Stage-Specific Expression of Dlx-5 during Osteoblast Differentiation: Involvement in Regulation of Osteocalcin Gene Expression

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Two homeotic genes, Dlx and Msx, appear to regulate development of mineralized tissues, including bone, cartilage, and tooth. Expression of Msx-1 and Msx-2 has been studied during development of the osteoblast phenotype, but the role of Dlx in this context and in the regulation of bone-expressed genes is unknown. We used targeted differential display to isolate homeotic genes of the Dlx family that are expressed at defined stages of osteoblast differentiation. These studies were carried out with fetal rat calvarial cells that produce bone-like tissue *in vitro*. We observed a mineralization stage-specific mRNA and cloned the corresponding cDNA, which represents the rat homolog of Dlx-5. Northern blot analysis and competitive RT-PCR demonstrated that Dlx-5 and the bone-specific osteocalcin genes exhibit similar up-regulated expression during the mineralization period of osteoblast differentiation. This expression pattern differs from that of Msx-2, which is found predominantly in proliferating osteoblasts. Several approaches were pursued to determine functional consequences of Dlx-5 expression on osteocalcin transcription. Constitutive expression of Dlx-5 in ROS 17/2.8 cells decreased osteocalcin promoter activity in transient assays, and conditional expression of Dlx-5 in stable cell lines reduced endogenous mRNA levels. Consistent with this finding, antisense inhibition of Dlx-5 increased osteocalcin gene transcription. Osteocalcin promoter deletion analysis and binding of the *in vitro* translation product of Dlx-5 demonstrated that repressor activity was targeted to a single homeodomain-binding site, located in OC-Box I (−99 to −76). These findings demonstrate that Dlx-5 represses osteocalcin gene transcription. However, the coupling of increased Dlx-5 expression with progression of osteoblast differentiation suggests an important role in promoting expression of the mature bone cell phenotype. (Molecular Endocrinology 11: 1681–1694, 1997)

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INTRODUCTION

Bone formation occurs during embryonic development and postnatal growth, but also during subsequent bone remodeling to support calcium homeostasis and adaptation to physical forces. Osteogenesis is additionally linked to osseous transplantation, implantation, or skeletal tissue repair. This remodeling requires continuous recruitment, proliferation, and differentiation of osteoprogenitor cells. Like other connective tissue-forming cells, osteoprogenitor cells originate from mesenchymal cells derived from the mesodermal germ cell layer (1). Subsequently, osteoblast precursor cells develop into mature osteocytic cells by progressing through a multistage developmental sequence (2). An understanding of regulatory mechanisms contributing to osteoblast differentiation is critical for gaining insight into the pathogenesis of bone-related diseases, as well as for the development of new treatment regimens.

Formation of the vertebrate body plan is controlled in part by homeodomain transcription factors that regulate the temporal appearance and location of preosseous tissues along the vertebral column (3, 4).

Thus, homeodomain proteins may directly mediate osteoblast differentiation by selectively activating and/or repressing genes that support development of skeletal tissues *in vivo*. Homeodomain proteins contain a 60-amino acid segment, the homeobox, which represents the DNA recognition domain (5, 6). Unlike the classic homeobox (Hox) genes, which are clustered at four chromosomal loci and specify anterior-posterior positional information along the vertebrate body axis, the homeodomain superclass of genes encompasses many atypical members. These genes include the Dlx and Msx genes, which have a dispersed chromosomal distribution [reviewed by Gehring *et al.*, 1994 (7)].

Several lines of *in vivo* evidence support the concept that the Dlx and Msx family of homeobox proteins may represent regulatory genes that preferentially support skeletal tissue differentiation. Expression of Dlx and Msx genes is primarily restricted to the epithelial-mesenchymal interaction site during apical ectodermal ridge (AER) development (8–11). The presence of Dlx and Msx homeodomain proteins during these transitions is critical for craniofacial (12–15), tooth (16, 17), brain, and neural development (18, 19). Developmental studies in mouse (20), rat (19), and chicken limb bud (9) have revealed that several Dlx family members are highly expressed in cartilage and in developing endochondral and membranous bone. Furthermore, results from genetic studies suggest that Dlx and Msx genes are directly involved in bone morphogenesis (10–12, 21–24).

Recent studies suggest the involvement of homeodomain proteins in regulation of osteoblast development and bone tissue-specific gene expression. For example, bone-specific expression of the osteocalcin (OC) gene is controlled by a principal multipartite promoter element (OC-box) that contains a homeodomain recognition motif (25–27). Similarly, homeodomain motifs have been implicated in the regulation of the collagen type I gene in osteoblasts (28, 29). Several studies have suggested that Msx-2, which binds the OC-box homeodomain motif of the OC gene, is a key regulator of OC gene expression (25, 27) and development of the bone cell phenotype (21, 26). Although Dlx appears to be important for skeletal formation, to date there is no direct evidence for a role of Dlx in osteoblast differentiation or regulation of osteoblast-restricted genes.

In this study we applied a homeobox-directed PCR approach that takes advantages of both mRNA differential display (30) for sensitivity and simplicity, and RNA finger printing (31) for reproducibility and specificity. Using this method, we provide the first evidence for differentiation-specific expression of a Dlx-5 homolog during rat calvarial osteoblast differentiation. Our findings suggest that Dlx-5 may directly support expression of the mature bone cell phenotype, and our results are consistent with the concept that multiple homeodomain proteins may provide stringent developmental and tissue-specific regulation of OC gene transcription.

RESULTS

Isolation of an Osteoblast Dlx-Related mRNA That Is Tissue Specific and Expressed in a Developmental Stage-Specific Manner

Our goal in this study was to isolate Dlx-related homeobox-containing genes that are expressed in a stage-specific manner during osteoblast differentiation. For this purpose RNA was prepared from primary cultures of calvarial-derived osteoblasts at two developmental periods: early stage proliferating cells (day 2) that do not express bone phenotypic genes and post-proliferative cultures with bone-like mineralized nodules (day 21) that express genes characteristic of differentiated osteoblasts (2). Dlx-related sequences in these RNA preparations were amplified using a homeobox-specific 5′-primer and a loosely conserved sequence of the 3′-end of the coding region of Dlx as the 3′-primer (see *Materials and Methods* for nucleotide sequences). This primer set provided high specificity for the Dlx family of proteins, as well as reproducibility in the representation of mRNAs by differential display.

Figure 1A shows the differential display pattern of mRNA from proliferating (P) and mineralized (M) rat osteoblast (ROB), or proliferating (P) and confluent (C) ROS 17/2.8 rat osteosarcoma cells (ROS). The *large arrowhead* indicates the band of interest that is preferentially expressed in mature rat osteoblast cultures having a mineralized matrix. The band is constitutively expressed in ROS 17/2.8 cells, present at similar levels in proliferating (day 2) and confluent (day 8) cells. This pattern of expression was reproduced by Northern blot analysis of RNA harvested from a ROB cell differentiation time course and confluent or proliferating ROS 17/2.8 cells when probed with the isolated reamplified mineralization-specific band from Fig. 1A. This analysis (Fig. 1B) demonstrates that the Dlx isolate is very weakly expressed in ROB proliferating cells (day 2 and day 6). Dlx expression was detected after confluency (day 10) and continued to increase throughout the experimental period. Equal levels of transcripts were found in the proliferating and confluent ROS 17/2.8 cells. Notably, we observed that there are two distinct transcripts in ROB cell RNA, as indicated by *arrowheads* in Fig. 1B, while only a single transcript is present in ROS cells.

