JOURNAL OF BONE AND MINERAL RESEARCH
Volume 17, Number 12, 2002

The Zinc Finger Transcription Factor Zfp60 Is a Negative Regulator of Cartilage Differentiation

BERNHARD GANSS and HIROAKI KOBAYASHI

ABSTRACT

The differentiation of many mesenchyme-derived cells, including cells that form bone and cartilage, is regulated at the level of gene transcription, but many of the factors involved in this regulation remain to be identified. In this study, a modified RNA fingerprinting technique was used to identify the KRAB domain zinc finger transcription factor Zfp60 as a candidate regulator of cell differentiation in mouse calvaria primary cultures. The highest expression of Zfp60 mRNA in vivo was found between embryonic day 11 (E11) and E15 during mouse embryonic development, coinciding with stages of active organ formation. The expression of Zfp60 mRNA and protein was analyzed further in mouse embryos during skeletal development. The most prominent expression was found in prehypertrophic chondrocytes, where it coincides with the expression of key regulators of chondrocyte maturation, Indian hedgehog (Ihh), and the parathyroid hormone–related peptide (PTHrP) receptor. Zfp60 mRNA was also found transiently expressed during chondrogenesis of C1 cells in vitro, preceding collagen type X expression and cellular hypertrophy. Overexpression of Zfp60 inhibited cartilage differentiation in the chondrogenic ATDC5 cell line. These results suggest a role for Zfp60 as a negative regulator of gene transcription, specifically during the development and/or differentiation of chondrocytes. (J Bone Miner Res 2002;17:2151–2160)

Key words: differential display, zinc finger, transcription factor, gene expression, cartilage differentiation

INTRODUCTION

MOST CELL TYPES that form the components of the motile apparatus such as bone, cartilage, and skeletal muscle cells originate, along with fibroblasts and adipocytes, from common mesenchymal precursor cells during embryonic development. The bone-forming cells (osteoblasts) in mammals are derived from two embryonic lineages. The craniofacial skeleton, in particular derivatives of the branchial arch, originates from cells of the cranial neural crest, and the axial and appendicular skeleton are derived from sclerotome mesenchyme and lateral plate mesoderm, respectively.[1] In addition to this ontogenetic difference, skeletal elements are formed by two mechanisms based on the appearance of an intermediary cartilage model (endochondral ossification) or lack thereof (intramembraneous ossification). Neural crest-derived skeletal elements such as calvaria, craniofacial bones, and parts of the clavicle generally are formed by intramembraneous ossification, and mesoderm-derived skeletal elements undergo mostly endochondral ossification.[2]

The cartilage anlage that precedes long-bone formation develops as a result of cell proliferation and differentiation under the control of transcriptional regulators such as Sox genes.[3] The differentiation of chondrocytes within the growth plate of long bones is regulated by complex regulatory mechanisms involving bone morphogenetic proteins (BMPs), parathyroid hormone–related peptide (PTHrP), Indian hedgehog (Ihh), and FGF signaling.[4,5] Although Sox genes have also been identified as transcriptional regulators in growth plate chondrocytes,[6] the precise transcriptional

The authors have no conflict of interest.

CIHR Group in Matrix Dynamics, Faculty of Dentistry, University of Toronto, Toronto, Ontario, Canada.

mechanisms that control cartilage hypertrophy remain unknown.

The differentiation of osteoblasts from mesenchymal stem cells is a multistage process.[7] The molecular mechanisms that govern this progressive development of the osteoblast phenotype are largely mediated by the action of transcriptional regulators such as the runt-domain transcription factor Cbfa1/Runx-2.[8–10] Cbfa1 is essential for osteoblast differentiation[11] and chondrocyte maturation,[12,13] suggesting that Cbfa1 is expressed in bipotential progenitor cells. Cbfa1 is necessary, but not sufficient for bone formation in some experimental systems.[14] Furthermore, functional differences between various isoforms of Cbfa1[15] and the discovery of coregulators for Cbfa1[16–18] imply that a complex genetic network for the precise regulation of osteoblast differentiation beyond the activity of Cbfa1 must exist. A transcription factor osterix (Osx) that acts downstream of Cbfa1 recently has been identified as a specific regulator of osteoblast differentiation without obviously affecting chondrocyte differentiation.[19] However, little is known about similar regulators of gene transcription that act downstream of Cbfa1 in chondrocytes.

Zinc finger transcription factors of the C2H2 (Krüppel) type constitute the largest family of transcription factors and regulate many fundamental cellular processes including cell differentiation, proliferation, organ development, and tumorigenesis.[20] Although it has been estimated that several hundred members of this gene family exist in the mammalian genome,[21,22] little is known about their specific biological functions and, surprisingly, few have been implicated in bone cell differentiation (Krox-20,[23] TGF-β inducible early gene [TIEG],[24–26] nuclear matrix protein/Cas-interacting zinc finger protein [NP/NMP4/CIZ],[27] AJ18,[28] and Osx[19]). Therefore, the aim of this study was to identify additional C2H2 zinc finger transcription factors as potential regulators of skeletal development.

A modified version of the differential display of mRNA by polymerase chain reaction (DD-PCR)/RNA fingerprinting procedure was developed and used to isolate five zinc finger gene fragments that are differentially expressed at two distinct stages in primary cultures of mouse calvaria cells, an established model system for bone cell differentiation. One of these fragments (Zif-27) was identified as part of the transcription factor Zfp60, which has been reported previously to be transiently expressed during muscle differentiation in vitro.[29] Here, the expression profile of Zfp60 mRNA and protein was analyzed with a particular emphasis on osteogenesis in vivo and during chondrogenesis in vitro. In addition, the effect of Zfp60 on cartilage differentiation in vitro was determined.

MATERIALS AND METHODS

DD-PCR/RNA fingerprinting

Mouse calvaria osteoblast cultures were established as described[30] and seeded at a density of 4×10^5 cells/dish in 100-mm cell culture dishes (Becton-Dickinson, Franklin Lakes, NJ, USA). Total RNA was isolated from cells[31] after 4 days (late proliferative stage), and after 14 days (differentiation stage) in culture. cDNA was synthesized from 3 μg of total RNA each using oligo$(dT)_{11}$NN primer (modification 1) or the degenerate primer ZiF-5′.1 (5′-ADGGCTTCTCNCCDGT-3′; modification 2) with Superscript II reverse transcriptase (RT; Gibco BRL, Burlington, Ontario) according to the manufacturer's instructions. ZiF-5′.1 is directed against the conserved "linker" region in C2H2-type zinc finger transcription factors. PCR amplification was performed with AmpliTaq DNA polymerase (Perkin-Elmer, Mississauga, Ontario) using primers DD-2 (5′-TCGTGGCCCT-3′) and ZiF-5′.3 (5′-TCNCCDGTGTG-3′) under "rapid PCR" conditions.[32] PCR products were labeled by the addition of 2 μl of ^{35}S-deoxyadenosine triphosphate (dATP; 10 μCi/μl; Amersham, Oakville, Ontario, Canada) and separated by electrophoresis on a 6% polyacrylamide gel. Gels were dried and exposed to Kodak XAR-5 film overnight. Bands of interest were excised and DNA was eluted, reamplified, and cloned into the plasmid pCR Script (Stratagene, PDI Bioscience, Aurora, Ontario, Canada) according to the manufacturer's instructions. Three independent clones from each isolated band were sequenced (Sequenase kit; Amersham-Pharmacia Biotech, Baie d'Urfé, Quebec, Canada).

