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Static Magnetic Fields-Induced Bone Sialoprotein (BSP) Expression Is Mediated Through FGF2 Response Element and Pituitary-Specific Transcription Factor-1 Motif

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Abstract Bone sialoprotein (BSP) is a sulfated and phosphorylated glycoprotein found almost exclusively in mineralized connective tissues. Recent studies on the developmental expression of BSP mRNA and temporo-spatial appearance of the protein during bone formation in vivo and in vitro have demonstrated that BSP is expressed by differentiated osteoblasts, and that it may function in the initial nucleation of hydroxyapatite crystals in de novo bone formation. Physical forces may play a fundamental role in the regulation of cell function in bone, but little is known about how cells are able to sense mechanical loads and signal transduction. Magnetic fields of sufficient magnitude have been shown to affect various biologic systems at organ, tissue, cellular, and subcellular levels. In the present study, rat osteosarcoma-derived osteoblast-like cells, UMR 106, were used to assess the effect of static magnetic fields (SMF) on gene transcription of BSP. In our culture system, application of 300 and 800 Gauss SMF increased BSP mRNA levels after 24 h stimulation. To determine the molecular basis of the transcriptional regulation of BSP gene transcription by SMF, we conducted transient transfection analyses with chimeric constructs of the rat BSP gene promoter linked to a luciferase (LUC) reporter gene. SMF (300 and 800 Gauss) increased expression of the construct (pLUC3; −116 to +60) after 24 h treatment. Further deletion analysis of the BSP promoter showed that a region within nt −116 to −84 was targeted by SMF, the effect of which was inhibited by the tyrosine kinase inhibitor herbimycin A (HA). Mutations (2 bp) were made in an inverted CCAAT box between nt −50 and −46, a cyclicAMP response element (CRE; between nt −75 and −68), a fibroblast growth factor-2 response element (FRE; −92 to −85), and a pituitary-specific transcription factor-1 motif (Pit-1; nt −111 to −105) within the pLUC3 construct. Transcriptional stimulation by SMF was almost completely abrogated in constructs that included 2-bp mutations in the FRE and Pit-1. Binding of nuclear proteins to a radiolabeled FRE was increased and that to a Pit-1 was decreased in nuclear extracts prepared from SMF-stimulated UMR 106 cells. Further, the stimulatory and inhibitory effects of SMF on FRE and Pit-1 DNA-protein complexes were completely abolished by HA treatment. These studies, therefore, show that SMF increases BSP transcription through a tyrosine kinase-dependent pathway and that the SMF effects are mediated through juxtaposed FRE and Pit-1 elements in the proximal promoter of the *BSP* gene. J. Cell. Biochem. 91: 1183–1196, 2004. © 2004 Wiley-Liss, Inc.

Key words: bone sialoprotein; gene regulation; mineralized tissues; static magnetic fields; ferritic magnet; transcription

Abbreviations used: BSP, bone sialoprotein; SMF, static magnetic field; LUC, luciferase; CRE, cyclicAMP response element; FRE, fibroblast growth factor-2 response element; AP-1, activator protein-1.

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Bone remodeling is controlled by a wide variety of systemic and local humoral factors. It is also established that mechanical strain plays a crucial role in bone remodeling [Klein-Nulend et al., 1997]. The absence of physical stress on the skeleton, as seen in immobilization or during space flight, can lead to an osteoporotic condition [Vico et al., 1987, 1988; Bikle et al., 1987]. A variety of mechanical stresses have been applied to bone cell cultures in vitro to examine the effect of mechanical forces on bone remodeling. Continuously applied compressive pressure suppressed alkaline phosphatase (ALPase) activity, collagen synthesis and calcification in MC3T3-E1 mouse osteoblast-like cells [Ozawa et al., 1990]. Intermittent hydrostatic compression promotes alkaline phosphatase, procollagen type I, and osteopontin (OPN) gene expression in MC3T3-E1 cells [Klein-Nulend et al., 1997]. Intermittent compressive force increased OPN expression in rat osteoblast-like ROS 17/2.8 cells [Kubota et al., 1993], and type I collagen and OPN expression in human osteoblast-like osteosarcoma cells, OHS-4 [Harter et al., 1995]. Magnetic fields are involved in part of mechanical stress. There are two major types of magnetic field: pulsing electromagnetic fields (PEMF) and static magnetic fields (SMF) [Takano-Yamamoto et al., 1992; Nagai and Ota, 1994]. The mechanisms of effects of PEMF are different from those of SMF. PEMF generate an electric current in the tissue, while SMF create only a magnetic field [Andrew and Bassett, 1982; Bruce et al., 1987]. PEMF has been used extensively for treatment of non-union fractures [Bassett et al., 1977, 1981]. SMF stimulation on the bone has a local effect to prevent the decrease in bone mineral density caused by surgical invasion or implantation [Yan et al., 1998].

Bone sialoprotein (BSP) is a 34-kDa protein that is highly sulfated, phosphorylated and glycosylated, and is expressed almost exclusively in mineralizing connective tissues [Oldberg et al., 1988; Ogata et al., 1995]. Studies on the developmental expression of BSP have shown that BSP mRNA is expressed at high levels by osteoblasts at the onset of bone formation [Chen et al., 1991, 1992; Ganss et al., 1999], and under steady-state conditions in vitro BSP nucleates hydroxyapatite crystal formation [Hunter and Goldberg, 1993] indicating a role for this protein in the initial mineralization of bone. Thus, regulation of the *BSP* gene appears to be important in the differentiation of osteoblasts and for bone matrix mineralization. BSP is also expressed in several pathologies in which mineralization occurs, including malignant breast cancer where it is associated with the formation of ectopic hydroxyapatite microcrystals [Ibrahim et al., 2000; Waltregny et al., 2000]. Thus, regulation of BSP gene transcription appears to be important in the differentiation of osteoblasts, for bone matrix mineralization and for pathologic mineralization. To study the transcriptional regulation of BSP, rat, human, and mouse BSP gene promoters were characterized [Kerr et al., 1993; Li and Sodek, 1993; Kim et al., 1994; Benson et al., 1999]. These promoters include an inverted TATA element [Li et al., 1995] overlapping a vitamin D response element [Kim et al., 1996], an inverted CCAAT box (−50 to −46), which is required for basal transcriptional activity [Kim and Sodek, 1999; Shimizu and Ogata, 2002]. In addition, a cyclicAMP response element (CRE; −75 to −68) [Samoto et al., 2002, 2003], a fibroblast growth factor-2 response element (FRE; −92 to −85) [Shimizu-Sasaki et al., 2001; Samoto et al., 2003], a pituitary-specific transcription factor-1 (Pit-1) motif (−111 to −105) which mediates the stimulatory effects of parathyroid hormone [PTH, Ogata et al., 2000], and a homeodomain binding element (HOX; −199 to −192) [Benson et al., 2000; Shimizu et al., 2003] have been characterized. Further upstream in the rat promoter a transforming growth factor-β activation element (−499 to −485) [Ogata et al., 1997; Shimizu et al., 2003] and a glucocorticoid response element (−920 to −906), overlapping an activator protein-1 (AP-1) site (−921 to −915) [Ogata et al., 1995; Yamauchi et al., 1996] have also been identified.