The reamplified band of interest was cloned into the pCR II vector and sequenced using Sp6 and T7 promoter primers. Sequence comparison of the 289-nucleotide clone with known sequences in data bases confirmed that the clone is a rat Dlx gene. It is identical with sequences published for rDlx (19) and rat Dlx-3 (32). Amino acid sequence comparison of our isolate and homeodomain sequences of known Dlx-3 and

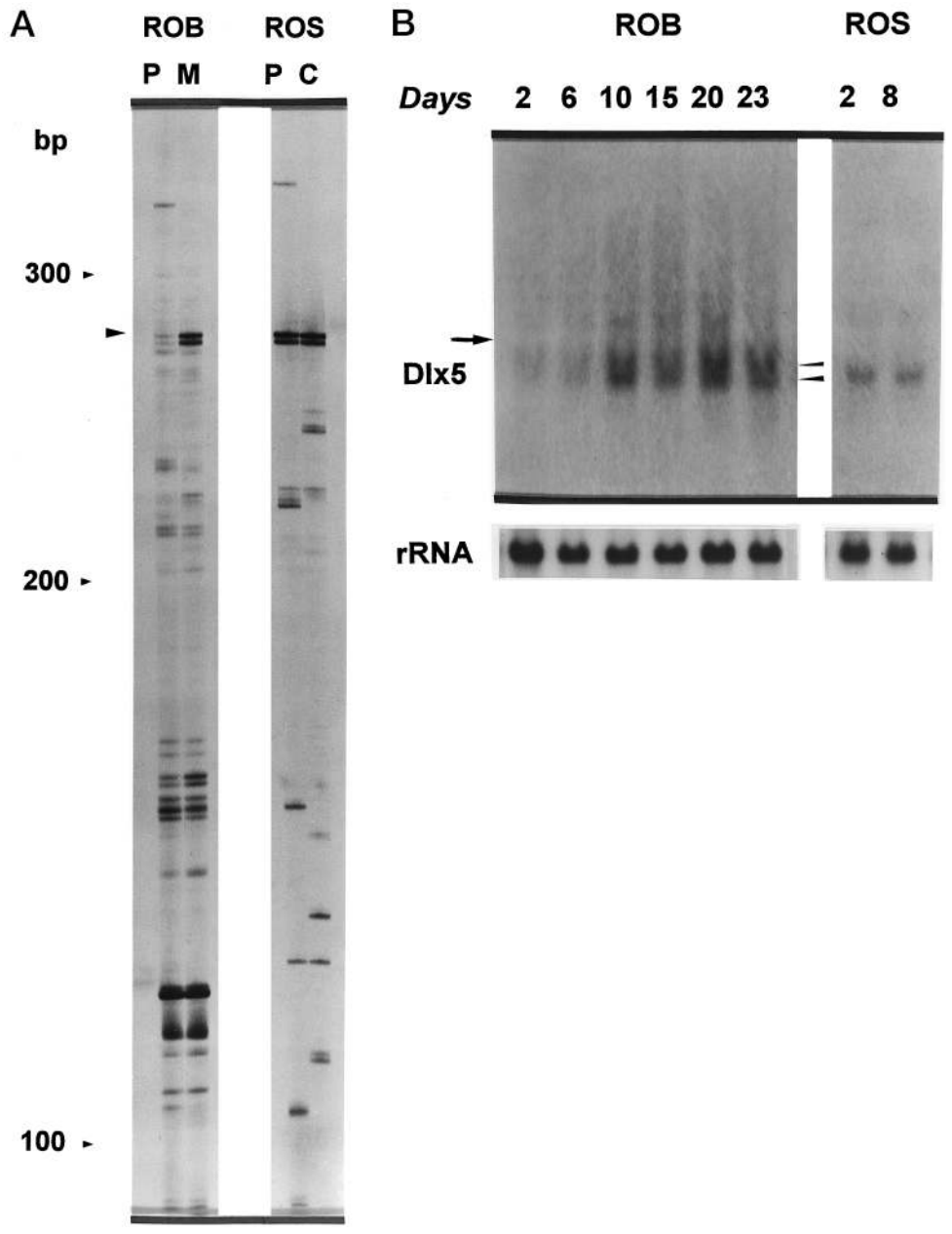

Fig. 1. Differential Display and Northern Expression of Cytoplasmic RNA from Primary Cultured Rat Osteoblasts (ROB) during *in Vitro* Differentiation and ROS 17/2.8 Rat Osteosarcoma Cells (ROS) during Density Inhibition

A, Reverse transcription and PCR conditions are described in *Materials and Methods*. The amplified product of interest is indicated by the *large arrow head*. The sizes of bands were calculated from a comigrating sequence reaction, and the *small arrows* indicate position of sized markers. P, Proliferating ROB and ROS at day 2; C, confluent ROS at day 8; M, mineralized ROB at day 21. B, The reamplified product of the differential displayed band was used as a probe for Northern hybridization. Twenty five micrograms of DNase I-treated total cellular RNA from ROB or ROS 17/2.8 cells harvested at different time points were loaded on each lane. The hybridization of two distinct transcripts was observed in the differentiated ROB cell and are indicated by the *arrowheads* to the *right* of the figure. The bands are designated Dlx-5 based on sequence analysis (see Fig. 2). The blots were probed for 28S ribosomal RNA to demonstrate similar RNA loading. The migration of the 18S ribosomal RNA is indicated by the *arrow* to the *left* of the figure.

Dlx-5 genes indicates that our gene is the rat homolog of Dlx-5 (Fig. 2). A 5′-primer that includes the translation start site of Dlx-5 was designed to obtain a full-length Dlx-5 coding region by RT-PCR of RNA from mineralized ROB cultures. The resulting clone was confirmed by sequencing and was subsequently used as a Dlx-5 cDNA. Northern analysis of RNA from a time course of differentiating ROB cells showed the same pattern of Dlx-5 expression when probed with this full length cloned cDNA (Fig. 3A) as the isolated differen-

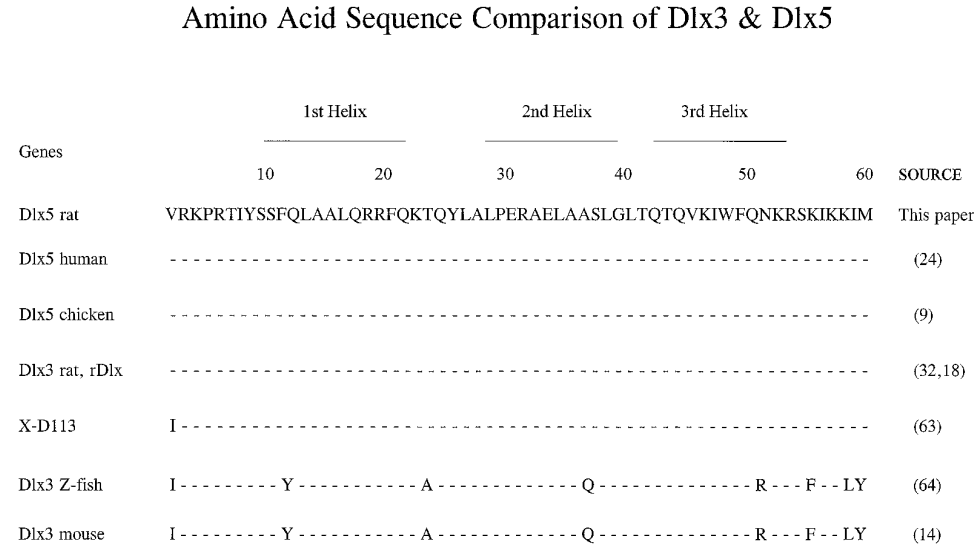

Fig. 2. Sequence Comparisons between the Homeodomains of Our Isolate, Dlx-5, and Dlx-3

Our cloned cDNA fragment has an identical homeobox amino acid sequence when compared with human and chicken Dlx-5 genes, rDlx and rat Dlx-3, but differs 10% from Dlx-3 of mouse or zebra fish. Helix domains of the homeobox are indicated.

tial display probe (shown in Fig. 1B). Other parameters of gene expression in this osteoblast differentiation time course (Fig. 3A), including histone H4 (H4) and OC, reflected a typical osteoblast developmental sequence of gene expression (2). Msx-2 mRNA levels are high in proliferating cells (day 2) and decrease after confluency (day 7 to day 21), although an increase in the transcripts, particularly the lower form, was observed from day 21 to day 28. The developmental up-regulation of Dlx-5 expression appears to parallel OC gene expression and osteoblast differentiation. Dlx-5 expression is not observed postproliferatively in rat osteoblasts cultured in the absence of both ascorbic acid and β-glycerol phosphate, conditions that do not support extracellular matrix mineralization (Fig. 3B). Figure 3B shows Dlx-5 expression is not detected in the nondifferentiated osteoblasts that are maintained for the same time period (26 days) as the mineralized cultures.