Northern blots

Total RNA was isolated from multiple tissues of 3- to 4-week-old mice (CD-1 strain; Charles River, St.-Constant, Quebec, Canada) by a one-step procedure[31] and enriched for polyA$^+$ RNA using the Oligotex mRNA kit (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer's instructions. The C1 cell line (a generous gift from Dr. Anne Poliard, Institut Pasteur, Paris, France) was grown in aggregates as described.[33] Total RNA was isolated at various time points, separated on a 1% formaldehyde gel, blotted onto a Hybond-N$^+$ nylon membrane (Amersham-Pharmacia Biotech), and cross-linked on a UV transilluminator. This blot was hybridized with a double-labeled (^{32}P-dATP and ^{32}P-deoxycytidine triphosphate [dCTP]) Zfp60 cDNA probe derived from the 3′-untranslated region (UTR) in ExpressHyb hybridization solution (Clontech, Palo Alto, CA, USA) as recommended by the manufacturer. A mouse embryo multiple tissue Northern blot (Clontech) was hybridized with a Zfp60 cDNA probe and with a mouse Runx2/Osf2-specific probe (plasmid pLA-Oa4 containing the first 336 bp of Osf2/Cbfa1 5′-untranslated and coding sequence,[34] provided by Dr. G. Karsenty, Baylor College of Medicine, Houston, TX, USA) as described previously. After washing (0.1× SSC, 0.1% SDS, at 63°C), blots were exposed to Kodak XAR-5 film for the times indicated. Radioactive probes were removed (0.1% SDS, 85°C, 30 min) and blots were rehybridized as described previously with a ^{32}P-dCTP–labeled GAPDH or β-actin cDNA probe to determine relative expression levels.

In situ hybridizations

For Zfp60, a 670-bp DNA fragment (nucleotides 2033–2703; GenBank accession no. U48721[29]) was amplified by PCR and subcloned into the *Sma*I site of pBluescript II (Stratagene). The vector pBluescript II-in situ hybridization (pBS-ISH), generated by replacing the multiple cloning site of pCR Script Amp SK(+) (Stratagene) between *Sac*I and

*Kpn*I with a single SrfI restriction site, was used to clone PCR-amplified DNA fragments of the mouse PTH/PTHrP receptor (nucleotides 558–964; Accession # X78936) and mouse Ihh (nucleotides 1691–1992; accession no. U85610). A mouse bone sialoprotein (BSP) fragment (nucleotides 607-1048; Accession # L20232) was cloned between the SrfI and *Hinc*II restriction sites in pCR Script Amp SK(+) (Stratagene). The vector pRK26,[35] containing a 346-bp cDNA fragment of mouse collagen X (nucleotides 1880–2225; accession no. Z21610), was obtained from Dr. Kathryn Cheah (University of Hong Kong). The orientation of all inserts was confirmed by sequencing. Plasmids (1 μg each), linearized with appropriate restriction enzymes, were used to prepare digoxigenin (DIG)-labeled (DIG RNA labeling kit; Roche Biochemicals, Laval, QC, Canada) or ^{35}S-uridine thiotriphosphate (UTP)–labeled[36] sense and antisense RNA transcripts. Mouse embryos were prepared as described.[37] In situ hybridization on sections (6 μm) of fixed, paraffin-embedded C57BL/6 mouse embryos (embryonic day 16.5 [E16.5]) was performed as described previously.[38] Tissues were counterstained with hematoxylin/eosin after hybridizations with radioactive probes or with eosin alone after hybridization with DIG-labeled probes.

Immunohistochemistry

A C-terminal amide conjugate of the synthetic peptide "EGDANRNITNKKE" to keyhole limpet hemocyanine (KLH; Alberta Peptide Institute, Edmonton, Alberta, Canada) was used to raise a polyclonal antiserum in white New Zealand rabbits according to a standard protocol (Cedarlane Laboratories, Hornby, Ontario, Canada). The antiserum was affinity-purified over a column containing the synthetic peptide coupled to Sepharose 4B (Amersham-Pharmacia Biotech) and concentrated by ultrafiltration (Centricon YM-30; Millipore, Bedford, MA, USA). Immunohistochemical detection of Zfp-60 was performed using the Vectastain elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Briefly, sagittal paraffin sections (12 μm) of E16.5 CD-1 mouse embryos (Charles River) were dewaxed, rehydrated, and incubated with the primary antibody (1:30 dilution) overnight at 4°C. Control experiments were performed without the addition of primary antibody. Tissues were then processed as recommended by the manufacturer (Vector Laboratories).

Cell culture and transfections

C1 cells were cultured as described.[33] Briefly, cells were seeded in untreated plastic dishes (100 mm) at a density of 3×10^5 cells/10 ml of DMEM with 10% FBS (Gibco Life Technologies, Burlington, Ontario, Canada) and grown in suspension for 9 days. The medium was then switched (designated day 0) to DMEM with 1% FBS and 1×10^{-6} M of dexamethasone (Dex) and changed every 3 days thereafter. Cell aggregates were embedded in paraffin and sections (6 μm) were stained with hematoxylin and eosin. RNA was prepared as described[31] and processed for Northern blot analysis.

The chondrogenic cell line ATDC5[39] (cell no. RCB0565; Riken Cell Bank, Tsukuba, Japan) was cultivated as recommended by the supplier. Cells were seeded in triplicate at a density of 4×10^4 cells/well in 48-well culture plates (Falcon; Becton Dickinson) and grown in maintenance medium (a 1:1 mixture of DMEM: Ham's F12 medium with 5% FBS; Gibco Life Technologies) for 48 h, at which point the cells had reached ~90% confluency. The maintenance medium was then replaced by differentiation medium containing insulin (10 μg/ml)/transferrin (10 μg/ml)/Na-selenite (10 ng/ml) supplement (ITS; Roche Biochemicals), without and with the addition of 10^{-8} M of PTHrP(1-34) (a generous gift from A. Karaplis, Montreal, Canada). Two other triplicate sets of cells were transfected with 0.1 μg or 0.3 μg each of a vector construct containing the full-length sequence of Zfp60 (nucleotides 127-2284; accession no. U48721) in pCMV5[40] or empty vector using Lipofectamine Plus reagent (Gibco Life Technologies) according to the manufacturer's instructions. Cells were cultured in differentiation medium for 14 days with a medium change every other day and then stained with Alcian blue for sulfated glycosaminoglycan deposits indicative of mature chondrocyte formation. The dye in the matrices was extracted with 200 μl of 6 M of guanidinium-HCl per well overnight at room temperature and the photometric absorption was determined at 595 nm. Statistical analysis was performed by Student's independent group *t*-test.

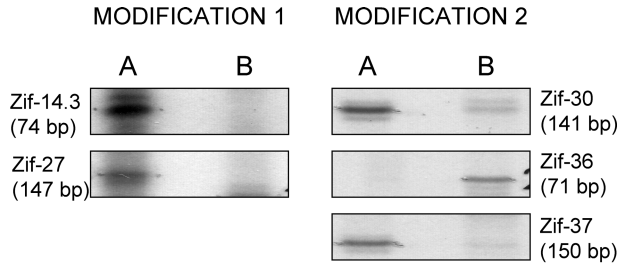

FIG. 1. Differential expression of five cDNA amplicons in cultures of mouse calvaria cells after (A) 4 days and (B) 14 days in culture. Fragments were generated by two modifications of the RNA fingerprinting procedure to target members of the C2H2 zinc finger gene family as described in the Materials and Methods section.

