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Original

Osteogenic transcription factors and proto-oncogene regulate bone sialoprotein gene transcription

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Abstract: Runt homeodomain protein 2 (Runx2), distalless 5 (Dlx5) and Smad1 are transcription factors that play critical roles in controlling the differentiation of osteoblasts and mineralization of bone. Proto-oncogene tyrosine-protein kinase, Src, is an enzyme encoded by the Src gene. The normal cellular gene is called cellular-Src (c-Src). Bone sialoprotein (BSP), a protein implicated in the initial mineralization of newly formed bone, is an early phenotypic marker of differentiated osteoblasts. In this study, we used overexpression plasmids with Runx2, Dlx5, Smad1 or c-Src inserts to search for the effects of these transcription factors and proto-oncogene on BSP gene expression using rat osteoblast-like ROS 17/2.8. When we used Runx2, Dlx5 or c-Src overexpression plasmids for the transfection, BSP and Runx2 mRNA levels were increased in ROS 17/2.8 cells. However, overexpression of Smad1 did not induce BSP and Runx2 mRNA. Transient transfection analyses were performed using chimeric constructs of the rat BSP gene promoter linked to a luciferase reporter gene. Transfection of ROS 17/2.8 cells with Runx2, Dlx5 or c-Src overexpression plasmid increased the luciferase activities of the constructs, pLUC3 (-116 to +60), pLUC4 (-425 to +60) and pLUC5 (-801 to +60). However, Smad1 overexpression had no effect on the luciferase activities. These results demonstrate that overexpression of Runx2, Dlx5 or c-Src stimulates BSP transcription, and suggest that Runx2, Dlx5 and c-Src might be crucial transcriptional regulators of mineralization and bone formation.
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Keywords: bone sialoprotein; transcription factors; proto-oncogene.

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Introduction

Transcription factors are vital to the process of transcriptional control of gene expression. In general, they need to be capable of binding to DNA in order to influence transcription, either positively or negatively. In fact, transcription factors are frequently classified on the basis of their DNA binding domains, and those domains that have been characterized so far include the basic helix-loop-helix (1), the basic-leucine zipper (2), the helix-turn-helix present in homeobox transcription factors (3), the two cysteine-two histidine zinc finger (4) found, for example, in the SP1 transcription factor family, and the multi-cysteine zinc finger (5) found in the steroid-thyroid hormone receptor family.

Runt homeodomain protein 2 (Runx2) is essential for development of the osteoblast phenotype (6-8). Runx2 is expressed in the early mesenchyme of developing skeletal tissues (embryonic age E9.5) (9,10), and studies of null mouse models have clearly established that Runx2 is required for osteoblast differentiation and bone formation at later stages of embryonic development. The mammalian homologues of drosophila distalless (Dlx) proteins are among the homeodomain proteins that also function in specification of skeletal structures (11-13). Dlx2 and Dlx3 contribute to craniofacial development but with distinct temporal and spatial patterns of expression

(14,15). Dlx2 also has an important role in mesenchymal condensation (16). Dlx5 and Dlx6 are essential for development of the jaw, axial and appendicular bones (13). Several *in vitro* studies have demonstrated that Dlx5, an inducer of mesoderm differentiation, is up-regulated during osteoblast differentiation (17) and has competence in inducing chondrocyte and osteoblast differentiation (18,19). Src is a proto-oncogene encoding a tyrosine kinase that belongs to a family of non-receptor tyrosine kinases known as Src family kinases. The importance of Src in bone cell physiology became apparent after phenotyping of the Src-knockout mouse, which shows decreased bone resorption and increased bone formation (20,21). Bone morphogenetic protein (BMP) receptor (BMPR) complexes are heterooligomers of type I (BMPR-I) and type II (BMPR-II) receptors and phosphorylate Smad1 and its related molecules upon ligand binding (22,23). Phosphorylated Smads, together with Smad4, are translocated into the nucleus where they may regulate the transcriptional activity of genes involved in osteoblastic differentiation (24).

