. The temporal expression of bone-related genes, such as type I collagen, alkaline phosphatase, and osteocalcin, define these stages of osteoblast maturation from the growth and matrix maturation periods to mineralization.

Cbfa1 is a component of an osteoblast-specific DNA-binding complex that has been well documented to be highly restricted to cells of the osteoblast lineage [Banerjee et al., 1997; Ducy et al., 1997]. Increases in Cbfa1 cellular protein levels have been documented during in vitro differentiation of rat calvarial-derived osteoblasts [Banerjee et al., 1997]. However, the regulated expression of Cbfa1 during development of the bone cell phenotype is unknown. The progression of osteoblast differentiation is highly influenced by transforming growth factor β1 (TGF-β1) [Breen et al., 1994] and steroid hormones including glucocorticoids such as dexamethasone (Dex), 1,25-dihydroxyvitamin D_3 (vitamin D_3) [Owen et al., 1991; Broess et al., 1995], and estrogens [reviewed in Lian et al., 1999]. As these osteotrophic factors regulate differentiation primarily of osteoprogenitors, Cbfa1 is a viable candidate for regulation by these osteogenic hormones. To address these questions, we carried out studies in human osteoblast-like cell lines that are competent to produce a mineralized matrix and exhibit different responses to TGFβ and steroid hormones [Bodine et al., 1997a].

Here we demonstrate that conditionally immortalized human bone cell lines, representing both early and late stage osteoblastic cells, express Cbfa1/AML-3 abundantly from growth to mineralization stages, and that Cbfa1 is associated with the nuclear matrix in transcriptionally active domains. Although the mRNA levels of Cbfa1 in these cell lines are unchanged, we show by antibody supershift assay and Western blot analyses that protein levels and protein-DNA binding complexes increase in response to glucocorticoids, but not vitamin D_3, 17β-estradiol, or TGF-β1. We also report for the first time species-related differences in hormonal regulation of Cbfa1 in humans (these studies) when compared to rats [Chang et al., 1998] and mice [Zhang et al., 1997].

MATERIALS AND METHODS

Isolation and Maintenance of Cell Lines

Human osteoblast (HOB) cell lines examined in this study were established from explant bone cell cultures derived from normal cortical bone fragments obtained from a femoral end of a 77-year-old woman who had undergone knee replacement surgery (03-CE6 and 03-CE10 cell lines) and from trabecular bone fragments from a 14-year-old girl (04-T8 cell line). Initially, bone fragments were treated as described by Robey and Termine [1985]. Osteoblasts were then immortalized by infection with adenovirus-*ori*-SV40 *ts*A 209 [Lei et al., 1992] and cloned as previously described [Bodine et al., 1996a; Bodine et al., 1996b]. Of the obtained HOB clones, approximately 5% expressed relatively high levels of ERα mRNA and contained functional estrogen receptors [Bodine et al., 1997a]. Two of these estrogen-responsive lines, HOB 03-CE6 [Bodine et al., 1997a] and HOB 03-CE10 [Bodine et al., 1997b], were used in the present studies.

Cultures were maintained in vented T-175 flasks at 34°C using growth medium. Growth medium consisted of phenol red-free Dulbecco's modified Eagle's medium (DMEM)/F-12 containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1% (v/v) penicillin-streptomycin and 2 mM GlutaMAX-1. Immunocytochemistry for the T antigen indicated that essentially all of the HOB cells expressed the *tsA* 209 mutant protein. Cells were passed at a ratio of ~1:3 twice a week using a solution of 0.05% (w/v) trypsin-0.53 mM ethylenediaminetetraacetic acid (EDTA). The flasks were allowed to become ~80% confluent before passing (~50,000 cells/cm^2).

Differentiation Protocol

Hormonal responsiveness and the ability of these osteoblasts to produce mineralized matrix were carried out at 39°C, the non-permissive temperature that inactivates the *ts*A 209 protein and induces expression of the osteoblast phenotype [Bodine et al., 1996a; Bodine et al., 1996b; Bodine et al., 1997a]. Cells in growth medium were seeded in either six-well or 100-mm plates at 0.084×10^6 cells/well or 0.5×10^6 cells/plate, respectively, and cultured until confluent (from four to six days) at 34°C. The cultures were then switched to 39°C in differentiation medium. Differentiation medium consisted of growth medium supplemented with 50 μg/ml ascorbic acid, and 10 nM menadione sodium bisulfite (vitamin K_3). Cells were fed 24 h before adding experimental medium, which consists of phenol red-free DMEM/F-12 supplemented with 2% (v/v) heat-inactivated charcoal-stripped FBS (HyClone, Logan, UT), 1% (v/v) penicillin-streptomycin, and 2 mM GlutaMAX-1. Cell cultures were then incubated for 48 h in experimental media in which the serum was replaced by 0.25% (w/v) bovine serum albumin (BSA). Cultures were supplemented either with 5 mM β-glycero-phosphate, 10^{-8} M $1,25(OH)_2D_3$ (kindly provided by M. Uskokovic, Hoffmann-La Roche, Nutley, NJ), 10^{-8} M 17β-estradiol, or 10^{-8} M Dex (Sigma, St. Louis, MO) when needed. Except where noted, tissue culture reagents were purchased from Gibco BRL (Grand Island, NY), while other reagents and chemicals were obtained from Sigma (St. Louis, MO) or VWR (Philadelphia, PA). Recombinant human TGF-β1 (2 ng/ml) was obtained from R&D Systems (Minneapolis, MN).

Biochemical Assays

Alkaline phosphatase activity (APase) was quantitated in cell layers from three wells/sample using p-nitrophenol phosphate as substrate and normalized to total protein as previously described [Owen et al., 1990]. Secreted osteocalcin was measured directly in a 48-h conditioned media by a radioimmunoassay method using Biomedical Technology Institute reagents (Stoughton, MA). To measure mineral accumulation, cell layers ($n = 3$) were extracted 3 h on ice in O.5 N HCl. Calcium content of each extract was determined by a colorimetric assay using reagents for the Sigma, procedure number 587.

Histochemistry

Cell layers were washed with cold phosphate-buffered saline (PBS), 2% (w/v) paraformaldehyde was added for 10 min, then cells were rinsed with 0.1 M cacodylate buffer prior to histochemical assay. To assess APase activity, fixed cell layers were incubated for 30 min at 37°C with 20 mg/ml naphthol AS-Mx phosphatase disodium salt and 40 mg/ml Fast Red TR salt in a pH 8.4 Tris buffer as described by Lowry et al. [Lowry et al., 1954].

Gene Expression

Total RNA was isolated from HOB cells using Trizol (GIBCO-BRL) according to the manufacturer's specifications. RNA (10 μg per lane) was separated in a 1% agarose-formaldehyde gel, transferred onto Zetaprobe membrane (Bio-Rad Labs, Hercules, CA), and hybridized to probes specific for PEBP2αA1 (GenBank Accession #D14636), the murine homologue of Cbfa1/AML-3. cDNA (obtained from Dr. Y. Ito, Kyoto University, Japan) [Ogawa et al., 1993], having 98.8% sequence homology with human Cbfa1 (GenBank Accession #AF001450) [Mundlos et al., 1997], was used as the specific probe. Hybridization was performed at 68°C and the blots washed extensively in buffer containing $0.1 \times$ SSC (15 mM sodium chloride, 1.5 mM sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) at 55°C. Data were analyzed using a Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The following DNA probes were used: human type I procollagen (GenBank Accession #NM000088) [Bernard et al., 1983], osteonectin (GenBank Accession #J03040) [Swaroop et al., 1988], human osteocalcin (GenBank Accession #X04143) [Celeste et al., 1986], human bone/liver/kidney-type alkaline phosphatase (GenBank Accession #NM000478) [Weiss et al., 1988], human histone H4 (GenBank Accession #M16707) [Pauli et al., 1987], and human TGF-β1 (GenBank Accession #M60315) [Celeste et al., 1990].

