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Scleraxis Messenger Ribonucleic Acid Is Expressed in C2C12 Myoblasts and Its Level Is Down-Regulated by Bone Morphogenetic Protein-2 (BMP2)

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Abstract We examined the mRNA expression of scleraxis, a non-myogenic helix-loop-helix type transcription factor in C2C12 myogenic cells. Scleraxis mRNA has been shown to be expressed in sclerotome and perichondrium of the embryos. We found that C2C12 cells express 1.2 kb scleraxis mRNA constitutively. Since BMP was reported to induce ectopic bone formation when implanted in muscle, we examined the effects of BMP on scleraxis expression. Scleraxis mRNA expression in C2C12 cells was suppressed by the treatment with BMP2. This suppression was observed at 200 ng/ml but not at the lower concentrations. BMP2 treatment suppressed scleraxis mRNA level within 24 h and lasted at least up to 48 h. Electrophoresis mobility shift assay showed that the proteins in the crude nuclear extracts prepared from C2C12 cells bound to an Scx-E-box sequence, CATGTG, which is preferentially recognized by scleraxis. This binding was competed out by 100-fold molar excess of cold Scx-E-box sequence but not by the one with mutations in the E-box. This band was supershifted by the addition of antiserum raised against scleraxis. BMP2 treatment suppressed the Scx-E binding activity in C2C12 cells. This suppression of the Scx-E-box binding activity was in parallel to the BMP2 suppression of the transcriptional activity of the Scx-E-CAT reporter gene transfected into C2C12 cells. These data indicated that although the default pathway for C2C12 cells is to differentiate into muscle cells, these cells do express non-myogenic transcription factor, scleraxis, whose expression is suppressed by BMP2. J. Cell. Biochem. 67:66–74, 1997.

Key words: scleraxis; C2C12 myoblasts; mRNA; BMP2; HLH-type transcription factor

Scleraxis is a helix-loop-helix (HLH) transcription factor that is expressed in sclerotome and perichondrium in embryos [Cserjesi et al., 1995; Liu et al., 1996]. Mesenchymal cells could give rise to muscle, cartilage, fat, and bone, and, therefore, it is postulated that certain cells at intermediate stages of differentiation could give rise to more than one type of cells [Taylor and Jones, 1979; Grigoriadis et al., 1988, 1990; Bennett et al., 1991; Poliard et al., 1995). Myogenic cell differentiation is under the control of four HLH type transcription factors called MDFs (Muscle Differentiation Factors) [Davis et al., 1987; Braun et al., 1989, 1990; Edmondson and Olson, 1989; Rhodes and Konieczny, 1989; Wright et al., 1989; Braun et al., 1990; Miner and Wold, 1990; Olson, 1993; Weintraub, 1993]. Overexpression of MDFs can convert non-muscle cells, such as chondrocytes, into myogenic cells [Weintraub et al., 1989]. This observation indicates that immature type cells do have certain plasticity. This plasticity would

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be based on the possible endogenous expression of multiple types of transcription factors, which are respectively necessary for the determination of different cell types.

C2C12 cells differentiate into muscle cells upon removal of growth stimulatory condition (serum) and, therefore, the default pathway of these cells is to become muscle cells [Blau et al., 1983]. These cells express HLH type transcription factors (HLH-TFs) and this HLH-TF machinery is operating in these cells. One such factor, Id1, is regulated by BMP and vitamin D, suggesting that BMP and vitamin D may modulate differentiation via the regulation of such negative type HLH-TF [Kawaguchi et al., 1992; Ogata et al., 1993].

BMP was first identified in bone matrix as an activity to induce ectopic bone formation when implanted in muscle or subcutaneous tissues, and its genes were cloned from a human osteosarcoma library [Urist, 1965; Urist et al., 1983]. More than ten BMP genes have been cloned to date and the human recombinant BMP sequences show that they are subfamily members of the TGFβ super family [Wozney et al., 1988]. BMPs are also expressed in many tissues other than skeletal tissues and are considered to play a key role in morphogenesis in axial structures and limbs of embryos, neurogenesis, formation of eye, gut, and glomerulus [Reddi, 1994]. Null mutation of BMPs or their receptors causes early embryonic death at around the time of the appearance of mesenchymal cells, suggesting that BMPs are the basic regulatory molecules in determination of mesenchymal cell fate and, hence, morphology of the body [Miner and Wold, 1990; Luo et al., 1995].

