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ARTICLES

HLH Transcription Factor Activity in Osteogenic Cells

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Abstract To examine possible mechanisms underlying osteoblast differentiation from mesenchymal stem cells, we investigated bHLH functional activity in cell lines representing different stages of osteoblast maturation. Interaction of nuclear proteins with oligonucleotides corresponding to various bHLH binding sequences (known as E-boxes) was determined in mobility shift assays. Both ADD-1 oligonucleotide, a binding site for transcription factor ADD-1, and OCE-1, an E-box from osteocalcin promoter, produced retarded bands after incubation with nuclear extracts from osteogenic cells. Cells at different stages of osteogenic maturation demonstrated similar patterns and intensity of binding, as did cells treated with different osteogenic inducers. Binding to ADD-1 and OCE-1 was not tissue-specific as it was also observed in fibroblastic 10T1/2 cells. MEF-1 oligonucleotide, the E-box sequence from the muscle creatine kinase enhancer, demonstrated no changes in binding with nuclear extracts from moderately differentiated (W-20) or relatively mature (ROS 17/2.8) cells under any conditions tested. However, in poorly differentiated RI-2J cells, which do not express osteogenic markers unless treated with dexamethasone, induction of differentiation was reflected in transient inhibition of binding to MEF-1. Inhibition of binding was not seen under differentiation-restrictive conditions. Promoter-reporter studies also demonstrated inhibition of MEF-1 driven CAT expression by dexamethasone under differentiation-permissive conditions in RI-2J cells. These data suggest that bHLH gene expression is not required for the early steps of osteogenesis; moreover, inhibition of bHLH protein binding to a MEF1-type E box might be an integral part of osteogenic commitment. J. Cell. Biochem. 65:1–10.

Key words: bHLH functional activity; osteoblast differentiation; gene expression; osteogenesis

During the past several years, data has accumulated indicating that osteogenesis begins in a multipotential stem cell of the mesenchymal lineage which is also a precursor for myoblasts, adipocytes and chondrocytes. Clonal cell lines can be derived from calvaria or bone marrow stromal cells which differentiate into not only osteoblasts but also myoblasts, adipocytes, and chondrocytes [Grigoriadis et al., 1990; Yamaguchi and Kahn, 1991; Dorheim et al., 1993; Gimble et al., 1994; Okuyama et al., 1995]. In vivo, bone morphogenetic proteins (BMPs) can induce bone formation in muscle, and in cultured myogenic cells treatment with BMP-2 blocks myogenic differentiation while at the same time induces osteoblastic features [Murray et al., 1993; Katagiri et al., 1994].

For myogenic cells, establishment of the differentiated phenotype is predominantly under the regulation of members of basic helix-loop-helix (HLH) gene family [Olson and Klein, 1994]. The myoblast-specific bHLH transcription factors such as MyoD, myogenin, and myf5, form heterodimers with the more ubiquitously expressed E2A gene products, E12 and E47. These heterodimers bind promoter and enhancer sequences (collectively called E-boxes) of tissue-specific genes and activate their transcription. Other members of the HLH family, including Id-1, Id-2, and twist, are dominant negative regulators which interrupt this process by dimerizing with E12 and E47 proteins, thus preventing MyoD/E2A dimer formation and differentiation [Benezra et al., 1990; Murray et al., 1992].

The presence of negative Id HLH regulators has been demonstrated in osteoblastic cells [Ogata and Noda, 1991; Ogata et al., 1993]. Moreover, Id expression was modulated by the

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agents known to promote osteogenesis [Glackin et al., 1992; Murray et al., 1992; Ogata et al., 1993] and the osteocalcin gene promoter was shown to contain an E-box sequence crucial for osteocalcin gene expression [Tamura and Noda, 1994]. These data suggest that HLH genes might be responsible for osteogenic commitment and/or subsequent differentiation in a manner similar to that in myogenic cells. We have therefore investigated the expression of HLH genes during osteogenic differentiation by examining the interaction of proteins with bHLH binding sites (E-boxes) using nuclear extracts from osteogenic cells and by assessing the functional activity of E-box-containing promoters in expression constructs transfected into various osteogenic cell lines.

MATERIALS AND METHODS

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated. Recombinant human BMP-2 and the murine stromal cell line W-20 [Thies et al., 1992] were generously provided by Genetics Institute (Andover, MA). Fetal bovine serum (FBS) was preselected by assaying for alkaline phosphatase (APase) activity and osteopontin (OP) mRNA and identifying those lots showing high levels with dexamethasone (Dex) but low levels in the absence of inducer. The selected serum was purchased from Atlanta Biologicals. ITS+™-Premix serum substitute was purchased from Collaborative Biomedical products and tissue culture media were obtained from Gibco BRL. ROS 17/2.8 osteosarcoma cells were donated by Dr. G. Rodan (Merck), and 10T1/2 mouse embryonic fibroblasts were purchased from the University of Pennsylvania Cell Center. Isolation of immortalized RI-2J cell line is described below.

Cell Culture

ROS 17/2.8, W-20, and 10T1/2 fibroblasts were maintained in DMEM with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in 5% CO_2. RI-2J cells were cultured in α-MEM with the same serum and antibiotic supplements.

Cell Immortalization

Rat bone marrow cells were immortalized with a retrovirus containing the large T antigen of SV-40 virus and the Neo® gene. Briefly, bone marrow cells were collected from femurs of 4–5-week-old Wistar rats as described previously [Leboy et al., 1991]. Cells were plated at a density of 1.5×10^6 cells/cm^2 in α-MEM with 15% FBS and 2 mM L-glutamine (day 0). On day 1, polybrene (2 µg/ml) was added and the incubation continued overnight. On day 2, culture medium was replaced with a retrovirus-containing supernatant. After 3 h incubation at 37°C with gentle mixing, virus-containing medium was substituted with regular culture medium. Selection for resistance to 500–1000 µg/ml G418 was started on day 5 and continued for 3 weeks. Neomycin-resistant clones were collected and screened for APase activity in the presence and absence of dexamethasone (Dex) or 1,25 dihydroxyvitamin D3.