Western Blot Analyses

Nuclear extracts (30 μg per lane) were resolved in 12% SDS-polyacrylamide gel electrophoresis 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 specifica-

tions. Western blot analyses were performed as described [Banerjee et al., 1997]. Rabbit polyclonal antibody specific for AML-3/Cbfa1 [Meyers et al., 1996] was used in these studies (a gift from S. Hiebert, Vanderbilt University). Membranes were incubated in a 1:100–150 dilution of the primary antibody (anti-rabbit IgG) in tris-buffered saline containing 1% BSA. Blots were separately incubated with goat polyclonal anti-actin antibody (Santa Cruz Biotech, Inc., Santa Cruz, CA; cat. #SC-1615) raised against a human actin peptide for use as control. Membranes were then incubated with secondary antibody for 45 min followed by chemiluminescent detection using an ECL (Amersham Life Sciences, Arlington Heights, IL) kit according to manufacturer's specifications. Membranes were exposed for 10 s to 1 min to Kodak (X-OMAT) AR films for detection of signals.

Analysis of Protein-DNA Interactions

Nuclear extracts were prepared from HOB cell lines as described previously [Hoffmann et al., 1994; Banerjee et al., 1996]. DNA-binding reactions were performed with 4 μg of nuclear extracts incubated with 10 fmol of ^{32}P end-labeled double-stranded AML binding site consensus oligonucleotide (5′-CGAGTATTGTGGT-TAATACG-3′) as the probe using conditions described previously [Banerjee et al., 1996; Banerjee et al., 1997]. Antisera supershift experiments were performed with 1–2 μl of gel supershift rabbit antiserum specific for AML-3/Cbfa1 [Meyers et al., 1996]. Protein-DNA complexes were resolved in 4% nondenaturing polyacrylamide gels. Gels were dried and exposed to Kodak films (Eastman Kodak, New Haven, CT) at −70°C for 6–12 h.

Indirect Immunofluorescence Analysis of Endogenous Cbfa1

Human osteoblasts were grown on glass coverslips (Fisher Scientific, Springfield, NJ) at 34°C in growth medium to 40% confluency before switching to the nonpermissive 39°C temperature. Cells were maintained for 48 h in differentiation medium, then either 4% paraformaldehyde in PBS was added to washed cell layers or the cells were subjected to in situ extraction of cytoskeletal and soluble chromatin to reveal the nuclear matrix intermediate filaments (see below). Coverslips were processed using protocols previously published by our laboratory [Zeng et al., 1997; Zeng et al., 1998].

The primary Cbfa1/AML-3 antibody at 1:300 dilution was incubated for 1–1.5 h at 37°C. Anti-RNA polymerase II_0 (B3 mouse IgM mAb) that recognizes the hyperphosphorylated large subunit (250 kDa) of RNA polymerase II [Mortillaro et al., 1996] was also used in these studies. The secondary antibody was incubated for 1 h at 37°C and was either a fluorescein isothiocynate (FITC)-conjugated goat anti-rabbit antibody (1:400, Jackson ImmunoResearch) to detect Cbfa1 or a Texas red-conjugated donkey anti-mouse antibody (1:400, Jackson ImmunoResearch) for anti-RNA Pol II detection. In situ nuclear matrices were prepared as described [Fey et al., 1984]. Briefly, cells on coverslips were washed in PBS and extracted twice in cytoskeletal (CSK) buffer [Fey et al., 1984] for 15 min each. CSK buffer contains 10 mM PIPES (1,4-Piper-azinebis-(ethanesulfonic acid)), pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 1.2 mM phenylmethylsulfonylfluoride, 1% vanadyl ribonucleoside complex. DNase I digestion was performed twice in digestion buffer (CSK buffer with 50 mM NaCl) containing 100 μg/ml DNase I for 30 min, followed by extraction in digestion buffer containing 0.25 M $(NH_4)_2SO_4$ for 10 min. DNA content was evaluated by 4′,6-diamidino-2-phenylindole (DAPI) staining (5 μg/ml 4′,6-diamidino-2-phenylindole in PBS containing BSA and 0.05% Triton X-100). Cells were mounted in Vectashield H-1000 (Vector Laboratories, Inc., Burlingame, CA). Primary antibody controls were processed in parallel using only the second antibody. Microscopic images were obtained by using a CCD camera interfaced with a digital microscope system (Carl Zeiss Inc., Thornwood, NY). Images were analyzed by using the Metamorph software (Universal Imaging Corporation, West Chester, PA).

RESULTS

Immortalized HOB Cell Lines Exhibit Different Phenotypic Properties

Our goal was to determine if Cbfa1 expression is regulated by growth factors and hormones that influence osteoblast differentiation. To address this question, we selected three cell lines that were conditionally immortalized by the SV40 temperature-sensitive T-antigen as previously described [Bodine et al., 1996a]. The

04-T8 cell line was immortalized following enzymatic digestion of trabecular bone from a 14-year-old girl. These cells exhibited fibroblastic-like features. Two additional cell lines, 03-CE6 (CE6) [Bodine et al., 1997a] and 03-CE10 (CE10) [Bodine et al., 1997b], were derived from the same cortical explant culture from a 77-year-old woman and characterized as cell lines exhibiting endogenous levels of functional estrogen receptors. The 04-T8 and CE6 cell lines grew as uniform multilayers, while more focal multilayers were formed in cultures of the CE10 cell lines. All cell lines proliferated at the permissive temperature of 34°C and exhibited cessation of growth at 39°C.

The cell lines exhibited low basal levels of APase activity and nondetectable osteocalcin at the permissive growth temperature. At 39°C (Fig. 1), which induces the osteogenic phenotype with cessation of cell growth, a significant ($P < 0.001$) increase in APase activity occurred in the 04-T8 cell line. APase activity/cell was stimulated by vitamin D_3 and Dex to the greatest extent in the CE10 and 04-T8 cell lines, with a slight increase in CE6 cells. On the other hand, 17β-estradiol upregulated enzyme activity the most in the CE6 cells. We have previously reported that 17β-estradiol increased APase mRNA levels in this cell line [Bodine et al., 1997a]. Osteocalcin is not detected at 34°C during the growth period (Fig. 1B), but appears in the 39°C control at low levels. Vitamin D_3-treated cultures resulted in a significant increase in synthesis/secretion of osteocalcin in 24 h in all cell lines with 04-T8 and CE6 cells exhibiting threefold higher levels than CE10 cells. Thus, based on these properties, the CE10 cell line appears to reflect an earlier stage osteoblast phenotype when compared to CE6 and 04-T8 cells.