Although BMP2 treatment directs mesenchymal cells to express genes encoding proteins related to osteoblastic phenotypes, molecular details of this phenomenon have not been elucidated. Based on the observation that mesenchymal cells can differentiate into osteogenic cells, we hypothesized that expression of certain positive transcription factors related to skeletogenesis may be induced or suppressed upon BMP treatment. The aim of this work was to examine whether a myogenic menchymal cell line, C2C12, expresses a non-myogenic transcription factor, scleraxis, and to examine the effects of BMP2 on the expression of this gene. We found that C2C12 cells express scleraxis gene constitutively and, furthermore, BMP2 treatment suppresses scleraxis expression in these cells.

MATERIALS AND METHODS

Cell Culture

C2C12 cells (myoblasts) were kindly provided by Dr. Nabeshima and were maintained in DMEM supplemented with 5% fetal bovine serum (FBS) [Blau et al., 1983]. All cultures were fed with fresh medium every 2–3 days. C2C12 cells were plated at 10,000–20,000 cells/cm^2 and cultured for several days to reach confluence before experiments. At this point, the cells did not form myotubes and hence were maintained in an immature condition. $TGF\beta_1$ was purchased from King Brewing Co. (Kurashiki, Japan).

RNA Preparation and Northern Analysis

Total RNA was extracted according to the acid guanidium thiocyanate-phenol-chloroform method [Chomczynski and Sacchi, 1987]. Total cellular RNA was subjected to Northern analysis by fractionation in 1.0% agarose gels containing 0.66 M formaldehyde. The RNAs were transferred to nylon filters (Hybond-N; Amersham Corp., Arlington Heights, IL) by electroblotting for 18 h [Thomas, 1980]. A 1.2-kb scleraxis cDNA probe was labeled with [α-^{32}P] dCTP by using random primers and Klenow fragment [Feinberg and Vogelstein, 1983]. Complementary DNA for glyceraldehyde-6-phosphate dehydrogenase (GAPDH) was a gift from Dr. Sevgi Rodan. Filters were hybridized to the probes in hybridization solution containing 50% formaldehyde, 5 × SSC (1 × SSC consists of 0.15 M NaCl and 10 mM sodium citrate), 5 × Denhardt's solution, 0.1% SDS and 50 μg/ml sheared and denatured herring sperm DNA, at 42°C overnight, were washed at 65°C in 0.2 × SSC, 0.1% SDS and were exposed to X-ray films at −70°C using intensifying screens.

Electrophoresis Mobility Shift Assays

Nuclear extracts were prepared according to the method described by Dignam [Dignam et al., 1983]. For DNA binding, a double-stranded 18-bp synthetic oligonucleotide containing Scleraxis binding site was used. The sequence of the upperstrand of this oligonucleotide is CCGAACACATGTGCCCGC (Scx-E), read 5′ to 3′. The core CANNTG sequence is underlined. This oligonucleotide was labeled by kinase reaction

using [γ-^{32}P] ATP (New England Nuclear Co., Boston, MA) and T_4 polynucleotide kinase, followed by removal of unincorporated label over sephadex G-50 columns. Approximately 20,000 cpm of the probe was incubated with 15 µg of nuclear protein for 15 min at 30°C in 20-µl reaction mixture, containing buffer D (20 mM Hepes, pH 7.5, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol), 5 µg of BSA (Sigma Chemical Co., St. Louis, MO), and 2 µg of poly (dI-dC) (Pharmacia, Piscataway, NJ). For competition experiments, 100-fold molar excess of double-stranded unlabeled competitors were included in the binding reactions. The upper strand sequences of the double-stranded competitors were CCGAACACATGTGCCCGC (Scx-E) [Cserjesi et al., 1995] and CCGAACAAATTTACCCGC (mScx-E). These double-stranded oligos were incubated with nuclear extracts for 15 min before the addition of the probe. The DNA-protein complexes were fractionated in a 4% polyacrylamide gel as described elsewhere [Tamura and Noda, 1994]. For the supershift experiments, 1 µl of 0.1 µg/µl IgG or antiserum, raised against scleraxis were added to the reaction mixture and were incubated for 20 min before electrophoresis.

Transient DNA Transfections and CAT Assays

Transfection of plasmid DNA into C2C12 cells was performed by lipofection using DNA-lipid complexes. The cells were cotransfected with 1 µg of the reporter plasmid and Scx expression vector [Cserjesi et al., 1995] or pBluescript SK (+) (pBS) as control [Gorman, 1985]. The cells were then exposed for 3 h, and were cultured in fresh DMEM supplemented with 5% FBS. After 24 h, the cell extracts were prepared for the analysis of chloramphenicol acetyltransferase (CAT) activity. Equivalent amounts of the cellular proteins were incubated in a reaction buffer (0.25 M Tris-HCl, pH 8.0, 40 mM acetyl CoA [Sigma Chemical Co.], [^{14}C] chloramphenicol [1.85 G Bq/mmol; Amersham Corp.]), overnight at 37°C. The acetylation was examined by thin layer chromatography (TLC) followed by autoradiography of the TLC plates. Quantitation of acetylation ratios was performed by a Bioimaging Analyzer (Fuji Film Inc., Tokyo, Japan).

