THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 278, No. 50, Issue of December 12, pp. 50563–50571, 2003

Printed in U.S.A.

Osteocrin, a Novel Bone-specific Secreted Protein That Modulates the Osteoblast Phenotype*

Received for publication, July 8, 2003, and in revised form, September 27, 2003
Published, JBC Papers in Press, October 1, 2003, DOI 10.1074/jbc.M307310200

Gethin Thomas‡§¶, Pierre Moffatt‡¶, Patrick Salois¶, Marie-Hélène Gaumond¶, Rock Gingras¶, Éric Godin¶, Dengshun Miao‖, David Goltzman‖, and Christian Lanctôt¶

From ¶Phenogene Therapeutics Inc., 416 de Maisonneuve West, Suite 1020, Montreal, Quebec H3A 1L2, Canada, and the ‖Calcium Research Laboratory, McGill University Health Centre, McGill University, Montreal, Quebec H3A 1A1, Canada

Although a number of secreted factors have been demonstrated to be bone regulators, none of these are unique to bone. Using a viral-based signal-trap strategy we have identified a novel gene we have termed "osteocrin." A 1280-bp mRNA encodes osteocrin producing a mature protein of 103 amino acids with a molecular mass of 11.4 kDa. Osteocrin shows no homology with any known gene except for two conserved sequence motifs reminiscent of dibasic cleavage sites found in peptide hormone precursors. Immunofluorescence and Western blot analysis confirmed the secretory nature of osteocrin. Two protein species were identified in the medium of cells overexpressing osteocrin, a full-length 11.4 kDa species and a processed ~5 kDa species. Mutation of the ^{76}KKKR^{79} dibasic cleavage site abolished the appearance of this smaller osteocrin fragment. By *in situ* hybridization in mouse embryos, osteocrin was expressed specifically in Cbfa-1-positive, osteocalcin-negative osteoblasts. Immunohistochemistry on adult mouse bone showed osteocrin localization in osteoblasts and young osteocytes. By Northern blot analysis, osteocrin expression was only detected in bone, expression peaking just after birth and decreasing markedly with age. In primary osteoblastic cell cultures osteocrin expression coincided with matrix formation then decreased in very mature cultures. Treatment of cultures with 1,25-dihydroxyvitamin D_3 resulted in a rapid dose-dependent down-regulation of osteocrin expression, suggesting direct regulation. Chronic treatment of primary cultures with osteocrin-conditioned media inhibited mineralization and reduced osteocalcin and alkaline phosphatase expression. These results suggest that osteocrin represents a novel, unique vitamin D-regulated bone-specific protein that appears to act as a soluble osteoblast regulator.

Bone is a dynamic tissue that is continually being modeled and remodeled through the coordinated actions of the bone forming osteoblasts and bone resorbing osteoclasts. Such remodeling is necessary to respond to the continually changing mechanical and regulatory demands placed upon the skeleton (1–4). The balance of the activity of the osteoblasts and osteoclasts is tightly regulated at both the systemic and local levels through the actions of a number of secreted molecules. Systemically, calciotropic hormones such as 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$)[1] and parathyroid hormone (PTH) can regulate both bone formation and resorption, acting on both osteoclastic and osteoblastic cell lineages (5–8), whereas $1,25(OH)_2D_3$ also acts via the intestine and kidney to regulate systemic calcium and phosphate availability (9, 10). Other hormones, such as growth hormone (11), estrogens (12), and thyroid hormone (13) also influence bone mass. More recently, it has been reported that the peptide hormone leptin exerts a strong systemic anti-osteogenic effect when activating the sympathetic nervous system through a hypothalamic relay (14, 15).

Remodeling occurs in discrete units, termed basic multicellular units, consisting of a cone of cutting osteoclasts followed by osteoblasts laying down a new collagenous matrix (16, 17). Osteoblasts also secrete abundant amounts of non-collagenous proteins, which play key roles in the maturation and mineralization of the bone matrix (18, 19). In addition to building bone, the osteoblasts are directly and indirectly involved in regulating bone metabolism. Osteoblasts secrete molecules that regulate osteoclastogenesis and/or osteoclast activity, such as receptor activator of NFκB ligand and osteoprotegerin (20) and also release growth factors into the bone matrix, such as members of the insulin-like growth factor, transforming growth factor, and fibroblast growth factor families (21). These factors are thought to be stored and released during successive remodeling cycles to control local bone formation and resorption (21–23). Insulin-like growth factor-1 and basic fibroblast growth factor, for example, act in a paracrine fashion stimulating osteoblast proliferation and activity (24–27).

It should be noted that none of the secreted bone regulatory proteins described to date are specifically expressed by bone cells. In fact, currently, only four highly bone-specific genes have been identified, two key transcription factors (Cbfa-1 (28) and osterix (29)) and two structural proteins (bone sialoprotein (30) and osteocalcin (31, 32)). To identify novel potential bone-specific regulatory molecules, we undertook to screen cDNA libraries from bone using a viral-based signal trap technology (33). We report here the identification of a novel gene specifically expressed in osteoblasts. The protein contains dibasic cleavage sites conserved in vertebrate homologues and reminiscent of those

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY395730, AY398681, AY398682, and AY398683.

‡ These authors contributed equally to this work.

§ To whom correspondence should be addressed: Phenogene Therapeutics Inc., 416 de Maisonneuve West, Suite 1020, Montreal, Quebec, H3A 1L2, Canada. Tel.: 514-288-9099; Fax: 514-288-1111; E-mail: gthomas@phenogene.com.

[1] The abbreviations used are: $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; PTH, parathyroid hormone; nt, nucleotide; RACE, rapid amplification of cDNA ends; RT, reverse transcription; aa, amino acids; βGP, β-glycerophosphate; PB, phosphate buffer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Cbja-1, core binding factor alpha-1.

TABLE I
Summary of primers used in PCR amplification under "Materials and Methods"

Primer identifier	Primer sequence 5′–3′
dT_{15} primer-linker	GAGATGAATTCCTCGAGCTTTTTTTTTTTTTTT
25–10G	AAACGCTCTGACTTCTCACAAGATG
18–30V	GAGATGAATTCCTCGAGC
24–10G	CCATCAGCCTCTGGAACTGGAGAG
24–10Gmut	CTT*GCTAGC*GGTCTCAAATACGTCATTC
25–10Gmut	TTC*GCTAGC*AGCTTCTCTGGCTTTGGG
18–131G	AGATGCTCGACTGGAGAT
19–9G	GAATCAATTAGCCTCTGGA
21–9G	GGAAAGAACTTCACTGAAGTG
19–14G	GCTCCTCAACGAATTGGTG
23–4V (degenerate)	ACI(GA)(TC)I(CAT)(GC)IGA(AG)G(CA)GAA(GA)TC(AG)GC
23–6V (degenerate)	CT(AG)TCIA(TAG)(GC)GG(AG)(GC)(AT)ICC(AG)AAICC
21–14G	AGATGCTGGACTGGAGATTGG
21–15G	TTCTACCAATCCGATCCATGG
18–9G	GTCATTGAGAGCAATGCC
19–12G	CAAGGTCATCCATGACAAC

found in hormonal precursors. Functionally, this novel protein appears able to modulate osteoblastic differentiation.

MATERIALS AND METHODS

Cloning of Full-length Mouse Osteocrin cDNA—We have previously developed a screening system that allows the rapid identification of nucleic acids encoding signal peptides from complex libraries of cDNA fragments (33). A library enriched in 5′ fragments of cDNAs derived from developing calvaria (33) was obtained using a protocol adapted from the "oligo-capping" method (34).

The original osteocrin fragment retrieved from this screening protocol comprised nt 1–430 of the corresponding full-length clone (Fig. 1), which was subsequently obtained using a modified 3′-RACE strategy as follows. Five μg of e15.5 mouse calvaria total RNA was reverse-transcribed with Superscript II™ (Invitrogen) using a dT_{15} primer-linker. cDNA was subjected to 25 cycles of PCR with the Titan™ RT-PCR kit (Roche) using forward primer 25–10G (see Table I for primer sequences) and reverse primer 18–30V, complementary to the 5′ portion of the dT_{15} primer-linker (underlined). The PCR products were cloned in place of green fluorescent protein into the pQBI25fc3 cytomegalovirus-based expression vector (qBiogene, Montreal, Canada) and the coding sequence of osteocrin amplified by RT-PCR using Titan™ and primers 25–10G and 24–10G. The cDNA encoding the mutant osteocrin protein (mut-osteocrin, 128 aa), in which the ^{76}KKKR^{79} was changed into ^{76}AS^{77}, was constructed by mismatch PCR. Briefly, the wild-type mouse osteocrin expression vector was used as a template for two separate amplifications with Vent DNA polymerase (New England Biolabs, Mississauga, Ontario, Canada) and the following primer pairs 25–10G (forward) and 24–10Gmut (reverse), and 25–10Gmut (forward) and 24–10G (reverse). Each PCR product generated was digested with NheI, purified, and rejoined with T4 DNA ligase. All PCR products were cloned into the pQBI25fc3 vector and verified by dye terminator cycle sequencing (DTCS™ sequencing kit, Beckman Coulter, Fullerton, CA) on a Beckman CEQ 2000 automated sequencer.