Oligonucleotides

Oligonucleotides corresponding to different E-box sequences were synthesized in the Nucleic Acid Facility of the University of Pennsylvania. The sequences of their top strands were as follows:

OCE-1: 5′ GGTACCTTGACCTATTGCGCACATGACCCCC 3′
ADD-1: 5′ GGTACCGATCCAATTGGGCAATCAGGAC 3′
μE_5E_2: 5′ GGTACCAGAACACCTGCAGCAGCTGGCAGGC 3′
MEF-1: 5′ GGTACCCCCCCCAACACCTGCTGCCTGAGCCC 3′.

Nuclear Extract Preparation and Mobility Shift Assay

Nuclear extracts were prepared from $1–5 \times 10^6$ cells as described by Schreiber et al [1989]. E-box-containing oligonucleotides were end-labeled with ^{32}P using T4 polynucleotide kinase. Binding was carried out with 5 µg of protein (1–2 µl of nuclear extract), 2 µg of Poly-d(I-C), and 3 µl of 5x binding mixture (60 mM Hepes, pH 7.9, 20 mM Tris-HCl, pH 7.9, 300 mM KCl, 5 mM EDTA, pH 8.0, 60% glycerol, and 5 mM DTT) in a total volume of 15 µl for 20 min at room temperature. Labelled oligonucleotides (0.05 pmols, $2–4 \times 10^4$ cpm/reaction) were added and incubation continued for additional 20 min. Protein-DNA complexes were separated on polyacrylamide gel (Acrylamide: BisAcrylamide = 29:1) for 2 h at 100 V. For each condition, at least three different preparations of nuclear extracts were tested.

RNA Isolation, Northern Blotting, and Hybridizations

Total RNA was extracted by acid guanidinium isothiocyanate-phenol-chloroform as described previously [Leboy et al., 1989]. Twenty µg RNA was denatured in glyoxal buffer at 50°C for 1 h, chilled on ice, and separated by electrophoresis on 1% agarose gel with circulating buffer (0.01 M Na_2HPO_4 pH 6.5). RNA was transferred to nylon membrane (DuPont GeneScreen Plus, NEN Research, Boston, MA) by alkaline blotting with 5 mM NaOH. Hybridization of probes to ^{32}P-labelled probes was performed in 3 × SSPE with 50% formamide. Plasmids containing the following cDNAs were employed to generate the desired probes: rat osteopontin [Yoon et al., 1987], rat alkaline phosphatase [Theide et al., 1988], rat bone sialoprotein [Chen et al., 1992], and rat collagen α1(I) [Genovese et al., 1984] were used to produce nick-translated probes with a kit from BRL and α-^{32}P-dCTP; rat osteocalcin [Yoon et al., 1988] and Id (pMH18ΔR) [Benezra et al., 1990] were used to prepare riboprobes with a kit from Promega Corporation and α-^{32}P-CTP. Hybridizations were performed overnight at 42°–48°C for nick-translated probes and at 65°C for riboprobes. Hybridization membranes were washed with 2 × SSC-0.1% SDS at room temperature, followed by 0.1 × SSC-0.1% SDS at room temperature and then 0.1 × SSC-0.1% SDS at hybridization temperature. The extent of hybridization of probe to membranes was determined by autoradiography using Kodak XOMAT AR film, and quantitated with a Molecular Dynamics densitometer.

Assays for Alkaline Phosphatase and PTH-Stimulated cAMP

Kinetic assays for alkaline phosphatase activity were carried out as described previously [Leboy et al., 1989]. Measurement of cAMP levels after PTH stimulation were performed using the Biotrak cAMP[^{125}I] assay system (Amersham) which is based on competition between cAMP produced and a known amount of ^{125}I-labelled cAMP for a cAMP-specific antibody. Cells cultured in 35 mm dishes were washed with HBSS and exposed to 200 ng/ml of rat (1-34) PTH (Sigma) for 2 min. The medium was then removed, the cell layer washed with 1 ml fresh HBSS, and the cells in HBSS were transferred to a microfuge tube. Cells were pelleted by centrifugation and the cAMP extracted with 65% ethanol. Extracts were evaporated to dryness in a vacuum oven and resuspended in the Amersham assay buffer for measurement of cAMP.

Transfection With MEF-CAT Constructs

Cells were cotransfected, using the $CaPO_4$ precipitation procedure [Ausubel et al., 1990], with a 5:1 mixture of plasmid containing five copies of the MEF-1 E-box upstream of the chloramphenicol acetyltransferase (CAT) reporter gene and plasmid containing the SV-40 virus promoter/β-galactosidase gene. After 48-72 h, cells were harvested in Tris HCl buffer, disrupted by sonication and centrifuged. All supernatants were tested for β-galactosidase activity with o-nitrophenyl-β-D-galactopyranoside substrate to determine transfection efficiency [Alam and Cook, 1990].

For CAT assays, cell extracts containing equal amounts of β-galactosidase activity were assayed with ^{14}C-chloramphenicol using silica gel TLC [Ausubel et al., 1990]. Relative amounts of unreacted CAT and acetylated CAT were then assessed by densitometry scanning of X-ray films.

RESULTS

Characterization of Cell Lines

To evaluate the activity of HLH genes over the course of osteogenesis, three cell lines which represented different stages in osteogenic differentiation were employed.