RESULTS

Scleraxis Gene Is Expressed in C2C12 Cells

To investigate whether scleraxis is expressed in C2C12 myoblasts, we conducted Northern analysis. We found that scleraxis mRNA was expressed constitutively in C2C12 cells as a 1.2-kb band (Fig. 1, lane 1). As serum depletion is known to trigger myogenic differentiation in these cells, the serum effect on the level of scleraxis mRNA was examined. The scleraxis mRNA level was similar in the cells cultured in the medium supplemented with 0.5 and 5% serum where initiation of myotube formation was observed while the level was slightly lower in the cells cultured in 15% serum where no myotube formation was initiated (data not shown). As BMP2 was reported to induce expression of osteoblastic phenotypes in mesenchymal cells, we examined the effect of BMP2 on scleraxis gene expression in the these cells. Twenty-four-hour BMP2 treatment (200 ng/ml) suppressed scleraxis mRNA expression several-fold in these cells (Fig. 1, lane 3). In addition, another family member, TGFβ (2 ng/ml), was also found to suppress scleraxis mRNA level (Fig. 1, lane 2). Co-treatment with TGFβ did not further suppress scleraxis mRNA level in BMP2-treated cells (Fig. 1, lane 4). GAPDH mRNA levels were not altered by the BMP2 or TGFβ treatment and served as control (Fig. 1, bottom).

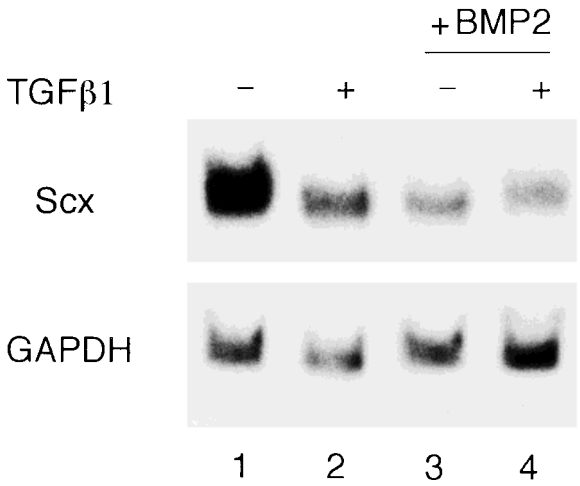

Fig. 1. Scleraxis gene is expressed in C2C12 myoblasts and its expression is regulated by BMP2 and TGFβ. C2C12 cells were grown to confluence and were cultured for 24 h in the absence (−) or presence (+) of 200 ng/ml BMP2 and/or 2 ng/ml TGFβ1. Northern blot analysis was conducted as described in Materials and Methods. The positions of Scleraxis (Scx) and glyceraldehyde-6-phosphate dehydrogenase (GAPDH) are indicated. The data represent one of two independent experiments with similar results.

Profiles of the BMP2 Effects on Scleraxis Gene Expression in C2C12 Cells

We examined the dose profile and the time course of the effect of BMP2 on scleraxis gene expression in C2C12 cells. The effects of BMP2 were observed at 200 ng/ml (Fig. 2), but not at the lower concentrations. The levels of the scleraxis message in the control cells gradually increased at 24 (Fig. 3, lane 2) and 48 h (Fig. 3, lane 4). The BMP2 effect on scleraxis mRNA expression was observed within 24 h (Fig. 3, lane 3) and this effect was still observed at 48 h (Fig. 3, lane 5).