RESULTS

Isolation of differentially expressed C2H2 zinc finger gene fragments from mouse calvaria cells

The primary culture of calvaria-derived cells of various organisms[30] is a well-established model system to investigate molecular and cellular events that occur during osteogenesis in vitro. In this report, a DD-PCR/RNA fingerprinting approach[41] was used to analyze the differential expression of C2H2 zinc finger transcription factors at early and advanced stages of osteogenesis in this system. Several differentially expressed cDNA fragments were identified with two modifications of the RNA fingerprinting protocol (Fig. 1). In modification 1, 2 out of 14 differentially expressed bands resembled fragments of C2H2 zinc finger genes, and 3 out of 7 differentially expressed bands were identified as C2H2 zinc finger gene fragments in modifica-

```
ZiF-14.3        RLCGKKYTRKDQLEYHIRKHTG
ZiF-27(Zfp60)    QCGSAFRLPYQLTQHQRIHYDVKPFQCKECGRAFVRSTGLRIHERIHTG
ZiF-30        QCKQCGKRFTCSSSLRKHERTHTGEKPYECKQCGKAFSCSSSFRKHERTHT
ZiF-36      CYECSECGKTFIKMSNLIRHQRIHTGE
ZiF-37           QCRKSFSKSSSLANHRRTHTGEKPYKCADCGKCFSERSKLITHQRVHTG
consensus KPYXCXXCXKXFXXXXXLXXHXRXHTGEKPYXCXXCXKXFXXXXXLXXHXRXHTG
            F                    K         F                 K
```

FIG. 2. Predicted amino acid sequence comparison of the five C2H2 zinc finger gene fragments isolated from mouse calvaria primary cultures. Positions of conserved cysteine and histidine residues are underlined and residues conforming to the C2H2 zinc finger consensus sequence (bottom line) are shown in bold type.

tion 2. An amino acid sequence alignment showed that all five isolated fragments conform to the C2H2 consensus sequence (Fig. 2). When used as probes in Northern blot analyses on RNA isolated from mouse calvaria primary cells, the five C2H2 zinc finger gene fragments hybridized to transcripts of different sizes, indicating that the fragments are indeed part of five independent transcripts (not shown). A database search (National Centre for Biotechnology Information Basic Local Alignment Search Tool [NCBI BLAST][42]) identified the fragment Zif-27 as part of the zinc finger transcription factor Zfp60.[29] The fragment ZiF-30 was found to be highly similar to part of the hypothetical human protein MGC2663 (Entrez protein database accession # NP_077011; NCBI), another Krüppel-Associated Box (KRAB)-domain zinc finger protein. ZiF-14.3, ZiF-36, and ZiF-37 did not show any homology with known genes beyond a general similarity to the C2H2 zinc finger gene consensus sequence and therefore may be considered as novel gene fragments.

Zfp60 mRNA expression during mouse development

The developmental expression of Zfp60 mRNA in comparison with Cbfa1/Runx-2/Osf2 was assessed by Northern blot on mRNA isolated from whole mouse embryos at E7, E11, E15, and E17 and from multiple tissues of 3- to 4-week-old mice (Fig. 3). Expression levels were low at E7 and increased transiently to maximal levels between E11 and E15, and expression at E17 was significantly reduced. Expression levels of Zfp60 and Cbfa1/Runx-2/Osf2 were found maximal during the onset of mineralization (Fig. 3A). In young adult mice (3–4 weeks of age) Zfp60 expression was detected at relatively low levels in multiple tissues (Fig. 3B). Highest mRNA levels were found in mineralizing tissues (mandible, long bone, and calvaria) and kidney, followed by brain, heart, lung, tongue, and skeletal muscle, and Zfp60 transcripts were almost undetectable in liver. In addition to the previously reported Zfp60 mRNA transcript of 4.2 kb,[29] a second less abundant transcript of 6.8 kb was detected in mouse embryos and most tissues of young adult mice.

Zfp60 mRNA and protein expression during osteogenesis in vivo

The expression of Zfp60 mRNA was analyzed by in situ hybridization on E16.5 mouse embryos to correlate its initial isolation from calvaria with early aspects of osteogenesis. Previous results[38] suggested that the expression Zfp60 in skeletal structures is transient in cells at the initiation stage of cartilage hypertrophy. To determine the localization of Zfp60 mRNA more precisely and at the cellular level, a DIG-labeled Zfp60 RNA antisense probe (670 nt) was used for in situ hybridization experiments (Fig. 4). The expression of Zfp60 mRNA was detected in epithelial cells (Fig. 4a) and in the center of cartilage condensations of developing metatarsal bones, where it is restricted to chondrocytes that have committed to hypertrophy, while proliferating chondrocytes and cells in the perichondrium are clearly negative for Zfp60 expression (Fig. 4d). The scapula at this developmental stage shows overt signs of mineralization and a clearly developed zone of chondrocyte maturation. Proliferating and hypertrophic chondrocytes can be readily distinguished from each other and from mineralized osteoid tissue. Expression of Zfp60 is detected in skeletal muscle tissue surrounding the scapula and in prehypertrophic chondrocytes but not in the perichondrium. A view of transitory chondrocytes in the scapula at higher magnification shows significant levels of Zfp60 expression in cells undergoing the transition from proliferating to hypertrophic cartilage cells (Fig. 4c). A similar expression pattern was also observed in transitory chondrocytes of the rib. Promi-

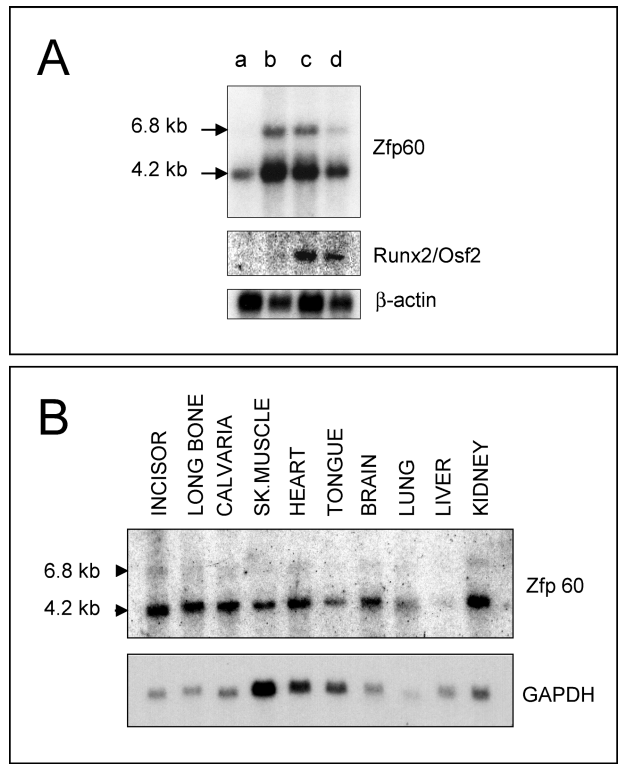

FIG. 3. Northern analysis of Zfp60 mRNA expression. (A) Each lane contains 2 μg of polyA$^+$ RNA from whole mouse embryos at E7 (a), E11 (b), E15 (c), and E17 (d) in panel and (B) ~1 μg of polyA$^+$ RNA isolated from the indicated tissues of 3- to 4-week-old mice in panel. Both blots were hybridized with the same cDNA probe derived from the 3′-UTR of Zfp60 and exposed for (A) 16 h or (B) 5 days. Blots were then rehybridized with a Runx2/Osf2 probe and normalized against (A) β-actin or (B) GAPDH.