Tissue specificity of Dlx-5 expression was assessed by examination of total cellular RNA from major organs and tissues of 3-month-old mice (Fig. 4). Northern blot analyses indicated that Dlx-5 was expressed in bone tissue and was absent in all the soft tissues we assayed. We also note that a single 1.5-kb transcript is observed in the mouse tissues, similar to ROS 17/2.8 cells.

Dlx-5 Selectively Represses OC Promoter Activity in Osseous Cells

It is known that the homeodomain protein Msx-2, which is expressed abundantly in proliferating osteoblasts, down-regulates OC transcription and synthesis (27). To investigate whether Dlx-5 can also regulate OC transcription, we examined the effect of forced expression of Dlx-5 on OC promoter activity. A cytomegalovirus (CMV) promoter containing plasmid expressing Dlx-5 was cotransfected with a plasmid containing a reporter gene under the control of various promoters. As a control, the promoter constructs were also transfected with a CMV plasmid lacking the Dlx-5 gene. Dlx-5 does not significantly influence activity of several eukaryotic promoters, including the thymidine kinase, histone, osteopontin, and the dimerized SP1-binding site (2$\times$ SP1) (Fig. 5A). Dlx-5 suppressed OC promoter activity in ROS 17/2.8 cells using both -1097 OC-choramphenicol acetyltransferase (CAT) (Fig. 5A) and the -1050 OC-Luc (data not shown) chimeric constructs. We note that Dlx-5 also inhibited SV40 promoter activity, further reflecting promoter selectivity of Dlx-5.

To test the effect of increased Dlx-5 expression on OC promoter activity in cells in which endogenous expression of Dlx-5 is lower than in ROS 17/2.8 cells, transient transfection assays were performed in several additional cell lines (Fig. 5B). Dlx-5 expression was increased by transient transfection of the CMV Dlx-5 construct, and control reactions were cotransfected with the empty CMV vector, lacking the Dlx-5 sequences. In proliferating ROB cells, a 2-fold reduction in activity is observed in OC promoter activity. The OC promoter is generally inactive in non-bone cell lines. Transient transfection studies in C2C12 mouse myoblast cells indicate that background levels of OC promoter activity are not altered by expression of Dlx-5, demonstrating that Dlx-5 does not activate OC promoter activity in non-bone cells. The OC promoter is also normally inactive in IMR-90 fibroblasts, but can be up-regulated by coexpression of the CBFA/PEβP2α/AML factor (core binding factor α, also known

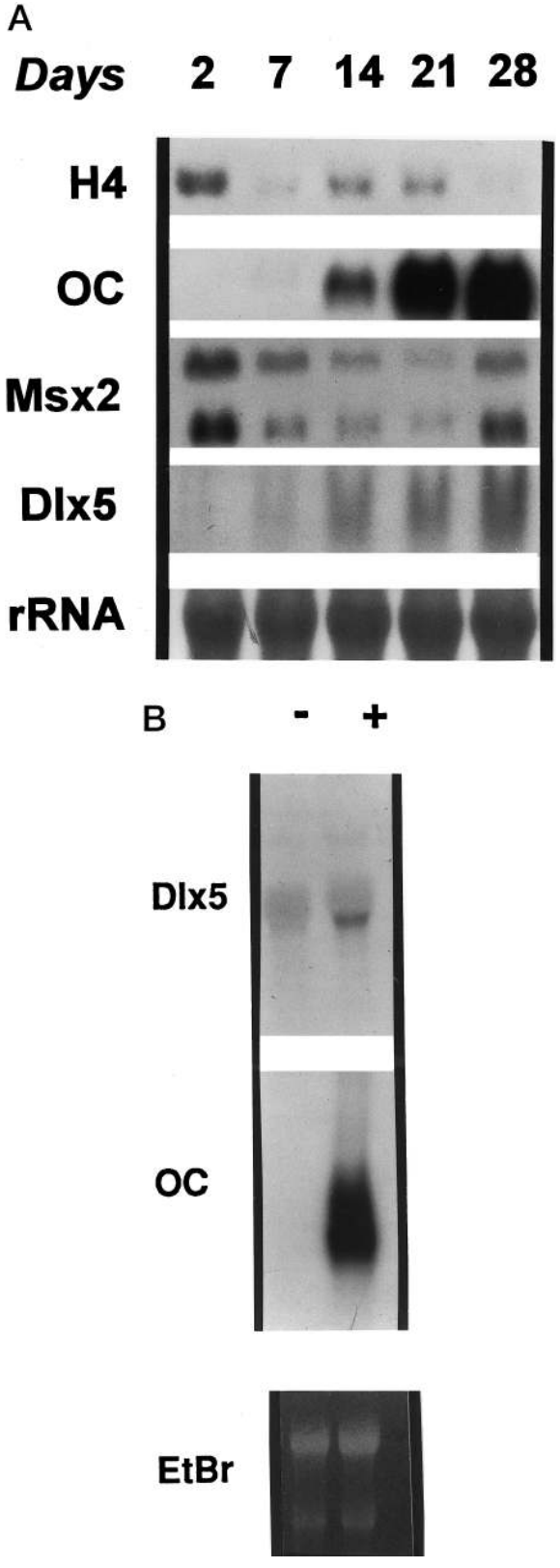

Fig. 3. Developmental Expression of Dlx-5 during *in Vitro* Osteoblast Differentiation

A, Total cellular RNA from ROB cells harvested at the indicated days was examined by Northern analysis for representation of histone H4 (H4) and OC gene expression (10 μg/lane) to assess the extent of ROB differentiation, as well as Msx-2 (10 μg/lane) and Dlx-5 (25 μg/lane). Consistency of RNA loading is reflected by hybridization to 28S ribosomal RNA; a representative blot (also used for Dlx-5) is shown. B, Northern blot analysis of osteoblasts cultured for 26 days in the presence (+) or absence (−) of ascorbic acid and β-glycerophosphate to promote or prevent differentiation, respectively. Note the absence of OC and Dlx-5 expression in nondifferentiated cells (−). Ethidium bromide staining of the ribosomal RNA (EtBr) shows intactness of RNA and consistency in loading (20 μg/lane). The agarose gels used here did not resolve the two Dlx-5 transcripts.

as polyoma enhancer binding protein and acute myelogenous leukemia factor) as demonstrated by Banerjee *et al.* (33). When activity is induced in IMR-90 cells by AML-1B expression, Dlx-5 coexpression down-regulates this AML-dependent level of OC promoter activity (Fig. 5C). Therefore, Dlx-5 consistently down-regulates OC promoter activity in all cell lines tested.

The Dlx-5-Responsive Element Is Localized to OC-Box I

To locate the Dlx-5-responsive element in the OC promoter (−1050 to +32), we searched for possible homeodomain-binding sites. There are four homeodomain core-binding sequences [(ATTA or TAAT located at −49 to −52, −86 to −89, −553 to −556, and −990 to −993, numbered according to Lian *et al.* (34)]. Four OC promoter-luciferase constructs, containing −1050, −637, −199, and −83 nucleotides or no promoter sequences, were chosen to assay for Dlx-5 activity in transient transfection assays in ROS 17/2.8 cells (Fig. 6A). Each of these constructs sequentially eliminates one of the four potential homeodomain-binding sites. This promoter deletion analysis indicates that a Dlx-5-responsive negative regulatory element is located between −199 and −83 nucleotides upstream from the transcription start site (Fig. 6A). The conserved promoter element, OC-Box I, which contains a functional homeodomain-binding site (25, 27), is located within this sequence.