Although PEMF have been used for treatment of non-union fractures, little is known about SMF's effects on bone metabolism. The aim of the present study was to evaluate the ability of SMF to regulate osteoblast specific gene expression such as BSP. In this study, we show that SMF stimulates BSP expression in osteoblast-like UMR 106 cells through FRE and Pit-1 elements in the rat BSP promoter.

MATERIALS AND METHODS

Materials

Cell culture media, fetal bovine serum (FBS), lipofectamine, penicillin and streptomycin, and

trypsin were obtained from Invitrogen (Tokyo, Japan). DyNAmo SYBR green qPCR Kit and M-MLV reverse transcriptase RNase H^- were from Finnzymes (Espoo, Finland). The pGL3-basic vector, pSV-β-galactosidase control vector, recombinant Rnasin, and random hexamer were purchased from Promega Co. (Madison, WI). Protein kinase inhibitors H89 and H7 were from Seikagaku Corporation (Tokyo, Japan), and a tyrosine kinase inhibitor, herbimycin A (HA), and guanidium thiocyanate were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Ferritic magnets (300 and 800 Gauss) were from Izumi Trading Corporation (Tokyo, Japan). All other chemicals were of analytical grade.

Cell Culture

The rat clonal cell line, UMR 106 cells (generously provided by Dr. T.J. Martin) were cultured at 37°C in 5% CO_2-air in alpha-minimum essential medium (α-MEM) supplemented with 10% FBS and used in these studies as an osteoblastic cell line that synthesizes BSP [Partridge et al., 1983; Samoto et al., 2003]. Rat stromal bone marrow cells (SBMC) [Pitaru et al., 1993], were kindly provided by Dr. S. Pitaru (Tel Aviv University, Israel). Cells were first grown to confluence in 60 mm tissue culture dishes in α-MEM medium containing 10% FBS, then cultured in α-MEM without serum and treated with SMF. Square ferritic magnets (300 Gauss; $10 \times 10 \times 1$ cm and 800 Gauss; $10 \times 10 \times 2$ cm) were placed on the culture dishes for SMF treatment. RNA was isolated from triplicate cultures and analyzed for expression of BSP mRNA by Real-time PCR as described below.

Real-Time PCR

Total RNA was extracted from UMR 106 cells with guanidium thiocyanate at 24 h after treatment with SMF as described previously [Ogata et al., 1997], 1 μg was used as a template for cDNA synthesis. cDNA was prepared using random hexamer and M-MLV reverse transcriptase RNase H^-. Quantitative real-time PCR was performed using the following primer sets: BSP-R-T forward, 5′-TCCTCCTCTGAAACGGTTTCC-3′; BSP-R-T reverse, 5′-CGAACTATCGCCATCTCCATT-3′; GAPDH-R-T forward, 5′-AGATGGTGAAGG-TCGGTGTC-3′; GAPDH-R-T reverse, 5′-ATT-GAACTTGCCGTGGGTAG-3′ using the SYBR Green qPCR kit in a DNA Engine Opticon 2 continuous fluorescence detection system (MJ Research, Inc., Waltham, MA). The expected size of the PCR products for BSP and GAPDH were 73 and 167 bp, respectively. The amplification reactions were performed in 20 μl of final volume containing 1× SYBR Green Master Mix, 0.25 μM primer mixture and 10 ng cDNA. To reduce variability between replicates, PCR premixes, which contain all reagents except for cDNA, were prepared and aliquoted into 0.2 ml thin-wall strip tubes (MJ Research, Inc.). The thermal cycling conditions were 40 cycles of the following protocol: 15 s denaturation at 95°C; 50 s annealing at 64°C, followed by 12 s extension at 77°C. Post-PCR melting curves confirmed the specificity of single-target amplification and—fold expression of BSP relative to GAPDH was determined in triplicate [Muller et al., 2002].

Transient Transfection Assays

Exponentially growing UMR 106 cells were used for the transfection assays. Twenty-four hours after plating, cells at 50–70% confluence were transfected using a Lipofectamine reagent [Rose et al., 1991]. The transfection mixture included 1 μg of a luciferase (LUC) construct [Ogata et al., 1995] and 2 μg pSV-β-galactosidase control vector (β-gal) as an internal control. Two days post-transfection, cells were deprived of serum for 12 h and treated with 300 or 800 Gauss of SMF for 24 h prior to harvesting. The LUC assay was performed according to the supplier's protocol (PicaGene, Toyo Inki, Tokyo, Japan) using a Luminescence reader BLR20 (Aloka) to measure the LUC activity. The protein kinase inhibitor H89 (5 μM) and H7 (5 μM) were used to inhibit protein kinase A and C. HA (1 μM) was used for tyrosine kinase inhibition [Shimizu-Sasaki et al., 2001; Samoto et al., 2002]. Oligonucleotide-directed mutagenesis by PCR was utilized to introduce dinucleotide substitutions using the quick change site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). All constructs were sequenced as described previously to verify the fidelity of the mutagenesis [Shimizu-Sasaki et al., 2001].