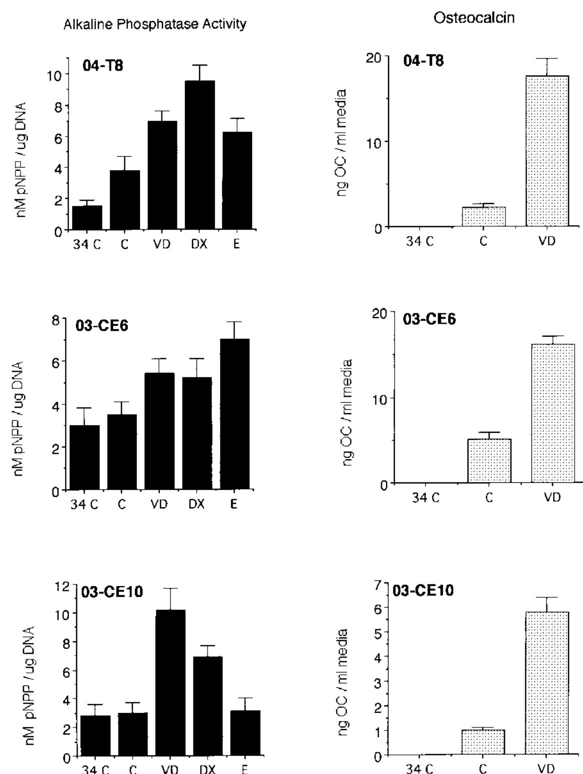

Fig. 1. Biochemical markers reflect the osteogenic phenotype in human osteoblast cell lines. Alkaline phosphatase activity (solid bars) in response to 10 mM vitamin D_3 (VD), dexamethasone (DX), and 17β-estradiol (E) was measured as nm of converted p-nitrophenol phosphate normalized to DNA in each of the indicated cell lines. Osteocalcin synthesis (grey bars) is quantitated in media collected 48 h after feeding by radioimmunoassay at 34°C, in 39°C control (C), and 10^{-8} M, $1,25(OH)_2D_3$-treated cells (VD) for 24 h. Values are mean ± SD for n = 3 wells from cell lines.

The 04-T8 and CE6 cell lines could be induced to produce a mineralized matrix in the presence of Dex (Fig. 2). In contrast, Dex blocked mineralization of the CE10 cell line (data not shown). Notably, continuous treatment with $1,25(OH)_2D_3$ (vitamin D_3) inhibited mineralization of the 04-T8 and CE6 cell lines (data not shown), but promoted mineralization of the matrix in the CE10 cell line (Fig. 2). The heavily mineralized nodules in the vitamin D_3-induced CE10 cell line have strikingly reduced APase levels, consistent with further differentiation to a later maturation stage osteoblast. We note that in the absence of hormone, mineral deposition of the ECM is not observed and the cultures cannot be maintained for more than two weeks [Bodine et al., 1997a], data not shown. Thus, the onset of mineral deposition provides a bone-like environment that supports cell maturation, continued viability, and gene expression.

Human Osteoblasts Express Cbfa1 That Associates With the Nuclear Matrix

Using specific antibodies recognizing the Cbfa1 gene products, we detected significant cellular levels of Cbfa1 in all cell lines by in situ immunofluorescence (e.g., 04-T8 and CE10 cells shown in Fig. 3). All cells in the field showed similar levels of endogenous Cbfa1 expression (Fig. 3A, left middle and left bottom panels). A punctate staining is observed in whole cells that is further resolved in the in situ nuclear matrix cell preparations (Fig. 3B). The negative DAPI staining reflects selective removal of cytoplas-

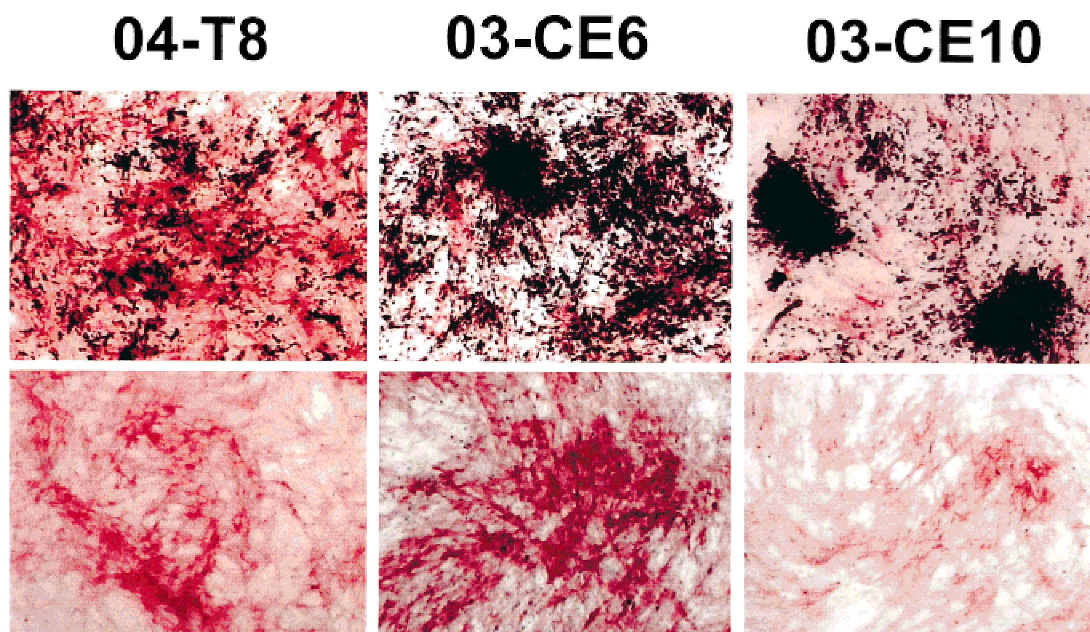

Fig. 2. Induction of mineral deposition by steroid hormones in human osteoblast (HOB) cells. The indicated HOB cell lines were grown at 34°C for seven days, then switched to 39°C for 15 days and maintained in differentiation medium with 10 mM β-glycerolphosphate, and 10^{-8} M $1,25(OH)_2D_3$ for 03-CE10 cells and 10^{-8} M dexamethasone for 03-CE6 and 04-T8 cell lines. Cells were fixed in 2.5% paraformaldehyde for histochemical detection of alkaline phosphatase (top and bottom rows), and mineral deposition with the von Kossa stain (top row).

mic and soluble nuclear cell components while retaining the nuclear matrix intermediate filaments (NMIF; Fig. 3B).

In Figure 4, Cbfa1 is visualized in discrete foci on the nuclear matrix throughout the nucleus of 04-T8 cells (top left panel). RNA polymerase II also associates with the nuclear matrix (top middle panel), and the yellow foci of merged Cbfa1 and RNA Pol II immunofluorescence demonstrate that a subset of Cbfa1 colocalizes with RNA Pol II (top right panel), reflecting sites of active transcription. The bottom left panel (DAPI) shows complete absence of chromatin material indicative of NMIF extraction. The same cell preparation is shown in phase contrast (bottom right panel). Thus, endogenous Cbfa1 associates with the nuclear matrix in human osteoblasts as reported previously for transfected Cbfa2 in human osteosarcoma cells [Zeng et al., 1997] and Cbfa1 in rat osteosarcoma cells [Tang et al., 1999].

Cbfa1 Expression Constitutive in HOB Cell Lines During Mineralization of the ECM

We examined the three human cell lines for modifications in the expression of osteoblast phenotypic genes reflecting the growth, matrix maturation, and mineralization stages during a five-week culture period (Fig. 5A and 5B). After switching cells to the nonpermissive temperature (day 6), complete cessation of cell growth occurred after 24 h. Mineral deposition begins after 2.5 weeks of plating for each of the cells maintained in their appropriate differentiation-promoting media, requiring 10^{-8} M Dex for 04-T8 and CE6 cells and 10^{-8} M vitamin D_3 for the CE10 cell line. Different profiles of expression of two phenotypic genes characterizing osteoblast differentiation are observed for each cell line.