To further confirm Dlx-5 suppressor activity on the OC gene and the location of the active element, an antisense experimental approach was carried out (Fig. 6B). The OC promoter-deletion series of OC-Luc constructs were cotransfected with the tetracycline-responsive Dlx antisense expression plasmid into the tetracycline-regulated transactivator (tTA) ROS 17/2.8 cell line (described in *Materials and Methods*). The control vector (without Dlx-5 antisense sequences) had no effect on the activity of the −1050 OC promoter. Consistent with the overexpression results, all constructs containing the OC-Box I (located at −99 to −76) were responsive to overexpression of antisense Dlx-5. OC promoter activity was induced by coexpression of antisense Dlx-5 (-Tet). No significant effect was

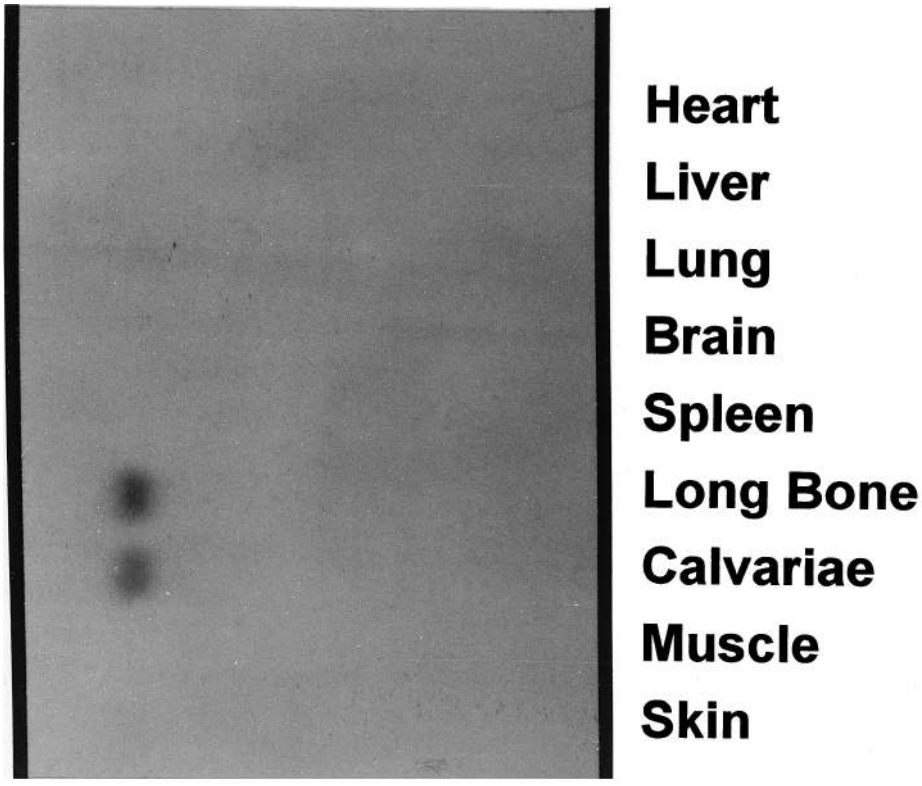

Fig. 4. Northern Analysis of Expression of Dlx-5 in 3-Month-Old Mouse Tissues

Twenty five micrograms of total cellular RNA from major organ and bone tissues were separated on a 1% agarose/formaldehyde gel and transferred overnight by capillary action onto Zetaprobe membrane. The blot was hybridized with the Dlx-5 cDNA probe.

observed in the shorter OC-Luc construct (−83 OC-Luc), which lacks the ATTA motif. These data suggest an active site for Dlx-5 interaction at the OC-Box (−99 to −76) in the OC promoter.

Dlx-5 Functional Activity Is Mediated by the Homeodomain Sequence within the OC-Box

To assay for binding specificity of Dlx-5 with OC-Box sequences, gel mobility shift assays were performed (Fig. 7). *In vitro* translation of both wild type Dlx-5 and mutant Dlx-5, which lacks the homeobox, produced the expected molecular masses (−35 kDa and −25 kDa, respectively) as determined by SDS-PAGE and autoradiography (data not shown). These *in vitro* translation products were incubated with a labeled oligonucleotide containing OC-Box sequences −99 to −76 (WT, Table 1). Nucleotide sequences of wild type OC-Box and mutant OC-Box oligonucleotides that were used as competitors are detailed in Table 1. Dlx-5 protein binds strongly to the OC-Box oligonucleotide (Fig. 7). Products of a control *in vitro* translation reaction without template and the homeobox-deleted Dlx-5 protein showed no binding (Fig. 7, lanes 1 and 2). Dlx protein-OC-Box sequence interactions in the presence of 50-fold excess of wild type or mutant oligonucleotide competitors are shown (Fig. 7, lanes 4–12). Wild type OC-Box (WT), homeobox binding consensus site (hbs), and mCC2 efficiently compete for Dlx-5 binding. The mT-T, mAG, and mCC1 oligonucleotides show very low affinity for Dlx-5, while the mAA, m8, and mutant homeobox consensus binding site (mhbs) show no competition for Dlx-5 binding. These data confirm the specificity of Dlx-5 interactions with the core ATTA homeodomain binding site within the OC-Box.

To further establish that this site within the OC-Box mediates Dlx-5 responsiveness, OC promoter (−351 to +32) constructs containing either wild type, mAA, or mAG mutant OC-Box sequences fused to the luciferase reporter were cotransfected with Dlx-5 or a control plasmid in ROS 17/2.8 cells. These mutations result in a significant decrease in promoter activity (Fig. 8). Forced expression of Dlx-5 significantly suppressed wild type −351-Luc OC promoter activity, but had no effect on activity of the constructs containing the mutated OC-Box sequences (Fig. 8). Thus mutations that did not compete or competed weakly with wild type OC-Box sequences in the gel mobility shift assays (Fig. 7) were also not responsive to Dlx-induced down-regulation of OC promoter activity. This finding confirms that the homeodomain-binding site within the OC-Box is the recognition sequence for functional Dlx-5 activity.