Gel Mobility Shift Assays

Confluent UMR106 cells in T-75 flasks treated for 24 h with 300 or 800 Gauss in α-MEM without serum were used to prepare nuclear extracts as described in our previous papers

[Ogata et al., 1997; Shimizu-Sasaki et al., 2001; Samoto et al., 2003] 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 aprotinin, pH 7.9). Protein concentration was determined by the Bradford [1976] method. Double-stranded oligonucleotides encompassing the inverted CCAAT (nt −61 to −37, 5′-CCGTGACCGTG**ATTGG**CTGCTGAGA), FGF2 response element (FRE; nt −98 to −79, 5′-TTTTCT**GGTGAGAA**CCCACA), and Pit-1 (nt −115 to −85: 5′-CGTG**TTGTAGT**TACGGATTTTCTGGTGAGAA) in the BSP promoter were prepared by Bio-Synthesis, Inc. (Lewisville, TX). For gel shift analysis the double-stranded-oligonucleotides were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Nuclear protein extracts (3 μg) were incubated for 20 min at room temperature (RT = 21°C) with 0.1 pM radiolabeled ds-oligonucleotide in buffer containing 50 mM KCl, 0.5 mM EDTA, 10 mM Tris-HCl (pH 7.9), 1 mM DTT, 0.04% Non-idet P-40, 5% glycerol, and 1 μg poly (dI-dC). Incubation mixtures were resolved by electrophoresis on 5% non-denaturing acrylamide gels (38:2 acrylamide/bis acrylamide) run at 150 V at RT. Following electrophoresis, the gels were dried and autoradiograms were analyzed by a Bio-Imaging analyzer (Fuji BAS2000, Tokyo, Japan)

Statistical Analysis

Triplicate samples were analyzed for each experiment and replicated to ensure consistency of the responses to SMF. Significant differences between control and SMF treatment were determined using the Student's *t*-test.

RESULTS

Stimulation of BSP mRNA Expression in UMR 106 Cells

BSP gene expression was investigated at 24 h after SMF stimulation by real-time PCR (Fig. 1). When osteoblastic UMR106 cells were exposed to 300 and 800 Gauss SMF, expression of BSP mRNA was increased ~2.2- and 2.7-fold at 24 h.

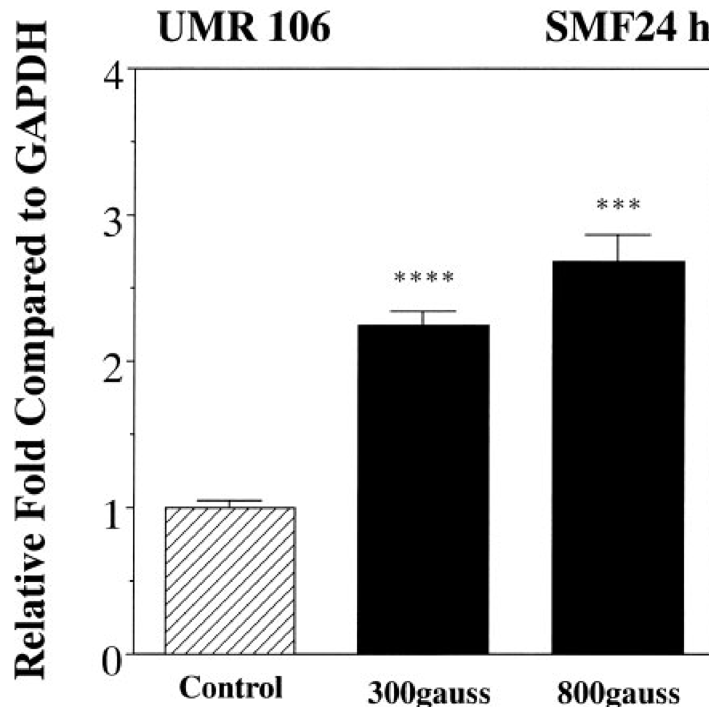

Fig. 1. Effect of static magnetic fields (SMF) on bone sialoprotein (BSP) mRNA levels. Relative gene expression for BSP generated from real-time PCR of UMR 106 cells treated with 300 and 800 Gauss SMF. The expression of GAPDH was also examined as a control. The relative amounts of mRNA of BSP to GAPDH were calculated. The experiments were performed in triplicate for each data point, and the standard errors are shown as error bars. Significant differences compared with controls are shown at the following probability levels: ****, $P < 0.01$; ***, $P < 0.02$.

Transient Transfection Analysis of Rat BSP Promoter Constructs

Transient transfection of chimeric constructs encompassing different regions of the rat BSP promoter ligated to a LUC reporter gene (pLUC3-pLUC5), were performed in UMR 106 cells. While the results of transfection assays indicated no increase in transcription at 12 h (Fig. 2A), a ~1.8-fold increase in transcription was seen after 24 h treatment by 300 and 800 Gauss (Fig. 2B) using the construct, pLUC3, which encompasses nucleotides −116 to +60, as well as in longer constructs (pLUC4; −425 to +60, pLUC5; −801 to +60). In shorter constructs (pLUC1; −18 to +60, pLUC2; −43 to +60), LUC activities were not increased by 300 and 800 Gauss SMF (data not shown). When transcriptional activity in response to 800 Gauss SMF was analyzed in normal rat SBMC [Pitaru et al., 1993], the transcriptional activity of pLUC3 was increased 1.9-fold (Fig. 2C). Included within the DNA sequence that is unique to this region (pLUC3; between nt −116 and +60) is an inverted CCAAT box (ATTGG; between nt −50 and −46), a possible CRE (between nt −75 and −68), FRE (between nt −92 and −85) and a Pit-1 motif (between nt −111 and −105) (Fig. 3). To more closely define the regulatory element between nt −116 and −43 that is utilized by SMF, we prepared a series of 5′-deletion constructs. Transcription by constructs −108 BSPLUC and −116 BSPLUC was increased by SMF, but no increase was seen with −84 BSPLUC (Fig. 4). Since protein kinases mediate SMF signaling, we also investigated the effects of the protein kinase C inhibitor H7, the protein kinase A inhibitor H89, and the tyrosine kinase inhibitor HA on SMF-mediated transcription. Although SMF induction of −116 BSPLUC promoter activity was inhibited by HA, no effects were observed for PKC and PKA (Fig. 5), indicating involvement of tyrosine kinase in the signaling pathway. Next, we introduced mutations in the possible response elements encoded within nt −116 to +60 of pLUC3 as shown in Figure 6. Whereas mutations in the CRE had little effect on SMF and mutation of the CCAAT box essentially abolished basal expression, mutations of the FRE and the Pit-1 significantly reduced the SMF effects on the transcriptional activities (Fig. 6). These results suggest that the FRE and the Pit-1 are required as functional *cis*-elements for up-regulation of BSP transcripion by SMF.