BSP is a prominent component of mineralized connective tissues and has been implicated in tissue mineralization. Studies on the developmental expression, tissue localization and structural properties of BSP in newly forming bone have shown that the expression of BSP is essentially restricted to differentiated cells in mineralizing tissues and that it might initiate hydroxyapatite formation during *de novo* bone formation (25-28). BSP is expressed by prostate, breast and lung cancers and associated with the formation of ectopic hydroxyapatite microcrystals in the tumor tissues (29). The rat, mouse and human BSP genes have been cloned and partially characterized (30-32). BSP gene promoters have an inverted TATA box (nt -24 to -19) (33) that overlaps a vitamin D response element (34), and an inverted CCAAT box (-50 to -46) required for basal gene transcription (35,36). In addition, a fibroblast growth factor 2 (FGF2) response element (FRE; -92 to -85) (37-40), a cyclic AMP (cAMP) response element (CRE; -75 to -68) (38,39,41), a pituitary-specific transcription factor-1 binding site (Pit-1; -111 to -105) (42), a transforming growth factor-β (TGF-β) activation element (-499 to -485) (43), and a homeodomain protein-binding site (HOX; -199 to -192) (40,44) have been characterized. Further upstream, a glucocorticoid response element (GRE) overlapping an activator protein 1 (AP1) site (45,46) has also been identified. The effect of FGF2 on BSP is mediated through FRE in the proximal promoter of the rat BSP gene. FGF2 also increases AP1 binding activity (37,47).

These studies demonstrate that Runx2, Dlx5 or c-Src induces BSP transcription in the proximal promoter of the rat BSP gene. Moreover, Runx2, Dlx5 transcription factors and Src tyrosine kinase appear to be key regulators of BSP transcription.

Materials and Methods

Reagents

Alpha minimal essential medium (α-MEM), fetal calf serum (FCS), Lipofectamine, penicillin, streptomycin and trypsin-EDTA were obtained from Invitrogen (Carlsbad, CA, USA). pGL3-basic and the pSV-β-galactosidase control vector were purchased from Promega (Madison, WI, USA). An EXScript RT reagent kit and SYBR Premix Ex Taq were purchased from Takara-bio (Tokyo, Japan). All chemicals used were of analytical grade.

Cell culture

Rat osteoblast-like ROS 17/2.8 cells were cultured at 37°C in 5% CO_2 air in α-MEM supplemented with 10% FCS. These cells were first grown to confluence in 100-mm tissue culture dishes in α-MEM medium containing 10% FCS, then cultured in α-MEM without serum for 12 h (45,48). Nuclear proteins were extracted using the method of Dignam et al. (49) with the addition of extra proteinase inhibitors (the extraction buffer was 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol (DTT), 25% (v/v) glycerol, 0.5 mM phenylmethyl-sulfonyl fluoride, 2 μg/mL leupeptin, 2 μg/mL pepstatin A, 1 μg/mL aprotin, pH 7.9).

Northern blotting

Exponentially growing ROS 17/2.8 cells were used for transfection using overexpression plasmids. Twenty-four hours after plating, cells at 40-60% confluence were transfected using Lipofectamine reagent. The transfection mixture included 2 μg of a control empty plasmid (pCMV5), Runx2 (kindly provided by Dr. G. Karsenty), Dlx5, Smad1 or c-Src expression plasmid (kindly provided by Dr. J. Sodek). Twelve hours after transfection, the medium was changed to α-MEM containing 10% FCS and cultured for 24 h, followed by culture in α-MEM without serum for 12 h. Total RNA was extracted with guanidium thiocyanate from triplicate cultures, and purified. Aliquots (20 μg) of total RNA were fractionated in 1.2% agarose gel and transferred onto a Hybond-N+ membrane. Hybridizations were performed at 42°C with a ^{32}P-labeled rat BSP cDNA probe. After hybridization, the membranes were washed four times for 5 min each time at 21℃ in 300 mM sodium chloride, 30 mM trisodium citrate, pH 7.0, containing 0.1% SDS. This was followed by two 20-min washes at 55°C in 15 mM