The mRNA profile (Fig. 5A) for osteonectin, an abundant noncollagenous protein contributing to formation of the bone ECM [Robey, 1996], shows maximal expression in each of the cell lines in the immediate postproliferative stage and then declines continuously during the mineralization stage. The 04-T8 cells expressed twofold greater levels of osteonectin compared to the CE6 and CE10 cell lines. The temporal expression of APase was more variable among the cell lines, but in general reached a peak level, then declined when the ECM mineralized. These observations of a temporal profile of

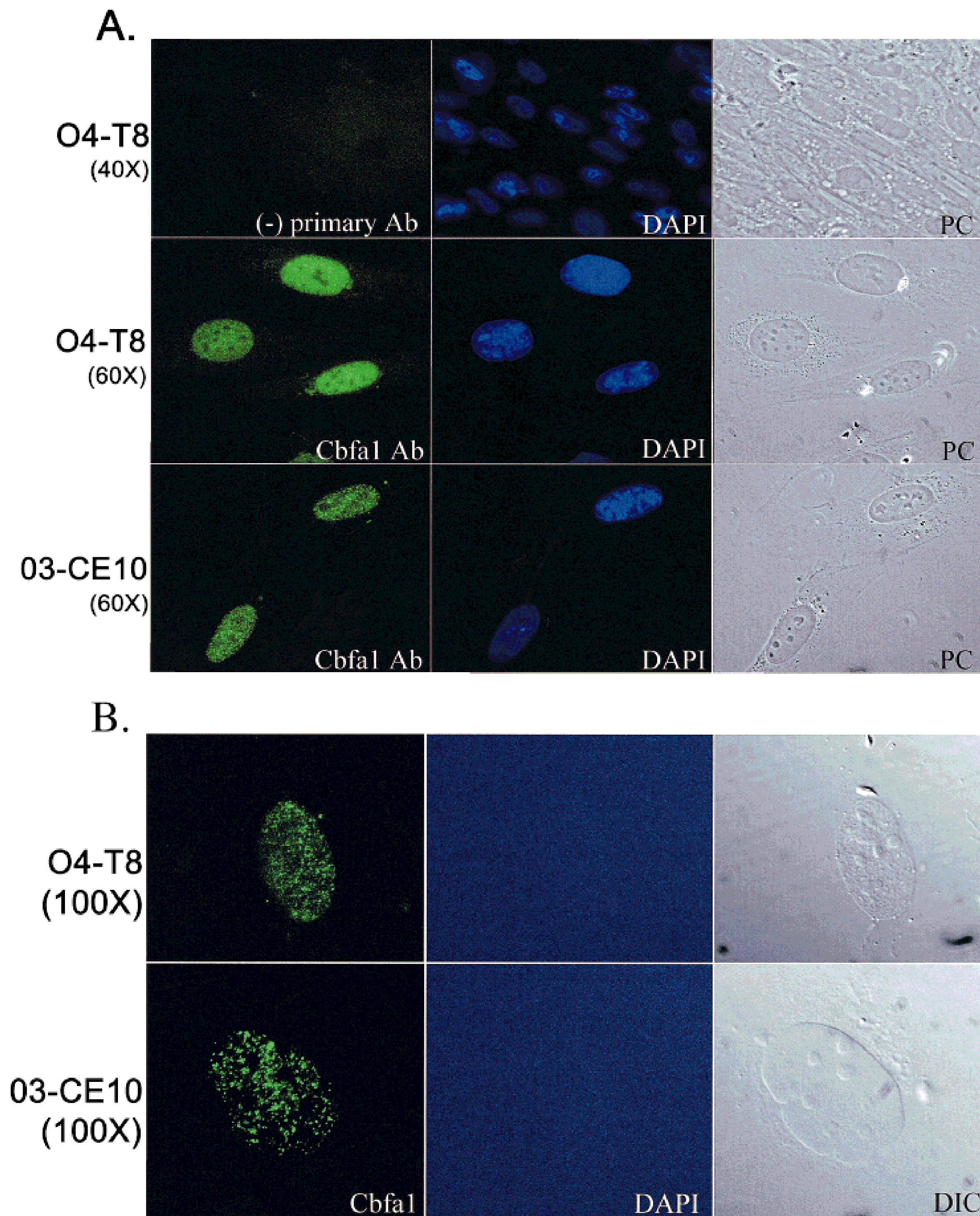

Fig. 3. Immunofluorescent detection of Cbfa1 in human osteoblast cell lines. **A:** Endogenous Cbfa1 levels in cultured whole cells of 04-T8 (top and middle rows) and 03-CE10 (bottom row). Cells were cultured at 39°C, and after 48 h, fixed in 4% paraformaldehyde (see Materials and Methods). Top panel indicated (-) primary antibody shows incubation of whole fixed 04-T8 cells with secondary antibody only. All left panels show cells incubated with or without Cbfa1 antibody; middle panels show cells stained with 4′,6-diamidino-2-phenylindole (DAPI); and right panels show phase contrast (PC) image to show all cells in field. Magnification of respective fields are indicated. **B:** Antibody reactions were carried out on in situ preparations of nuclear matrix intermediate filaments (left panels; see Materials and Methods), DAPI staining (middle panel) demonstrating complete digestion of soluble chromatin, and differential interference contrast microscopy (DIC; right panel) at 100 × magnification.

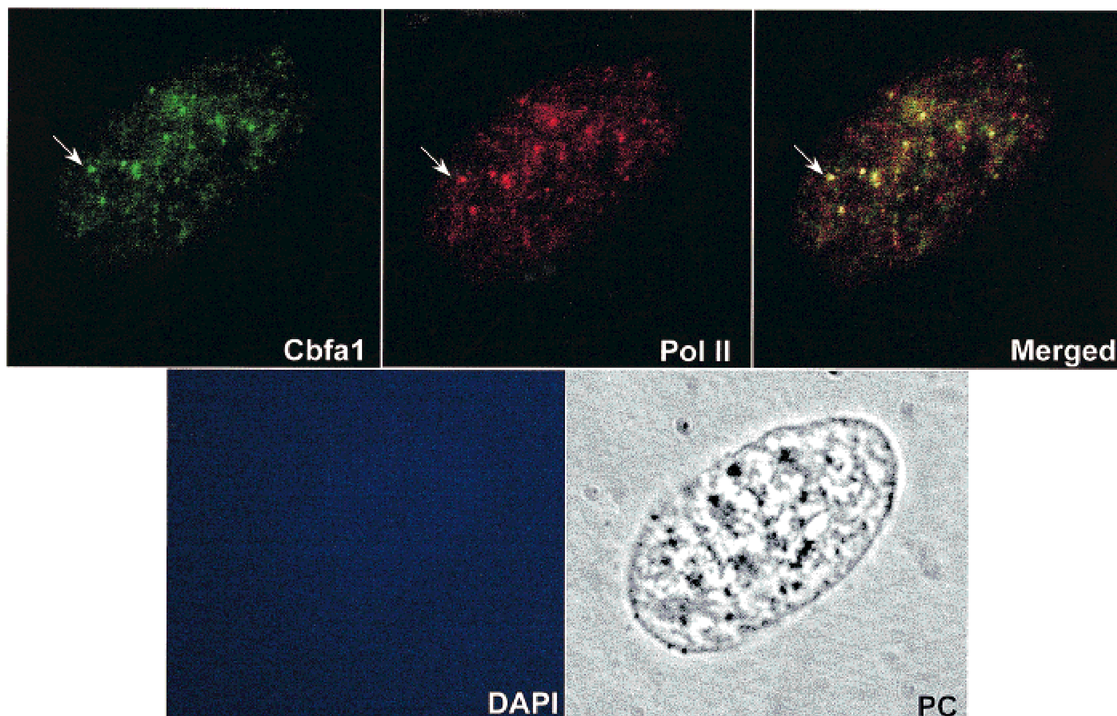

Fig. 4. Nuclear matrix association of Cbfa1 in transcriptionally active RNA polymerase II domains. The 04-T8 cells were prepared for in situ nuclear matrix detection of Cbfa1 after two days of culture at 39°C. Top left panel: FITC detection of Cbfa1 following incubation with Cbfa1 primary antibody (indicated Cbfa1). Top middle panel: Texas red detection following incubation with RNA polymerase II primary antibody (indicated Pol II). Top right panel: merged profiles of Cbfa1 and RNA Pol II shows red, green, and yellow foci (indicated Merged). Lower left panel: Complete removal of chromatin by with 4′,6-diamidino-2-phenylindole staining (DAPI). Bottom right panel: Phase contrast image (PC). Arrowhead indicates foci that colocalize. Magnification, 100 ×.