The immortalized rat stromal cell line RI-2J was the least differentiated cell line used in these studies. At early times in culture without inducer, these cells expressed mRNA for collagen α1(I) but the levels of alkaline phosphatase (APase) and osteopontin (OP) mRNA were negligible. With prolonged cultivation PTH-dependent cAMP production was detected, but induction of OP and APase mRNA required treatment with Dex (Table I). Although BMP-2, like Dex, acts on primary stromal cells to induce the full spectrum of osteoblastic markers [Rickard et al., 1994], BMP-2 treatment of RI-2J cells induced only APase, but not OP mRNA. No osteocalcin or bone sialoprotein mRNA was detected under any culture conditions. Thus, RI-2J cells can, in the presence of Dex, show some properties characteristic of preosteoblasts, but they appear unable to differentiate further.

TABLE I. Characterization of RI-2J Cell Line*

	OP mRNA	AP mRNA	Collagen I mRNA	PTH-induced cAMP[a] @ day 1	PTH-induced cAMP[a] Confluent
Control	1	1	1	1.06	1.48
Dex (10^{-8} M)	5.1 ± 1.0	26.3 ± 23.7	2.1	1.15	1.25
BMP-2 (100 ng/ml)	0.9 ± 0.7	20.0 ± 21.2	ND	1.23	1.66

*Total RNA was prepared from day 3 pre-confluent cultures. Twenty μg of each RNA sample was separated on glyoxal gels and hybridized to probes as described in Materials and Methods. Films of Northern blots were scanned with a Molecular Dynamics densitometer and the values for Control cultures set to 1.0. ND = Not determined.
[a]Results are presented as a ratio of cAMP amount in PTH-treated vs untreated cultures. cAMP values ranged from 65–71 fmol cAMP/dish for day 1 samples from non-PTH treated cells to 106–114 fmol cAMP/dish for confluent cultures treated with BMP. All assays were performed with duplicate samples.

TABLE II. Cell Lines Used in the Study

Cell line	Source	Inducer	Properties PTH[a]	OP[b]	AP[b]	BSP[b]	OC[b]	Ca^{2+} [c]
ROS 17/2.8	Rat osteosarcoma	none	+	+	+	+	+	+
		Dex	+	+	+	+	+	+
		BMP-2	+	+	+	+	+	+
W-20	Mouse clonal bone marrow	none	(+)	(+)	(+)	−	−	−
	stromal cell line	Dex	ND*	(+)	(+)	−	−	−
		BMP-2	+	+	+	−	+	−
RI-2J	Rat bone marrow stromal	none	(+)	−	−	−	−	−
	cells immortalized with	Dex	+	+	+	−	−	−
	SV-40 large T	BMP-2	+	−	+	−	−	−

*ND = Not determined. (+) = Expressed only after confluency.
[a]cAMP response to PTH stimulation.
[b]mRNA expression.
[c]Alizarin Red stain for Ca^{2+}.

W-20 is a non-immortalized mouse bone marrow stromal cell line [Thies et al., 1992]. Relatively undifferentiated in sparse cultures, at confluency these cells express high levels of OP and APase mRNA with or without Dex. BMP-2 further enhances APase mRNA expression and induces appearance of OC mRNA and PTH responsiveness. These cells do not express bone sialoprotein mRNA under any culture conditions (Table II).

The ROS 17/2.8 cells demonstrated properties previously reported for this osteosarcoma cell line [Rodan et al., 1988]. Subconfluent cultures expressed substantial levels of OP and APase mRNA, and osteocalcin mRNA was present in the absence of VitD. With confluency, levels of these mRNAs increased and bone sialoprotein mRNA was detected. In addition, the cells produced mineralized nodules in the presence of β-glycerophosphate. Dex enhanced expression of APase and bone sialoprotein, while VitD elevated levels of OP, APase, and osteocalcin.

Together, the selected cell lines encompassed a broad spectrum of osteogenic differentiation (Table II), with fibroblastic cells initially expressing none of the osteoblast markers (low density RI-2J cells), preosteoblasts showing low levels of early osteoblast markers (Dex-treated RI-2J, low density W-20 cells), and mature mineralizing osteoblastic cells (confluent ROS 17/2.8 osteosarcoma cells).

Binding of Nuclear and Cytoplasmic Proteins From Osteogenic Cells to E-Box Sequences

The various members of the bHLH family of transcription factors bind as dimers to different E-box sequences. Therefore, in an attempt to characterize the HLH profile of osteogenic cells we used several E-box oligonucleotides known to interact with various HLH proteins and to be expressed in different mesenchymal derivates: 1) MEF-1 (from muscle creatine phosphokinase enhancer) which preferentially binds complexes containing MyoD family proteins as well as ubiquitous E2A gene products [Braun et al.,

1989]. 2) μE_5E_2-the binding site for E2A gene products E_{12} and E_{47} from the immunoglobulin promoter region [Wilson et al., 1991]. 3) ADD–the binding site for an HLH-zip protein (ADD-1) expressed in adipogenic cells [Tontonoz et al., 1993]. 4) OCE-1-an E-box sequence from the osteocalcin gene promoter reported to bind HLH-type proteins in an Id-dependent fashion and crucial for OC gene expression [Tamura and Noda, 1994].