Gel Mobility Shift Assays

To determine how the nuclear proteins that bind to the FRE and Pit-1 elements might be modulated by SMF, double-stranded oligonucleotides were end-labeled and incubated with equal amounts (3 μg) of nuclear proteins extracted from confluent UMR 106 cells that were either not treated (control) or treated with 300 and 800 Gauss SMF for 24 h. When the FRE and Pit-1 were used as probes, the formation of FRE–protein complexes was increased by SMF (Fig. 7, lanes 1–3). On the other hand, the formation of Pit-1–protein complexes was decreased by SMF (Fig. 8, lanes 1–3). When we used the inverted CCAAT sequence as a probe, the DNA-NF-Y protein complex [Kim and Sodek, 1999; Shimizu and Ogata, 2002] did not change after SMF stimulation (Fig. 9, lanes 1–3). Since the results of transient transfection assays indicate that the effects of SMF are mediated through a tyrosine kinase-dependent pathway, we used tyrosine kinase inhibitor HA together with SMF to prepare the nuclear extracts. While HA had no effect on the CCAAT DNA-NF-Y protein complex (Fig. 9, lanes 4–6), HA completely abolished the stimulatory and the inhibitory effects of SMF on the formation of FRE and Pit-1 DNA-protein complexes (Fig. 7 and 8, lanes 4–6).

DISCUSSION

These studies have shown for the first time that SMF increases expression of *BSP* gene in osteoblastic UMR 106 cells. Transduction of the SMF signaling is mediated through a tyrosine kinase, which target nuclear proteins that bind to FRE and Pit-1 elements in the proximal promoter of the *BSP* gene.

Mechanical loading plays an important role in regulating bone metabolism. Increased mechanical loading increases bone formation and decreases bone resorption [Harter et al., 1995; Schmidt et al., 1998]. However, the mechanism by which bone cells sense and respond to their physical environment is still poorly understood. The sensitivity of bone tissue to mechanical loading has been proposed to be involved a variety of cellular biophysical signals including loading-induced electric fields, matrix strain, and fluid flow [You et al., 2001].

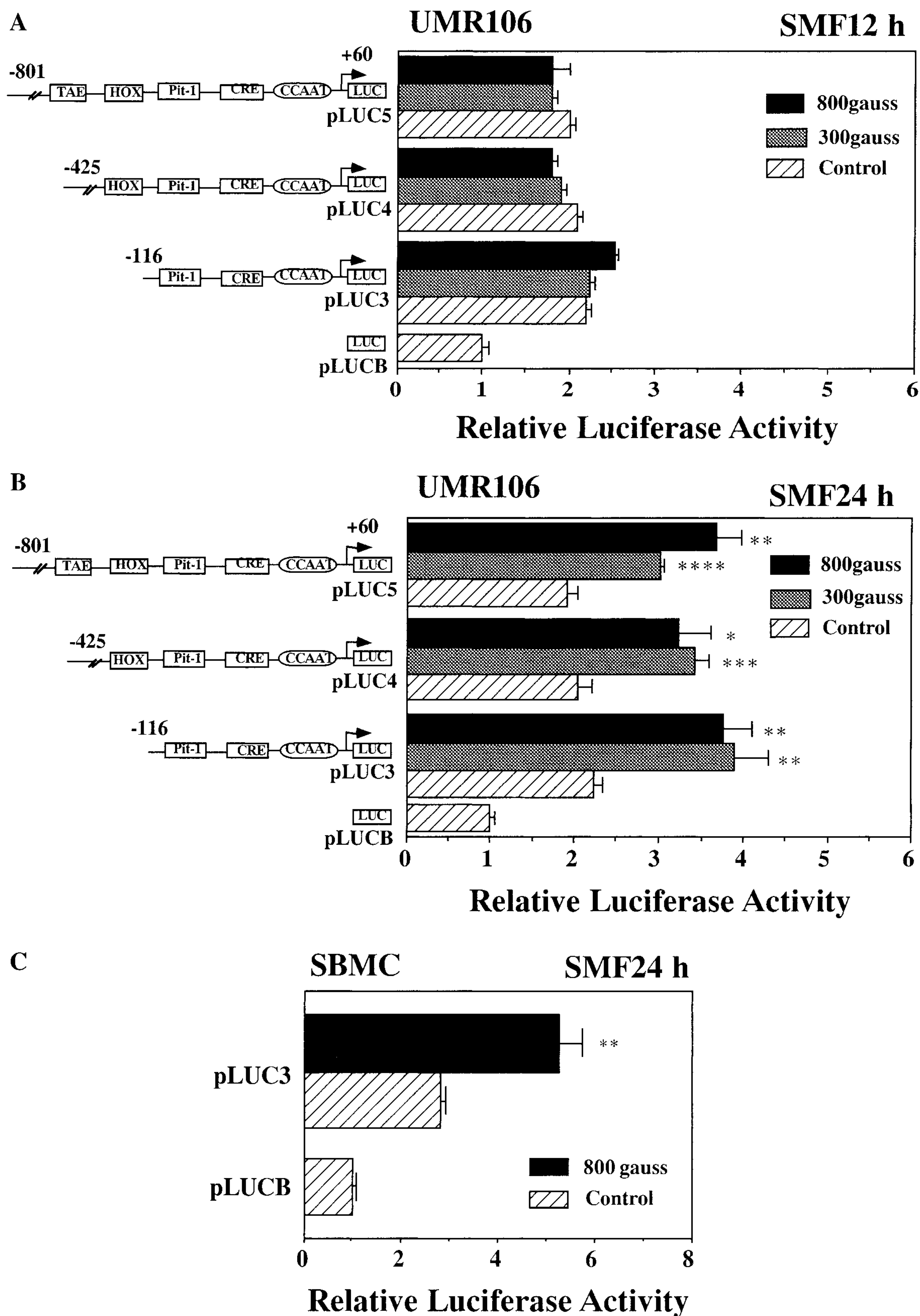

Fig. 2.

Bruce et al. [1987] reported no significant effect of SMF on fracture healing in the rabbit radius. Yan et al. [1998] reported that long-term local SMF stimulation of bone has a local effect to prevent a decrease in bone mineral density. Ali Darendeliler et al. [1995] showed that SMF increased in both the organization and amount of new bone deposited in the area of tension between the orthodontically moved maxillary incisors of the guinea pig.