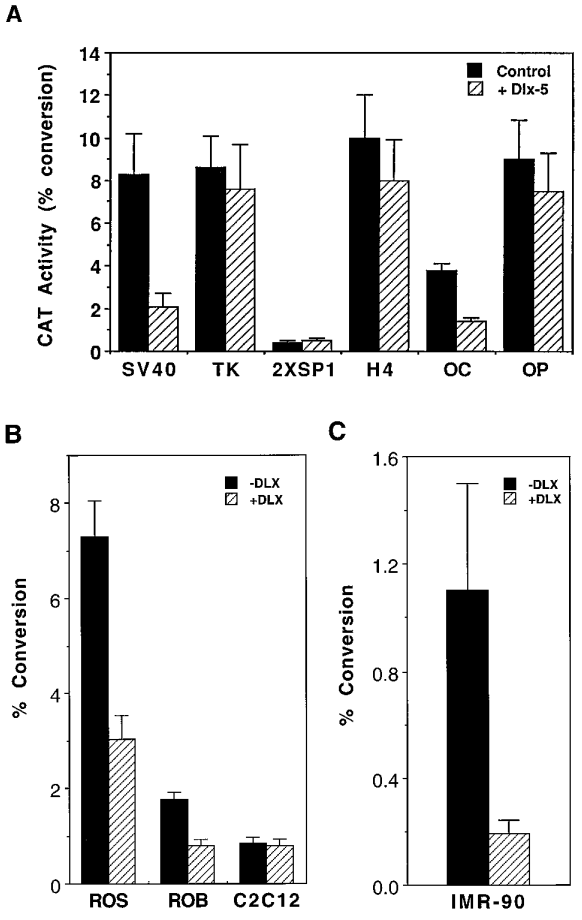

Fig. 5. Regulation of Target Promoter Activity by Dlx-5

A, A construct expressing Dlx-5 driven by a CMV promoter (+ Dlx-5, *hatched bars*) or lacking the Dlx-5 sequences (control, *solid bars*) was cotransfected into ROS 17/2.8 cells along with plasmids containing the CAT reporter gene fused to various eukaryotic promoters: thymidine kinase (TK); histone H4 (H4); osteopontin (OP); OC; a dimerized SP1 binding site (2XSP1); and the SV40 promoter. Cells were assayed for CAT expression 48 h after transfection. B, Activity of the −1097 OC promoter-CAT construct was assayed in the presence (+Dlx, *hatched bars*) or absence (−Dlx, *solid bars*) of a Dlx-5-expressing construct in two cell lines (ROS 17/2.8 osteosarcoma cells and C2C12 myoblasts) as well as primary rat osteoblasts. Promoter activity is reported as percent conversion of chloramphenicol. C, Activity of reporter construct containing 108 nucleotides of the OC proximal promoter and a CBFA/AML-1 binding site was measured in IMR-90 cells. To activate the promoter construct, 1 μg of a CMV construct expressing AML-1B was cotransfected in the presence (*hatched bars*) of 4 μg Dlx-5 expression construct or 4 μg vector alone, lacking Dlx-5 sequences (*solid bars*).

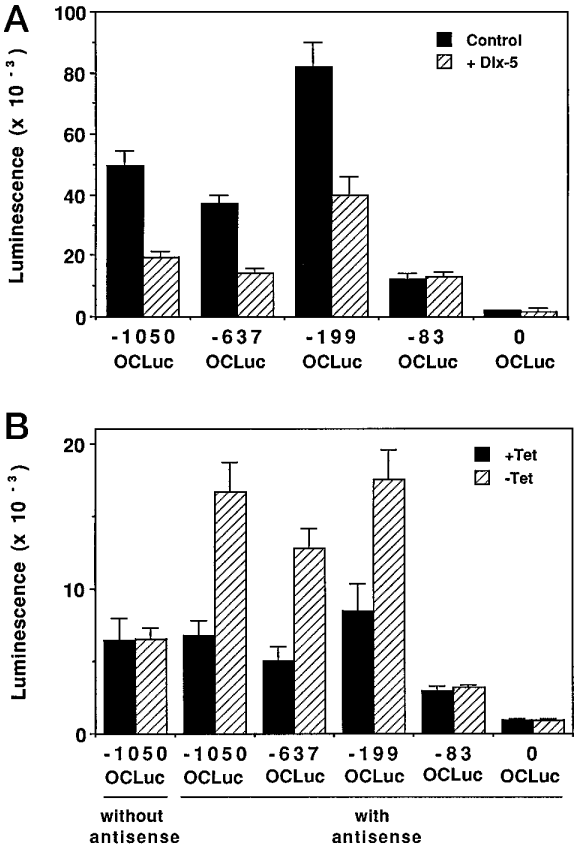

Fig. 6. Functional Analysis of Deletion Mutants of the OC Promoter in Response to Forced or Antisense Expression of Dlx-5

A, A series of OC promoter-luciferase chimeric DNA constructs that sequentially eliminate each of the four potential homeodomain motifs were cotransfected with CMV/Dlx-5 (*hatched bars*) or a control CMV plasmid without Dlx-5 sequences (*solid bars*) into ROS 17/2.8 cells. Values are the mean of six independent transfections. Standard deviation is indicated by lines *above* each *bar*. Transfection efficiencies were monitored by cotransfecting the OP-CAT plasmid. B, OC promoter-luciferase chimeric DNA constructs were transiently cotransfected with pUHD-30-anti-Dlx-5 expressing Dlx-5 antisense sequences, or pUHD-30 without Dlx-5 antisense sequences into ROS 17/2.8 cells having a stably integrated tetracycline-responsive transactivator (+Tet represents control activity when media contains 1 μg/ml tetracycline). Dlx-5 antisense expression is activated in cells transfected with antisense plasmid by withdrawal of tetracycline from media (− Tet); this procedure had no effect in the absence of antisense sequences on (−1050) OC-Luc. *Solid column*, + tetracycline; *hatched column*, − tetracycline.

Dlx-5 Regulates Endogenous Expression of the Bone-Specific OC Gene

We have established that the OC gene represents a target for Dlx-5 transcriptional regulation, which occurs via OC-Box I, a primary tissue-specific promoter element (26, 35). To address directly whether Dlx-5 can modulate endogenous OC gene expression, we created cell lines conditionally expressing Dlx-5. ROS 17/2.8 cells expressing the tetracycline regulated transactivator were stably transfected with a Dlx-5 expression plasmid controlled by tetracycline. A cell line that exhibited clear up-regulation of Dlx-5 by tetracycline was selected and fur-

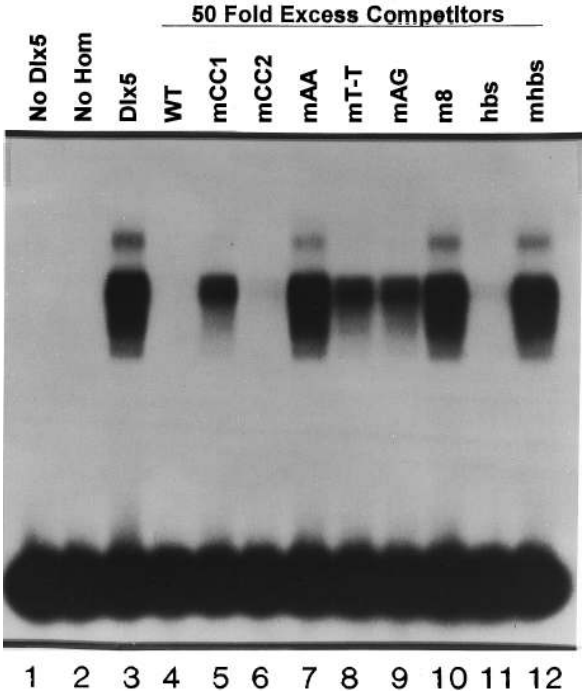

Fig. 7. Binding Activity of *in Vitro* Transcribed and Translated Dlx-5 with Wild Type OC-Box and Several Mutants

Gel mobility shift assay of γ-^{32}P end-labeled OC-Box oligonucleotide bound with Dlx-5 synthesized by *in vitro* translation. Lane 1 (no Dlx-5) shows binding of an *in vitro* translation reaction that did not contain a template; lane 2 (no Hom), binding activity of mutant Dlx-5 lacking the homeobox; lane 3, WT, binding of *in vitro* translated WT Dlx-5; lanes 4–12 show various effects of DNA competitors on Dlx-5 binding including several mutant oligonucleotides described in Table 1.

Table 1. Sequences of Wild Type and Mutant OC-Box and Homeodomain Consensus Binding Site of Oligonucleotides

Mutant Name		Mutant Sequences	
WT		ATGACCCCAATTAGTCCTGGCAG	
mCC1	−99	------gg----------	−76
mCC2		------gt----------	
mAA		--------gg----------	
mT-T		----------a---c---------	
mAG		----------tc---------	
m8		------gactgctc---------	
hbs		G--T--------GT	
mhbs		G--T---cgg--GT	

ther studied. Endogenous expression of different genes was assayed by Northern analysis in the presence or absence of tetracycline (Fig. 9). Tetracycline levels were used that have been shown not to influence the levels of OC biosynthesis in cells lacking the Dlx-5 construct (data not shown). When tetracycline is removed, expression of Dlx-5, fibronectin, and collagen type I is up-regulated. Strikingly, elevation of Dlx-5 expression results in down-regulation of OC expression. This finding supports our observations in transiently transfected cells. For comparison, histone H4, osteopontin, and alkaline phosphatase expression is not altered by the tetracycline-related up-regulation of Dlx-5 expression.