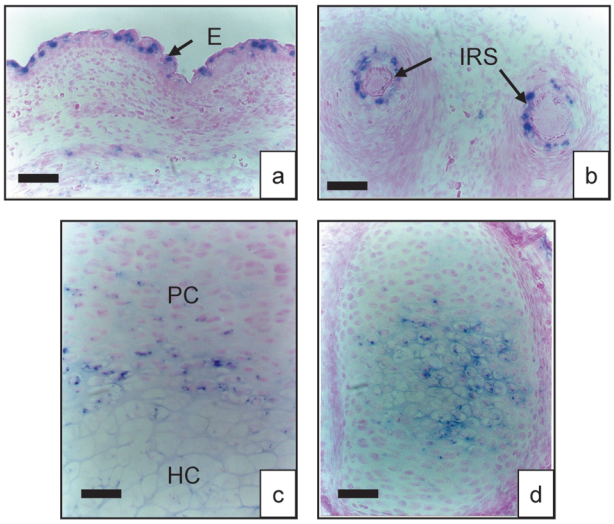

FIG. 4. Zfp60 mRNA expression in various tissues of 16.5 dpc mouse embryos as determined by in situ hybridization with a DIG-labeled cRNA probe. Expression at cellular resolution is observed in (a) skin epithelium, (b) inner root sheath cells of developing whisker follicles, and (c) in transition stage chondrocytes of the scapula and (d) in the center of metacarpal cartilage condensations [(a and b) bar = 100 μm; (c and d) bar = 40 μm]. E, epithelium; HC, hypertrophic cartilage; IRS, inner root sheath; PC, proliferating chondrocytes.

nent signals were also detected in cells of the inner root sheath in developing whisker follicles (Fig. 4b).

A direct comparison of Zfp60 mRNA expression with that of known marker genes of chondro-/osteogenesis in transition stage chondrocytes of the rib revealed that expression of Zfp60 overlaps with the expression of two known regulators of cartilage hypertrophy, Ihh, and the PTH/PTHrP receptor (PTHrP-R) in prehypertrophic chondrocytes, but not, or to a lesser degree, with type X collagen (ColX) as a marker of mature chondrocytes or BSP as a marker of osteoblasts (Fig. 5). The Zfp60 signal in the matrix of the lower hypertrophic area (Fig. 5a) is artifactual, because hybridization with several sense cRNA probes resulted in similar background staining (not shown).

A polyclonal antiserum against a partial sequence of Zfp60 was used to confirm the expression of Zfp60 protein predominantly in epithelial cells, prehypertrophic cartilage cells, and cells that actively participate in morphogenesis at sites of epithelial-mesenchymal interactions such as inner root sheath cells of developing whisker follicles (Fig. 6). A control experiment without the use of the primary Zfp60 antiserum did not reveal positive signals in any tissues at this developmental stage (not shown).

Expression of Zfp60 during chondrogenesis in vitro

C1 cells undergo chondrogenic differentiation when grown in aggregates in the presence of 10^{-6} M of Dex for extended periods of time.[33] The expression of Zfp60 during chondrogenesis in this system was analyzed by Northern blot and compared with the expression of ColX mRNA, a marker of hypertrophic chondrocytes (Fig. 7). Results from this experiment indicate that Zfp60 is expressed at all stages of chondrogenic differentiation in C1 cells. However, its temporal expression is transient and reaches a maximum level at day 10 in culture, coinciding with a prehypertrophic status of the cells. In contrast, ColX mRNA can only be detected between days 25 and 53 with a maximal expression at day 45. Similar to in vivo data the maximal expression of Zfp60 thus precedes the onset of chondrocyte hypertrophy and the concomitant increase in ColX mRNA expression in mature chondrocytes in this system.

Effects of Zfp60 on chondrocyte differentiation in vitro

The expression data in vivo and in vitro suggest a role for Zfp60 in chondrocyte maturation and/or differentiation. Thus, the effect of Zfp60 on chondrogenesis in vitro was investigated by transient transfection assays. The teratocarcinoma-derived cell line ATDC5 was grown for 14 days under conditions that stimulate cartilage differentiation.[39] In medium containing insulin, transferrin, and sodium selenite the cells differentiated into Alcian blue–positive cartilage nodules (Fig. 8A). PTHrP(1-34), a critical signaling molecule in cartilage differentiation,[43] significantly reduced chondrogenic differentiation when added during the 14-day culture period at a concentration of 1×10^{-8} M, indicating that the differentiation program of these cells is regulated in a manner similar to the situation in vivo (Fig. 8A). Transient transfection of ATDC5 cells with a pCMV5-Zfp60 expression vector at two concentrations in triplicate experiments at the beginning of the 14-day culture period inhibited chondrocyte differentiation to an extent comparable with PTHrP treatment, whereas transfection with an empty vector had no obvious effect on the formation of cartilage nodules after 14 days (Fig. 8A). Photometric quantitation (Fig. 8B) of Alcian blue staining intensity revealed a significant reduction ($p < 0.01$) of cartilage matrix formation only in cells in which chondrocyte differentiation was inhibited by continuous administration of PTHrP or transient overexpression of Zfp60.

DISCUSSION

The proper development of the skeleton is coordinated by a complex molecular network of signaling molecules, transcription factors, and extracellular matrix components. However, many regulators of gene transcription involved in bone cell differentiation remain to be identified. The primary culture of calvaria-derived cells is a well-established experimental system to study the molecular regulation of bone formation in vitro. However, these cells are multipotential and can differentiate into several mesenchyme-derived cell phenotypes such as muscle, fat, and cartilage in vitro.[44] In fact, a chondrogenic cell line (CFK2) has been derived from rat calvaria primary cultures.[45] Two modifications of the differential display technique,[46] designed to amplify fragments of C2H2 zinc finger transcription factors that are differentially expressed between proliferation and differentiation stages, were applied successfully in mouse calvaria primary cultures. Modification 1 produced 14 differentially expressed bands that were selected for further

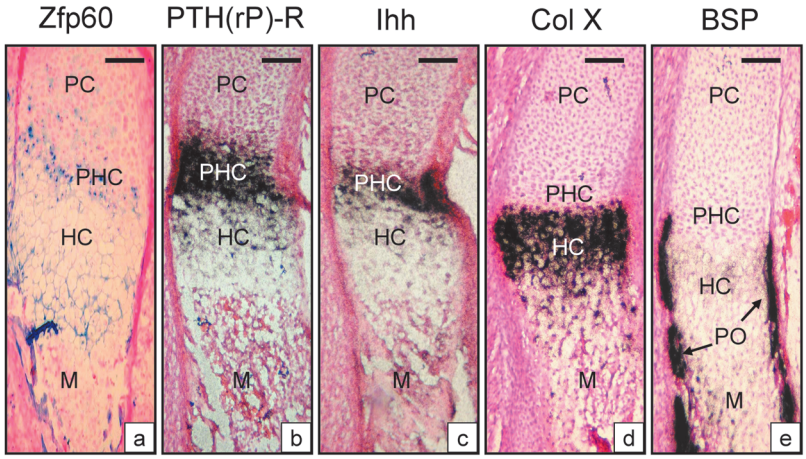

FIG. 5. Expression analysis of Zfp60 mRNA by in situ hybridization in comparison with other markers of chondrogenesis/osteogenesis in areas of cartilage differentiation of developing ribs. Tissue sections of mouse embryos at E16.5 were hybridized to (a) DIG-UTP and (b–e) ^{35}S-UTP-labeled antisense cRNA probes for (a) Zfp60, (b) the PTH/PTHrP receptor, (c) Ihh, (d) ColX), and (e) BSP. PC, proliferating chondrocytes; PHC, prehypertrophic chondrocytes; HC, hypertrophic chondrocytes; PO, periosteum; M, mineralized bone (bar = 100 μm).

analysis. Twelve of these bands (86%) were ultimately recognized as PCR artifacts and only two contained conserved sequences of C2H2 zinc finger genes. In modification 2, four out of seven differentially displayed bands (57%) were artifacts and the remaining three bands contained zinc finger gene fragments. Notably, although three independent clones were sequenced from each amplified band, the three sequences were not identical in all cases. The differential expression of the zinc finger gene fragments suggests that they may be involved in bone cell differentiation. All gene fragments were subjected to a database search (BLAST, NCBI).