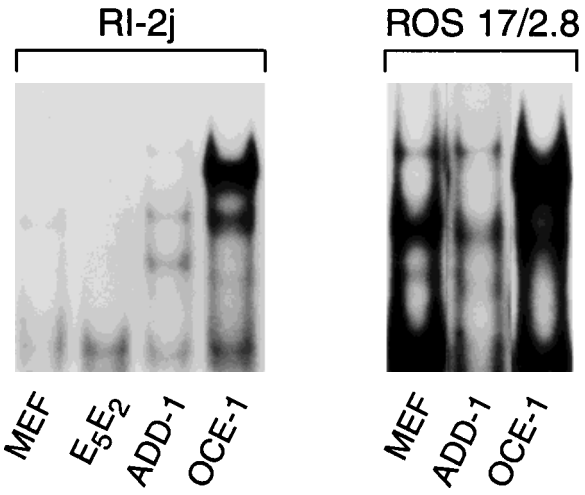

Fig. 1. Binding of nuclear proteins from osteoblastic cells to ^{32}P-labelled E-box oligonucleotides. Mobility shift assay with nuclear extracts from RI-2J (**left panel**) and ROS 17/2.8 cells (**right panel**) produces retarded bands with each of the E-box sequences tested. While one band in the middle appears to be common to MEF-1, ADD-1, and OCE-1, others are unique to each oligonucleotide.

E-box binding with different oligonucleotides. To assess the E-box binding capacity of osteoblastic cells with these four oligonucleotides, nuclear extracts from ROS 17/2.8 and RI-2J cells were prepared and used in mobility shift assays. Both extracts produced retarded bands with all of the oligonucleotides. The binding of nuclear proteins to MEF-1, ADD, and OCE-1 showed both a band common to all of the oligonucleotides and some bands which were unique to each oligonucleotide (Fig. 1). Specificity of binding was analyzed by competition experiments. With ADD, OCE-1 and MEF-1, the DNA-protein bands disappeared in the presence of 50x excess of cold oligonucleotide identical to that used as a probe; results with ADD and OCE-1 oligos are shown in Figure 2. Cross competition experiments were also performed, using different E-box oligonucleotides as cold competitors for binding to a single radioactive oligonucleotide. These analyses demonstrated competition for some bands among different E-boxes, arguing that similar proteins participated in their formation. When the μE_5E_2 oligonucleotide was radioactively labelled, the bands were not competed by a 50x excess of either μE_5E_2 or any of the other oligos tested (data not shown); thus binding to the μE_5E_2 DNA appeared nonspecific. In addition,

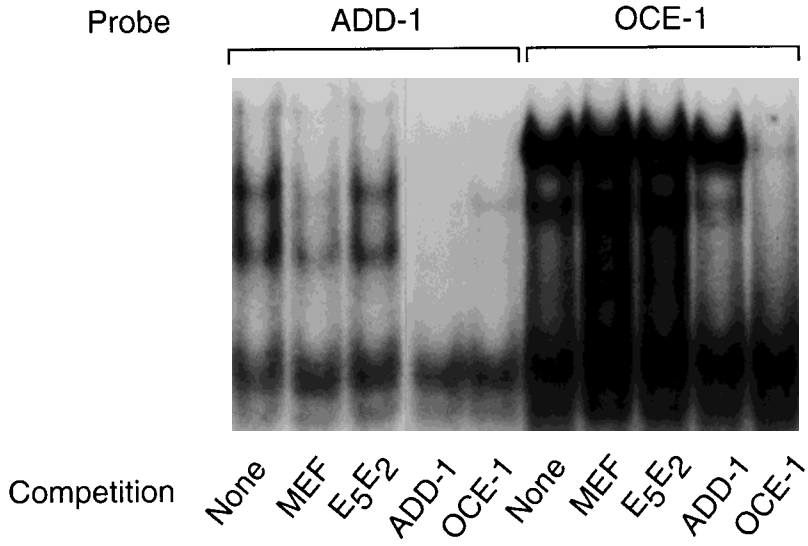

Fig. 2. Specificity of E-box binding to ADD-1 and OCE-1 oligonucleotides. Competition experiments were performed using 50x molar excess of cold oligonucleotides as competitors. In each case specific binding was totally eliminated by the presence of a cold oligonucleotide identical to the one used as a probe. Various degrees of cross-competition between different E-box sequences were also observed; both MEF-1 and OCE-1 competed for ADD-1 binding proteins while ADD-1 diminished intensity of one of the OCE-1 bands.

cold μE_5E_2 did not interfere with formation of any of the bands formed by other three oligonucleotides (Fig. 2). These results argue against the presence of functionally active E12/E47 heterodimer in RI-2J cells.

Expression of HLH-like activity in osteoblastic cells of different differentiation status. To detect whether osteogenic differentiation is accompanied by changes in the binding ability of HLH proteins, we used two approaches. Fibroblastic cells and cell lines which represent different stages in osteogenesis were compared with respect to protein binding to different E-box sequences. In addition, we examined whether E-box binding was altered under the influence of factors and conditions known to induce, promote, or modulate osteogenesis (confluency, Dex, BMP-2).

Differences in the maturation status of various osteogenic cell lines, cultured without inducers, were not reflected in differences in E-box binding. Nuclear extracts from fibroblastic 10T1/2, low differentiated RI-2J, modestly differentiated W-20 and highly differentiated ROS 17/2.8 cells all produced similar patterns of binding with all oligonucleotides tested (Fig. 3). Consistent with these observations, addition of BMP-2 or Dex to W-20 and ROS 17/2.8 cells did not cause a detectable difference in binding to any of the E-box oligonucleotides tested despite the ability of the factors to modulate the differentiation status of these cells. Similarly, neither BMP nor Dex altered the pattern of RI-2J nuclear protein binding to ADD and OCE-1 oligonucleotides (data not shown).