Cloning of Vertebrate Homologues of Osteocrin—Human and rat osteocrin were cloned by standard RT-PCR starting from 50 ng of bone marrow poly(A) RNA (Clontech Inc, Palo Alto, CA) or 5 μg UMR106 osteosarcoma cell total RNA (ATCC, Manassas, VA), respectively. RT-PCR was performed with Titan™ with a common forward primer (18–131G) and specific reverse primers (human, 19–9G; rat, 21–9G). For snake osteocrin, total RNA was extracted from the vertebrae and surrounding muscles of a 2-month-old (~130 g) Burmese python using Trizol™ (Invitrogen) according to the manufacturer's protocol. First-strand cDNA was synthesized using dT_{15} primer-linker and Superscript II™. cDNA was then subjected to 33 cycles of PCR using rTaq DNA polymerase (NEB) with forward primer 23–4V and reverse primer 23–6V. 3′-RACE was then performed as described above with forward primer 19–14G and reverse primer 18–30G.

Production of Antibodies—A synthetic peptide (^{120}CMDRIGRNRL-SNSRG^{133}) corresponding to the last 14 residues of human osteocrin preceded by an extra cysteine, was conjugated to activated keyhole limpet hemocyanin via sulfhydryl groups. The peptide/carrier complex was mixed with complete Freund's adjuvant and injected into rabbits according to standard protocols (Affinity Bioreagents, Golden, CO).

Cell Culture and Transfections—Primary calvarial osteoblasts were cultured from 19–20-day-old embryonic rats based on a previously described protocol (35). Briefly, calvaria were subjected to 5 sequential

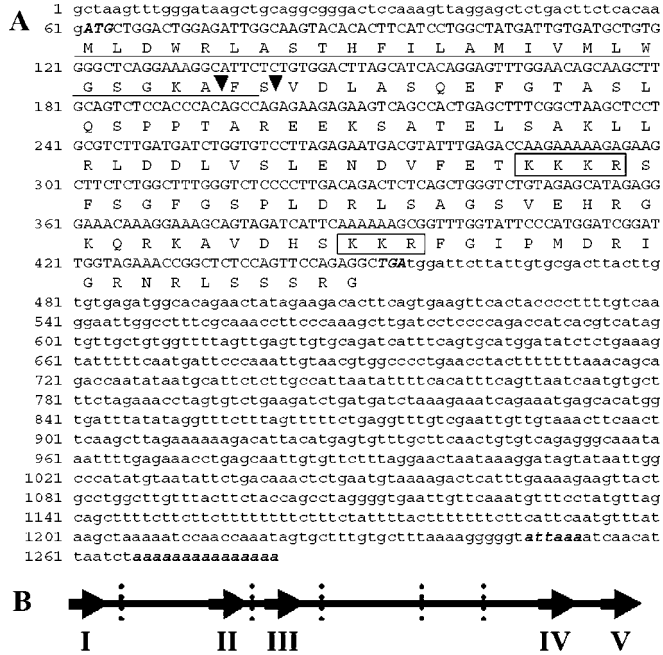

FIG. 1. **Full-length cDNA, deduced amino acid sequence, and genomic structure of mouse osteocrin.** *A*, the full-length mouse cDNA is 1280 bp and contains an open reading frame of 393-bp flanked by 61 and 811 bp of untranslated sequences at the 5′ and 3′ ends, respectively. The ATG start codon, the TGA stop codon and the polyadenylation signal and site are in *boldface*. The mouse gene produces a protein of 130 residues containing a signal peptide (*underlined*) with the two predicted signal peptidase cleavage sites marked by *arrowheads*. The two putative dibasic cleavage sites, KKKR and KKR, are *boxed*. *B*, the osteocrin genomic locus on chromosome 16 is ~44 kb containing 5 exons. The *dashed lines* denote gaps in the publicly available mouse genomic sequence. Note the exons are not drawn to scale.

20 min digests in 1 mg/ml collagenase (Worthington, Lakewood, NJ)/0.25% trypsin (Roche)/0.05% EDTA). Cells from digests 2–5 were pooled and seeded at 10000 cells/cm^2 in α-minimal essential medium containing 10% (v/v) fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 40 mg/ml gentomycin, 20 mM HEPES, 2 mM glutamine, 50 μg/ml ascorbic acid (all Invitrogen) with or without 10 mM β-glycerophosphate (βGP) (Sigma) depending on experimental conditions. Media was changed after 2 days and every 2–3 days thereafter. Cultures were treated with 1,25$(OH)_2D_3$ (Sigma) or ethanol vehicle at various concentrations and for differing time periods as noted for each experiment. For transfections experiments, HEK293 (qBiogen) and UMR106 cells were cultured in Dulbecco's modified Eagle's medium with 10% (v/v) fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. Cells were transiently transfected with 500 ng of plasmid DNA/10^6 cells using Effectene™ (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer's instructions. Media was changed after 6 h, and cells were left for an additional 16 h before washing with phosphate-buffered