Effect of BMP2 on the Binding Activity in the Nuclear Extracts of C2C12 to Scleraxis Recognition Sequence

We examined the expression of scleraxis in C2C12 cells by the electrophoresis mobility shift assay (EMSA). EMSA was carried out by using an E box (Scx-E) sequence (CATGTG) as a probe that is recognized preferably by scleraxis [Cserjesi et al., 1995]. As shown in Figure 4, nuclear extracts prepared from C2C12 cells were incubated with Scx-E box probe. Retardation of the band was observed on EMSA (Fig. 4, lane 2). This band was supershifted by the addition of

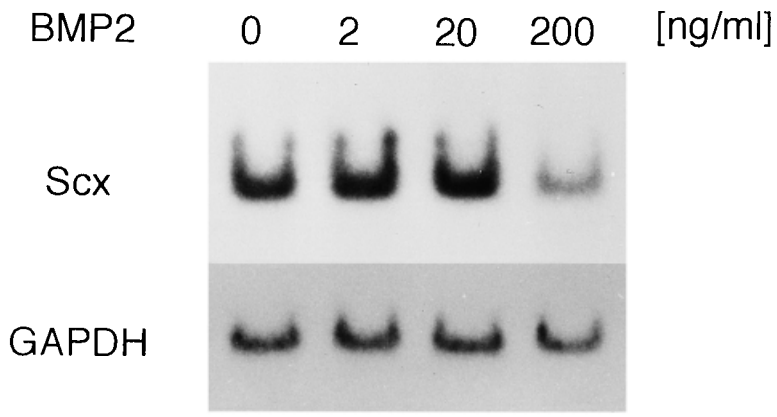

Fig. 2. Dose-dependence of the BMP2 effects on Scleraxis mRNA expression in C2C12 cells. C2C12 cells at confluence were treated with the indicated doses of BMP2 for 24 h. Northern blot analysis was conducted as described in Materials and Methods. The positions of Scx and GAPDH are indicated.

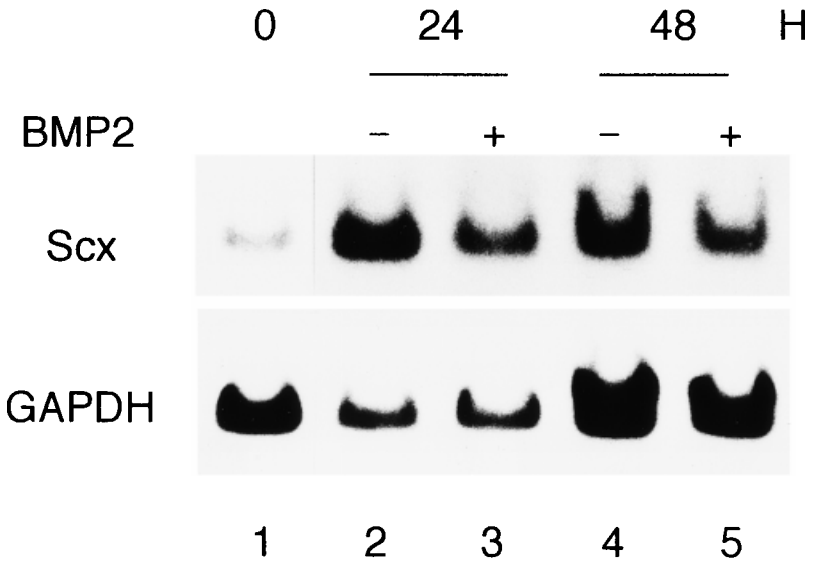

Fig. 3. Time course of the BMP2 effects on Scleraxis mRNA expression in C2C12 cells. Confluent C2C12 cells were cultured for the indicated periods of time (h) in the absence (−) or presence (+) of 200 ng/ml BMP2. Northern blot analysis was conducted as described in Materials and Methods. The positions of Scx and GAPDH are indicated.