BSP is a unique marker of early osteogenic differentiation that can regulate the formation of mineral crystals. In UMR 106 cells, SMF (300 and 800 Gauss) stimulated BSP promoter activity (pLUC3) ~1.8-fold (Fig. 2B), which is comparable with the increases in BSP mRNA levels of ~2.2- and 2.7-fold by real-time PCR (Fig. 1). SMF also induced BSP transcription in SBMC (Fig. 2C), indicating that increased BSP expression occurs in normal osteoprogenitor cells and is not a specific feature of transformed UMR 106 cells. From transient transfection assays, we initially located the SMF-responsive region to the proximal promoter (nt −116 and −43; Fig. 2B) of the *BSP* gene, which encompasses an inverted CCAAT box (nt −50 and −46), a CRE (nt −75 and −68), a FGF2 response element (FRE; nt −92 and −85), and a Pit-1 (nt −111 and −105) motif (Fig. 3). The results of LUC analyses using 5′-deletion constructs between nt −116 and −43 in the BSP promoter show that the SMF effects are targeted to a region encompassed by nt −116 and −84 (Fig. 4). Although mutation of the CRE was without effect, mutation of the CCAAT element resulted in the loss of basal transcriptional activity, as reported previously [Kim and Sodek, 1999; Shimizu and Ogata, 2002]. As a consequence the involvement of the inverted CCAAT was difficult to ascertain. However, the lack of SMF-induced transcription with constructs −84 and −60BSPLUC (Fig. 4) indicates that the CCAAT is not a target of SMF regulation. In comparison, mutations in the FRE and Pit-1 sites suggest that they are required for the induction of BSP expression by the SMF. The involvement of the FRE and Pit-1 elements is further supported by gel shift analyses in which proteins from nuclear extracts formed complexes with the FRE and Pit-1 elements that were increased and decreased by SMF (Figs. 7 and 8). In addition, tyrosine kinase inhibitor HA completely abolished the stimulatory and inhibitory effects of SMF on the formation of FRE and Pit-1 DNA-protein complexes. The nuclear factor binding to the FRE element, which is regulated by tyrosine kinase (Src) and MAP kinase, has yet to be characterized and is the focus of our studies because of its potential role in regulating basal and FGF2-induced expression of BSP in osteoblasts [Shimizu-Sasaki et al., 2001], as well as mediating the SMF effects. Pit-1 is a suppressor element in the rat BSP gene promoter, which is the target of PTH stimulated transcription of the *BSP* gene [Ogata et al., 2000]. PTH acts through a protein kinase A pathway involving cAMP to stimulate BSP transcription by blocking the action of a Pit-1-related nuclear protein that suppresses BSP transcription by binding a cognate element in the BSP promoter. Western blot results using the anti-Pit-1 antibodies showed the presence of an immunoreactive 30 kDa protein in UMR 106 cells (data not shown). A combination of transcription assays and gel mobility shifts has revealed that SMF increases FRE binding activity and abrogates the suppressor activity of a Pit-1-like protein that binds to a Pit-1 element in the proximal promoter of the *BSP* gene.

Osteopontin is one of the target molecules in the mechanical stress-induced regulation of bone metabolism [Kubota et al., 1993; Harter et al., 1995; Klein-Nulend et al., 1997; You et al., 2001]. Osteocalcin and type I collagen are significantly reduced in rats after 7 days of spaceflight [Patterson-Buckendahl et al., 1985]. These results show that mechanical strain is

Fig. 2. SMF up-regulates BSP promoter activity in UMR-106 cells. **A**, **B**: Transient transfections of UMR 106 cells in the presence or absence of SMF (300 and 800 Gauss) for 12 h (A) and 24 h (B), were used to determine transcriptional activity of chimeric constructs that included various regions of the BSP promoter ligated to a luciferase (LUC) reporter gene. The results of transcriptional activity obtained from three separate transfections with constructs; pLUC basic (pLUCB) and pLUC3 to pLUC5 have been combined and the values expressed with standard errors. Significant differences compared to controls are shown at the following probability levels: *, $P < 0.1$; **, $P < 0.05$; ***, $P < 0.02$; ****, $P < 0.01$. **C**: Transient transfections of rat stromal bone marrow cells (SBMC) in the presence or absence of SMF (800 Gauss) for 24 h were used to determine transcriptional activity of chimeric constructs that included the BSP promoter ligated to a LUC reporter gene. The results of transcriptional activity obtained from three separate transfections with constructs; pLUC basic (pLUCB) and pLUC3 have been combined and the values expressed with standard errors. Significant difference compared with control at the $P < 0.05$ level is shown (**).