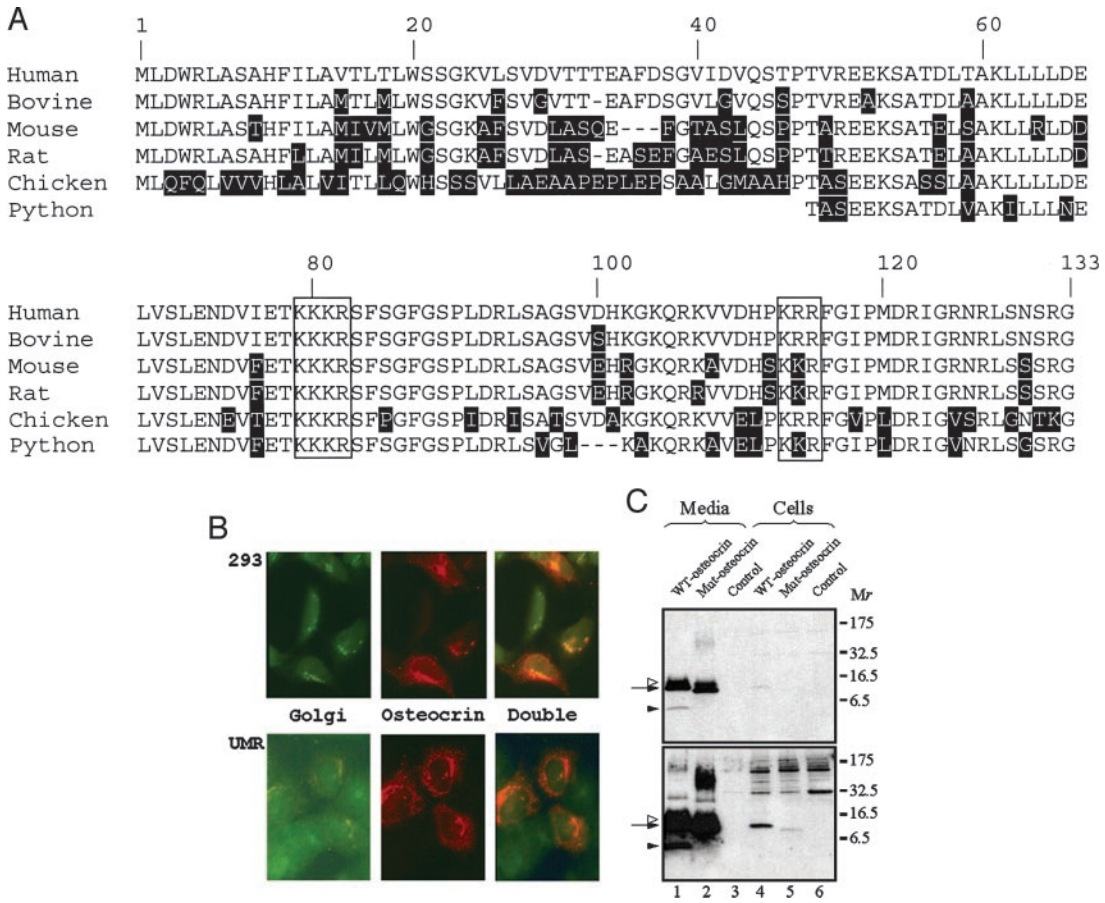

FIG. 2. **Predicted osteocrin protein structure and secretion.** *A*, predicted amino acid sequences from human, bovine, mouse, rat, chicken, and snake (partial) cDNAs (see "Materials and Methods"). The two dibasic cleavage sites, ^{76}KKKR^{79} and ^{110}KKR^{112}, are well conserved (*boxed*). *Shaded* residues represent non-conserved residues from the human sequence. Note that the C-terminal half of the protein is highly conserved. *B*, immunofluorescent localization with a human osteocrin-specific antibody demonstrates overexpressed osteocrin is clearly visible in the secretory apparatus of HEK293A fibroblasts and UMR106 osteosarcoma cells (×400) colocalizing with the 58K Golgi protein-specific antibody. *C*, the majority of osteocrin is detected in the medium of HEK293 cells by Western blot when transiently transfected (*lanes 1* and *2*). Residual protein in the secretory apparatus is detected in the cell lysate (*lanes 4* and *5*). Two exposures of the Western blot are shown to illustrate the underrepresented smaller processed fragments, with the *lower panel* representing a longer exposure. A doublet can be seen at the expected full-length size of 11.4 kDa and smaller processed fragments at ~5 kDa (*lane 1*). Mutation of the KKKR site to AS abolishes processing of the protein leaving only full-length 11.4 kDa bands (*lane 2*). Equal proportions of media or cell lysate from cultured cells were loaded. *WT*, wild type; *Mut*, mutated.

saline and accumulation in serum-free Dulbecco's modified Eagle's medium for 48 h for analysis of secreted products. For conditioned media treatment of primary osteoblast cultures, media from osteocrin or mock-transfected HEK293 cells were diluted 1:6 in normal culture media and replenished every 2–3 days from day 2 until the termination of the experiment at day 18. To measure ^{45}Ca uptake, $^{45}CaCl_2$ (1μCi/ml)(Amersham Biosciences) was added to the medium for the last 48 h of the experiment. Medium was collected and the cells scraped into 5% (v/v) trichloroacetic acid. Cell/matrix-associated ^{45}Ca incorporation was calculated as a percentage of the total input in the cellular and medium fractions.

Immunofluorescence and Western Analysis—For immunofluorescence detection, cells grown on coverslips were fixed with 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.2, and permeabilized with 0.5% Triton X-100™ in PB for 4 min. Cells were then blocked with PB supplemented with 5% (w/v) dried milk for 30 min. Cells were then incubated for 1 h with a mix of 1/100 dilution (v/v) of goat anti-human osteocrin antisera and a 1/50 dilution (v/v) of mouse anti-rat Golgi 58K protein antibody (Sigma) in PB. Cells were then incubated in a mix of goat anti-rabbit coupled to AlexaFluor 594 and goat anti-mouse AlexaFluor 488 (both Molecular Probes, Eugene, OR) in PB for 1 h. Cells were washed twice and observed by fluorescence microscopy. All steps were performed at room temperature.

For Western blotting, protein in the conditioned medium was precipitated by adding trichloroacetic acid to a final concentration of 10% (w/v) and incubated on ice for 1 h. After centrifugation at 12000 × *g* for 20 min at 4 °C, the pellet was washed once with chilled acetone, dried briefly, and resuspended directly in 2× Laemli loading buffer (Bio-Rad, Mississauga, Ontario, Canada) and boiled. For cell extracts, cells were rinsed with phosphate-buffered saline and solubilized in lysis buffer (50 mM Tris-HCL, pH8.0, 150 mM NaCl, 2 mM EDTA, 1% IGEPAL-630™, and 1% (v/v) protease inhibitor mixture (Sigma)). Cell debris and insoluble material were pelleted by centrifugation at 12000 × *g* for 5 min at 4 °C. The soluble proteins were mixed with 1 volume of 2× Laemli loading buffer and boiled. Proteins were electrophoresed on a 16.5% denaturing Tris-Tricine polyacrylamide gel (Bio-Rad) and transferred onto 0.2 μm nitrocellulose (Protran, Schleicher, and Schuell, Keene, NH) according to standard Western blot protocols. All subsequent incubations were performed at room temperature. The membrane was incubated in a 1/800 dilution (v/v) of osteocrin-antiserum in Tris-buffered saline, 0.1% Tween 20 with 2.5% (w/v) dried milk followed by goat anti-rabbit horseradish peroxidase (Sigma) diluted 1/30000 in TBST with 2.5% (w/v) dried milk and the signal visualized with ECL™ reagent (Amersham Biosciences).

In Situ Hybridization—*In situ* hybridization was performed on mouse embryonic tissue sections from e16.5 to e17.5 as described previously (36). The mouse cRNA antisense probes used were as follows: an 815-bp cbfa-1 fragment (nt 931–1746 of GenBank™ accession number AF10284), an 187-bp osteocalcin fragment (nt 1–187 of GenBank™ accession number U11542), and a 431-bp osteocrin fragment (nt 1–431 of sequence shown in Fig. 1*A*). Cbfa-1 and osteocalcin were exposed for 21 days and osteocrin for 30 days.

Northern and RT-PCR Analysis—RNA was isolated from whole bones or osteoblastic cell cultures using Trizol™ with glycogen (5 μg/ml) as carrier according to the manufacturer's instructions. For RT-PCR, cDNAs were generated with Superscript II™ reverse transcriptase and oligo-dT_{18} priming, and PCR amplification carried out with gene-specific primers using rTaq DNA polymerase (New England

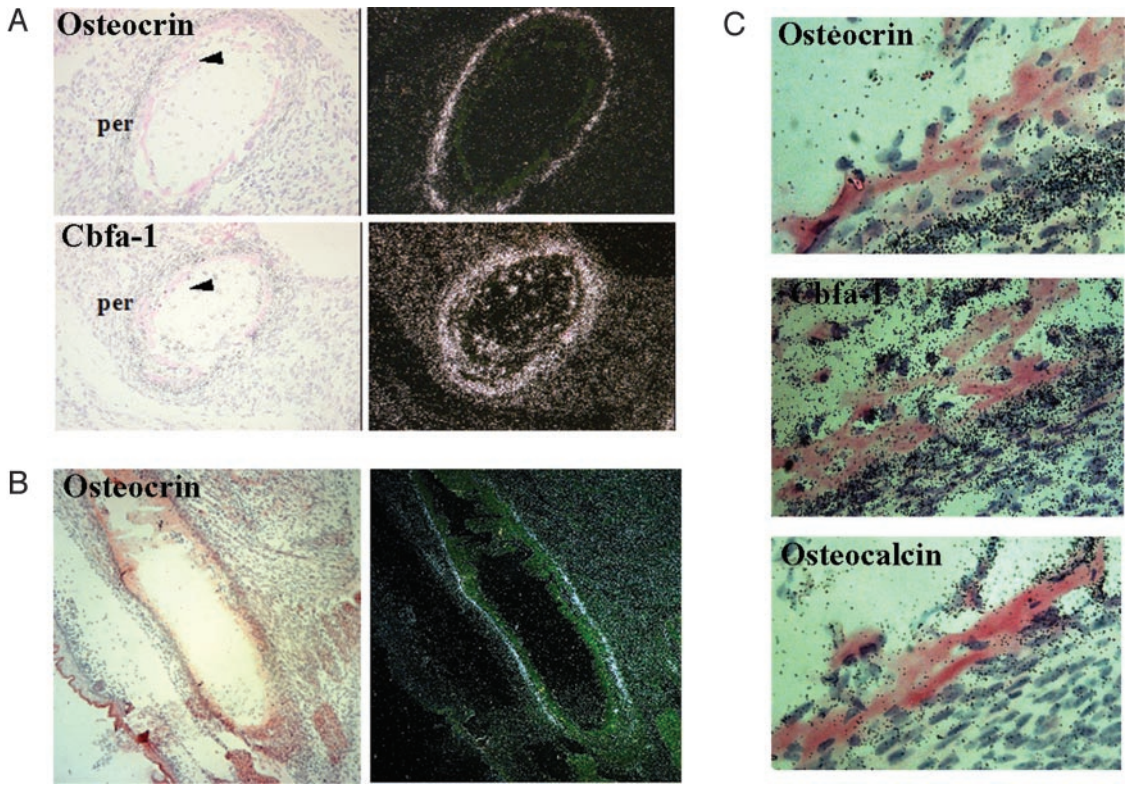

FIG. 3. **Osteocrin is expressed in osteoblasts.** *A*, *in situ* localization of osteocrin transcripts in e16.5 mouse ribs. Osteocrin is localized within a subset of Cbfa-1-positive cells in a subperiosteal layer (*per*). Active mature osteoblasts are signified by an *arrowhead* (×200). *B*, expression of osteocrin in whole e17.5 fore limb. Osteocrin is clearly localized in the osteoblasts on the periphery of the bone (×100). *C*, in e16.5 mouse tibiae, osteocrin co-localizes with a subset of Cbfa-1-positive cells, exclusive from the more mature osteocalcin-positive cells found on the bone surface and in the trabecular bone (*arrowheads*) (×400).