MEF-1 was the only E-box containing oligonucleotide which showed differences in binding of nuclear proteins associated with differentiation of the cells. As shown in Figure 4, the intensity of binding of RI-2J nuclear extract to MEF-1, relatively low at the beginning of culture period (Fig. 1), was further inhibited by treatment with Dex, an agent capable of inducing the initial steps of differentiation. In seven independent assays, the intensity of binding to MEF-1 in control cells was 3.6 ± 0.8-fold higher than that in Dex-treated cultures. This inhibition was transient and was seen only during the initial culture period (days 1–3). At later stages of culture, Dex showed either no effect or elevated binding to the MEF-1 oligonucleotide.

Several experiments were performed to ascertain whether the inhibition of MEF-1 binding indeed paralleled the induction of differentiation or was a nonspecific response of these cells to Dex. RI-2J cells were cultured under conditions which either permitted or restricted the Dex-induced differentiation. Differentiation-permissive conditions were growth in 10% FBS throughout the culture period; under these con-

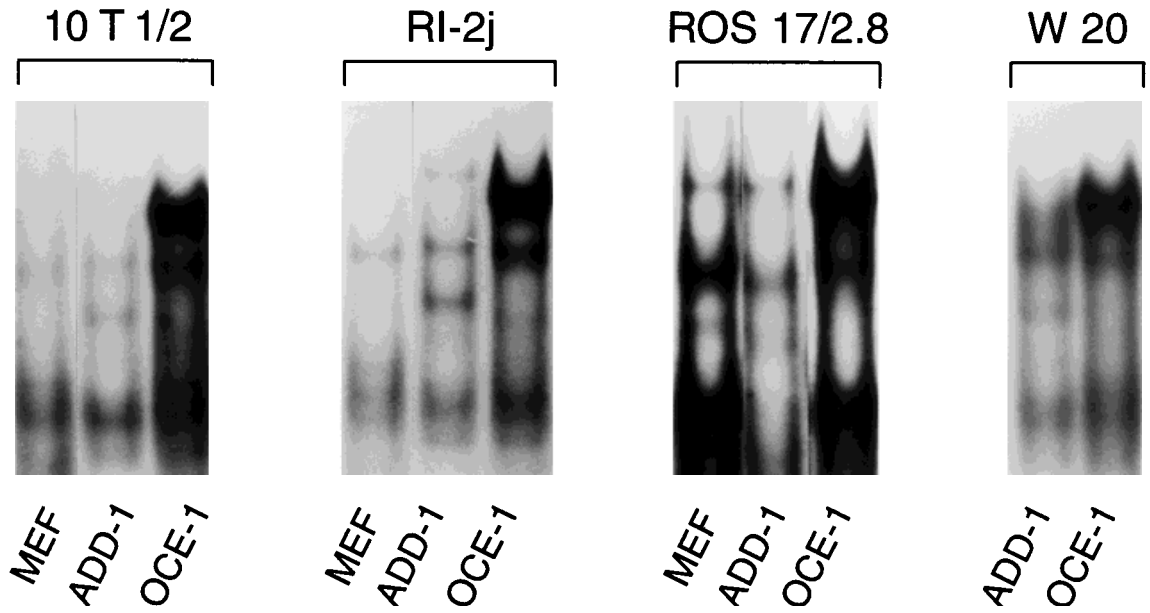

Fig. 3. Binding to E-box oligonucleotides is not tissue-specific. Similar patterns of binding of nuclear proteins to the panel of E-box-containing oligonucleotides were observed with nuclear extracts from fibroblastic cells (10T1/2) and osteogenic cells at different stages of maturation.

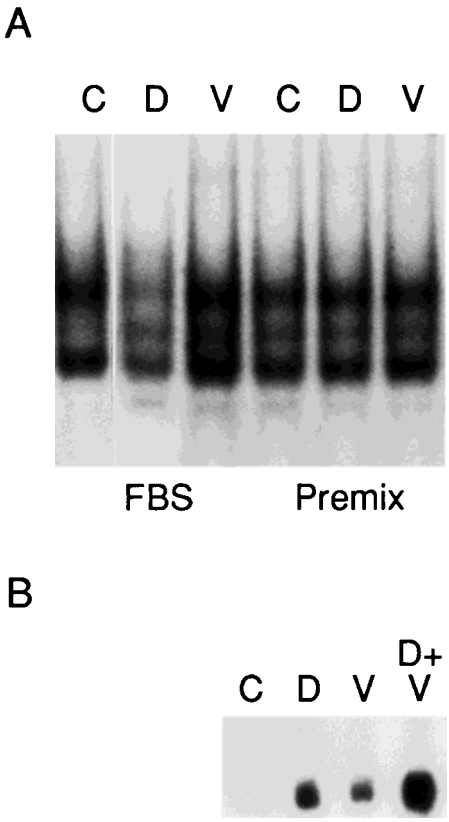

Fig. 4. Binding of nuclear proteins to MEF-1 sequence in RI-2J cells is down-regulated under conditions which induce differentiation of these cells. **A:** Induction of differentiation is accompanied by inhibition of binding to MEF-1 sequence. Under differentiation-restrictive conditions (ITS-Premix serum substitute instead of FBS in the culture medium) inhibition of binding is not detected. For each set of conditions, at least three different batches of nuclear extracts were tested. **B:** Northern blot hybridization for OP mRNA indicates that RI-2J cells cultured with FBS show Dex-induced expression of OP.

TABLE III. Comparison of Osteoblastic Differentiation of RI-2J Cells in Parallel Cultures in the Presence of Either FBS or Premix (Control = 1)*

	FBS		Premix	
Inducer	OP	AP	OP	AP
Control	1.0	1.0	1.0	1.0
10^{-8} M Dex	26.7	4.1	2.2	1.0

*20 ug of each RNA sample was separated on glyoxal gels and hybridized to probes as described in Materials and Methods. Films of Northern blots were scanned with a Molecular Dynamics densitometer and the values for control cultures set to 1.0.