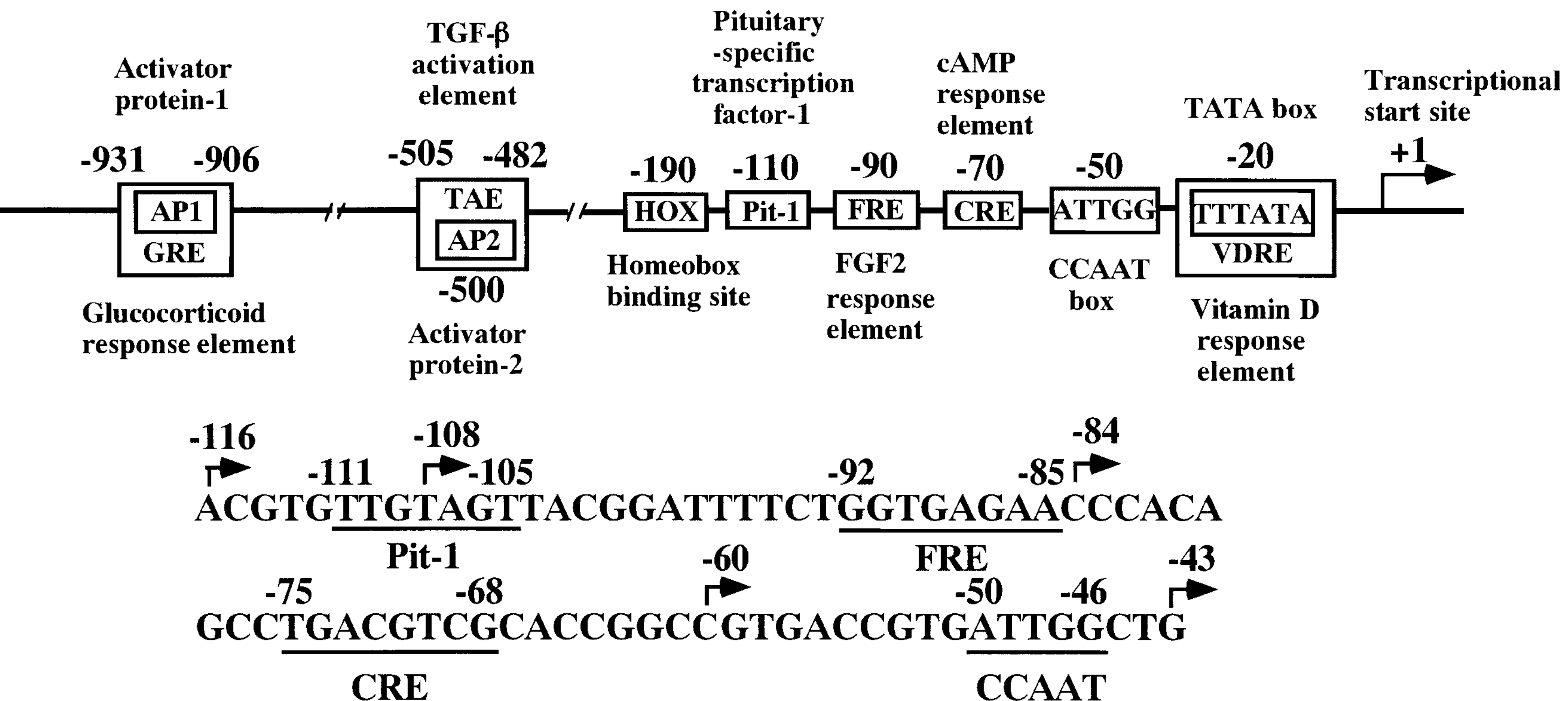

Fig. 3. Regulatory elements in the proximal rat BSP promoter. The positions of the inverted TATA and CCAAT boxes, a cyclicAMP response element (CRE), an FGF2 response element (FRE), a pituitary-specific transcription factor-1 (Pit-1), a homeobox-binding site (HOX), a TGF-β activation element (TAE) overlapping with AP2, glucocorticoid response elements (GRE) overlapping the AP1, and a vitamin D response element (VDRE) that overlaps the inverted TATA box are shown in the proximal promoter region of the rat *BSP* gene. The numbering of nucleotides is relative to the transcription start site (+1).

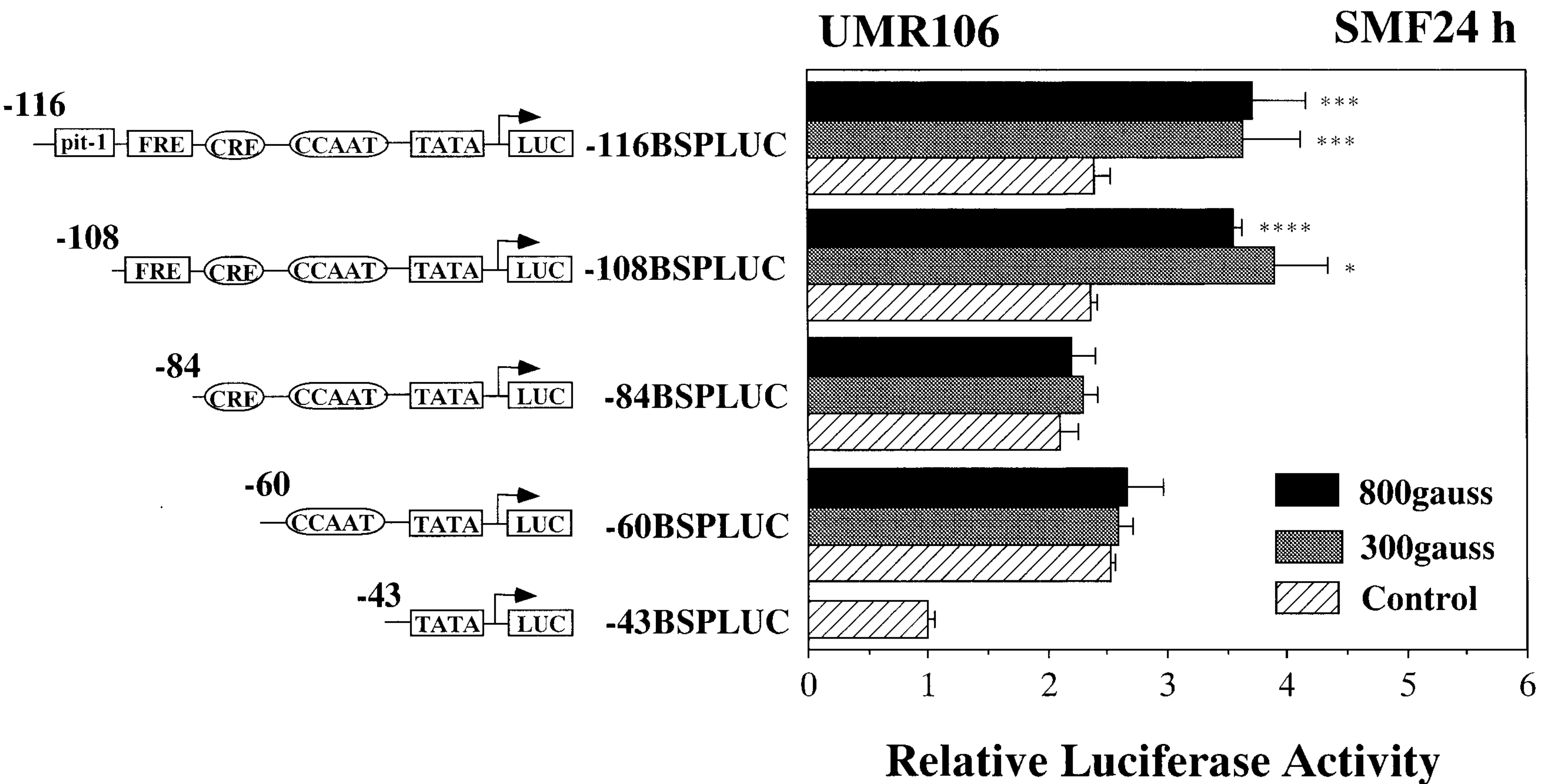

Fig. 4. Fine 5′-deletion mapping of the nt −116 to −43 element in the BSP promoter. A series of rat BSP promoter 5′-deletion constructs were analyzed for relative promoter activity after transfection into UMR 106 cells and examined for induction in the presence of SMF (300 and 800 Gauss) for 24 h. The results of transcriptional activity obtained from three separate transfections with constructs; −43 BSPLUC (−43 to +60), −60 BSPLUC (−60 to +60), −84 BSPLUC (−84 to +60), −108BSPLUC (−108 to +60), −116 BSPLUC (−116 to +60) have been combined and the values expressed with standard errors. Significant differences compared to controls are shown at the following probability levels: *, $P < 0.1$; ***, $P < 0.02$; ****, $P < 0.01$.