Biolabs). Gene-specific primers and conditions were as follows: osteocrin, forward 21–14G and reverse 21–15G (annealing temp = 52 °C, 28 cycles, product 378-bp); GAPDH, forward 18–9G and reverse 19–12G (annealing temp = 56 °C, 33 cycles, product 398-bp).

Northern blots were generated on nylon membranes (Osmonics, Westborough, MA) by standard methods (37). Filters were prehybridized for 4 h and hybridized overnight in Church buffer (38) at 65 °C. The rat osteocrin cDNA probe corresponded to the full coding sequence (Fig. 1*A*). For rat osteocalcin, a cDNA corresponding to the full coding sequence (GenBank™ accession number M25490) was generated by PCR. A 681-bp mouse fragment corresponding to GenBank™ accession number J04806 was cloned for the osteopontin cDNA probe. For alkaline phosphatase, a 341-bp fragment corresponding to GenBank™ accession number NM013059 was generated. A mouse GAPDH cDNA probe corresponding to −21 to 956 bp of GenBank™ accession number M32599 was generated by PCR. Probes were labeled with [α-^{32}P]dCTP using a standard random priming protocol (37).

Immunohistochemistry—Immunohistochemistry was as described previously (39). Briefly, decalcified tibial sections were stained with primary osteocrin antibody or preimmune rabbit serum overnight at room temperature followed by biotinylated goat anti-rabbit IgG (Sigma) and stained using the avidin-biotin-peroxidase complex technique.

RESULTS

Identification and Cloning of Osteocrin—We previously reported the application of a novel technology, based on a signal-trap, to identify cDNAs encoding secreted and membrane-bound proteins (33). Screening of a mouse e15.5 calvarial bone library identified a 433-bp fragment encoding a putative translation product containing a signal peptide deriving from a novel unannotated gene. A modified 3′-RACE strategy was used to obtain the full-length cDNA. Sequences from at least five different clones were aligned to reconstitute the full-length cDNA sequence shown in Fig. 1*A*. This mouse cDNA is 1280-bp containing an open reading frame of 393 bp flanked by 61 bp and 826 bp of untranslated sequences at the 5′ and 3′ ends, respectively. A polyadenylation signal is found 14 bp upstream of a poly(A) stretch. The putative initiator ATG codon is found in an adequate Kozak context (AAG*ATG*C). Delineation of the corresponding genomic locus was enabled by BLAST searches of the mouse genome sequence (www.ensembl.org). The gene is located at chromosome 16B2 (syntenic to human chromosome region 3q28). Covering ~44 kb, the mouse gene locus is unusually large for a 1280 mRNA derived from only 5 exons (Fig. 1*B*).

Protein Structure and Secretion of Osteocrin—The novel mouse cDNA encoded a protein of 130 residues containing a signal peptide (Fig. 1*A*, *underlined*). The SignalP v2.0 server (40) predicted two possible signal peptidase cleavage sites, at Ala-25 and Ser-27 (Fig. 1*A*, *arrowheads*). Initial mass spectrometric analyses on a semi-purified preparation gave a size of 11371 Daltons favoring Ser-27 as the predominant cleavage site. Such a size also matched exactly the predicted molecular weight from the sequence suggesting no post-translational modifications. The mature 11.4-kDa protein has an isoelectric point of 10.02. Extensive *in silico* analysis suggested that the protein presented no strong homology with any known protein or protein domains. However, two sequence motifs reminiscent of dibasic cleavage sites found in peptide hormone precursors were evident: KKKR at position 76–79 and KKR at position 110–112 (Fig. 1*A*, *boxed*). Such putative cleavage sites could create peptides of 48, 30, and 18 aa, respectively. Because of these features and its bone-specific expression (see "Restricted Expression of Osteocrin"), we tentatively named this novel gene osteocrin.

To further investigate the significance of the putative processing sites in mouse osteocrin, we determined whether the dibasic sites were conserved in other species. Rat and human full-length cDNAs and a partial cDNA from snake were cloned using RT-PCR. In addition, *in silico* searches (GenBank™)