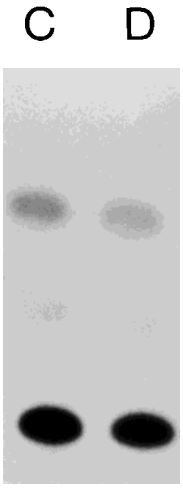

Fig. 5. CAT activity in extracts from RI-2J cells transfected with MEF-1-containing promoter-reporter construct. RI-2J cells were transiently transfected with a CAT construct containing the MEF-1 sequence and cultured for 2 days in the presence or absence of Dex (10^{-8} M) under differentiation-promoting conditions. Inhibition of CAT activity accompanied Dex-induced differentiation.

ditions Dex (with or without Vitamin D) induced both OP mRNA (Fig. 4B, Table III) and APase mRNA (Table III). Restrictive conditions involved replacing the FBS with ITS-Premix on day 1; this results in negligible OP or APase induction even after prolonged culture (Table III). As shown in Figure 4A, inhibition of binding in the presence of Dex was only seen under differentiation-permissive conditions. Thus, decreased nuclear protein binding to the MEF-1 oligonucleotide is associated with early stages of osteogenic induction in RI-2J cells.

Activity of E-Box Containing Promoter With Osteogenic Inducers

To test the functional activity of bHLH proteins in an osteogenic cell line and their capacity to regulate transcription in response to osteogenic inducers, RI-2J cells were transiently transfected with plasmids containing an E-box sequence linked to the reporter gene chloramphenicol acetyltransferase (CAT); the CAT reporter plasmid contained five MEF-1 sites linked to an alkaline phosphatase TATA box [Wilson et al., 1991]. The RI-2J cells were then cultured for two additional days with and without Dex. As shown in Figure 5, diminished CAT activity was seen when osteogenesis was induced by Dex. Like the DNA binding experiments, these CAT assays suggest that when the RI-2J cell line was induced to undergo osteogenesis with Dex, the early stages of induction (day 3) were associated with low levels of functional bHLH proteins.

Expression of a Negative Regulator of HLH Protein Function, Id, in the Course of Osteogenesis

bHLH proteins show diminished DNA binding when complexed with Id proteins. Therefore, expression of Id mRNA under conditions which produced different intensities of nuclear extract binding to MEF-1 was investigated. The low density RI-2J cells, which show limited expression of osteoblastic markers, contained significant amounts of Id mRNA; this was further increased by treatment with Dex (Table IV). When cells were cultured in differentiation restrictive conditions with ITS-Premix, Dex no longer enhanced Id expression. Thus, the pattern of Id expression in Dex-treated low density RI-2J cells was inversely correlated with MEF-1 binding. These results suggest that high Id (and the consequent low DNA binding by bHLH proteins) is associated with early stages of osteogenesis.

DISCUSSION

In myogenesis, where the role of HLH genes in governing differentiation is well established, the activity of these transcription factors is under strict developmental regulation. Some members of the MyoD family, e.g., myogenin, are not expressed until after induction of differentiation [Hollenberg et al., 1993; Olson and Klein, 1994]. Transcription of others such as MyoD is turned on after commitment of the multipotential progenitor cell into myoblasts, but their ability to bind DNA is blocked by the presence of large amounts of negative regulators (Id-twist family) [Jen et al., 1992]. Forced expression of any of these proteins in multipotential cells or mesenchymal cell lines is sufficient to activate myogenesis, and appearance of functionally active, DNA-binding HLH proteins is a hallmark of differentiating myotubes.

TABLE IV. Relative Levels of Id mRNA in RI-2J Cells Treated With Various Osteogenic Inducers*

Culture conditions	Days in culture	Id Message level (Control = 1.0)	Inhibition of binding[a]
Control	3	1.0	
Dex	3	2.3	+
Control	8	1.0	
Dex	8	2.1	–

*RNA was prepared from cells cultured with 10^{-8} M Dex. Films of Northern blots were scanned with Molecular Dynamics densitometer.

[a]Absence or presence of inhibition of MEF-1 binding in nuclear extracts produced from parallel cultures.

If osteogenesis were regulated by HLH genes in a manner identical to that in myogenesis, one would expect that different stages in osteogenesis would be associated with altered patterns of HLH binding activity. In addition, if changes in the extent of osteogenesis were achieved via changes in expression and/or activity of HLH transcription factors, it would be anticipated that agents which induce and/or modulate differentiation would induce binding of new HLH proteins to their recognition sequences. Contrary to these hypotheses, the studies reported here did not demonstrate appearance of novel bands under the influence of BMP and/or dexamethasone. Except for MEF-1, there were also no qualitative differences in binding among nuclear extracts from cells which represented different stages of osteogenic differentiation (Fig. 3). Moreover, nuclear extracts from fibroblastic 10T1/2 cells produced similar mobility bands with the same intensity (Fig. 3).

While our results would suggest that osteogenesis is not associated with elevated expression of HLH transcription factors, several alternative possibilities must be considered: 1) The similarities in mobility shift patterns might result from different proteins of the same class with similar molecular weights and heterodimerization patterns. 2) Protein-protein interactions which modulate activity of HLH transcription factors might be disrupted during nuclear extract preparation, allowing in vitro binding to E-box sequences which is normally suppressed in vivo. 3) Subtle differences in the amount rather than the type of functional HLH proteins may be sufficient for activation of different genes. This form of regulation was demonstrated in homeodomain genes during early development [Pankratz et al., 1990].