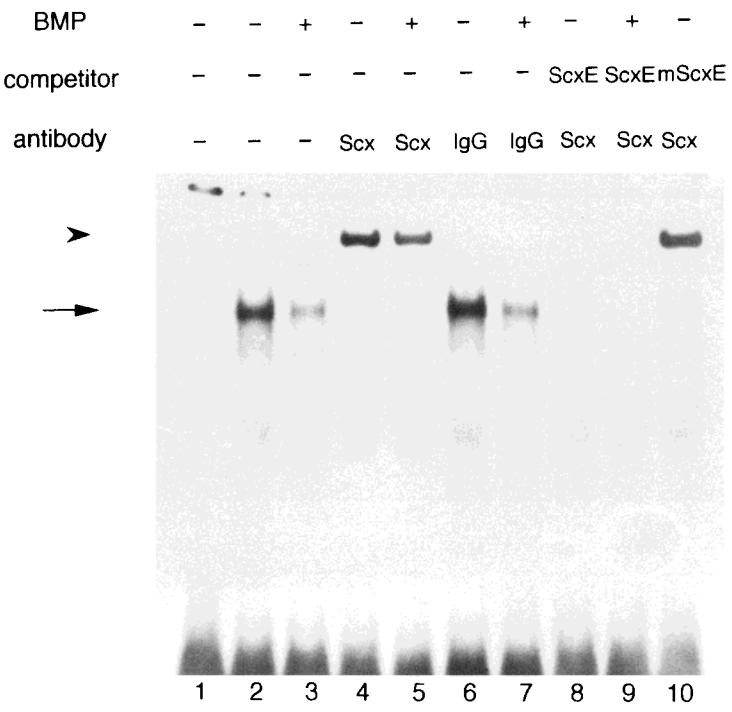

Fig. 4. Effects of BMP2 on Scx-E box binding activity in the nuclear extracts of C2C12 cells. Electrophoretic mobility shift assays (EMSA) was performed as described in Materials and Methods. **Lane 1,** free Scx-E box probe alone. Equal amounts of nuclear protein prepared from C2C12 cells cultured for 24 h in the presence (**lanes 3, 5, 7, 9**) or absence (**lanes 2, 4, 6, 8, 10**) of 200 ng/ml BMP2 were incubated with radiolabeled Scx-E probe. For supershift experiment, the reaction was incubated with antiserum against scleraxis (lanes 4, 5, 8, 9, and 10) or non-specific IgG (lanes 6 and 7). For competition, 100-fold molar excess of cold Scx-E sequence (lanes 8 and 9) and mScx-E sequence, which contains mutation in E-box (lane 10), respectively, were incubated with nuclear extracts in the binding reaction for 15 min before the addition of the probe. Radiolabeled Scx-E box probe (20,000 cpm) was used in each lane, and protein-DNA complexes were resolved by EMSA. The arrow indicates Scx-E box specific protein-DNA complex and the arrowhead indicates the supershifted band.

the antiserum (Fig. 4, lane 4), but not by IgG (Fig. 4, lanes 6 and 7) and was competed out by the presence of 100-fold molar excess of the unlabeled Scx-E box probe (Fig. 4, lane 8), but not by mutated competitor, mScx-E, in which base mutations were introduced into the E-box core region (from CATGTG to AATTTA) (Fig. 4, lane 10). We then examined whether BMP2 effects on scleraxis mRNA expression correlates with the level of DNA binding activity in the nuclear extracts of C2C12. In the nuclear extracts of the C2C12 cells cultured in the presence of BMP2 at 200 ng/ml for 24 h, the DNA binding activity to Scx-E box was suppressed (Fig. 4, lanes 3 and 7) compared to the control (Fig. 4, lanes 2 and 6). The suppressed binding activity was supershifted by the addition of antiserum against scleraxis (Fig. 4, lane 5) and was also competed out by the addition of 100-fold molar excess of unlabeled Scx-E (Fig. 4, lane 9). These results indicated that BMP2 suppression of Scx mRNA abundance correlated well with the decrease in Scx-E box binding activity in the nuclear extracts of C2C12 cells that contain scleraxis.

Effects of BMP2 on the Transcriptional Activity of Scx-E-CAT Construct

To determine whether BMP2 suppression of scleraxis expression could alter Scx-E-box-mediated transcription in C2C12 cells, a CAT assay was conducted. We first cotransfected scleraxis expression vector transiently in C2C12 cells with a CAT reporter gene containing tandem copies of the scleraxis-binding site sequences (Scx-E) inserted upstream to the thymidine kinase basal promoter [Cserjesi et al., 1995]. The basal level of the transcription of the CAT reporter gene in C2C12 cells in the presence of the cotransfected pBS plasmid (Fig. 5, lanes 1 and 2) was observed in these cells. Cotransfection with a scleraxis expression vector enhanced the CAT activity (Fig. 5, lanes 5 and 6). In this system, BMP2 treatment suppressed the Scx-E box-CAT activity (Fig. 5, lanes 3 and 4), indicating that BMP2 suppres-