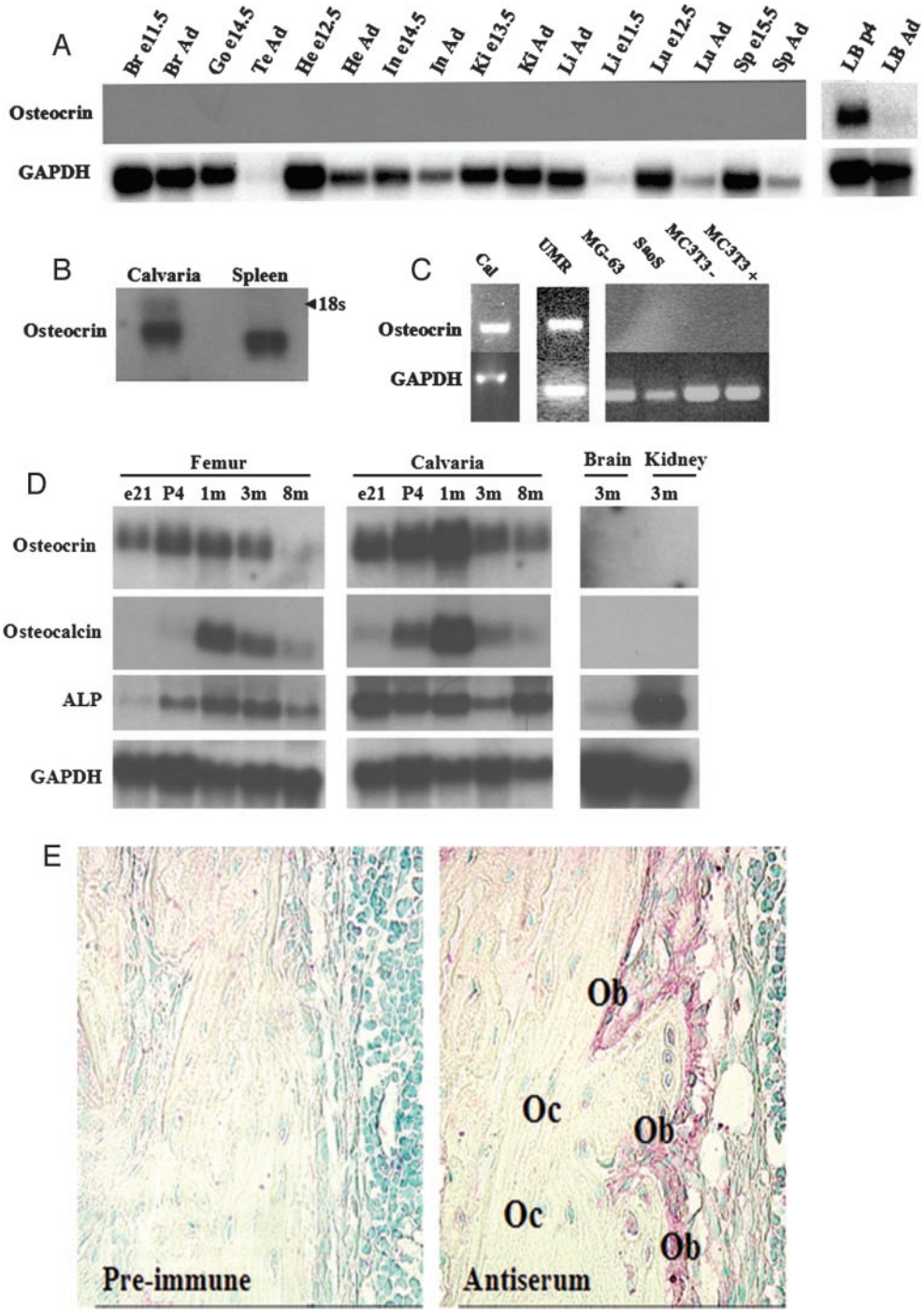

FIG. 4. **Osteocrin is a bone-specific gene.** *A*, Northern blot showing bone specificity of mouse osteocrin expression. Osteocrin is only detected in neonate and adult long bones and calvaria. No message was detected in embryonic (*e*) or adult (*ad*) non-bone tissues. Five μg total RNA was loaded. *Br*, brain; *Go*, gonads; *Te*, testes; *He*, heart; *In*, intestine; *Ki*, kidney; *Li*, liver; *Lu*, lung; *Sp*, spleen. *B*, osteocrin in detected by Northern blot in rat spleen. The transcript size of adult rat spleen osteocrin is smaller than that for adult rat calvaria. Fifteen μg of total RNA was loaded. The position of the 18 S ribosomal RNA band is marked. *C*, osteocrin expression was detected in embryonic calvariae and UMR106 cells by RT-PCR. No expression was detected in MG-63, SaoS-2, undifferentiated (−), or differentiated (+) MC3T3.E1 cells. *D*, Northern blot showing osteocrin expression in femora and calvariae in embryonic (*e21*), newborn (*p4*), growing (1 month, *1m*), adult (3 month, *3m*), and aged (8-month, *8m*) rats. In femora, osteocrin expression was highest in newborn rats decreasing significantly in aged rats. Osteocrin expression peaks at 1-month in calvaria with a less marked decrease in older rats. Twenty-five μg of total RNA was loaded. *ALP*, alkaline phosphatase. For Figs. 4, *A–D*, GAPDH represents a loading control. *E*, immunohistochemistry with an osteocrin-specific antibody showing localization of osteocrin protein to active osteoblasts (*Ob*) in adult mouse tibia. The protein is absent from the mature osteocytes (*Oc*). No staining is visible in the sections stained with pre-immune serum (×400).

identified a single bovine EST (accession number BF045261) and two chicken ESTs (accession numbers ChEST861c21 and ChEST83i24) (41). Overall amino acid homology between different species is high (Fig. 2*A*), for example human and mouse sequences are 74% identical (84% similar) and human and chicken sequences are 59% identical (71% similar). Further, the dibasic cleavage sites are conserved across species (Fig. 2*A*) with the C-terminal half of the protein from the KKKR dibasic site being the most conserved. For instance, the C-terminal portion of the human protein (residues 116–133) differs by only

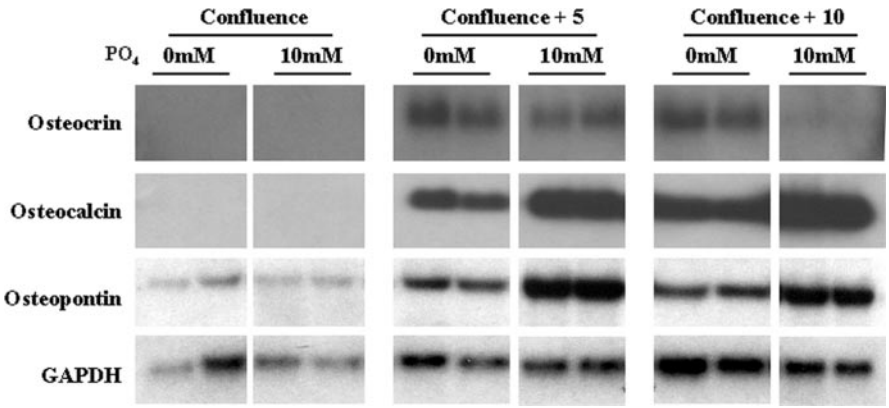

FIG. 5. **Osteocrin is expressed during osteoblast matrix production and maturation.** Northern blot of a time course of calvarial primary osteoblast differentiation at confluence and 5 and 10 days post-confluence in the presence and absence of 10 mM βGP (PO_4). Osteocrin is expressed at 5 and 10 days post-confluence but not at confluence. Cultures maintained in 10 mM βGP until 10 days post-confluence exhibit a marked down-regulation in osteocrin expression. Osteocalcin and osteopontin expression are highest at 10 days post-confluence and are increased by βGP treatment. Twenty μg of total RNA was loaded. GAPDH represents a loading control. Duplicate samples are shown.

3 amino acids from that of the snake protein (C-terminal 90% similarity, full-length similarity of 79%).

Functionality of the predicted signal peptide in osteocrin was confirmed by immunofluorescence in HEK293 human fibroblasts and UMR106 rat osteosarcoma cells transfected with a vector expressing osteocrin. Labeling was clearly localized in the secretory apparatus of transfected cells, confirmed by colocalization with the Golgi apparatus-specific Golgi 58 kDa protein (Fig. 2*B*). Western blot analysis of transfected HEK293 cells demonstrated that osteocrin was constitutively secreted and accumulated in the culture medium of transfected cells (Fig. 2*C*, *lane 1*). Similar accumulation (data not shown) and localization (Fig. 2*B*) was seen in UMR106 osteoblasts transfected with the same vector. Some osteocrin was also detected in the cellular extract, most likely residual from the secretory apparatus (Fig. 2*C*, *lane 4*). No signal was detected in the mock-transfected HEK293 cells (Fig. 2*C*, *lane 3*). Three bands were visible in the media. Two closely separated bands can be seen at ~11.4 kDa, which might arise from alternate or incomplete processing of the signal peptide, as described above. The third band, at ~5 kDa, could represent the 51-aa fragment generated by processing at the ^{76}KKKR^{79} dibasic cleavage site (Fig. 2*C*, *lane 1*). To test this hypothesis, we substituted an alanine and a serine for the ^{76}KKKR^{79} site. Due to the replacement of 4 charged residues (KKKR) with two uncharged residues (AS) in the mutated osteocrin, the mutated molecule migrated at a slightly lower position than the native molecule (Fig. 2*C*, *lanes 1 versus 2* and *4 versus 5*). Interestingly, the band at ~5 kDa was no longer detected in the medium from cells transfected with the mutated osteocrin cDNA (Fig. 2*C*, *lane 2*), suggesting this dibasic cleavage site is functional. Difficulties of visualization of small protein fragments may result in this band being underrepresented. However, longer exposure demonstrated the smaller band could still not be detected in the mutant osteocrin-transfected cells (Fig. 2*C*, *lane 2*, *lower panel*).

Restricted Expression of Osteocrin—In situ hybridization using a probe corresponding to the coding sequence of mouse osteocrin revealed a very defined and restricted expression pattern in embryonic mice. Expression first appears at e13.5 in the bone rudiments (data not shown). Osteocrin expression highlighted the periosteum in a subset of Cbfa-1-positive cells of the developing e16.5 ribs (Fig. 3*A*) and the e17.5 fore limb (Fig. 3*B*). Closer examination of e16.5 tibiae demonstrated that osteocrin was expressed in periosteal osteoblasts that are Cbfa-1-positive but osteocalcin-negative (Fig. 3*C*).

By Northern blot analysis, osteocrin message could not be

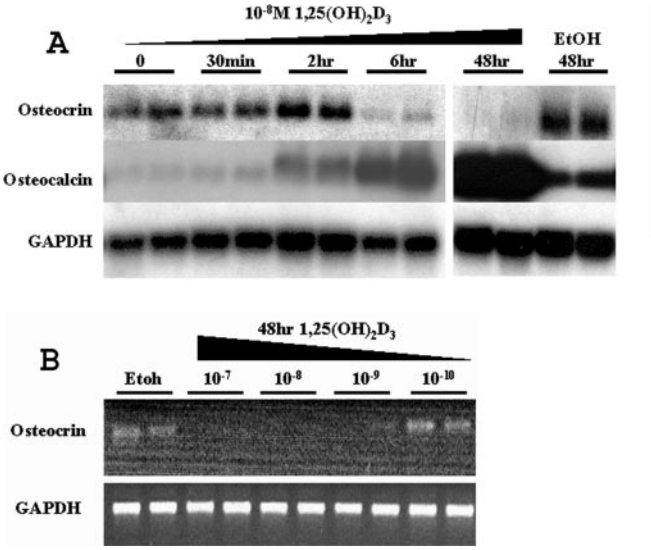

FIG. 6. **Osteocrin is down-regulated by $1,25(OH)_2D_3$ treatment.** Effects of $1,25(OH)_2D_3$ treatment on osteocrin expression in 6 day post-confluent primary calvarial osteoblast cultures. *A*, Northern blot of cultures treated with 10^{-8} M $1,25(OH)_2D_3$ over a 48 h time course. Osteocrin expression is down-regulated after 6 h of treatment and abolished after 48 h of treatment. Osteocalcin expression is up-regulated after 2 h of $1,25(OH)_2D_3$ treatment. Twenty μg of total RNA was loaded. *B*, dose response of osteocrin expression to 48 h $1,25(OH)_2D_3$ treatment by RT-PCR. Osteocrin was down-regulated by 10^{-7} M, 10^{-8} M, and 10^{-9} M but not 10^{-10} M $1,25(OH)_2D_3$. GAPDH represents a loading control. Duplicate samples are shown.

detected in tissues other than bone (Fig. 4*A*) except for a smaller sized transcript detected in rat spleen (Fig. 4*B*). Cloning and sequencing of this isoform suggested it is transcribed from an alternate promoter upstream of the third exon of the gene. The significance of this rat isoform is unclear because it does not contain an open reading frame. Furthermore, no such transcript was detected in mouse spleen.