While new bands did not appear in association with osteogenic differentiation, inhibition of binding to MEF-1 E-box during the early steps of Dex-induced osteogenesis was apparent (Fig. 4A). This inhibition was evident only under conditions that promoted differentiation (RI-2J cells treated with Dex in the presence of FBS), and was not present either in more mature cells (ROS 17/2.8) or in cells not capable of Dex-induced osteogenesis (10T1/2 fibroblasts, data not shown). Thus, there was a parallel between induction of osteogenic differentiation and inhibition of HLH binding to MEF, an E-box known to bind HLH heterodimers contain-

ing myo-specific transcription factors of the MyoD family. This raises the possibility that the commitment of multipotential cells to osteogenesis requires suppression of myogenesis via inhibition of myo-specific HLH protein expression or DNA binding.

Inhibition of myo-specific MEF binding could be achieved either by diminished expression of the MyoD family of transcription factors or by increase in expression of Id, an inhibitor of their DNA binding. Indeed, early stages of osteogenesis (low density RI-2J cells) which displayed diminished MEF-1 binding also showed high Id mRNA expression which was further elevated by Dex treatment (Table IV). Similar elevation of Id mRNA was reported with Dex-treated MC3T3-E1 osteoblastic cells by Ogata and Noda [1991]. Both our results and those of Ogata and Noda show that Dex elevation of Id expression is sustained in later stages of osteoblast differentiation. However, in more differentiated cells such as confluent Dex-treated RI-2J cells or ROS 17/2.8, the correlation between Dex-elevated Id expression and inhibition of MEF-1 binding was no longer observed. It may be that increased Id message level is not paralleled by an increase in Id protein in differentiated cells. This possibility is in accord with the results of Jen et al. [1992]. These authors showed that after stable transfection of Id gene under a strong promoter, high levels of Id mRNA were sustained for a long period of time in culture, but Id protein was detected by antibodies only for a short period of time after transfection and subsequently disappeared. Consistent with this possibility, Id-transfected cell lines produced in our laboratory demonstrate suppressed MEF-1 binding early after transfection, but suppression is lost several weeks in culture despite continued high expression of Id mRNA [Kazhdan, I., unpublished observations].

Most of the previous studies of HLH gene expression in osteoblasts have utilized either the rat osteosarcoma cell line ROS 17/2.8 [Kawaguchi et al. 1992; Tamura and Noda, 1994] or the mouse pre-osteoblast line MC3T3-E1 [Glackin et al., 1992; Murray et al., 1992]. Our results, which focus on early stages of osteogenesis, are generally consistent with the data from these groups. One exception concerns the specificity of binding to the OCE-1 sequence reported by Noda to be dependent on osteoblastic differentiation [Tamura and Noda, 1994]. These authors demonstrated little binding to the OCE-1 sequence with nuclear extracts of untreated 10T1/2 cells, while treatment with high doses of BMP-2 both induced osteoblastic proteins and significantly enhanced nuclear extract binding to OCE-1. Our studies of binding to OCE-1 oligonucleotide indicated similar mobility shift patterns with nuclear extracts from fibroblastic 10T1/2 cells and a panel of osteoblastic cells. We therefore were unable to demonstrate any differences in OCE-1 binding which were correlated with osteoblast differentiation.

In summary, the results of our studies suggest that the first steps of osteoblast differentiation are characterized by low functional activity of MEF-1 binding proteins and their inhibition by an effective inducer of differentiation (Fig. 4A). This inhibition seems connected to the induction of differentiation, as it was not seen under conditions which interfered with induction (Fig. 4A, with Premix), nor was it observed with cells showing more mature osteoblast characteristics (W-20 and ROS 17/2.8 cells). Cells not capable of Dex-induced osteogenesis (10T1/2 fibroblasts) also failed to demonstrate Dex inhibition of binding to MEF-1 oligonucleotide. These results are consistent with the hypothesis that, while HLH proteins might be necessary for optimal transcription of the genes characteristic of the later stages of osteoblast differentiation, they are not responsible for the initial activation of these genes in bone formation.

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REFERENCES

Alam J, Cook JL (1990): Reporter genes: Application to the study of mammalian gene transcription. Anal Biochem 188:245–254.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1990): "Current Protocols in Molecular Biology." New York: Wiley.

Benezra R, Davis RL, Lockshin D, Turner DL, Weintraub H (1990): The protein Id: A negative regulator of helix-loop-helix DNA binding proteins. Cell 61:49–59.

Braun T, Buschhausen DG, Bober E, Tannich E, Arnold HH (1989): A novel human muscle factor related to but distinct from MyoD induces myogenic conversion in 10T1/2 fibroblasts. EMBO J 8:701–709.

Chen J, Shapiro HS, Sodek J (1992): Developmental expression of bone sialoprotein mRNA in rat mineralized connective tissues. J Bone Miner Res 7:987–997.

Dorheim M-A, Sullivan M, Dandapani V, Wu X, Hudson J, Segarini PR, Rosen DM, Aulthouse AL, Gimble JM (1993): Osteoblastic gene expression during adipogenesis in hematopoietic supporting murine bone marrow stromal cells. J Cell Physiol 154:317–328.

Genovese C, Rowe D, Kream B (1984): Construction of DNA sequences complementary to rat α1 and α2 collagen mRNA and their use in studying the regulation of type I collagen synthesis by 1,25-dihydroxyvitamin D. Biochemistry 23:6210–6216.

Gimble JM, Wanker F, Wang C-S, Bass H, Wu X, Kelly K, Yancopoulos GD, Hill MR (1994): Regulation of bone marrow stromal cell differentiation by cytokines whose receptors share the gp130 protein. J Cell Biochem 54:122–133.

Glackin CA, Murray EJB, Murray SS (1992): Doxorubicin inhibits differentiation and enhances expression of the helix-loop-helix genes Id and mTwi in mouse osteoblastic cells. Biochem Intl 28:67–75.