ZiF-27 was subsequently identified as a fragment of the transcription factor Zfp60, a KRAB-domain zinc finger protein that has been implicated previously in myogenic cell differentiation.[29] The C2H2 zinc finger domain in this class of proteins generally exhibits sequence-specific DNA binding, while KRAB boxes are potent transcriptional repression domains[47] that interfere with gene transcription by binding to the heterochromatin protein 1 (HP-1) complex[48] through the corepressor protein KAP-1.[49] The differential expression in bone-forming cells and the presence of 19 consecutive C2H2 zinc finger motifs and an N-terminal KRAB domain in Zfp60 suggest that this factor acts as a transcriptional repressor during osteogenesis.

In this study, the mRNA expression of Zfp60 during mouse embryonic development was analyzed by Northern blot and found to be maximal between E11 and E15, a period of active organogenesis by epithelial-mesenchymal interactions. In addition to the previously reported major transcript of 4.2 kb,[29] a second minor transcript of 6.8 kb was detected that paralleled the expression of the 4.2-kb transcript. Because a probe derived from the 3′-UTR of Zfp60 was used in these blots, it is unlikely that the 6.8-kb signal represents an unrelated transcript. It is presently unclear whether these two Zfp60 transcripts result from alternative splicing or the use of alternative polyadenylation or transcription initiation sites. Multiple tissue Northern blots performed on various adult mouse tissues failed to detect any significant tissue-specific differences in expression levels between the two transcripts and it is not clear if those transcripts fulfill different biological functions. Earlier studies[50,29] have suggested a role for Zfp60 as a transcriptional regulator during myogenesis. However, although the first muscle-specific mRNAs (β-cardiac and embryonic skeletal myosin heavy chain [MHC] transcripts) are not expressed until E9–E10 in the developing mouse,[51] Zfp60 transcripts could be detected as early as E7 and throughout embryonic development in multiple tissues, therefore suggesting a more general function of Zfp60 in organogenesis

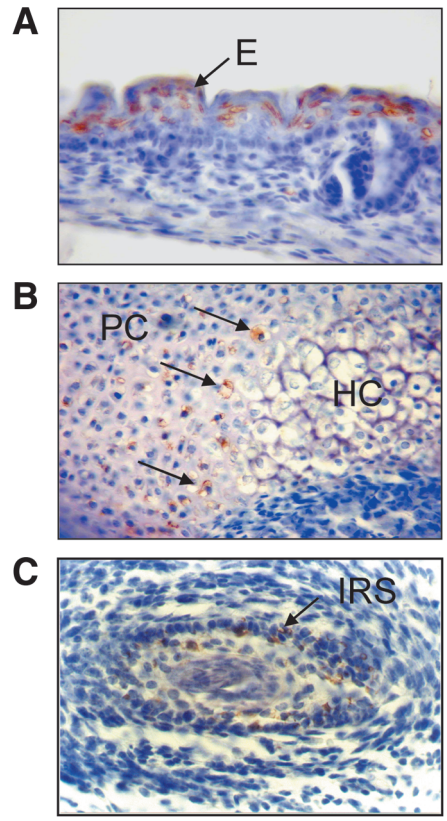

FIG. 6. Localization of Zfp60 protein expression in 16.5 dpc mouse embryos by immunostaining with a polyclonal antiserum raised against a Zfp60-specific peptide. The most intense signals (brown color) were found in skin (A) epithelium, (B) prehypertrophic chondrocytes, and (C) the inner root sheath of whisker follicles. E, epithelium; HC, hypertrophic cartilage, IRS, inner root sheath; PC, proliferating chondrocytes.

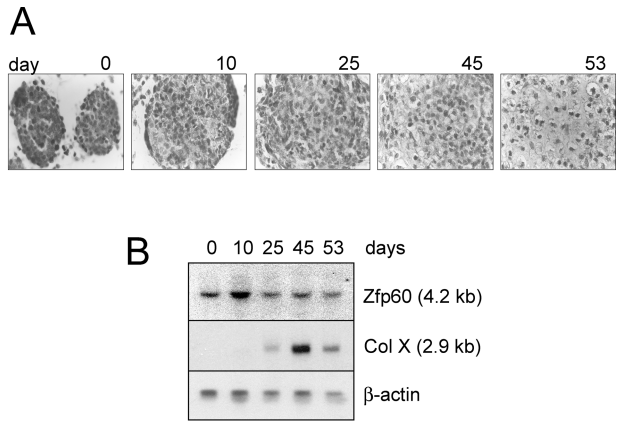

FIG. 7. Morphological changes and expression of Zfp60 mRNA during chondrogenic differentiation of C1 cells. (A) Sections of C1 cell aggregates were stained with hematoxylin and eosin after the indicated times to reveal cell morphology. Cells in the center of aggregates show the first morphological signs of hypertrophy after 25 days in culture. (B) Northern analysis of Zfp60 and ColX mRNA expression was performed in duplicate experiments. Twenty micrograms of total RNA per lane, isolated from C1 cell aggregates after the indicated times in culture, were hybridized sequentially with ^{32}P-labeled cDNA probes for Zfp60, ColX, and β-actin. The transcript sizes of the hybridization signals for Zfp60 and ColX are indicated.