By high cycle number RT-PCR (40 cycles), we could detect expression of osteocrin in muscle, kidney, testes, and heart, albeit at very low levels (data not shown). Osteocrin expression was detected in the UMR106 rat osteosarcoma cell line but not in undifferentiated and mineralizing mouse MC3T3 osteoblasts or human MG-63 and SaOS-2 osteosarcoma cell lines (Fig. 4*C*).

In bone, expression of osteocrin was highest in embryos and neonates, peaking at 4 days of age in both calvaria and long bones and decreasing steadily with age to very low levels in 8-month-old long bones (Fig. 4*D*). The age-related decrease was

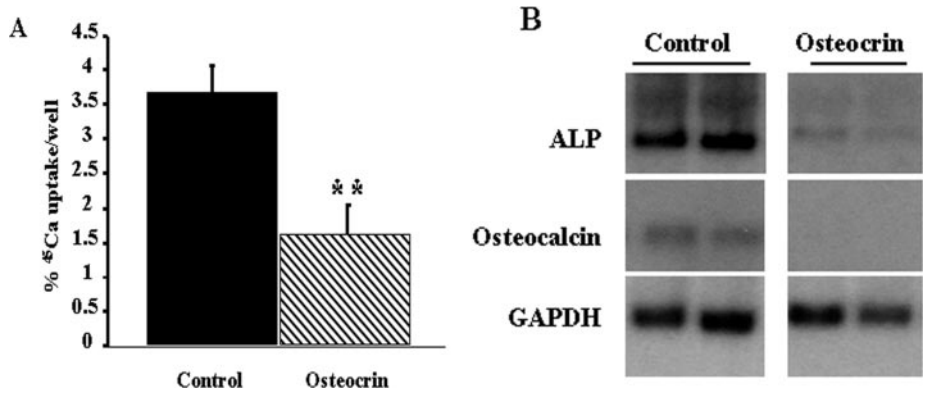

FIG. 7. **Osteocrin regulates the osteoblast phenotype.** Primary calvarial osteoblastic cultures were treated with conditioned media from osteocrin or empty vector (control) transfected HEK293A cells from day 2 to 10 days post-confluence. *A*, ^{45}Ca uptake was reduced 60% by osteocrin conditioned-media. Data is expressed as the mean ± S.E. of the % ^{45}Ca incorporated into the cultures. *, $p < 0.01$. *B*, Northern blot demonstrating total repression of osteocalcin and a marked reduction in alkaline phosphatase expression in osteocrin treated cultures. Fifteen μg of total RNA was loaded. GAPDH represents a loading control. Duplicate samples are shown.

less marked in calvaria by 8 months. Partitioning of long bones into midshaft (diaphysis and metaphysis) and epiphyseal regions demonstrated predominant expression of osteocrin in the diaphysis and metaphysis (data not shown). Maximal expression of osteocrin is reached earlier than that seen for osteocalcin, a marker of osteoblast activity but coincided more with that of tissue-nonspecific alkaline phosphatase. The relative expression levels of osteocrin were very low, as shown by a significantly longer exposure time (72 h) compared with osteocalcin (3 h).

Immunohistochemical localization using antibodies raised against the C-terminal region of osteocrin indicated that the protein was primarily synthesized by cuboidal osteoblasts on the endosteal surface of adult mouse long bones (Fig. 4*E*). Flattened preosteoblasts and newly formed osteocytes were also labeled, whereas mature entombed osteocytes were not.

Regulation of Osteocrin Expression in Calvarial Cultures—As expected from the *in situ* data, expression of osteocrin was evident in Northern blot analysis of primary calvarial osteoblastic cells (Fig. 5). Expression was first observed in conjunction with matrix formation between confluence and 5 days post-confluence. This point coincided with the onset of osteocalcin expression and significant up-regulation of osteopontin expression. Osteocrin expression was maintained through the mineralization phase 10 days post-confluence. Interestingly, primary osteoblasts cultured with 10 mM βGP for 10 days post-confluence exhibited decreased osteocrin expression, corresponding with the development of a very differentiated phenotype in these cultures. This down-regulation was, however, not observed for osteocalcin and osteopontin.

The bone-specific nature of the expression of this novel gene led us to investigate whether it was regulated by any of the major calciotropic hormones. Both PTH (10^{-8} M) and dexamethasone (10^{-8} M), a synthetic glucocorticoid, had no effect on osteocrin expression (data not shown). However treatment of mature cultures of rat primary osteoblasts with 10^{-8} M $1,25(OH)_2D_3$ in the absence of βGP showed significant reduction after only 6 h and completely abolition of osteocrin expression by 48 h (Fig. 6*A*). Osteocalcin expression was up-regulated in a similar time frame after 2 h of $1,25(OH)_2D_3$ treatment (Fig. 6*A*). Dose response showed that expression was repressed by 10^{-7} M and 10^{-8} M, to a lesser extent by 10^{-9} M but not by 10^{-10} M $1,25(OH)_2D_3$ (Fig. 6*B*). Such time and dose response characteristics suggest direct regulation of osteocrin by $1,25(OH)_2D_3$.

Osteocrin Blunts Terminal Differentiation of Osteoblasts—The biological activity of osteocrin products was assessed using conditioned medium from HEK293 fibroblasts transiently transfected with an expression vector for osteocrin. Primary osteoblasts were chronically treated with conditioned media from osteocrin- or empty vector-transfected cells from day 2 in culture (pre-confluence) until 10 days post-confluence (the mineralization phase). We estimated by direct enzyme-linked immunosorbent assay that the concentration of osteocrin products in conditioned media was in the order of 5 μg/ml. Treatment with osteocrin-containing medium resulted in a 60% decrease in mineralization as measured by ^{45}Ca uptake relative to empty vector (3.67% *versus* 1.61%, $p < 0.01$) (Fig. 7*A*). Further, osteocalcin expression was almost completely abolished and alkaline phosphatase expression markedly reduced by treatment with osteocrin-containing medium (Fig. 7*B*).

DISCUSSION

This report describes a novel secreted bone-specific protein that we have termed osteocrin. A cleavable signal peptide is found at the N terminus of the 130 aa mouse protein. Accordingly, the bulk of osteocrin is secreted in the culture medium by transiently transfected fibroblasts and osteoblast-like cells. Osteocrin presents no homology to any known protein or protein domain. However, there are two dibasic cleavage sites for members of the mammalian subtilisin/Kex20-like endoprotease family (42, 43). These putative processing sites are conserved in the osteocrin molecule from terrestrial vertebrates, from snake to human. Further, homology across species is particularly high in the C-terminal half of the molecule, between the putative cleavage sites. The presence and evolutionary conservation of the dibasic sites found in osteocrin are suggestive of processing similar to that seen for hormones such as proopiomelanocortin (44), PTH (45), and parathyroid hormone-related protein (46, 47). In addition, it is noteworthy that all the species of cloned osteocrin have a glycine residue at the C terminus. This raises the possibility that osteocrin products may be amidated, another feature found in many peptide hormones (48, 49). We have shown that processing of osteocrin occurs in transfected fibroblasts producing an immunoreactive fragment of ~5 kDa. Importantly, this fragment is not produced when the dibasic site at position 76–79 is mutated, indicating that it arises from cleavage at this site. Processing of the overexpressed osteocrin is not complete with significant amounts of the 11.4-kDa signal peptide-cleaved form of the protein being released. However, previous studies (50, 51) have indicated that prohormone overexpression can lead to a saturation of processing.

Osteocrin appears to be expressed in a subset of osteoblasts. It is expressed in bone from an early developmental stage

(e13.5) being found in the Cbfa-1-positive osteoblast population though it does not co-express with the mature osteoblastic marker osteocalcin. In adult bone, the protein is found in active osteoblasts and early osteocytes but not in terminal osteocytes, again suggesting expression in a subset of cells. Further, in adult bone, osteocrin appears to be localized to the endosteal surface, which is purported to be more active (52, 53).