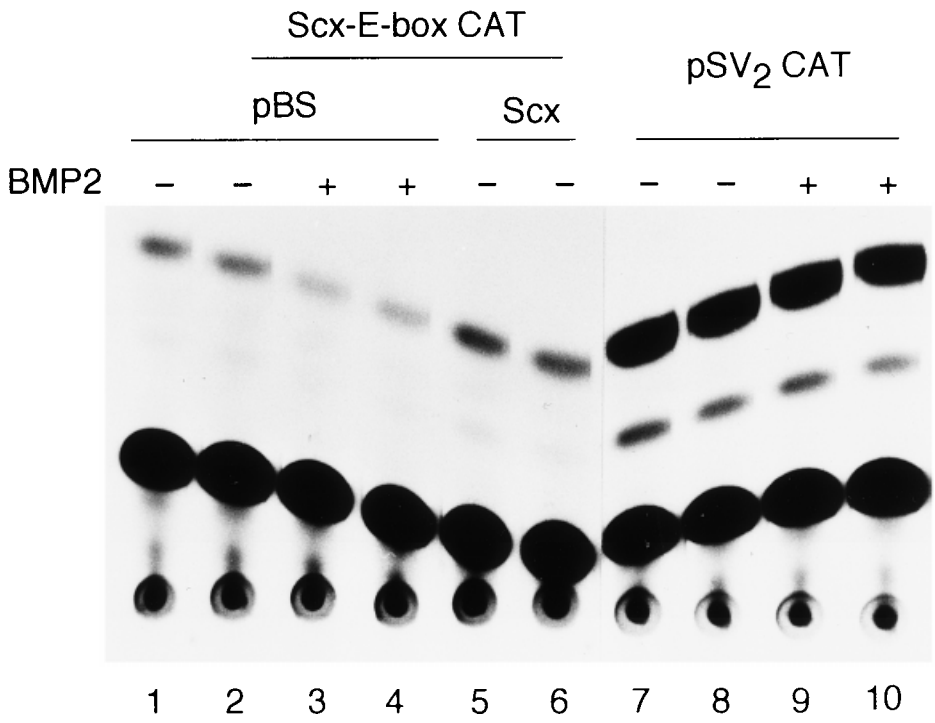

Fig. 5. Effects of BMP2 on the transcriptional activity of Scx-E-CAT reporter gene in C2C12 cells. C2C12 cells were cotransfected with 1 µg of reporter plasmid, Scx-E-CAT (**lanes 1–6**) and 1 µg of expression vectors containing Scx cDNA (lanes 5, 6) or 1 µg of pBluescript SK(+) (pBS) (lanes 1–4), or were transfected with pSV2CAT alone as positive controls (**lanes 7–10**). Cells were incubated in DMEM supplemented with 5% FBS in the absence (−) or presence (+) of 200 ng/ml BMP2 for 24 h. CAT activity was determined as described in Materials and Methods.

sion of the scleraxis mRNA level as well as the suppression of Scx-E box binding activity in the nuclear extracts to Scx-E-box sequence are translated into the repression of the Scx-E-box-dependent transcription activity. The BMP2 suppression of Scx-E-box-CAT activity was specific to this sequence since BMP2 treatment per se did not affect the pSV2CAT expression (Fig. 5, lanes 7, 8 vs. 9, 10).

DISCUSSION

In this paper, we report that C2C12 cells express scleraxis mRNA and that BMP2 suppresses the scleraxis mRNA expression in C2C12 cells. BMP2 treatment also suppresses binding activity to Scx-E-box in the nuclear extracts prepared from C2C12 cells. Furthermore, BMP treatment suppressed the transcriptional activity of the CAT reporter constructs, containing Scx-E-box sequence which is recognized preferentially by scleraxis.

Scleraxis is a basic helix-loop-helix transcription factor that prefigures chondrogenesis during mouse embryogenesis. During mouse embryogenesis, scleraxis transcripts are first detected between day 9.5 and 10.5 post coitum in the body wall and limb buds, followed by high levels of expression within mesenchymal precursors of the axial and appendicular skeletons and cranial mesenchymal tissues in advance of chondrogenesis [Cserjesi et al., 1995].

Constitutive expression of scleraxis in C2C12 cells is contrary to our prediction and suggests that these cells are still in the stages close to rather pluripotent levels, and, therefore, the cells could still differentiate into not only myogenic cells as their default path but also into non-myogenic cells under certain conditions or upon certain stimuli. It has been postulated that during the course of determination of mesenchymal cell fate, there could be certain precursor cells in intermediate stages, which are either tri- or bi-potential before they finally become mono-potential precursors. It is predicted from our study that the cells in these intermediate levels may still express a mixture of transcription factors responsible for the final determination of the fate of different types of cells, such as myoblasts or skeletal cells.

Bone morphogenetic proteins (BMPs) have been shown to possess bone- and cartilage-inducing activity in vivo as well as in vitro and certain members of the BMP family are expressed at the sites of bone formation in the embryos [Reddi, 1994]. BMP2 not only inhibits myogenic differentiation in C2C12 myoblasts but also converts their differentiation pathway into that of osteoblast lineage [Katagiri et al., 1994]. However, little is known of the genetic mechanisms that direct cartilage and bone formation or of the mechanisms whereby the BMPs stimulate these processes. Interestingly, we found that BMP-2 did not enhance but suppressed scleraxis expression in C2C12 cells. If scleraxis is the early marker of chondroblast cell lineage, suppression of its expression by BMPs may mean to shift the direction of the cell fate towards osteogenic cells rather than chondrocytes. This speculation may require further analysis especially by using null mutation animals.