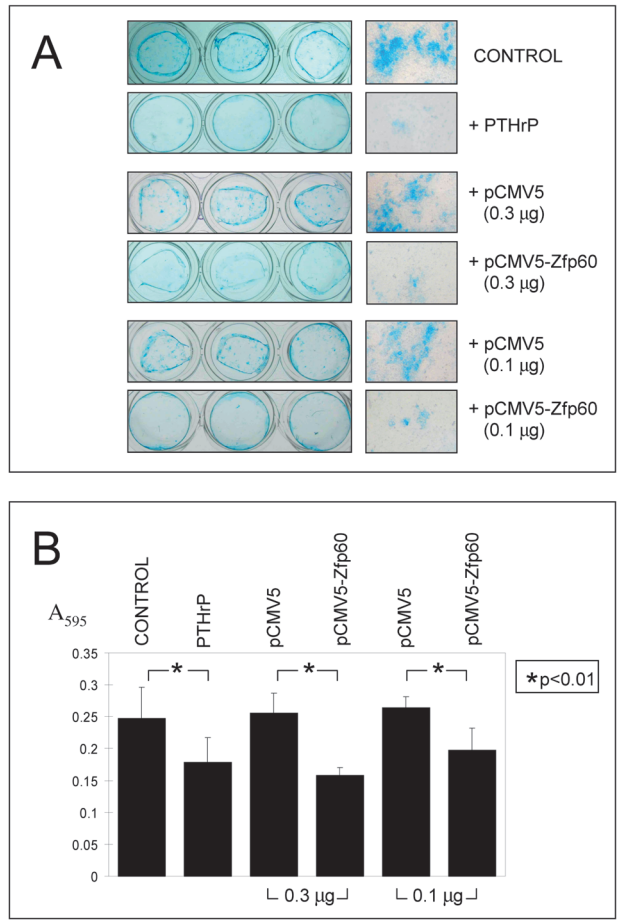

FIG. 8. Effect of PTHrP treatment and transient Zfp60 overexpression on chondrogenic differentiation of ATDC5 cells. (A) After an initial growth period of 48 h in 48-well plates in maintenance medium, cells were switched to differentiation medium without further additives (control) or with 10^{-8} M of PTHrP(1-34). Additional triplicate cells were transfected with 0.3 μg or 0.1 μg of the empty expression vector pCMV5 or a pCMV5-Zfp60 expression vector. Cells were maintained in differentiation medium for 14 days and then stained with Alcian blue (left column). Representative views of Alcian blue–stained cartilage nodules at higher magnification are shown (right column). (B) Alcian blue staining of the wells shown in panel A was quantified by dye elution in 6 M of guanidinium hydrochloride and absorption measurement at 595 nm (A_{595}). The inhibition of cartilage differentiation by PTHrP treatment and transient Zfp60 overexpression is statistically significant ($p < 0.01$).

rather than a specific role in the regulation of muscle differentiation. The mRNA expression levels of Zfp60 decline after the completion of organ formation, and in 3- to 4-week-old mice Zfp60 expression is maintained at low levels in multiple tissues, including bones, teeth, brain, kidney, and lung.

To further investigate the role of Zfp60 in osteogenesis, we analyzed the expression of Zfp60 mRNA on sections from E16.5 mouse embryos. The following processes are observed simultaneously at this developmental stage[52]: (a) the formation of a chondrocyte model for future long bones by cartilage condensation; (b) the formation of bone structures by intramembraneous ossification, for example, calvaria; and (c) the differentiation and calcification of chondrocytes in epiphyseal areas of predominant bones formed by endochondral ossification (e.g., humerus, femur, and scapula). Previous results revealed the expression of Zfp60 mRNA mainly in epithelial cells, skeletal muscle, and chondrogenic cells in a transient fashion.[38] However, Zfp60 expression was undetectable in neural tissue, cardiac or smooth muscle, lung, liver, kidney, testis, or tissues of endodermal origin. Hybridization with a DIG-labeled RNA probe (Fig. 5) allowed Zfp60 transcripts to be detected at a higher resolution, albeit at the expense of sensitivity. In skeletal tissues, Zfp60 expression was found in prehypertrophic cartilage cells of the basisphenoid and metatarsal/metacarpal bones, scapula, and rib, but not in proliferating or hypertrophic chondrocytes. This transitory expression pattern, which is reminiscent of Ihh and BMP-6 expression in the developing scapula,[53] suggests that Zfp60 expression in skeletal structures is confined to cells at the initiation stage of cartilage hypertrophy. The heterogeneity of calvaria-derived cultures may explain why Zfp60, although initially isolated from calvaria cells, is predominantly found in prehypertrophic areas of cartilage in vivo.

The control of cartilage hypertrophy, a crucial step in the development and growth of endochondral bones, is coordinated by the controlled proliferation and differentiation of chondrocytes and osteoblasts. The differentiation of growth plate chondrocytes is controlled by a complex molecular network that includes a negative feedback loop between the signaling molecules Ihh and PTHrP.[54–56] Ihh is expressed in cartilage cells that have committed to hypertrophy. It stimulates the expression of PTHrP in the perichondrium, which delays differentiation of growth plate chondrocytes by maintaining them in a proliferative state. The coexpres-

sion of Zfp60 with Ihh and the PTHrP-R in growth plate chondrocytes suggests that Zfp60 may be involved in the regulation of cartilage differentiation via the PTHrP/Ihh regulatory loop, possibly by affecting the transcriptional rate of PTHrP and/or Ihh target genes. In addition, Zfp60 is expressed in epithelial cells and at sites of early epithelial-mesenchymal interactions, as is PTHrP. Prominent Zfp60 signals were detected with a DIG-labeled probe in cells of the inner root sheath in developing whisker follicles (Fig. 4b). This result, in conjunction with earlier findings that the overexpression of PTHrP in the skin of mice interferes with hair follicle development,[57] further suggests a functional relationship between PTHrP and Zfp60. The regulatory network of cartilage differentiation and hypertrophy includes numerous other molecular signaling cascades such as the FGF/FGF receptor system (reviewed by Xu et al.[58]), BMP signaling pathways,[59] and complex interactions between them (reviewed by Poliard et al.[60]). Although we report here that the expression pattern of Zfp60 overlaps with the PTH/PTHrP-R during embryonic development, Zfp60 may be involved as a transcription factor in multiple signaling pathways to regulate chondrocyte hypertrophy.

The expression of Zfp60 was consequently investigated in several chondrogenic systems in vitro that had been established from rats (CFK-2[45]; C5.18[44]), and mice (ATDC5[39]; C1[33]). C1 cells undergo a chondrogenic differentiation program and sequentially express various types of connective tissue collagens, similar to the events observed in vivo.[61] Northern blot analyses confirmed that Zfp60 expression precedes the expression of ColX in chondrogenic C1 cells, which suggests that Zfp60 largely regulates the expression of target genes before chondrocytes become hypertrophic and undergo terminal differentiation. mRNA transcripts for Zfp60 could not be detected by Northern blot on 20 μg of total RNA in CFK-2, C5.18, or ATDC5 cells, presumably because Zfp60 expression levels are below the detection limit in these cells. Zfp60 transcripts could be detected by RT-PCR in ATDC5 cells (not shown) but not in C1 or C5.18 cells, potentially because of primer sequence mismatches between species.

Finally, experiments were performed to investigate the effect of Zfp60 overexpression on cartilage differentiation in vitro. The expression profiles and activities of known regulators of cartilage differentiation in the chondrogenic ATDC5 cell line[39] are similar to those observed during cartilage differentiation in vivo.[62,63] Therefore, ATDC5 cells are an appropriate model for the analysis of early and late-phase differentiation events during chondrogenesis.[64] Because ATDC5 cells, unlike C1 cells, begin to form cartilage nodules as signs of cellular hypertrophy within 14 days in monolayer culture, these cells were used to overexpress Zfp60 under the control of the cytomegalovirus (CMV) promoter during the early stage of chondrogenic differentiation. The early overexpression of Zfp60 resulted in a significantly impaired rate of cellular hypertrophy and cartilage matrix production. This effect was observed in transient transfection experiments, where it seems unlikely that the majority of cells maintain the expression of Zfp60 over the entire culture period of 14 days. We therefore hypothesize that Zfp60 stimulates the expression of a secreted inhibitor of differentiation at the prehypertrophic stage, possibly by suppressing the transcription of another factor that has an antagonistic effect on this hypothetical secreted factor. To test this hypothesis, an inducible expression system for Zfp60 in stably transfected ATDC5 cells currently is being developed to allow the identification of such (secreted) target genes.

In summary, this study has identified Zfp60, a member of the KRAB-C2H2 zinc finger gene family of transcription factors, as a negative regulator of cartilage differentiation in vitro. In vivo, Zfp60 is coexpressed with the regulatory molecules Ihh and the PTHrP-R in growth plate chondrocytes. Further studies, including the generation of Zfp60-deficient mice and the identification of upstream regulators and downstream targets for this potential transcriptional repressor, will help to determine the precise role of Zfp60 in the regulation of cartilage hypertrophy and skeletal growth.