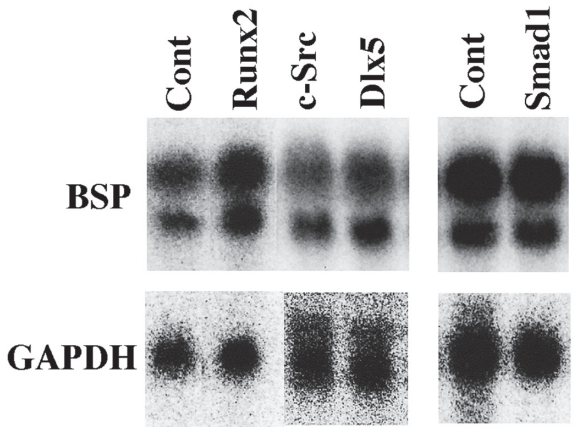

Fig. 1 Effects of Runx2, Dlx5, c-Src or Smad1 overexpression on BSP mRNA levels. ROS 17/2.8 cells were transfected with Runx2, Dlx5, c-Src, Smad1 or pCMV5 (Control) expression plasmids. Total RNA was then extracted, and the expression of BSP and GAPDH mRNA in the cells was measured by Northern blotting.

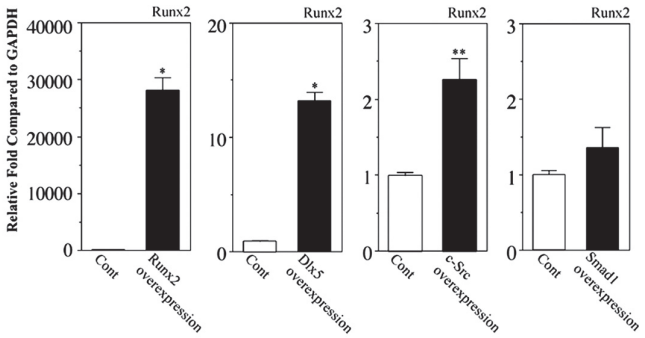

Fig. 2 Effects of Runx2, Dlx5, c-Src or Smad1 overexpression on Runx2 mRNA levels. ROS 17/2.8 cells were transfected with the Runx2, Dlx5, c-Src or Smad1 expression plasmid. Total RNA was then extracted, and the expression of Runx2 and GAPDH mRNA in the cells was measured by real-time PCR.

sodium chloride, 1.5 mM trisodium citrate, pH 7.0, and 0.1% SDS. The hybridized bands, representing the two polyadenylated forms (1.6 and 2.0 kb) of rat BSP mRNA, were scanned using a Bio-imaging analyzer (Fuji BAS 2500, Tokyo, Japan).

Real-time PCR

Total RNA (1 μg) was used as a template for cDNA synthesis. cDNA was prepared using the EXScript RT reagent Kit. Quantitative real-time PCR was performed using the following primer sets: Runx2 forward; 5′-CAAGTGGCCAGGTTCAACGA-3′, Runx2 reverse; 5′-TGTGAAGACCGTTATGGTCAAAGTG-3′,GAPDH forward;5′-GACAACTTTGGCATCGTGGA-3′, GAPDH reverse; 5′-ATGCAGGGATGATGTTCTGG-3′, using the SYBR Premix Ex Taq in a TP800 thermal cycler dice real-time system (Takara-bio, Tokyo, Japan). The amplification reactions were performed in a final volume of 25 μL containing 2 x SYBR Premix EX Taq (12.5 μL), 0.2 μM forward and reverse primers and 25 ng cDNA. To reduce variability between replicates, PCR premixes containing all reagents except for cDNA were prepared and aliquoted into 0.2-mL Hi-8-tubes (Takara-bio). The thermal cycling conditions were 10 s at 95°C and 40 cycles of 5 s at 95°C and 30 s at 60℃. Post-PCR melting curves confirmed the specificity of single-target amplification, and fold expressions of Runx2 relative to GAPDH were determined in triplicate.