It is intriguing that although BMP and TGFβ do not have the same ability to induce osteogenesis in C2C12 cells, i.e., BMP being stimulatory and TGFβ not being, both cytokines suppress scleraxis gene expression in these cells. What is known to be common between the two cytokines is the suppression of the myogenic differentiation. However, scleraxis is not a muscle specific transcription factor such as Myo D. This apparent paradox could be explained if scleraxis is required for the differentiation of chondrocytes. BMP suppression of scleraxis could mean that apparent BMP function is to let the cells become more osteogenic cells and not to become chondrogenic cells. TGFβ suppression of scleraxis also could mean that TGFβ may inhibit chondrogenic differentiation of these cells. It is known that TGFβ can suppress chondrogenic differentiation in some types of cells although it could enhance it in the other types. In the case of C2C12 cells, it is not known whether TGFβ treatment modulates their chondrogenic differentiation. Our results on TGFβ suppression of scleraxis expression predict that differentiation of C2C12 cells towards chondrocytes would not occur in the presence of TGFβ.

We observed similar levels of scleraxis in C2C12 cells cultured in 0.5, 1, 10, and 15% serum with a slight suppression only in 15% (data not shown), although initiation of myotube formation was observed at the lower serum concentrations. These observations suggested no apparent correlation between the levels of scleraxis mRNA and the differentiation of C2C12 cells at least at the relatively early time point we examined. This observation may reveal the presence of certain populations of scleraxis-expressing cells at least in some early stages of myogenic differentiation in cultures. Apparent similar levels of scleraxis could be explained since a majority of cells would still not be myogenic cells.

Regulatory roles played by myogenic HLH-type transcription factors such as MyoD and myogenin have been thought to promote myogenic differentiation in C2C12 cells. In analogy to such HLH-type transcription factors, scleraxis could be a HLH-type transcription factor playing a role in the precursors for the cells in skeletal cell lineages, more likely of chondrocytes. Simultaneous expression of the transcription factors responsible for different types of cell lineages has been observed during the transition between the diverse differentiation steps in other systems such as hematopoietic cells. In this case, transcription factors that are required at both early and late stages of differentiation have been shown to be expressed in the immature blood-cell precursors. Scleraxis expression in C2C12 cells may be in a situation similar to that seen in hematopoietic cells. Obviously, it is important to determine whether scleraxis is involved in regulation of any lineage specific genes. At this point, however, since scleraxis knock out mice have not yet been reported, no physiological target gene is known.

As observed in the case of hierarchy of the four myogenic helix-loop-helix type differentiation factors, scleraxis may be participating in the network of helix-loop-helix type transcription factor system, including Id and possibly as yet identified members, which are located upstream and/or down-stream of skeletogenic cytokines such as BMP2 and TGFβ. We have previously reported that certain members of HLH-type transcription factors, such as Id1 [Kawaguchi et al., 1992], HES1 [Matsue et al., 1997], and ADD-1 (SREBP-1) [Sawada and Noda, 1996] are expressed in osteoblasts and their expression is under the control of calcitropic cytokines and hormones including TGFβ, 1.25(OH)2 vitamin D3, BMP, and glucocorti-

coid. Our observation on BMP and TGFβ regulation of scleraxis in C2C12 cells could add further that not only relatively mature skeletal cells but also their possible precursors, such as C2C12 cells, could be the important stages of the cells in skeletal cell differentiation where scleraxis may play its role in modulation of the expression of the phenotypes.

A final decision on the cell fate in the pluripotent mesenchymal cells may be determined by the balance among the levels of diverse transcription factors, which include the members of the family of tissue-specific master regulatory genes for different types of cells (myoblasts, adipocytes, chondrocytes, osteoblasts, etc.) and non-tissue specific type transcription factors.

REFERENCES

Bennett JH, Joyner CJ, Triffitt JT, Owen ME (1991): Adipocytic cells cultured from marrow have osteogenic potential. J Cell Sci 99:131–139.

Blau HM, Chiu CP, Webster C (1983): Cytoplasmic activation of human nuclear genes in stable heterocaryons. Cell 32:1171–1180.

Braun T, Buschhausen DG, Bober E, Tannich E, Arnold HH (1989): A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversionin 10T1/2 fibroblasts. EMBO J 8:701–709.

Braun T, Bober E, Winter B, Rosenthal N, Arnold HH (1990): Myf-6, a new member of the human gene family of myogenic determination factors: Evidence for a gene cluster on chromosome 12. EMBO J 9:821–831.