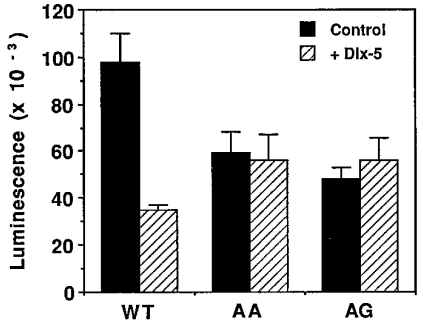

Fig. 8. Functional Analysis of Dlx-5 Activity on Wild Type and OC-Box Promoter Mutations

Expression of (−1050) OC-Luc WT and the OC-Box mutant constructs in ROS 17/2.8 cells in the presence (+ Dlx-5, *hatched columns*) or absence (control, *solid bars*) of Dlx-5 expressing plasmid. Cells were assayed for luciferase activity 48 h after transfection. Overexpression of Dlx-5 had no effect on promoter activity of the OC-Box mutant constructs. Values are mean and SD of six independent transfection experiments. Transfection efficiencies were monitored by cotransfection of OP-CAT and assaying CAT activity.

DISCUSSION

In this study we have characterized a differentially displayed Dlx homeodomain gene, which is selectively up-regulated during development of the osteoblast phenotype. The Dlx gene we identified in differentiated osteoblasts was already reported as rDlx (19) cloned from chondrosarcoma cells, and Dlx-3 in rat (32). However, our comparison of amino acid similarities in the homeodomain, as well as 5′- and 3′-flanking sequences indicates that rDlx, Dlx-3, and the Dlx gene described here all represent the rat homolog of Dlx-5.

Dlx-5 Represses OC Gene Transcription

Our identification of a Dlx-5-responsive *cis*-acting promoter element in the bone-specific OC gene provides the first evidence for a Dlx-5 responsive target gene. We have presented multiple converging lines of evidence that indicate that Dlx-5 functions to repress OC gene transcription. For example, transient coexpression of Dlx-5 decreases OC promoter activity, whereas antisense Dlx-5 mRNA expression increases OC gene transcription. This down-regulatory effect of Dlx-5 overexpression on OC promoter activity is observed in both osseous (ROS 17/2.8) and nonosseous (IMR-90) cells and is also observed in cells in which endoge-

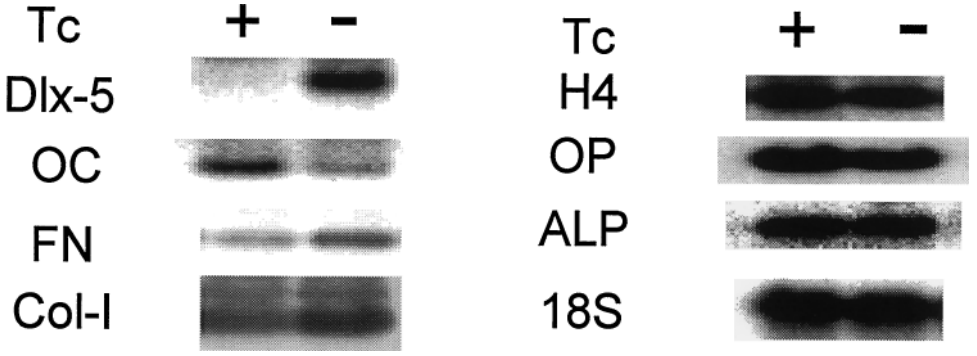

Fig. 9. Effects of Dlx-5 Overexpression on Osteoblast Proliferation and Differentiation Markers

ROS 17/2.8 cells containing the gene for the tetracycline-responsive transactivator were stably transfected with a Dlx-5 expression plasmid controlled by this transactivator. Stable cell lines with demonstrated significant changes of Dlx-5 expression in the presence or absence of tetracycline were selected by Northern analysis. Cells were maintained in media containing tetracycline (TC+) or harvested 48 h after the removal of tetracycline (TC−). Ten micrograms of total cellular RNA were analyzed for the expression of OC, fibronectin (FN), collagen type I (COL-I), histone H4 (H4), osteopontin (OP), alkaline phosphatase (AP), and 18S ribosomal RNA (18S) as a loading control.

nous Dlx-5 mRNA levels are low or below the level of detection (*e.g*. proliferating ROB and IMR-90 cells). Moreover, conditional expression of Dlx-5 in ROS 17/2.8 cells results in repression of endogenous OC gene expression. Thus, our data suggest that the OC gene represents a bona fide cellular target for Dlx-5.

Our data show that the levels of Dlx-5 mRNA, and consequently its inherent repressive function, are up-regulated when OC gene expression is induced to maximal levels during osteoblast maturation in the mineralization period. Induction of OC gene expression is determined in part by a multiplicity of potent *trans*-activators that mediate basal tissue-specific transcription (36). In addition, increased OC gene expression appears to be the combined effect of transcriptional up-regulation and mRNA stabilization (37). Therefore, we suggest that Dlx-5 may attenuate this potentially hyperactive level of OC gene expression in differentiated osteoblasts to permit physiological control of OC biosynthesis. This function would maintain a suboptimal tissue-specific basal transcription rate, which would render the gene responsive to enhancement by other gene-regulatory signaling mechanisms required for bone renewal (*e.g*. vitamin D_3). Furthermore, several lines of evidence indicate that negative regulation of OC may be important for proper bone development (38–40). However, apart from a role for Dlx-5 in regulating OC gene transcription, it is plausible that other target genes exist in which Dlx-5 may perhaps repress or activate gene transcription in conjunction with other transcription factors and depending on promoter context.

We and others have previously shown that the Msx-2 homeodomain protein is also a repressor of OC gene transcription (25–27). The results presented here reveal that Dlx-5 and Msx-2 recognize the same homeodomain motif located in the tissue-specific promoter element OC-box I. Interestingly, Msx-2 is expressed at maximal levels in proliferating osteoblasts, whereas maximal Dlx-5 expression is restricted to differentiated osteoblasts. The stringent negative regulation of OC expression by Msx-2 in the proliferation period and by Dlx-5 later in differentiated osteoblasts is consistent with the concept that OC biosynthesis is tightly controlled at specific stages of osteoblast maturation to facilitate developmental modifications in extracellular matrix composition, mineralization, and maintenance of the bone cell phenotype.

Recently, Abate-Shen and colleagues (41) have shown that Dlx-2, which recognizes the same DNA motif as Dlx-5, can activate transcription of chimeric promoter constructs. Furthermore, Dlx-2 and Dlx-5 are each capable of abrogating Msx-2-dependent repression by direct protein/protein interactions, which results in mutual interference of Msx and Dlx binding activity. These results may have important ramifications for understanding the functional effects of modulations in Msx-2 and Dlx-5 levels during osteoblast differentiation. For example, our data show that during the postproliferative period, but before the onset of extracellular matrix mineralization, there is a brief temporal overlap in Dlx-5 and Msx-2 expression. At this time, Dlx-5 and Msx-2 may form heterodimers and mutually negate repressive transcriptional effects, which could result in transient derepression of OC gene transcription coinciding with induction of OC gene expression.

Dlx-5 Expression and Osteoblast Differentiation

The rat Dlx-5 gene is expressed in developing cartilage, discrete neuronal tissues, and teeth (19). The mouse homolog of the gene is also expressed in all developing skeletal elements (20), with Dlx-5 expression proceeding along the mineralization front during long bone growth. Similarly, the chick homolog of Dlx-5 plays an important role in limb bud development and cartilage differentiation (9). These data suggest

that Dlx-5 genes have important and evolutionary conserved roles in tissue development of cartilage and bone. Our findings provide an important demonstration that Dlx-5 expression correlates with osteoblast differentiation and suggest Dlx-5 may be involved in maturation of the bone cell phenotype. We show that maximal expression of Dlx-5 occurs in the final stages of osteoblast differentiation *in vitro* when the extracellular matrix mineralizes. This pattern of Dlx-5 expression may reflect a general role of Dlx-5 in lineage commitment and progression of osteoblast differentiation.