ACKNOWLEDGMENTS

We thank Mairi MacGillivray and Sela Cheifetz for helpful comments on this study and Jaro Sodek for his support.

REFERENCES

1. Karsenty G 1998 Genetics of skeletogenesis. Dev Genet **22:** 301–313.
2. Hall BK, Miyake T 2000 All for one and one for all: Condensations and the initiation of skeletal development. Bioessays **22:**138–147.
3. Lefebvre V, Li P, de Crombrugghe B 1998 A new long form of Sox5 (L-Sox5), Sox6, and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. EMBO J **17:**5718–5733.
4. Minina E, Wenzel HM, Kreschel C, Karp S, Gaffield W, McMahon AP, Vortkamp A 2001 BMP and Ihh/PTHrP signaling interact to coordinate chondrocyte proliferation and differentiation. Development **128:**4523–4534.
5. Wang Q, Green RP, Zhao G, Ornitz DM 2001 Differential regulation of endochondral bone growth and joint development by FGFR1 and FGFR3 tyrosine kinase domains. Development **128:**3867–3876.
6. de Crombrugghe B, Lefebvre V, Behringer RR, Bi W, Murakami S, Huang W 2000 Transcriptional mechanisms of chondrocyte differentiation. Matrix Biol **19:**389–394.
7. Aubin J, Liu F 1996 The osteoblast lineage. In: Bilezikian J, Raisz L, Rodan G (eds.) Principles of Bone Biology, Academic Press, San Diego, CA, USA, pp. 51–67.
8. Franceschi RT 1999 The developmental control of osteoblast-specific gene expression: Role of specific transcription factors and the extracellular matrix environment. Crit Rev Oral Biol Med **10:**40–57.
9. Karsenty G 1998 Transcriptional regulation of osteoblast differentiation during development. Front Biosci **3:**D834–D837.
10. Yamaguchi A, Komori T, Suda T 2000 Regulation of osteoblast differentiation mediated by bone morphogenetic proteins, hedgehogs, and Cbfa1. Endocr Rev **21:**393–411.
11. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T 1997 Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell **89:**755–764.
12. Inada M, Yasui T, Nomura S, Miyake S, Deguchi K, Himeno M, Sato M, Yamagiwa H, Kimura T, Yasui N, Ochi T, Endo N, Kitamura Y, Kishimoto T, Komori T 1999 Maturational

disturbance of chondrocytes in Cbfa1-deficient mice. Dev Dyn **214:**279–290.
13. Enomoto H, Enomoto-Iwamoto M, Iwamoto M, Nomura S, Himeno M, Kitamura Y, Kishimoto T, Komori T 2000 Cbfa1 is a positive regulatory factor in chondrocyte maturation. J Biol Chem **275:**8695–8702.
14. Lee MH, Javed A, Kim HJ, Shin HI, Gutierrez S, Choi JY, Rosen V, Stein JL, van Wijnen AJ, Stein GS, Lian JB, Ryoo HM 1999 Transient upregulation of CBFA1 in response to bone morphogenetic protein-2 and transforming growth factor beta1 in C2C12 myogenic cells coincides with suppression of the myogenic phenotype but is not sufficient for osteoblast differentiation. J Cell Biochem **73:**114–125.
15. Harada H, Tagashira S, Fujiwara M, Ogawa S, Katsumata T, Yamaguchi A, Komori T, Nakatsuka M 1999 Cbfa1 isoforms exert functional differences in osteoblast differentiation. J Biol Chem **274:**6972–6978.
16. Hess J, Porte D, Munz C, Angel P 2001 AP-1 and Cbfa/runt physically interact and regulate parathyroid hormone-dependent MMP13 expression in osteoblasts through a new osteoblast-specific element 2/AP-1 composite element. J Biol Chem **276:** 20029–20038.
17. Kim WY, Sieweke M, Ogawa E, Wee HJ, Englmeier U, Graf T, Ito Y 1999 Mutual activation of Ets-1 and AML1 DNA binding by direct interaction of their autoinhibitory domains. EMBO J **18:**1609–1620.
18. McCarthy TL, Ji C, Chen Y, Kim KK, Imagawa M, Ito Y, Centrella M 2000 Runt domain factor (Runx)-dependent effects on CCAAT/enhancer-binding protein delta expression and activity in osteoblasts. J Biol Chem **275:**21746–21753.
19. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B 2002 The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell **108:**17–29.
20. Dang DT, Pevsner J, Yang VW 2000 The biology of the mammalian Kruppel-like family of transcription factors. Int J Biochem Cell Biol **32:**1103–1121.
21. Bellefroid EJ, Lecocq PJ, Benhida A, Poncelet DA, Belayew A, Martial JA 1989 The human genome contains hundreds of genes coding for finger proteins of the Kruppel type. DNA **8:**377–387.
22. Tupler R, Perini G, Green MR 2001 Expressing the human genome. Nature **409:**832–833.
23. Levi G, Topilko P, Schneider-Maunoury S, Lasagna M, Mantero S, Pesce B, Ghersi G, Cancedda R, Charnay P 1996 Role of Krox-20 in endochondral bone formation. Ann NY Acad Sci **785:**288–291.
24. Subramaniam M, Harris SA, Oursler MJ, Rasmussen K, Riggs BL, Spelsberg TC 1995 Identification of a novel TGF-beta-regulated gene encoding a putative zinc finger protein in human osteoblasts. Nucleic Acids Res **23:**4907–4912.
25. Hefferan TE, Subramaniam M, Khosla S, Riggs BL, Spelsberg TC 2000 Cytokine-specific induction of the TGF-beta inducible early gene (TIEG): Regulation by specific members of the TGF-beta family. J Cell Biochem **78:**380–390.
26. Hefferan TE, Reinholz GG, Rickard DJ, Johnsen SA, Waters KM, Subramaniam M, Spelsberg TC 2000 Overexpression of a nuclear protein. TIEG, mimics transforming growth factor-beta action in human osteoblast cells. J Biol Chem **275:**20255–20259.
27. Thunyakitpisal P, Alvarez M, Tokunaga K, Onyia JE, Hock J, Ohashi N, Feister H, Rhodes SJ, Bidwell JP 2001 Cloning and functional analysis of a family of nuclear matrix transcription factors (NP/NMP4) that regulate type I collagen expression in osteoblasts. J Bone Miner Res **16:**10–23.
28. Jheon AH, Ganss B, Cheifetz S, Sodek J 2001 Characterization of a novel KRAB/C2H2 zinc finger transcription factor involved in bone development. J Biol Chem **276:**18282–18289.
29. Perez M, Rompato G, Corbi N, De Gregorio L, Dragani TA, Passananti C 1996 Zfp60, a mouse zinc finger gene expressed transiently during in vitro muscle differentiation. FEBS Lett **387:**117–121.
30. Ecarot-Charrier B, Glorieux FH, van der Rest M, Pereira G 1983 Osteoblasts isolated from mouse calvaria initiate matrix mineralization in culture. J Cell Biol **96:**639–643.
31. Chomczynski P, Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem **162:**156–159.
32. Liu LZ, Shearn A 1995 Rapid PCR for RNA differential display in a conventional heat block thermal cycler. Biotechniques **19:**44–46.
33. Poliard A, Nifuji A, Lamblin D, Plee E, Forest C, Kellermann O 1995 Controlled conversion of an immortalized mesodermal progenitor cell towards osteogenic, chondrogenic, or adipogenic pathways. J Cell Biol **130:**1461–1472.
34. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G 1997 Osf2/Cbfa1: A transcriptional activator of osteoblast differentiation. Cell **89:**747–754.
35. Kong RY, Kwan KM, Lau ET, Thomas JT, Boot-Handford RP, Grant ME, Cheah KS 1993 Intron-exon structure, alternative use of promoter and expression of the mouse collagen X gene, Col10a-1. Eur J Biochem **213:**99–111.
36. Zeller R, Rogers M 1994 In situ hybridization to cellular RNA. In: Ausubel F, Brent R, Kingston R, Moore D, Seidman J, Smith J, Struhl K (eds.) Current Protocols in Molecular Biology. John Wiley & Sons, New York, NY, USA, pp. 14.3.1–14.3.16.
37. Zeller R 1998 Fixation, embedding, and sectioning of tissues, embryos, and single cells. In: Ausubel F, Brent R, Kingston R, Moore D, Seidman J, Smith J, Struhl K (eds.) Current Protocols in Molecular Biology. John Wiley & Sons, New York, NY, USA, pp. 14.1.1–14.1.7.
38. Ganss B, Cheifetz S 2001 Zif27/Zfp60: A member of the zinc finger gene family expressed at sites of cartilage formation in the mouse. In: Goldberg M, Boskey A, Robinson C (eds.) Chemistry and Biology of Mineralized Tissue. American Academy of Orthopaedic Surgeons, Rosemont, IL, USA, pp. 45–48.
39. Atsumi T, Miwa Y, Kimata K, Ikawa Y 1990 A chondrogenic cell line derived from a differentiating culture of AT805 teratocarcinoma cells. Cell Differ Dev **30:**109–116.
40. Andersson S, Davis DL, Dahlback H, Jornvall H, Russell DW 1989 Cloning, structure, and expression of the mitochondrial cytochrome P- 450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. J Biol Chem **264:**8222–8229.
41. McClelland M, Mathieu-Daude F, Welsh J 1995 RNA fingerprinting and differential display using arbitrarily primed PCR. Trends Genet **11:**242–246.
42. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ 1990 Basic local alignment search tool. J Mol Biol **215:**403–410.
43. Amizuka N, Warshawsky H, Henderson JE, Goltzman D, Karaplis AC 1994 Parathyroid hormone-related peptide-depleted mice show abnormal epiphyseal cartilage development and altered endochondral bone formation. J Cell Biol **126:**1611–1623.
44. Grigoriadis AE, Heersche JN, Aubin JE 1990 Continuously growing bipotential and monopotential myogenic, adipogenic, and chondrogenic subclones isolated from the multipotential RCJ 3.1 clonal cell line. Dev Biol **142:**313–318.
45. Bernier SM, Goltzman D 1993 Regulation of expression of the chondrocytic phenotype in a skeletal cell line (CFK2) in vitro. J Bone Miner Res **8:**475–484.
46. Liang P, Pardee AB 1992 Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science **257:**967–971.
47. Margolin JF, Friedman JR, Meyer WK, Vissing H, Thiesen HJ, Rauscher FJ III 1994 Kruppel-associated boxes are potent transcriptional repression domains. Proc Natl Acad Sci USA **91:**4509–4513