expressed genes modulated during synthesis and mineralization of the ECM indicate that the immortalized cell lines can further differentiate during progressive development of a mineralized matrix.

Expression of Cbfa1 was examined by Northern blot analysis in each cell line during the stages of growth, matrix maturation reflected by osteonectin and APase expression, and mineralization (Fig. 5B). Multiple transcripts of various sizes were detected. A 6.0-kb and a higher transcript predominated at all time points examined from the immediate postproliferative to the final mineralization stage. Densitometric scanning of the transcripts did not reveal significant selective modifications during the osteoblast maturation periods (data not shown). Thus, while expression of the phenotypic genes such as osteonectin and APase were changing, Cbfa1 mRNA expression levels remained constitutive.

Glucocorticoids Upregulate Cbfa1 Protein and DNA Binding Activity

The steroid hormones, including the glucocorticoid dexamethasone, 17β-estradiol, and $1,25(OH)_2D_3$ have significant effects on the expression of osteoblast growth and differentiation markers and represent important regulators of bone development and tissue organization. We addressed the potential regulation of Cbfa1 by these regulatory hormones in the three human osteoblast cell lines. The responsiveness of the cell lines to these agents was examined by assaying osteonectin mRNA, which is expressed at high levels during the matrix maturation period (Fig. 6, left panels). Osteonectin levels did not change in response to the hormones in the CE6 cell line. In contrast, osteonectin expression is regulated by dex and vitamin D_3 in the 04-T8, and by Dex in the CE10 cell lines. However, the responses were differ-

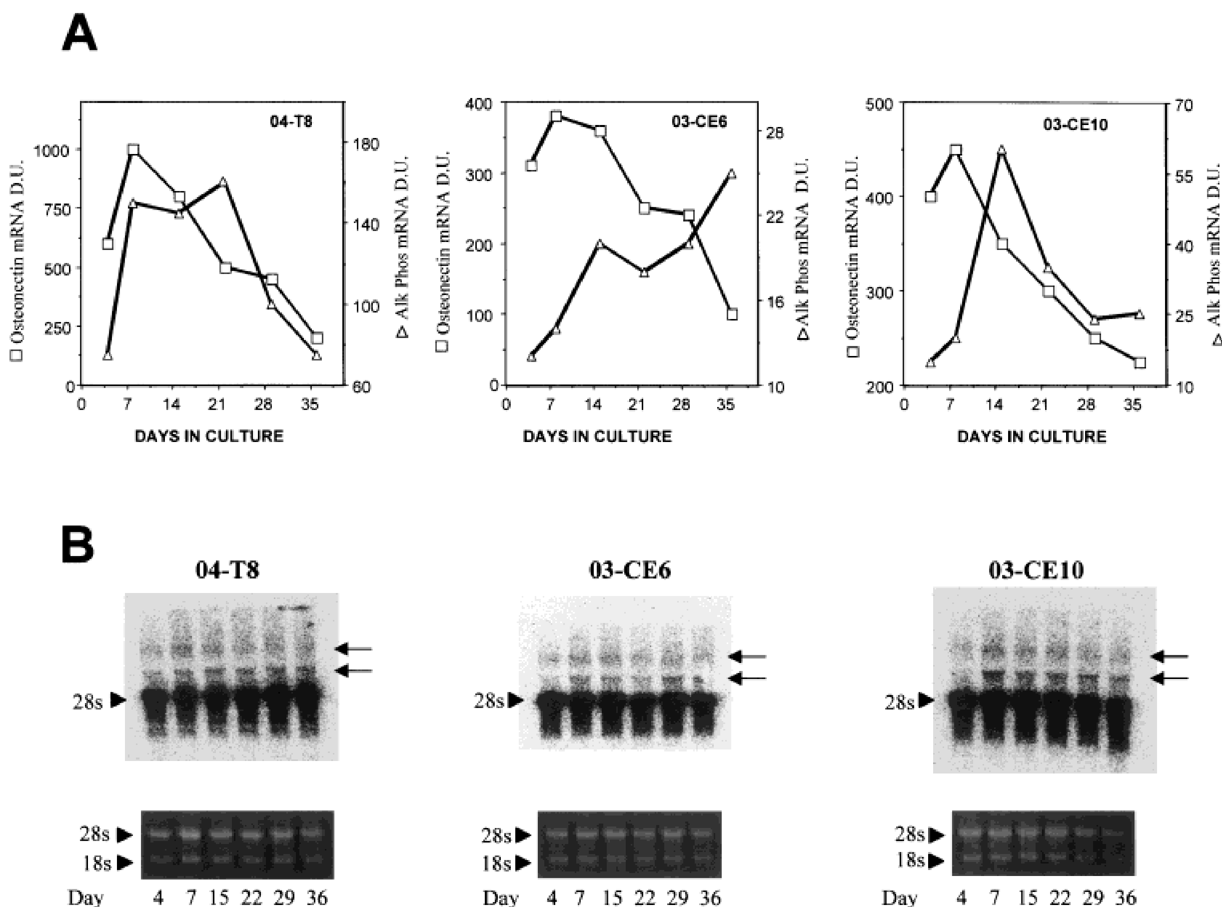

Fig. 5. mRNA levels of phenotypic genes during osteoblast differentiation. **A:** Expression of osteoblast phenotypic genes osteonectin (ON) and alkaline phosphatase (AP) was examined by Northern blot analysis in 04-T8, 03-CE6, and 03-CE10 cell lines. Ten micrograms of total RNA isolated from a time course (days 7, 14, 21, 28, and 35) of HOB-04-T8, HOB-03-CE6, and HOB-03-CE10 cells was run for a long time on 1% agarose/formaldehyde gels to fully resolve the region above 28S and blotted onto nylon membranes. RNA was hybridized with partial cDNA probes specific for human AP and ON genes as indicated. The blot was analyzed in a PhosphoImager after 30 h exposure. Data were analyzed in a gel documentation system and mRNA levels plotted are normalized to the 28S ribosomal band. **B:** Northern blot analysis of Cbfa1 transcripts during osteoblast differentiation in human osteoblast cell lines. Ten micrograms of total cellular RNA from days 4, 7, 15, 22, 29, and 36 was resolved on 1% agarose/formaldehyde gel as described in Materials and Methods and hybridized with a full-length Cbfa1 cDNA probe. Cbfa1 transcripts are indicated by arrows. Ethidium bromide staining of same gel shown on lower panels.

ent; for example, Dex increased osteonectin 2.5-fold in CE10 cells and decreased osteonectin mRNA by 50% in 04-T8 cells, while vitamin D_3 increased osteonectin mRNA levels threefold in 04-T8 cells. These differences in hormone responsiveness among the three cell lines provides an opportunity for determining if Cbfa1 expression is regulated by these steroid hormones and in relation to a parameter that reflects production of a bone-like ECM. Cbfa1 cellular RNA for all three cell lines was examined by Northern blot analysis in response to these hormones (Fig. 6, right panels). We used total cellular RNA from the same experiments in which we observed striking differences in osteonectin expression in response to the hormones. However, no significant changes in Cbfa1 mRNA levels were observed.