Of interest, the *runt* domain containing CBFA/AML factors were shown to regulate bone tissue-specific expression of the OC gene (42–46). More recently the CBFA1/AML-3 transcription factor was shown to be abundantly expressed in bone and less in thymus, but no other soft tissues (42, 45), and is a key regulator of bone formation in the developing embryo (46–48) and osteoblast differentiation *in vitro* (42). CBFA1/AML-3 appears slightly later in the formation of the mouse skeleton (9–12 days) (46–48), followed by the homeodomain proteins, Msx-2 and Dlx-5 (11, 19, 41, 49–51). Both Dlx-5 and Msx-2 are coexpressed in similar zones during early embryological development (*e.g*. the AER) at approximately 8.5 days (8, 10), and their expression is prominent along the anterior margin of the limb bud mesenchyme. CBFA1/AML-3 expression peaks at 12.5 days in mesenchymal condensations of developing bone structure (46, 47) before the first ossification center at 14.5 days. Dlx-5, Msx-2, and CBFA1/AML-3 may control osteogenesis analogous to the regulators of myogenesis (*e.g*. MyoD, myf-5, myogenin, and MRF4) (52, 53) and adipocyte differentiation (*e.g*. PPAR γ and C/EBPs)(54–56). Each of these sets of factors together control lineage determination and/or execution of the final differentiation program. Msx, Dlx, and AML genes may provide a cascade of factors that contribute to the initial formation and development of the skeleton and to osteoblast differentiation and maturation during postnatal bone formation. Thus, Dlx-5 appears to be a component of the combinatorial mechanism that controls formation and differentiation of skeletal tissues and may contribute to the progression of osteoblast differentiation.

MATERIALS AND METHODS

Cell Culture and Tissue Isolation

Rat osteoblasts (ROB) were prepared from calvaria of 21-day fetal rats as described by Owen *et al*. (57). Briefly, ROB cells were plated at a density of 6.5×10^5 cells per 100-mm dish and grown in MEM (GIBCO, Grand Island, NY) supplemented with 10% FCS. Osteoblast mineralization was enhanced by the addition of 50 mg/ml ascorbic acid and 10 mM β-glycerophosphate in medium on day 5 after plating. Osteoblast-like rat osteosarcoma cells, ROS 17/2.8 (58), were cultured at a density of 4.0×10^5 cells per 100-mm dish in F-12 medium (GIBCO) supplemented with 5% FCS. C2C12 mouse myoblasts were plated for transfection at 4×10^5 cells per 100-mm disk in DMEM (GIBCO) supplemented with 10% FCS. IMR-90 rat fibroblasts were plated for transfection at 6×10^5 cells per 100-mm dish in Basal Medium Eagle (BME, GIBCO) supplemented with 10% FCS. Soft tissues and bone were harvested from a 3-month-old male mouse and stored at −70 C. Calvaria and long bone were cleaned of periosteum and marrow.

RNA Isolation and Purification

Total cellular RNA was extracted with TriZol (GIBCO/BRL, Gaithersburg, MD) (59) according to the manufacturer's instructions. To extract cytoplasmic RNA, harvested cells were resuspended in lysis buffer (50 mM Tris-Cl, pH 8.0, containing 100 mM NaCl, 5 mM $MgCl_2$, and 0.5% Nonidet P-40), incubated for 5 min on ice, and centrifuged at $15{,}000 \times g$ for 2 min at 4 C. RNA was extracted from the supernatant with TriZol. Frozen tissues were ground and resuspended in TriZol solution to extract the RNA. Extracted RNA (100 μg) was incubated for 30 min at 37 C with 20 U of RNasin (Promega, Madison, WI) and 20 U of RNase free DNase I (Promega, Madison, WI) in 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$. Samples were phenol/chloroform extracted, chloroform extracted, and then ethanol precipitated. RNA was resuspended in DEPC (diethylpyrocarbonate)-treated water. The RNA integrity was assessed by the 28S/18S ribosomal RNA ratio after electrophoresis in 1% agarose/5.5% formaldehyde gels.

Differential Display

Differential display analysis was carried out according to Ref. 30 with modifications as described by Zhao *et al*.) (60). Briefly, RNA was prepared from ROB cells harvested on day 2 (proliferating cultures) and day 21 (mineralized cultures). Total RNA (300 ng) was reverse transcribed in a 30-μl reaction mixture with 300 U of superscript murine Moloney leukemia virus reverse transcriptase (GIBCO/BRL) and 100 U of RNasin in the presence of 2.5 mM of random hexamer and 20 mM deoxynucleoside triphosphate (dNTP) for 60 min at 40 C. Control reactions were performed in the absence of reverse transcriptase. Two microliters of the reverse transcription product were amplified with the GeneAmp kit (Perkin-Elmer Cetus, Norwalk, CT) in the presence of 0.5 μM 3′-primer and 5′-primer with 2 μM dNTPs and 0.5 μl ^{35}S-αdATP (NEN, Boston, MA). To detect Dlx family members, a 5′-primer (5′-ANCNCAGGTSAAAATCTGG-3′) was designed from very highly conserved homeobox sequences, and 3′-primers were designed from the loosely conserved 3′-end of Dlx coding regions (5′-GGCAGGTGGGAATTGATTGA-3′; D3). The buffer, $MgCl_2$, and *Taq* polymerase concentrations were as suggested by the manufacturer (Perkin-Elmer Cetus). The temperature profile was as follows: one cycle of 94 C for 1 min, 42 C for 4 min, and 72 C for 1 min, followed by 35 cycles as follows: 94 C for 1 min, 60 C for 2 min, and 72 C for 1 min, and a final 5-min elongation at 72 C. Amplified cDNAs were separated on a 6% 29:1 polyacrylamide denaturating gel. The gel was dried and exposed for 24–48 h to BioMax film (Kodak, Rochester, NY). The cDNA bands of interest were excised and eluted with 100 μl of Tris-EDTA. The eluted cDNA was ethanol precipitated. One half of the recovered cDNA was reamplified under the same PCR conditions as the first PCR reaction in the absence of isotope and increased dNTP concentrations to 20 μM. Ten of 50 μl of the PCR product were electrophoresed in a 1% agarose gel to estimate molecular weight and concentration of cDNA in the PCR product. The remaining samples were stored at minus]20 C for screening and cloning. The PCR product was cloned into the pCR II vector using the TA-cloning system (Invitrogen, San Diego, CA). The cloned cDNA insert was sequenced with Sequenase Version 2.0 (USB, Cleveland,

OH). The nucleotide sequences obtained were compared with known sequences by searching GenBank and EMBL databases (March 1996) with the Fasta program (Genetic Computer Group, Madison, WI).

Northern Blot Analysis

Total cellular RNA (20–30 μg) was separated on a 1% agarose/5.5% formaldehyde gel and transferred to Zetaprobe membrane (Bio-Rad, Melville, NY) using 20× NaCl-sodium citrate (SSC) buffer. RNA was cross-linked to filters by UV irradiation for 1 min and stored until use. DNA probes, either PCR product or cloned cDNA of Dlx-5 (this paper), human histone H4 cDNA (pFO 002) (61), rat OC (62), and rat Msx-2 (25) were labeled with α-[^{32}P]dCTP (3,000 Ci/mmol; NEN, Boston, MA) using the random primer technique (63). The blot was prehybridized in 50% formamide, 5 × SSPE (0.18 M NaCl, 0.01 M NaH_2PO_4, 0.001 M Na_2EDTA, pH 7.7), 5 × Denhardt's solution, 0.1% SDS, and 100 μg/ml salmon sperm DNA at 42 C for 3 h. For hybridization, 10^6 cpm/ml of heat denatured radioactive DNA probe was added and incubated at 42 C overnight. After hybridization, the blots were washed three times in 2 × SSC/0.1% SDS at room temperature for 15 min each, twice in 0.1 × SSC/0.1% SDS at room temperature for 20 min each, and twice in 0.1× SSC/0.1% SDS at 42 C for 20 min each. Blots were exposed to Kodak XAR film at −70 C with intensifying screens.