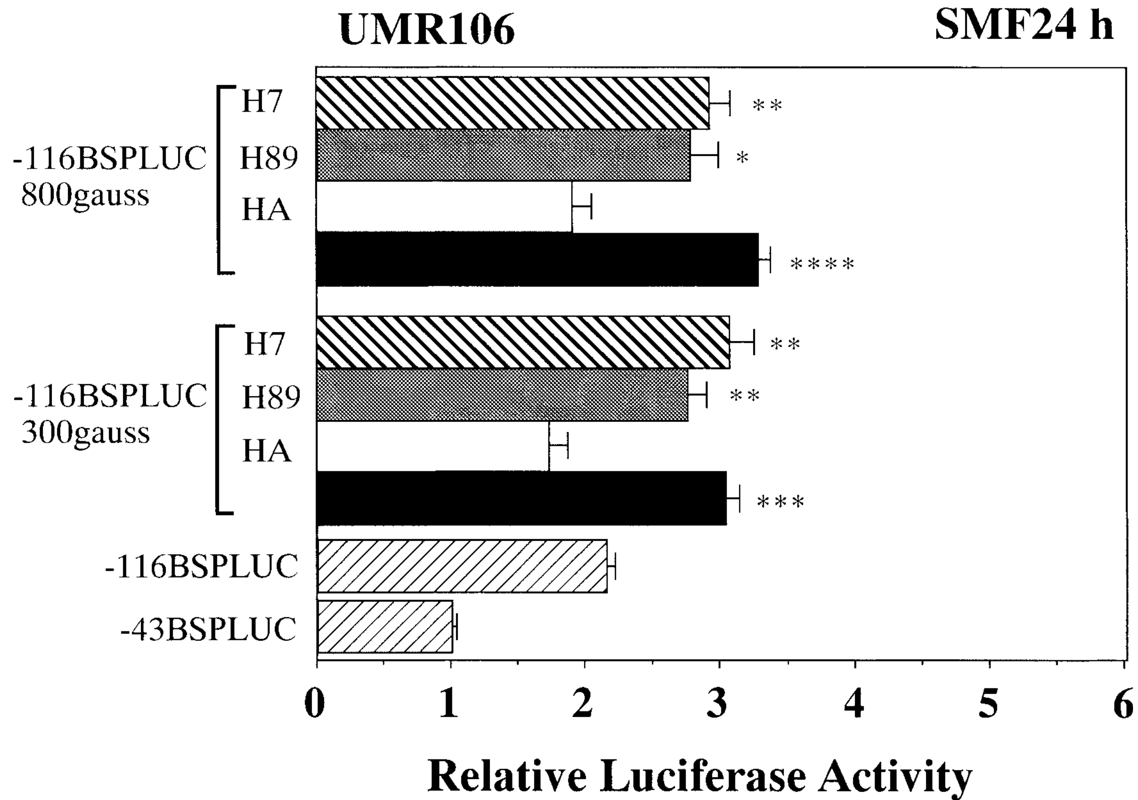

Fig. 5. Effect of kinase inhibitors on transcriptional activation by SMF. Transient transfection analysis of −116BSPLUC in the presence or absence of SMF (300 and 800 Gauss) for 24 h in UMR 106 cells is shown together with the effects of the PKC inhibitor (H7, 5 μM), PKA inhibitor (H89, 5 μM), and tyrosine kinase inhibitor (herbimycin A; HA, 1 μM). The results obtained from three separate transfections were combined and the values expressed with standard errors. Significant differences compared to controls are shown at the following probability levels: *, $P < 0.1$; **, $P < 0.05$; ***, $P < 0.02$; ****, $P < 0.01$.

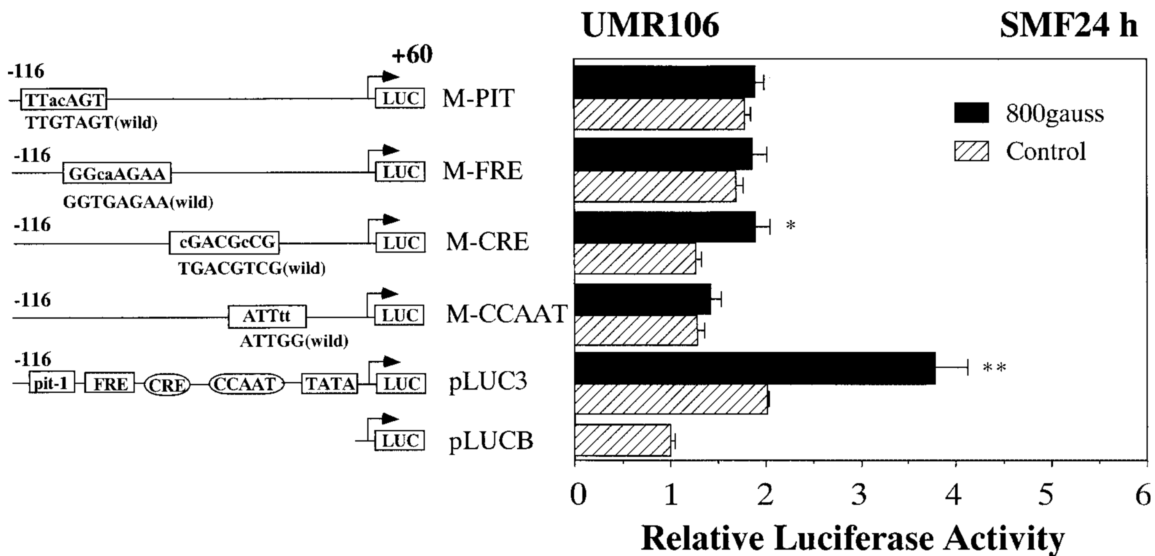

Fig. 6. Site mutation analysis of LUC activities in response to SMF. Dinucleotide substitutions were made within the context of the homologous −116 to +60 (pLUC3) BSP promoter fragments. M-CCAAT (ATT**tt**), M-CRE (**c**GACG**c**CG), M-FRE (GG**ca**AGAA), and M-PIT (TT**ac**AGT) were analyzed for relative promoter activity after transfection into UMR 106 cells and examined for induction in the presence of SMF (800 Gauss) for 24 h. The results of transcriptional activity obtained from three separate transfections with constructs were combined and the values expressed with standard errors. Significant differences compared to controls are shown at the following probability levels: *, $P < 0.1$; **, $P < 0.05$.