To further pursue mechanisms by which the steroid hormones or TGF-β1 may influence Cbfa1 activity, we examined interactions of Cbfa1 with promoter recognition sequences (Fig. 7). Cbfa1 forms an osteoblast-specific DNA binding complex (OBSC). We used nuclear extracts from each of the three human osteoblast cell lines treated with the steroid hormones and TGF-β1, and determined DNA binding activity of Cbfa1 using a probe analo-

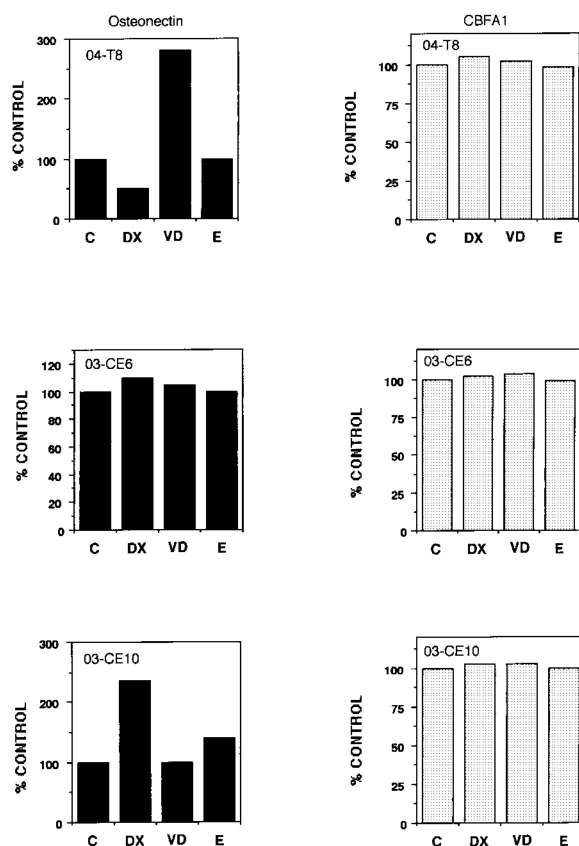

Fig. 6. Steroid hormone effects on expression of osteonectin and Cbfa1 in human osteoblast (HOB) cells. Northern analysis was performed with total RNA isolated from HOB-03-CE10, HOB-03-CE6, and HOB-04-T8 cells treated for 48 h at 39°C as described in Materials and Methods with 10^{-7} M dexamethasone (DX), 10^{-8} M 1,25$(OH)_2D_3$ (VD), and 10^{-8} M 17β-estradiol (E), along with untreated control (C). RNA was resolved on 1% agarose/formaldehyde gels and hybridized with cDNA probes specific for osteonectin and Cbfa1 as indicated. Data were analyzed in a gel documentation system. Graphs represent percent expression of RNA obtained from untreated control (C) compared to treated cells using the average value from two determinations.

gous to the rat osteocalcin promoter Cbfa binding sequence [Banerjee et al., 1997]. Figure 7A, 7B, and 7C show the representation of Cbfa1-containing DNA binding complexes in each of the human osteoblast cell lines. We have previously characterized this complex in rat osteoblasts and ROS 17/2.8 cells. The complex in mature osteoblasts consists of multiple bands, each of which are Cbfa-specific, proven by cross-competition analyses [Banerjee et al., 1997; Lindenmuth et al., 1997]. The partial supershift of the OBSC complex obtained by Cbfa1 antibody [Meyers et al., 1996] indicates that the remainder of the unshifted complex may contain Cbfa gene products in which the antigenic epitope is either absent [Stewart et al., 1997; Geoffroy et al., 1998] or not exposed to the antibody. No modifications of the Cbfa1 complex were observed with either TGF-β1- or 17β-estradiol-treated nuclear extracts. TGF-β1 has indirect effects on Cbfa1 mRNA expression [Lee et al., 1999] and does not appear to influence Cbfa1 DNA binding activity. Although the CE6 and CE10 cell lines are estrogen responsive [Bodine et al., 1997a], 17β-estradiol has no significant effect on Cbfa1 DNA binding activity. Only cells treated with Dex exhibited a striking increase (from threefold to fourfold) in Cbfa1-containing osteoblast-specific complexes. Examination of similarly treated nuclear extracts from differentiated primary rat osteoblasts (day 22; Fig. 7D) and mouse MC3T3 cells (day 14; Fig. 7E) also revealed differences in the levels of the Cbfa1-containing complexes in response to Dex and vitamin D_3. Rat osteoblast exhibited a significant decrease in the Cbfa1 complex in response to Dex. Only in mouse osteoblasts is the Cbfa1 DNA binding complex decreased in response to vitamin D_3. A previous study reported complete loss of osteoblast-specific DNA binding activity in response to vitamin D_3 in primary mouse osteoblasts [Zhang et al., 1997].

To determine if the Dex-induced increase in Cbfa protein-DNA complexes correlated to the representation of Cbfa1 protein levels, we carried out Western blot analysis of nuclear extracts from control, Dex-, and vitamin D_3-treated cell lines. Figure 8 reveals that in each of the HOB cell lines the cellular protein level of Cbfa1 is increased from fivefold to 10-fold following Dex treatment. This increase in response to dexamethasone in human osteoblasts contrasts to previous reports of Dex-mediated suppression of Cbfa1 in rat osteoblasts [Ji et al., 1998], which we also confirmed in these studies (see rat osteoblasts in Fig. 8). Blots were separately incubated with human actin antibody, which shows equivalent levels of actin expression in control and treated extracts. For rat osteoblasts, a nonspecific band ~80 kd is used as negative control. No effect by vitamin D_3 was observed in rat and human cells; however, a notable decrease in Cbfa1 protein is observed in the 10^{-8} M 1,25$(OH)_2D_3$-treated MC3T3 cells. Even in the 04-T8 cell line in which osteoblast phenotypic genes are induced several-fold by vitamin D_3, this hormone did not affect cellular