Grigoriadis AE, Heersche JNM, 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.

Hollenberg SM, Cheng PF, Weintraub H (1993): Use of a conditional MyoD transcription factor in studies of MyoD trans-activation and muscle determination. Proc Natl Acad Sci USA 90:8028–8032.

Jen Y, Weintraub H, Benezra R (1992): Overexpression of Id protein inhibits the muscle differentiation program: in vivo association of Id with E2A proteins. Genes Dev 6:1466–1479.

Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N, Ikeda T, Rosen V, Wozney JM, Fujisawa-Sehara A, Suda T (1994): Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J Cell Biol 127:1755–1766.

Kawaguchi N, DeLuca HF, Noda M (1992): Id gene expression and its suppression by 1,25-dihydroxyvitamin D_3 in rat osteoblastic osteosarcoma cells. Proc Natl Acad Sci USA 89:4569–4572.

Leboy PS, Vaias L, Uschmann B, Golub E, Adams SL, Pacifici M (1989): Ascorbic acid induces alkaline phosphatase, type X collagen and calcium deposition in cultured chick chondrocytes. J Biol Chem 264:17281–17286.

Leboy PS, Beresford JN, Devlin C, Owen ME (1991): Dexamethasone induction of osteoblast mRNAs in rat marrow stromal cell cultures. J Cell Physiol 146:370–378.

Murray SS, Glackin CA, Winters KA, Gazit D, Kahn AJ, Murray EJ (1992): Expression of helix-loop-helix regulatory genes during differentiation of mouse osteoblastic cells. J Bone Miner Res 7:1131–1138.

Murray SS, Murray EJB, Glackin CA, Urist MR (1993): Bone morphogenetic protein inhibits differentiation and affects expression of helix-loop-helix regulatory molecules in myoblastic cells. J Cell Biochem 53:51–60.

Ogata T, Wozney JM, Benezra R, Noda M (1993): Bone morphogenetic protein 2 transiently enhances expression of a gene, Id (inhibitor of differentiation), encoding a helix-loop-helix molecule in osteoblast-like cells. Proc Natl Acad Sci USA 90:9219–9222.

Ogata T, Noda M (1991): Expression of Id, a negative regulator of helix-loop-helix DNA binding proteins, is down-regulated at confluence and enhanced by dexamethasone in a mouse osteoblastic cell line, MC3T3E1. Biochem Biophys Res Commun 180:1194–1199.

Okuyama R, Yanai N, Obinata M (1995): Differentiation capacity towards mesenchymal cell lineages of bone marrow stromal cells established from temperature-sensitive SV-40 T-antigen gene transgenic mice. Exp Cell Res 218:424–429.

Olson EN, Klein WH (1994): bHLH factors in muscle development: dead lines and commitments, what to leave in and what to leave out. Genes Dev 8:1–8.

Pankratz MJ, Seifert E, Gerwin N, Billi B, Nauber U, Jackle H (1990): Gradients of *kruppel* and *knirps* gene products direct pair-rule gene stripe patterning in the posterior region of the Drosophila embryo. Cell 61:309–317.

Rickard DJ, Sullivan TA, Shenker BJ, Leboy PS, Kazhdan I (1994): Induction of rapid osteoblast differentiation in rat bone marrow stromal cell cultures by dexamethasone and BMP-2. Dev Biol 161:218–228.

Rodan GA, Heath JK, Yoon K, Noda M, Rodan SB (1988): Diversity of the osteoblastic phenotype. In: Evered D, Harnett S (eds): "Cell and Molecular Biology of Vertebrate Hard Tissues." Chichester: John Wiley and Sons, pp 78–91.

Schreiber E, Matthias P, Muller MM, Schaffner W (1989): Rapid detection of octamer binding proteins with "mini-extracts" prepared from a small number of cells. Nucl Acids Res 17:6419–6426.

Tamura M, Noda M (1994): Identification of a DNA sequence involved in osteoblast-specific gene expression via interaction with helix-loop-helix (HLH)-type transcription factors. J Cell Biol 126:773–782.

Theide M, Yoon K, Golub EE, Noda M, Rodan GA (1988): Structure and expression of rat osteosarcoma (ROS 17/2.8) alkaline phosphatase: Product of a single copy gene. Proc Natl Acad Sci USA 85:319–323.

Thies RS, Bauduy M, Ashton BA, Kurtzberg L, Wozney JM, Rosen V (1992): Recombinant human bone morphogenetic protein-2 induces osteoblastic differentiation in W-20-17 stromal cells. Endocrinology 130:1318–1324.

Tontonoz P, Bum Kim J, Graves RA, Spiegelman BM (1993): ADD1: A novel helix-loop-helix transcription factor associated with adipocyte determination and differentiation. Mol Cell Biol 13:4753–4759.

Wilson RB, Kiledjian M, Shen C, Benezra R, Dymecki SM, Desiderio SV, Kadesch T (1991): Repression of immunoglobulin enhancers by the helix-loop-helix protein Id: Implications for B-lymphoid-cell development. Mol Cell Biol 11:6185–6191.

Yamaguchi A, Kahn AJ (1991): Clonal osteogenic cell lines express myogenic and adipocytic developmental potential. Calcif Tissue Intl 49:221–225.

Yoon K, Buenaga R, Rodan GA (1987): Tissue specificity and developmental expression of rat osteopontin. Biochem Biophys Res Commun 148:1129–1136.

Yoon K, Rutledge SJ, Buenaga RF, Rodan GA (1988): Characterization of the rat osteocalcin gene: stimulation of promoter activity by 1,25-dihydroxyvitamin D3. Biochem 27:8521–8526.