Plasmids

Complementary DNA containing 95% of Dlx-5 coding sequences, including the translation start site, was obtained by RT-PCR and cloned into the pCR II vector (Invitrogen, San Diego, CA). Dlx-5 sequences were then subcloned into pcDNA I/Amp (Invitrogen, San Diego, CA) using the *Xba*I/*Hin*dIII restriction sites and designated pDlx-5. For expression in eukaryotic cells, Dlx-5 sequences were removed from pDlx-5 by *Eco*RI digestion and placed in the pUHD10–3 vector, which contains the tTA responsive element (64). This clone is designated pUHD10–3/Dlx-5. Homeobox-deleted Dlx-5 was produced by PCR amplification of part of the Dlx-5 clone, as previously described by Zarlegna *et al.* (65), and designated pDlx-5-Del. Clones were confirmed by restriction digestion and sequencing.

OC promoter deletion constructs in the luciferase vector, −1050 OC-Luc, −637 OC-Luc, −199 OC-Luc, and −83 OC-Luc, were constructed as previously described by Towler *et al.* (27). OC promoter/OC-Box mutant constructs in the pGL2 luciferase vector were subcloned from previously constructed promoter mutants (25) as follows: Promoter fragments were removed by *Bgl*II/*Hin*dIII (−351 to +32 of OC promoter) or *Xho*I/*Hin*dIII (−1097 to +32 of OC promoter) digestions and placed into pGL2-luc (Promega, Madison, WI). The plasmids used for tTA stable transfection, pUHD 15–1, pUHD 13–3, pUHD 10–3, and pSV2Neo, were the kind gift of Dr. Gossen (64). RSV-luc and CMV-luc reporter plasmids contain the luciferase gene (66) in the pGL2 vector (Promega). 205 H4CAT is described by Ramsey-Ewing *et al.* (67) as F0108CAT and contains 205 nucleotides of the H4 proximal promoter. TKCAT is pBLCAT2, as described by Luckow and Schütz (68). The SV40CAT is described by Gorman *et al.* (69) as pSV2CAT. Construction of the VDRE tetramer construct is described in Blanco *et al.* (70). The osteopontin (OP)-CAT chimeric gene construct pOPCAT is described in Ref. 71 and contains 776 nucleotides of the proximal osteopontin promoter. The AML-1B expression construct has been described by Meyers *et al.* (72), and construction of the AML/108CAT reporter gene plasmid (pTGRECAT) has been documented by Banerjee *et al.* (73).

Stable Transfection of Tetracycline-Regulated Transactivator

Stable transfection of tTA into ROS 17/2.8 rat osteosarcoma cells was done via the calcium phosphate coprecipitation method (74). Briefly, ROS 17/2.8 cells were plated at a density of 0.65×10^6 cells per 100-mm plate 24 h before transfection. pUHD 15–1 plasmid (18.6 μg) was cotransfected with 1.4 μg pSV2Neo. G418-resistant colonies were selected by adding 150 μg/ml of G418 into the media for 2–3 weeks. Success of stable transfection of tTA, pUHD 15–1, was screened by transient transfection of pUHD 13–3, which contains a tTA-responsive element controlling luciferase. Clone 316 showed 20-fold induction of luciferase activity when tetracycline (1 μg/ml) was removed and was therefore chosen for further experiments.

Clone 316 was subject to second round stable transfection with pUHD10–3 Dlx-5 and a Tk-hygromycin vector (75) using the calcium phsophate coprecipitation method (74). The transfected cells were maintained for 2 to 3 weeks in selection medium containing F12 (GIBCO, BRL, Grand Island, NY), 5% FCS, 150 μg/ml G418, 200 μg/ml hygromycin B (Calbiochem, La Jolla, CA), and 1 μg/ml tetracycline. Viable colonies were subcultured under the same conditions and used for experiments and screened by Northern analysis for tetracyclin-controlled regulation of Dlx-5 expression. To determine the effect of Dlx-5 overexpression on OC gene expression, cells were cultured at a density of 6×10^5 cells per 100-mm plate and maintained in selection medium. Seven days after plating, Dlx-5 expression was induced by removal of tetracycline from the medium 48 h before harvesting.

Transfection Assays

Cells were plated at a density of 4–6 × 10^5 cells per 100-mm plate for transient transfection experiments. ROS 17/2.8, ROB, C2C12, and tTA stably transfected ROS 17/2.8 cells were transfected by the diethylaminoethyl-dextran method (74). IMR-90 cells were transfected by the HEPES/calcium phosphate method (74) with a 1-min 10% dimethylsulfoxide shock. The total amount of exogenous DNA was maintained at 20 μg/plate consisting of 2 μg luciferase construct, 8 μg CAT construct, 4 μg of a Dlx construct, and 6 μg Salmon sperm DNA. The IMR-90 cells were transfected with 1 μg AML-1B expression plasmid and 5 μg Salmon sperm DNA. All plasmid DNA was prepared using Qiagen Maxi Kits (Qiagen Inc., Chatsworth, CA) and checked for supercoiled structure on 1% agarose/0.045 M Trizma base/0.045 M boric acid/1.25 mM EDTA (TBE) gels. Plasmids of similar quality were used for comparison of relative expression in each experiment. Cells were harvested 48 h after transfection.

Luciferase and CAT Assays

Luciferase activity was determined using the luciferase assay system (Promega, Madison, WI). The cell pellets were treated with 1× reporter lysis buffer (0.25 M Tris-HCl, pH 8.0, 0.1% Triton X-100, Promega, Madison, WI), and luminescence was measured on a Monolite 2010 (Analytical Luminescence Laboratory, San Diego, CA). CAT activity was determined as previously described (74). The samples were incubated with 0.25 μCi (1 Ci = 37 gigabecquerels) of [^{14}C]chloramphenicol (Dupont, Boston, MA) for 4–12 h, extracted with ethyl acetate, and separated by chromatography. Results were evaluated using a β-scope 603 blot analyzer from Betagen (Mountain View, CA).

In Vitro Transcription and Translation of Dlx5 Protein

Plasmids containing wild type Dlx-5 and homeobox-deleted Dlx-5 sequences in the pCR II vector were linearized by

restriction digest and used as templates for *in vitro* transcription with the Sp6 promoter. These transcripts were used for translation of protein using the TNT TM Coupled Reticulocyte lysate system (Promega, Madison, WI).

Gel Mobility Shift Assay

Wild type OC-Box oligonucleotide was end labeled with ^{32}P γ-dATP by using T4 polynucleotide kinase. The probe and mutant oligonucleotide used as competitors are in Table 1. Gel mobility shift assays were performed by binding *in vitro* transcribed and translated Dlx-5 protein (5 μl) to a labeled, double-strand DNA probe in the presence or absence of 50-fold molar excess of competitor for 10 min at room temperature. The binding reaction mixtures contained 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5% glycerol, 5% sucrose, 0.2 mM EDTA, 7.4 mM $MgCl_2$, 500 μg BSA per ml, 0.1% Nonidet P-40, 50 μg poly(deoxyinosinic-deoxycytidylic)acid per ml, and 10 mM dithiothreitol. Protein-DNA complexes were separated at 4 C on a 6.5% polyacrylamide gel containing 0.5× TBE buffer.

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