Transient transfection assays

Exponentially growing ROS17/2.8 cells were used for the transfection assays. Twenty-four hours after plating, cells at 40-60% confluence were transfected using Lipofectamine reagent. The transfection mixtures included 1 μg of luciferase (LUC) constructs (45), and 2 μg β-Gal plasmid as an internal transfection control. Two micrograms of control empty plasmid (pCMV5), Runx2, Dlx5, c-Src or Smad1 expression plasmid was used for the overexpression experiments. Twelve hours after transfection, the medium was changed to α-MEM containing 10% FCS and cultured for 24 h, followed by culture in α-MEM without serum for 12 h. The luciferase assays were performed in accordance with the supplier's protocol (PicaGene, Toyo Inki, Tokyo, Japan) using a luminescence reader (Acuu FLEX Lumi 400; Aloka, Tokyo, Japan).

Statistical analysis

Quadruplicate or triplicate samples were analyzed for each experiment, and experiments were replicated to ensure the consistency of the responses to overexpression. Significant differences between control and treatment groups were determined using one-way ANOVA.

Results

The results of Northern blotting showed that BSP mRNA levels were increased by overexpression of Runx2, Dlx5 or c-Src, whereas Smad1 overexpression did not change the level of BSP mRNA (Fig. 1). Runx2 gene expression was increased by Runx2, Dlx5 or c-Src overexpression. However, Smad1 overexpression was unable to induce the expression of Runx2 mRNA (Fig. 2). To determine the effects of Runx2, Dlx5, c-Src or Smad1 overexpression on BSP transcription, we used luciferase constructs that

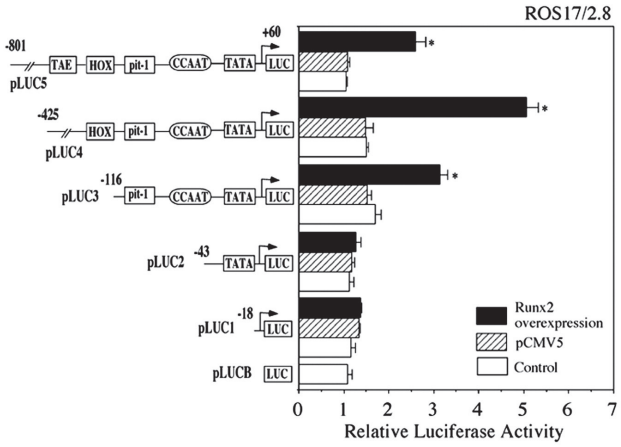

Fig. 3 Runx2 overexpression up-regulates rat BSP promoter activity. The transcriptional activities of pLUC3 (-116 to +60), pLUC4 (-425 to +60) and pLUC5 (-801 to +60) were increased by Runx2 overexpression in ROS 17/2.8 cells. The data for transcriptional activity obtained from four separate transfections with constructs, pLUC basic (pLUCB) and pLUC1 to pLUC5, have been combined, and the values expressed with standard errors *($P < 0.01$).