Temporally, peak expression of osteocrin in bone was just after birth with expression levels decreasing in aged bones. Such a reduction in expression with age is consistent with a role in osteoblast formation and/or activity, which is known to decrease with age (54–57). Age-related decreases in expression have been described for osteocalcin (56, 58) and another mature osteoblast marker, phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PHEX), an endopeptidase involved in phosphate regulation (59). Consistent with its earlier expression during osteoblast differentiation however, osteocrin levels diminish before those of osteocalcin. The age-related decrease in osteocrin expression is less marked in calvaria than long bones, possibly reflecting the fact that calvarial bones play a more protective role and as such, respond differently to physiological and regulatory demands, such as PGE_2 or bed-rest (60–63) than load-bearing long bones. Differences in gene expression have been previously described in osteoblasts and osteoclasts from long bones and calvaria (64).[2]

In vitro, osteoblasts display a very defined maturational phenotype (65). After an initial proliferative phase, the osteoblasts reach confluence and start to lay down a collagenous matrix. This is followed by the maturation of the matrix as a number of non-collagenous proteins are secreted into the matrix. Finally the matrix is mineralized forming a bone like layer. In this model, osteocrin expression coincides with the production and maturation of the bone matrix and is maintained at a constant level during this maturation period, suggesting a role in the active osteoblast. In contrast, other markers of osteoblast differentiation, such as osteocalcin, display a more linear expression pattern, with expression continuing to increase during the mineralization phase of the osteoblasts (65, 66).

Expression of osteocrin is rapidly and dose-responsively down-regulated by $1,25(OH)_2D_3$. Changes in RNA levels occur within 6 h of treatment, similar to other directly up-regulated (osteocalcin (67) and osteopontin (68)) and down-regulated (bone sialoprotein (69) and Cbfa-1 (70)) vitamin D responsive genes. All these genes are regulated by $1,25(OH)_2D_3$ through well defined vitamin D response elements (71, 72). Preliminary *in silico* analysis of the 5′ upstream regions of the human and mouse osteocrin genes revealed putative VDREs ~4.6 and 5.6 kb upstream of Exon 1, respectively. Whether these motifs are functional or modulate the $1,25(OH)_2D_3$ repression of osteocrin has yet to be confirmed.

In very mature primary osteoblastic cultures, terminal differentiation occurs (in this case induced by long-term culture in the presence of 10 mM βGP), resulting in apoptosis or the assumption of a more osteocytic phenotype (73–75). Such terminal differentiation results in a significant reduction in osteocrin expression, whereas reductions in expression of osteoblast markers such as osteocalcin or alkaline phosphatase (both expressed in osteocytes) do not occur. Thus, there appears to be a defined window of osteocrin expression reflecting osteoblast activity. Taken together with vitamin D regulation, these results suggest a role for osteocrin in the modulation of the mature osteoblast phenotype. This hypothesis is supported by the marked inhibition of mature osteoblastic functions observed upon treatment of primary osteoblasts with osteocrin-conditioned media. Mineralization was reduced together with expression of the mineralization-associated genes, alkaline phosphatase and osteocalcin. The concentration of osteocrin in the conditioned medium may represent supraphysiologic levels, but it has been suggested that local concentrations of other bioactive molecules such as insulin-like growth factor-1 and fibroblast growth factor 2 could be similarly high in the bone matrix (76). Osteocrin may therefore act directly on osteoblasts via autocrine or paracrine pathways.

In summary, osteocrin is a novel bone-active molecule that has no homology to any known protein family. The bone-specific expression pattern, direct-regulation by vitamin D and ability to modulate *in vitro* osteoblast function suggests that osteocrin represents a novel soluble regulator of bone metabolism. Further elucidation of the function and activity of osteocrin may provide new avenues for bone therapeutic approaches.

Acknowledgments—We thank members of our laboratory for help and advice. We also thank Jacques Drouin (Institut de recherches cliniques de Montréal) and Antonio Nanci (Université de Montréal) for helpful discussions.

[2] G. P. Thomas, unpublished data.

REFERENCES

1. Vaananen, H. K. (1993) *Ann. Med.* **25,** 353–359
2. MacDonald, B. R., and Gowen, M. (1993) *Baillieres. Clin. Rheumatol.* **7,** 421–443
3. Frost, H. M. (2000) *J. Bone Miner. Metab.* **18,** 305–316
4. Turner, C. H. (1992) *Bone* **13,** 403–409
5. Feldman, D. (1999) *Am. J. Med.* **107,** 637–639
6. Ishizuya, T., Yokose, S., Hori, M., Noda, T., Suda, T., Yoshiki, S., and Yamaguchi, A. (1997) *J. Clin. Invest.* **99,** 2961–2970
7. Kodaira, K., Mizuno, A., Yasuda, H., Shima, N., Murakami, A., Ueda, M., and Higashio, K. (1999) *Gene* **230,** 121–127
8. Karaplis, A. C., and Goltzman, D. (2000) *Rev. Endocr. Metab. Disord.* **1,** 331–341
9. Bortell, R., Owen, T. A., Bidwell, J. P., Gavazzo, P., Breen, E., van Wijnen, A. J., DeLuca, H. F., Stein, J. L., Lian, J. B., and Stein, G. S. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 6119–6123
10. Norman, A. W. (1992) *Curr. Opin. Rheumatol.* **4,** 375–382
11. Ohlsson, C., Bengtsson, B. A., Isaksson, O. G., Andreassen, T. T., and Slootweg, M. C. (1998) *Endocr. Rev.* **19,** 55–79
12. Manolagas, S. C., Kousteni, S., and Jilka, R. L. (2002) *Recent Prog. Horm. Res.* **57,** 385–409
13. Robson, H., Siebler, T., Shalet, S. M., and Williams, G. R. (2002) *Pediatr. Res.* **52,** 137–147
14. Amling, M., Pogoda, P., Beil, F. T., Schilling, A. F., Holzmann, T., Priemel, M., Blicharski, D., Catala-Lehnen, P., Rueger, J. M., Ducy, P., and Karsenty, G. (2001) *Adv. Exp. Med. Biol.* **496,** 85–94
15. Takeda, S., Elefteriou, F., Levasseur, R., Liu, X., Zhao, L., Parker, K. L., Armstrong, D., Ducy, P., and Karsenty, G. (2002) *Cell* **111,** 305–317
16. Parfitt, A. M., Mundy, G. R., Roodman, G. D., Hughes, D. E., and Boyce, B. F. (1996) *J. Bone Miner. Res.* **11,** 150–159
17. Parfitt, A. M. (2001) *J. Bone Miner. Res.* **16,** 1583–1585
18. Robey, P. G. (1996) *Connect Tissue Res.* **35,** 131–136
19. Gorski, J. P. (1998) *Crit. Rev. Oral. Biol. Med.* **9,** 201–223
20. Suda, T., Takahashi, N., Udagawa, N., Jimi, E., Gillespie, M. T., and Martin, T. J. (1999) *Endocr. Rev.* **20,** 345–357
21. Linkhart, T. A., Mohan, S., and Baylink, D. J. (1996) *Bone* **19,** 1S-12S
22. Rosen, C. J., and Donahue, L. R. (1998) *Proc. Soc. Exp. Biol. Med.* **219,** 1–7
23. McCarthy, T. L., Ji, C., and Centrella, M. (2000) *Crit. Rev. Oral. Biol. Med.* **11,** 409–422
24. Zhang, M., Xuan, S., Bouxsein, M. L., von Stechow, D., Akeno, N., Faugere, M. C., Malluche, H., Zhao, G., Rosen, C. J., Efstratiadis, A., and Clemens, T. L. (2002) *J. Biol. Chem.* **277,** 44005–44012
25. Zhao, G., Monier-Faugere, M. C., Langub, M. C., Geng, Z., Nakayama, T., Pike, J. W., Chernausek, S. D., Rosen, C. J., Donahue, L. R., Malluche, H. H., Fagin, J. A., and Clemens, T. L. (2000) *Endocrinology* **141,** 2674–2682
26. Canalis, E., Centrella, M., and McCarthy, T. (1988) *J. Clin. Invest.* **81,** 1572–1577
27. McCarthy, T. L., Centrella, M., and Canalis, E. (1989) *Endocrinology* **124,** 301–309
28. Ducy, P., Zhang, R., Geoffroy, V., Ridall, A. L., and Karsenty, G. (1997) *Cell* **89,** 747–754
29. Nakashima, K., Zhou, X., Kunkel, G., Zhang, Z., Deng, J. M., Behringer, R. R., and de Crombrugghe, B. (2002) *Cell* **108,** 17–29
30. Oldberg, A., Franzen, A., and Heinegard, D. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 8819–8823
31. Poser, J. W., Esch, F. S., Ling, N. C., and Price, P. A. (1980) *J. Biol. Chem.* **255,** 8685–8691
32. Lian, J. B., Hauschka, P. V., and Gallop, P. M. (1978) *Fed. Proc.* **37,** 2615–2620
33. Moffatt, P., Salois, P., Gaumond, M. H., St-Amant, N., Godin, E., and Lanctot, C. (2002) *Nucleic Acids Res.* **30,** 4285–4294
34. Maruyama, K., and Sugano, S. (1994) *Gene* **138,** 171–174
35. Aronow, M. A., Gerstenfeld, L. C., Owen, T. A., Tassinari, M. S., Stein, G. S., and Lian, J. B. (1990) *J. Cell. Physiol.* **143,** 213–221
36. Lanctot, C., Lamolet, B., and Drouin, J. (1997) *Development* **124,** 2807–2817