48. Lechner MS, Begg GE, Speicher DW, Rauscher FJ III 2000 Molecular determinants for targeting heterochromatin protein 1-mediated gene silencing: Direct chromoshadow domain-KAP-1 corepressor interaction is essential. Mol Cell Biol **20:**6449–6465
49. Friedman JR, Fredericks WJ, Jensen DE, Speicher DW, Huang XP, Neilson EG, Rauscher FJ III 1996 KAP-1, a novel corepressor for the highly conserved KRAB repression domain. Genes Dev **10:**2067–2078
50. Passananti C, Felsani A, Caruso M, Amati P 1989 Mouse genes coding for "zinc-finger"-containing proteins: Characterization and expression in differentiated cells. Proc Natl Acad Sci USA **86:**9417–9421.
51. Lyons GE, Ontell M, Cox R, Sassoon D, Buckingham M 1990 The expression of myosin genes in developing skeletal muscle in the mouse embryo. J Cell Biol **111:**1465–1476.
52. Kaufman M (ed.) 1995 The Atlas of Mouse Development. Academic Press, San Diego, CA, USA, pp. 320, 335.
53. Bitgood MJ, McMahon AP 1995 Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. Dev Biol **172:**126–138.
54. Lanske B, Karaplis AC, Lee K, Luz A, Vortkamp A, Pirro A, Karperien M, Defize LH, Ho C, Mulligan RC, Abou-Samra AB, Juppner H, Segre GV, Kronenberg HM 1996 PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. Science **273:**663–666.
55. Vortkamp A, Lee K, Lanske B, Segre GV, Kronenberg HM, Tabin CJ 1996 Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. Science **273:** 613–622.
56. Wallis GA 1996 Bone growth: Coordinating chondrocyte differentiation. Curr Biol **6:**1577–1580.
57. Wysolmerski JJ, Broadus AE, Zhou J, Fuchs E, Milstone LM, Philbrick WM 1994 Overexpression of parathyroid hormone-related protein in the skin of transgenic mice interferes with hair follicle development. Proc Natl Acad Sci USA **91:**1133–1137.
58. Xu X, Weinstein M, Li C, Deng C 1999 Fibroblast growth factor receptors (FGFRs) and their roles in limb development. Cell Tissue Res **296:**33–43.
59. Grimsrud CD, Romano PR, D'Souza M, Puzas JE, Reynolds PR, Rosier RN, O'Keefe RJ 1999 BMP-6 is an autocrine stimulator of chondrocyte differentiation. J Bone Miner Res **14:**475–482.
60. Vortkamp A 2001 Interaction of growth factors regulating chondrocyte differentiation in the developing embryo. Osteoarthritis Cartilage **9**(Suppl A)**:**S109–S117.
61. Poliard A, Ronziere MC, Freyria AM, Lamblin D, Herbage D, Kellermann O 1999 Lineage-dependent collagen expression and assembly during osteogenic or chondrogenic differentiation of a mesoblastic cell line. Exp Cell Res **253:**385–395.
62. Ito H, Akiyama H, Shigeno C, Nakamura T 1999 Bone morphogenetic protein-6 and parathyroid hormone-related protein coordinately regulate the hypertrophic conversion in mouse clonal chondrogenic EC cells, ATDC5. Biochim Biophys Acta **1451:**263–270.
63. Akiyama H, Shigeno C, Hiraki Y, Shukunami C, Kohno H, Akagi M, Konishi J, Nakamura T 1997 Cloning of a mouse smoothened cDNA and expression patterns of hedgehog signalling molecules during chondrogenesis and cartilage differentiation in clonal mouse EC cells, ATDC5. Biochem Biophys Res Commun **235:**142–147.
64. Shukunami C, Ishizeki K, Atsumi T, Ohta Y, Suzuki F, Hiraki Y 1997 Cellular hypertrophy and calcification of embryonal carcinoma-derived chondrogenic cell line ATDC5 in vitro. J Bone Miner Res **12:**1174–1188.

Address reprint requests to:
Bernhard Ganss, Ph.D.
CIHR Group in Matrix Dynamics
Faculty of Dentistry
University of Toronto
FitzGerald Building, Room 239
150 College Street
Toronto, Ontario M5S 3E2, Canada

Received in original form August 21, 2001; in revised form May 2, 2002; accepted July 1, 2002.