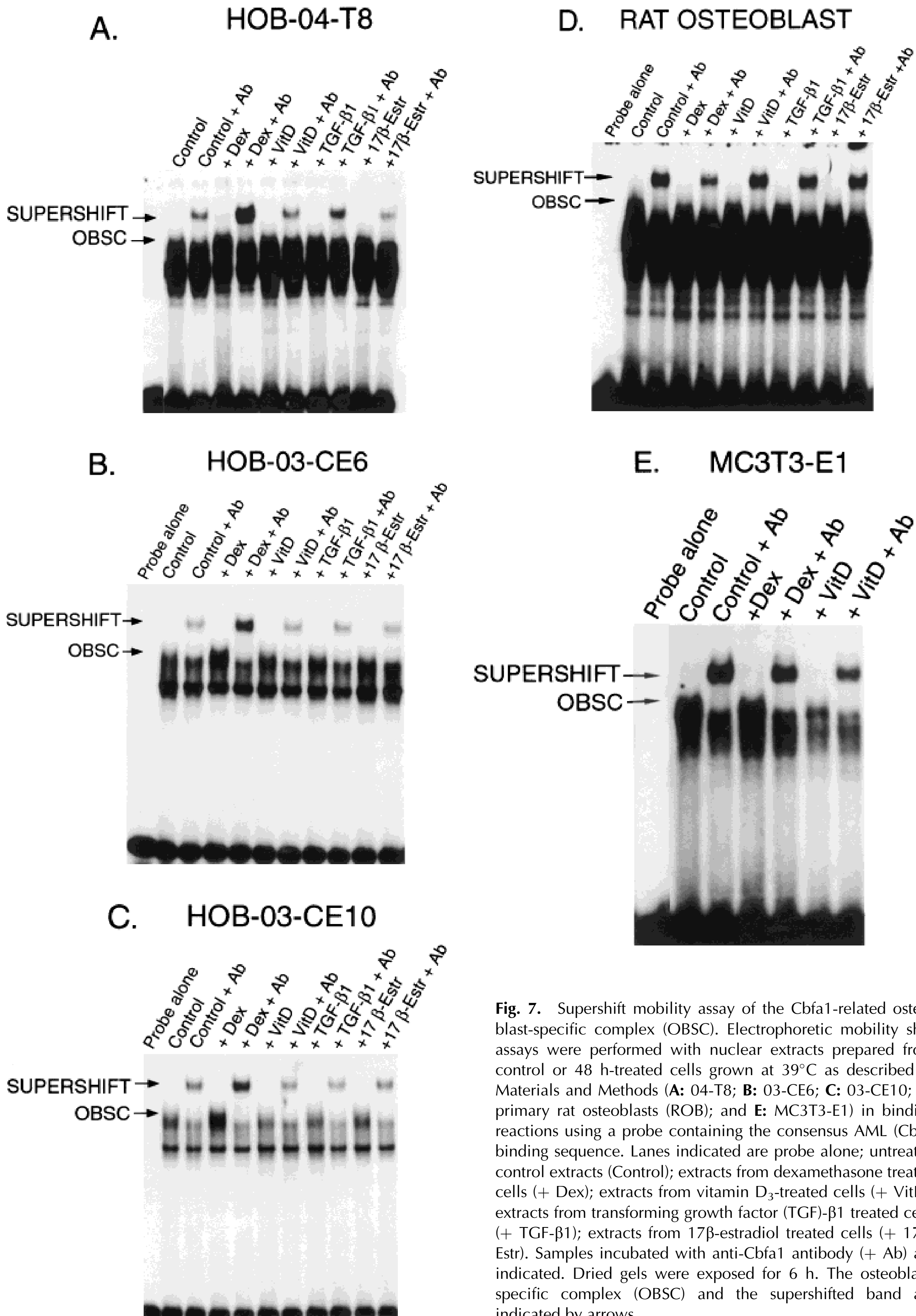

Fig. 7. Supershift mobility assay of the Cbfa1-related osteoblast-specific complex (OBSC). Electrophoretic mobility shift assays were performed with nuclear extracts prepared from control or 48 h-treated cells grown at 39°C as described in Materials and Methods (**A:** 04-T8; **B:** 03-CE6; **C:** 03-CE10; **D:** primary rat osteoblasts (ROB); and **E:** MC3T3-E1) in binding reactions using a probe containing the consensus AML (Cbfa) binding sequence. Lanes indicated are probe alone; untreated control extracts (Control); extracts from dexamethasone treated cells (+ Dex); extracts from vitamin D_3-treated cells (+ VitD); extracts from transforming growth factor (TGF)-β1 treated cells (+ TGF-β1); extracts from 17β-estradiol treated cells (+ 17β-Estr). Samples incubated with anti-Cbfa1 antibody (+ Ab) are indicated. Dried gels were exposed for 6 h. The osteoblast-specific complex (OBSC) and the supershifted band are indicated by arrows.

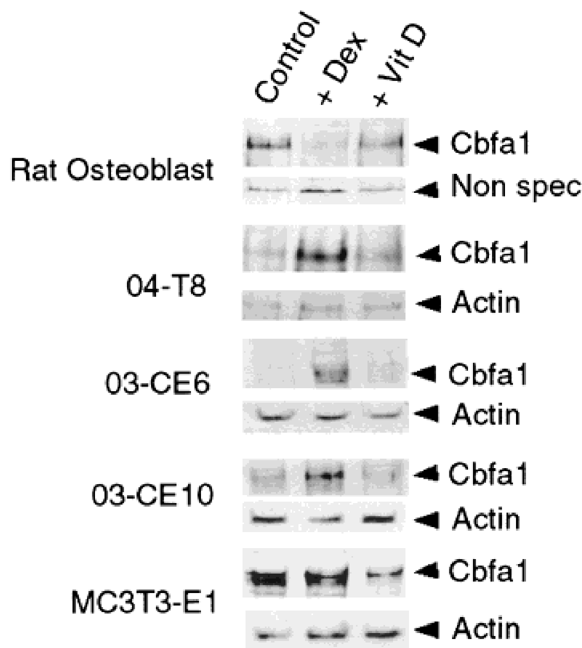

Fig. 8. Regulation of Cbfa1 cellular protein in human osteoblast (HOB) cells by glucocorticoids. The presence of 62-kDa AML-3 (Cbfa1) immunoreactive proteins was determined by Western blot analyses performed on rat osteoblast, mouse osteoblast (MC3T3-E1), and human osteoblast (03-CE6, 03-CE10, and 04-T8) nuclear extracts obtained from cells treated with dexamethasone (Dex) and $1,25(OH)_2$vitamin D_3 (Vit D) as indicated in Figure 7. Untreated cells were used as controls. Samples (30 μg per lane) were resolved on 10% polyacrylamide gels and blotted onto nitrocellulose membranes. Blots were incubated with IgG fractionated antibody specific for AML-3 (Cbfa1) and signals were detected by chemiluminescence. A nonspecific low molecular weight band (80 kDa) was selected for rat osteoblasts to demonstrate equal protein loading and transfer. All other blots of the cell lines were incubated with a β-actin antibody (lower panel of each cell line).

protein content of Cbfa1. It is also noteworthy that Cbfa1 expression is also upregulated by dexamethasone in the CE10 cell line, which produced a mineralizing matrix in response to $1,25(OH)_2D_3$, but not by the glucocorticoid.

DISCUSSION

Genetic analysis of Cbfa1 has clearly indicated a key role for Cbfa1 in both commitment and development of skeletal lineage cells responsible for production of calcified cartilage and bone tissue [Komori et al., 1997; Otto et al., 1997]. Our studies have examined expression and regulation of Cbfa1 human osteoblast cell lines that have been conditionally immortalized at different stages in development of the osteoblast phenotype. The multiple RNA transcripts we observe by Northern analyses may represent the several splice variants of the Cbfa1 gene that have been characterized. Exon skipping and the several options for splice variants at the 5′ end of the coding region would lead to shorter transcripts [Stewart et al., 1997; Geoffroy et al., 1998; Xiao et al., 1998]. The most predominant transcript is likely the alternative spliced Cbfa1 isoform (initiated at MASNS) abundant in skeletal lineage cells [Stewart et al., 1997; Thirunavukkarasu et al., 1998].