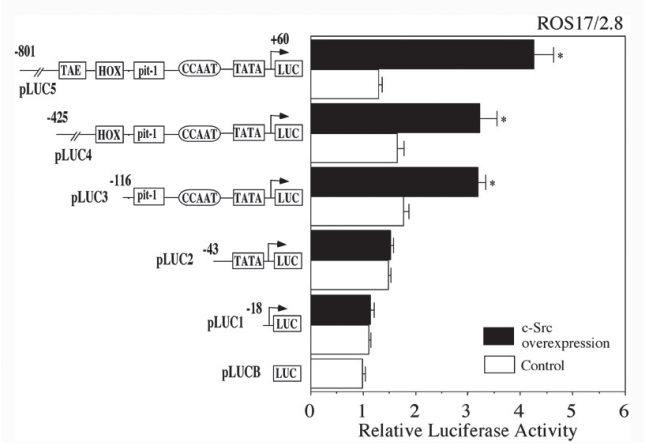

Fig. 5 c-Src overexpression up-regulates rat BSP promoter activities. The transcriptional activities of pLUC3 (-116 to +60), pLUC4 (-425 to +60) and pLUC5 (-801 to +60) were increased overexpression with c-Src in ROS 17/2.8 cells. The results of transcriptional activities obtained from four separate transfections with constructs, pLUC basic (pLUCB) and pLUC1 to pLUC6, have been combined and the values expressed with standard errors *($P < 0.01$).

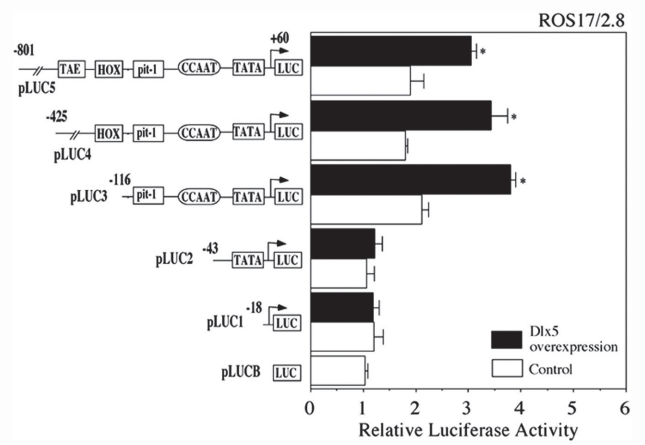

Fig. 4 Dlx5 overexpression up-regulates rat BSP promoter activity. The transcriptional activities of pLUC3 (-116 to +60), pLUC4 (-425 to +60) and pLUC5 (-801 to +60) were increased by overexpression of Dlx5 in ROS 17/2.8 cells. The data for transcriptional activity obtained from four separate transfections with the constructs, pLUC basic (pLUCB) and pLUC1 to pLUC5, have been combined, and the values expressed with standard errors *($P < 0.01$).

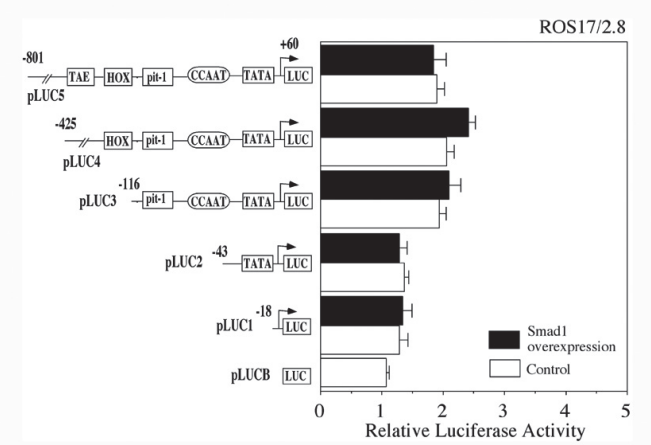

Fig. 6 Luciferase assays using rat BSP promoter constructs and a Smad1 overexpression plasmid. The transcriptional activities of BSP promoter constructs were not increased by overexpression of Smad1 in ROS 17/2.8 cells. The data for transcriptional activity obtained from four separate transfections with the constructs, pLUC basic (pLUCB) and pLUC1 to pLUC6, have been combined, and the values expressed with standard errors *($P < 0.01$).

included various regions of the rat BSP gene promoter transfected into ROS17/2.8 cells (Figs. 3-6). While not being influenced by Smad1 overexpression (Fig. 6), the luciferase activities of the BSP promoter constructs (pLUC3; -116 to +60, pLUC4; -425 to +60 and pLUC5; -801 to +60) were increased by overexpression of Runx2, Dlx5 or c-Src (Figs. 3-5). Runx2 increased pLUC4 activity maximally (Fig. 3), Dlx5 induced pLUC3, 4 and 5 activities at almost the same level (Fig. 4), and c-Src stimulated pLUC5 activity maximally (Fig. 5).