37. Sambrook, J., Fritsch, E., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., pp. 7.45–7.52, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
38. Church, G. M., and Gilbert, W. (1984) *Proc. Natl. Acad. Sci. U. S. A* **81,** 1991–1995
39. Miao, D., Bai, X., Panda, D., McKee, M., Karaplis, A., and Goltzman, D. (2001) *Endocrinology* **142,** 926–939
40. Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) *Protein Eng.* **10,** 1–6
41. Boardman, P. E., Sanz-Ezquerro, J., Overton, I. M., Burt, D. W., Bosch, E., Fong, W. T., Tickle, C., Brown, W. R., Wilson, S. A., and Hubbard, S. J. (2002) *Curr. Biol.* **12,** 1965–1969
42. Creemers, J. W., Jackson, R. S., and Hutton, J. C. (1998) *Semin. Cell Dev. Biol.* **9,** 3–10
43. Nakayama, K. (1997) *Biochem. J.* **327,** 625–635
44. Seidah, N. G., Benjannet, S., Hamelin, J., Mamarbachi, A. M., Basak, A., Marcinkiewicz, J., Mbikay, M., Chretien, M., and Marcinkiewicz, M. (1999) *Ann. N. Y. Acad. Sci.* **885,** 57–74
45. Hendy, G. N., Bennett, H. P., Gibbs, B. F., Lazure, C., Day, R., and Seidah, N. G. (1995) *J. Biol. Chem.* **270,** 9517–9525
46. Liu, B., Goltzman, D., and Rabbani, S. A. (1995) *Am. J. Physiol.* **268,** E832–838
47. Orloff, J. J., Reddy, D., de Papp, A. E., Yang, K. H., Soifer, N. E., and Stewart, A. F. (1994) *Endocr. Rev.* **15,** 40–60
48. Bradbury, A. F., and Smyth, D. G. (1987) *Biosci. Rep.* **7,** 907–916
49. Harris, R. B. (1989) *Arch Biochem. Biophys.* **275,** 315–333
50. Wang, T. C., Koh, T. J., Varro, A., Cahill, R. J., Dangler, C. A., Fox, J. G., and Dockray, G. J. (1996) *J. Clin. Invest.* **98,** 1918–1929
51. Lee, A. V., Darbre, P., and King, R. J. (1994) *Mol. Cell. Endocrinol.* **99,** 211–220
52. Pfeiffer, S., Lazenby, R., and Chiang, J. (1995) *Am. J. Phys. Anthropol.* **96,** 89–92
53. Mohan, S., Kutilek, S., Zhang, C., Shen, H. G., Kodama, Y., Srivastava, A. K., Wergedal, J. E., Beamer, W. G., and Baylink, D. J. (2000) *Bone* **27,** 471–478
54. Nishida, S., Endo, N., Yamagiwa, H., Tanizawa, T., and Takahashi, H. E. (1999) *J. Bone Miner. Metab.* **17,** 171–177
55. Sutherland, M. S., Rao, L. G., Muzaffar, S. A., Wylie, J. N., Wong, M. M., McBroom, R. J., and Murray, T. M. (1995) *Osteoporos. Int.* **5,** 335–343
56. Liang, C. T., Barnes, J., Seedor, J. G., Quartuccio, H. A., Bolander, M., Jeffrey, J. J., and Rodan, G. A. (1992) *Bone* **13,** 435–441
57. Chan, G. K., and Duque, G. (2002) *Gerontology* **48,** 62–71
58. Frenkel, B., Capparelli, C., Van Auken, M., Baran, D., Bryan, J., Stein, J. L., Stein, G. S., and Lian, J. B. (1997) *Endocrinology* **138,** 2109–2116
59. Meyer, R. A., Jr., Young, C. G., Meyer, M. H., Garges, P. L., and Price, D. K. (2000) *Calcif. Tissue Int.* **66,** 282–287
60. Kobayashi, Y., Goto, S., Tanno, T., Yamazaki, M., and Moriya, H. (1998) *Calcif. Tissue Int.* **62,** 426–436
61. Sodek, K. L., Tupy, J. H., Sodek, J., and Grynpas, M. D. (2000) *Bone* **26,** 189–198
62. Suponitzky, I., and Weinreb, M. (1998) *J. Endocrinol.* **156,** 51–57
63. Baron, R. (1993) in *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism* (Favus, M. J., ed) 2nd Ed., pp. 3–9, Lippincott-Raven, Philadelphia
64. Everts, V., Korper, W., Jansen, D. C., Steinfort, J., Lammerse, I., Heera, S., Docherty, A. J., and Beertsen, W. (1999) *FASEB J.* **13,** 1219–1230
65. Owen, T. A., Aronow, M., Shalhoub, V., Barone, L. M., Wilming, L., Tassinari, M. S., Kennedy, M. B., Pockwinse, S., Lian, J. B., and Stein, G. S. (1990) *J. Cell. Physiol.* **143,** 420–430
66. Oberbauer, A. M., Cruickshank, J., Thomas, A., Stumbaugh, A., Evans, K. D., Murray, J. D., and Egan, A. R. (2001) *Growth Dev. Aging* **65,** 3–13
67. Morrison, N. A., Shine, J., Fragonas, J. C., Verkest, V., McMenemy, M. L., and Eisman, J. A. (1989) *Science* **246,** 1158–1161
68. Noda, M., Vogel, R. L., Craig, A. M., Prahl, J., DeLuca, H. F., and Denhardt, D. T. (1990) *Proc. Natl. Acad. Sci. U. S. A* **87,** 9995–9999
69. Kim, R. H., Li, J. J., Ogata, Y., Yamauchi, M., Freedman, L. P., and Sodek, J. (1996) *Biochem. J.* **318,** 219–226
70. Drissi, H., Pouliot, A., Koolloos, C., Stein, J. L., Lian, J. B., Stein, G. S., and van Wijnen, A. J. (2002) *Exp. Cell Res.* **274,** 323–333
71. Haussler, M. R., Whitfield, G. K., Haussler, C. A., Hsieh, J. C., Thompson, P. D., Selznick, S. H., Dominguez, C. E., and Jurutka, P. W. (1998) *J. Bone Miner. Res.* **13,** 325–349
72. Jones, G., Strugnell, S. A., and DeLuca, H. F. (1998) *Physiol. Rev.* **78,** 1193–1231
73. Meleti, Z., Shapiro, I. M., and Adams, C. S. (2000) *Bone* **27,** 359–366
74. Adams, C. S., Mansfield, K., Perlot, R. L., and Shapiro, I. M. (2001) *J. Biol. Chem.* **276,** 20316–20322
75. Lynch, M. P., Capparelli, C., Stein, J. L., Stein, G. S., and Lian, J. B. (1998) *J. Cell. Biochem.* **68,** 31–49
76. Hauschka, P. V., Mavrakos, A. E., Iafrati, M. D., Doleman, S. E., and Klagsbrun, M. (1986) *J. Biol. Chem.* **261,** 12665–12674

Osteocrin, a Novel Bone-specific Secreted Protein That Modulates the Osteoblast Phenotype

Gethin Thomas, Pierre Moffatt, Patrick Salois, Marie-Hélène Gaumond, Rock Gingras, Éric Godin, Dengshun Miao, David Goltzman and Christian Lanctôt

J. Biol. Chem. 2003, 278:50563-50571.
doi: 10.1074/jbc.M307310200 originally published online October 1, 2003