Chomczynski P, Sacchi N (1987): Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Cserjesi P, Brown D, Ligon K, Lyons G, Olson EN (1995): Scleraxis: A basic helix-loop-helix protein that prefigures skeletal formation during mouse embryogenesis. Development 121:1099–1110.

Davis RL, Weintraub H, Lassar AB (1987): Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell 51:987–1000.

Dignam JD, Lebovitz RM, Roeder RG (1983): Acurate transcription initiation by RNA polymerase II in a soluble extract from isolated mamalian nuclei. Nucleic Acids Res 11:1475–1489.

Edmondson DG, Olson EN (1989): A gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. Genes Dev 3:628–640.

Feinberg AP, Vogelstein B (1983): A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13.

Gorman C (1985): Vectors used in mammalian cell expression. In Glover DM (ed): DNA Cloning, volume II. A Practical Approach. Oxford: IRL Press, pp 144–147.

Grigoriadis AE, Heersche JNM, Aubin JE (1988): Differentiation of muscle, fat, cartilag, and bone from progenitor cells present in a bone-derived clonal cell population: Effect of dexamethasone. J Cell Biol 106:2139–2151.

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.

Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N, Ikeda T, Rosen V, Wozney JM, Sehara AF, Suda T (1994): Bone morphogenetic protein-2 coverts 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 D3 in rat osteoblastic osteosarcoma cells. Proc Natl Acad Sci USA 89:4569–4572.

Liu Y, Nifuji A, Olson E, Noda M (1996): Sclerotome-related helix loop Helix type transcription factor (scleraxis) mRNA is expressed in osteoblasts and its level is enhanced by type-beta transforming growth factor. J Endocrin 151:491–499.

Luo G, Hofmann C, Bronckers AL, Sohocki M, Bradley A, Karsenty G (1995): BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. Genes Dev 9:2808–2820.

Matsue M, Kageyama R, Denhardt DT, Noda M (1997): Helix-loop-helix type transcription factor (HES-1) is expressed in osteoblastic cells, suppressed by 1,25(OH)2vitamin D3, and modulates 1,25(OH)2vitamin D3 enhancement of osteopontin expression. Bone 20:(in press).

Miner JH, Wold B (1990): Herculin, a fourth member of the MyoD family of myogenic regulatory genes. Proc Natl Acad Sci USA 87:1089–1093.

Ogata T, Wozney J, Benezra R, DeLuca HF, Noda M (1993): A differentiation promoting factor, bone morphogenetic protein-2 enhances expression of a helix-loop-helix molecule, Id (Inhibitor of Differentiation) in proliferating osteoblast-like cells. Proc Natl Acad Sci USA 90:9219–9222.

Olson EN (1993): Signal transduction pathways that regulate skeletal muscle gene expression. Mol Endocrinol 7:1369–1378.

Poliard A, Nifuji A, Lamblin D, Plee E, Forest C, Kelllermann O (1995): Controlled conversion of an immortalized masodermal progenitor cell towards osteogenic, chondrogenic, or adipogenic pathways. J Cell Biol 130:1461–1472.

Reddi AH (1994): Bone and cartilage differentiation. Curr Opin Genet Dev 4:737–744.

Rhodes SJ, Konieczny SF (1989): Identification of MRF4: A new member of the muscle regulatory factor gene family. Genes Dev 3:2050–2061.

Sawada Y, Noda M (1996): An adipogenic basic helix-loop-helix-leucine zipper type transcription factor (ADD1) mRNA is expressed and regulated by retinoic acid in osteoblastic cells. Mol Endocrinol 10:1238–1248.

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 126:773–783.

Taylor SM, Jones PA (1979): Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. Cell 17:771–779.

Thomas P (1980): Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc Natl Acad Sci USA 77:5201–5205.

Urist MR (1965): Bone: Formation by autoinduction. Science 150:893–899.

Urist MR, DeLange RJ, Finerman GAM (1983): Bone cell differentiation and growth factors. Science 220:680–686.

Weintraub H (1993): The MyoD family and myogenesis: Redundancy, networks, and thresholds. Cell 75:1241–1244.

Weintraub H, Tapscott SJ, Davis RL, Thayer MJ, Adam MK, Lassar AB, Miller AD (1989): Activation of muscle specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. Proc Natl Acad Sci USA 86:5434–5438.

Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA (1988): Novel regulators of bone formation: Molecular clones and activities. Science 242:1528–1534.

Wright WE, Sassoon DA, Liu VK (1989): Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. Cell 56:607–617.