Discussion

In this study, we showed that overexpression of an osteogenic transcription factor or proto-oncogene such as Runx2, Dlx5 or c-Src in osteoblast-like cells increased the levels of Runx2 and BSP mRNA. Whereas Runx2, Dlx5 or c-Src overexpression induced BSP promoter activities, they regulated BSP transcription in different

ways, being mediated through different promoter regions (Figs. 3-5). On the other hand, Smad1 overexpression had no effect on the level of Runx2 mRNA, expression of BSP mRNA, or BSP promoter activity (Figs. 1, 2, and 6).

We have previously reported that FGF2 increased the transcription of BSP via FRE in the proximal promoter of the rat BSP gene (37), and that Runx2, Dlx5 and Smad1 are transcription factors that bind to FRE (39,40). FGF2 induced expression and transactivation of Runx2 (50). The AP1 binding site overlapping with GRE (AP1/GRE site) is another target of FGF2 regulation of BSP gene transcription (47), and the formation of FRE- and AP1/GRE-protein complexes is increased by FGF2. Moreover, the results of supershift assays showed that c-Jun, c-Fos, Dlx5, Smad1 and glucocorticoid receptor complexes were AP1/GRE binding proteins (47). BMP2 increased Dlx5 in mouse 2T3 osteoblasts and primary fetal rat calvarial osteoblasts (51). BMP2-induced Runx2 expression is mediated by Dlx5 (52). Furthermore, Dlx5 reverses Msx2 inhibition of osteocalcin promoter activation by FGF2/forskolin (53). Runx2 and Dlx5 might regulate BSP gene transcription via FRE and AP1/GRE in the rat BSP gene promoter. Therefore, Runx2 and Dlx5 could be important transcription factors for BSP transcription.

Transcription of the BSP gene is stimulated by v-Src acting through an inverted CCAAT box (35). Src inhibitor accelerates the differentiation of human bone marrow-derived mesenchymal stromal cells into osteoblasts (54). Whereas Src kinase plays a positive role in osteoclast survival and resorption activity, Src may negatively regulate osteoblast maturation (54,55). The significance of Src tyrosine kinase signaling in bone development is suggested by the osteopetrotic phenotype of Src null mice (20).

Smad1, 5 and 8 are activated by BMP receptors and transduce BMP signaling (22). TGF-β1 regulation of vimentin gene expression during the differentiation of C2C12 myoblasts requires Smads, AP1 and SP1 as transcription factors (56). We have previously identified a TGF-β activation element in the rat BSP gene promoter that mediates the stimulatory effects of TGF-β1 on BSP transcription (43). Whereas Smad1 interacted with FRE and AP1/GRE in the rat BSP gene promoter (40,47), Smad1 overexpression did not increase the level of Runx2 mRNA, expression of BSP mRNA or BSP promoter activity. A further study to clarify the reason for these discrepant findings is required.

In summary, we have shown that overexpression of Runx2, Dlx5 or c-Src in osteoblast-like cells increases the levels of Runx2 and BSP mRNA. Runx2, Dlx5 or c-Src overexpression induced BSP gene transcription that was mediated through different promoter regions. Since BSP is expressed specifically in mineralized connective tissues, Runx2, Dlx5 and c-Src are crucial osteogenic transcription factors or proto-oncogenes for bone formation, and it is conceivable that they might contribute to cell-specific expression of the BSP gene during the formation of bone extracellular matrix.

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