The distinct human osteoblast cell lines exhibit different properties of committed osteoblasts, and each are competent to produce a mineralized matrix and to further differentiate into mature osteoblasts. The 04-T8 cell line expresses osteoblast phenotypic markers approximately twofold greater than the estrogen-responsive cell lines. The CE10 line has been characterized as less mature than the CE6 cell line on the basis of two- to threefold lower basal levels of osteocalcin synthesis (see Fig. 1), a marker of mature osteoblasts. Furthermore, induced differentiation of CE10 by $1,25(OH)_2D_3$ is consistent with properties exhibited by an early-stage osteoblast [Owen et al., 1991; Broess et al., 1995; Gerstenfeld et al., 1996]. In conclusion, each of these cell lines possess osteoblast phenotypic properties including the ability to synthesize a matrix competent for mineralization, but show distinct differences in hormonal responsiveness as a result of their maturational stage. They apparently are immortalized at a stage that reflects the heterogeneity of osteoblast subpopulation in vivo [Aubin and Liu, 1996; Lian and Stein, 1996].

In each of these immortalized cell lines, Cbfa1 mRNA expression appears constitutive throughout stages of postproliferative maturation and mineralization, while other osteoblast phenotypic genes are being temporally expressed. This finding is in contrast to the temporal increases in Cbfa1 mRNA and protein during the differentiation of fetal rat primary osteoblasts [Merriman et al., 1995; Banerjee et al., 1997]. Therefore, constitutive expression of Cbfa1 in HOB cells may reflect immortalization of the differentiated cells, species differences, or adult versus fetal origin. Together these findings support the concept that Cbfa1 expression, which is required for the onset of osteogenesis and osteoblast differentiation [Banerjee et al.,

1997; Ducy et al., 1997; Komori et al., 1997], is present throughout the stages of osteoblast phenotype development from committed osteoprogenitors [Lee et al., 1999; Gori et al., 1999; Gronthos et al., 1999] to mature osteocytes.

The three human osteoblast cell lines we selected for these studies also exhibited distinct responses in expression of some genes to the steroid hormones, glucocorticoid, and $1,25(OH)_2D_3$ as noted above, reflecting their immortalization at a distinct stage of osteoblast maturation. However, in each cell line, dexamethasone increased cellular levels of Cbfa1 by Western blot analyses and functional DNA binding activity by gel mobility shift assay. Gel mobility shift analyses is a discriminating assay for determining DNA binding activity in nuclear extracts of cells in response to hormones. This response was similar (twofold to threefold) in each of the cell lines, and thus the increase in Cbfa DNA binding activity in response to glucocorticoids can be considered to be independent of the stage of maturation of the osteoblast. The striking increase in Cbfa1 protein is in contrast to any change in mRNA transcripts, suggesting that Cbfa1 factors in osteoblasts may be in part regulated at the posttranscriptional level. Recent studies have shown Cbfa1 is phosphorylated in osteoblasts, contributing to its enhancer activity [Xiao et al., 2000]. Furthermore, Cbfa1 functional protein is being examined by Western blot analysis and DNA binding activity by using nuclear extracted proteins that likely represent functionally modified transcription factors. The dissociation between the absence of responsiveness of Cbfa1 mRNA to hormones and the striking changes observed at the protein level reflect multiple levels of control for gene regulation. Examples of glucocorticoid effects on posttranscriptional regulation have been reported [Shalhoub et al., 1998]. The biologic implications of our findings of dexamethasone's increase in Cbfa activity can be related to several consequences of glucocorticoid on bone cell activities.

We have observed in these studies species differences in glucocorticoid regulation of Cbfa1 activity. Dexamethasone modestly decreases Cbfa1 DNA binding activity in the rat, confirming our Western analysis and others [Chang et al., 1998], but does not affect mouse Cbfa1 protein. However, glucocorticoids appear to significantly contribute to increased protein levels and DNA binding activity in human osteoblasts. Glucocorticoids have complex effects on bone remodeling. Dexamethasone is a potent inducer of osteogenic colony formation in marrow mesenchymal cells and promotes osteoblast differentiation in human [Cheng et al., 1996] and rat [Malaval et al., 1994; Shalhoub et al., 1992], but not mouse-derived cells [Frenkel et al., 1997; Bellows et al., 1998; Aslam et al., 1999]. The Dex-induced increase in Cbfa1 may also be functionally related to osteoclast activity and bone resorption. Recently, O'Brien et al. [1998] reported a Cbfa regulatory element in the mouse osteoprotegerin ligand (OPGL) promoter, also known as the osteoclast differentiation factor (ODF) or RANKL. In Cbfa1 null mice, osteoblastic expression of OPGL is undetected [Gao et al., 1998]. Thus, glucocorticoids stimulate ODF/OPGL and can inhibit expression of its neutralizing receptor osteoprotegerin, which inhibits osteoclast activity [Vidal et al., 1998; Hofbauer et al., 1999]. Therefore, glucocorticoid regulation of Cbfa1 is a contributing factor coupling bone formation to bone resorption. The pharmacologic use of glucocorticoid treatment in humans [reviewed in Manolagas and Weinstein, 1999; Canalis, 1996] may lead to deregulation of the Cbfa1 axis.

A key component of Cbfa1 functional activity is the association of Cbfa factors with the nuclear matrix. The nuclear matrix functions as the scaffold of the nucleus to concentrate and target transcription factors. It also modifies the chromatin organization of genes for interaction with basal transcriptional machinery (RNA polymerase II) and is involved in RNA processing. We have shown in several nonosseous cell types and osteosarcoma cell lines, including ROS 17/2.8 and human SaOS2 cells, that Cbfa1 is localized within the nuclear matrix subnuclear compartment [Zeng et al., 1998; Tang et al., 1999]. Furthermore, we have identified a conserved 31 amino acid nuclear matrix targeting sequence (NMTS) residing in the C terminus of all Cbfa factors [Zeng et al., 1998]. Deletion of the NMTS in transfection experiments results in significant loss of transcription enhancer activity, even though the DNA binding runt homology domain is not altered. The role of Cbfa1 in gene expression is usually documented by colocalization of Cbfa and the hyperphosphorylated form of RNA Pol II at subcellular foci that are transcriptionally active. We show in these studies that in conditionally immorta-

lized human osteoblasts, Cbfa1 associates with the nuclear matrix in discreet domains that also colocalize with RNA polymerase II. In human osteoblasts treated with glucocorticoids, we did not observe an increase in the number of Cbfa1 foci or modifications in the distributions of foci colocalizing with RNA polymerase II (data not shown). However, in situ immunofluorescence may not provide the level of discrimination required to definitively interpret a negative finding.

In conclusion, by in situ immunofluorescence, Western blot analyses, and sensitive antibody supershift gel mobility assays, we have determined the expression and subnuclear distribution of Cbfa1 in human osteoblast cell lines exhibiting properties that reflect different stages of differentiation. Our results show the following:

1. Endogenous Cbfa1 is functionally associated with the nuclear matrix in osteoblasts and is present in transcriptionally active RNA Pol II domains.
2. Cbfa1 protein and DNA binding activity in human osteoblasts is upregulated by glucocorticoids, but not in rodent cells.
3. There is discordance of Cbfa1 mRNA and protein expression, suggesting post-transcriptional control.
4. Glucocorticoid regulation of Cbfa1 occurs at all stages of osteoblast maturation.

We suggest that the constitutive levels of Cbfa1 observed in early (03-CE10), mid-stage (03-CE06), and more mature (04-T8) immortalized human osteoblast cell lines may serve a separate function from progression of differentiation of mesenchymal precursors. Cbfa1 steady state mRNA levels in committed osteoblasts and during later mineralization stages may be necessary, after differentiation, to sustain the osteoblast phenotype.

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