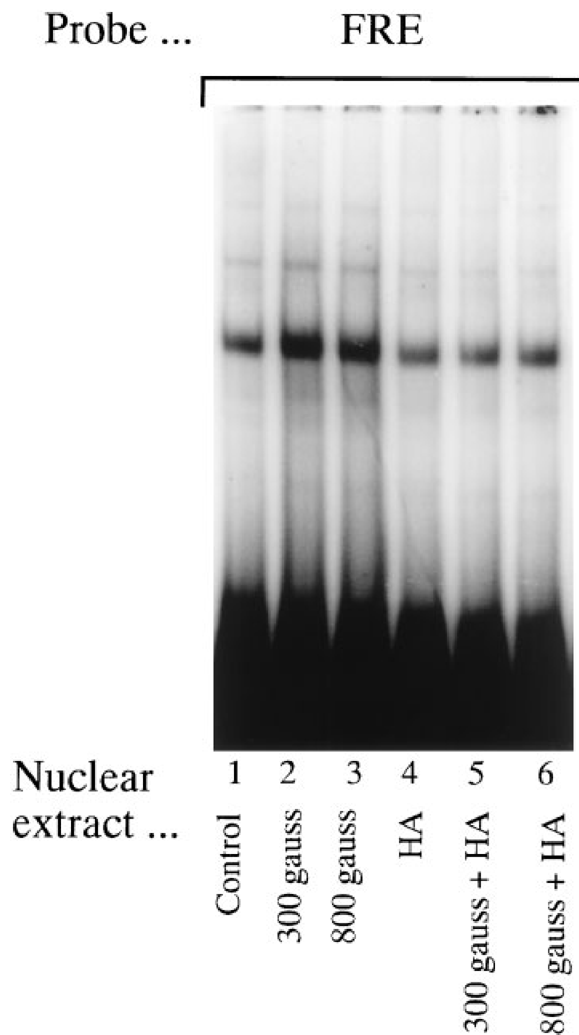

Fig. 7. SMF up-regulates a nuclear protein that recognizes the FRE. Radiolabeled double-stranded FRE (−98 TTTTCT**GGTGA-GAA**CCCACA −79) oligonucleotides were incubated for 20 min at 21°C with nuclear protein extracts (3 μg) obtained from UMR 106 cells incubated without (**lane 1**) and with 300 Gauss SMF (**lane 2**), 800 Gauss (**lane 3**), HA (**lane 4**), 300 Gauss + HA (**lane 5**), 800 Gauss + HA (**lane 6**) SMF for 24 h. DNA–protein complexes were separated on 5% polyacrylamide gel in low-ionic-strength tris-borate buffer, dried under vacuum, and exposed to an imaging plate for quantitation using an image analyzer.

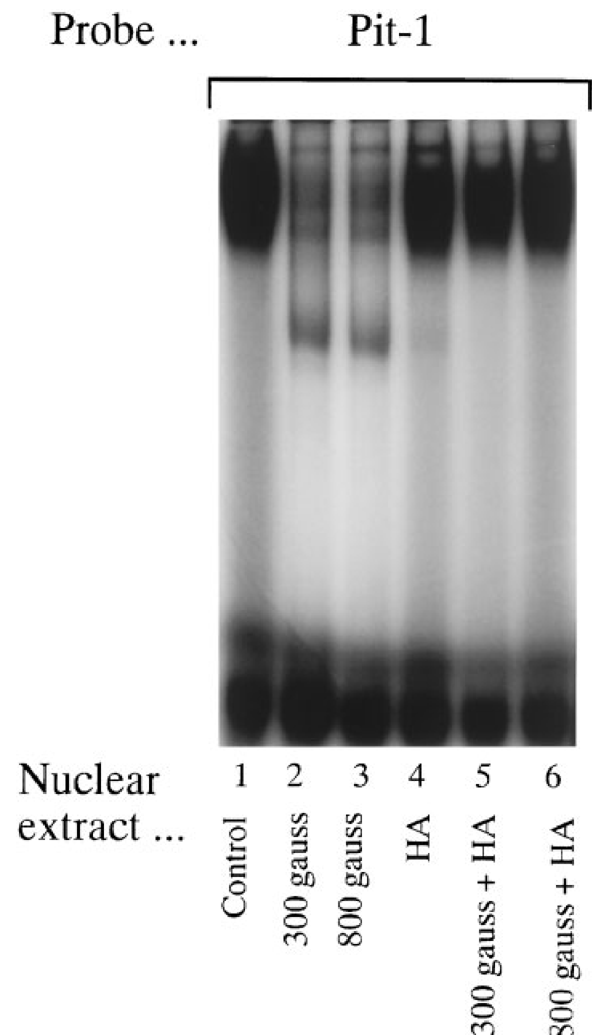

Fig. 8. SMF decreases the DNA binding activity recognized by Pit-1 sequence. Radiolabeled double-stranded Pit-1 (−115 CGTG**TTGTAGT**TACGGATTTTCTGGTGAGAA −85) oligonucleotide was incubated for 20 min at 21°C with nuclear protein extracts (3 μg) obtained from UMR 106 cells incubated without (**lane 1**) and with 300 Gauss SMF (**lane 2**), 800 Gauss (**lane 3**), HA (**lane 4**), 300 Gauss + HA (**lane 5**), 800 Gauss + HA (**lane 6**) SMF for 24 h. DNA–protein complexes were separated on 5% polyacrylamide gel in low-ionic-strength tris-borate buffer, dried under vacuum, and exposed to an imaging plate for quantitation using an imaging analyzer.

crucial for maintenance of bone volume and bone metabolism. Previous studies using cardiac myocytes have shown that mechanical stretching is mediated through a serum response element (SRE; CCATATTAGG) in c-*fos* promoter [Sadoshima and Izumo, 1993]. Platelet-derived growth factor (PDGF) B chain promoter contains a fluid shear-stress-responsive element (SSRE; GAGACC) and mechanical stress induced PDGF gene expression is mediated through SSRE in endothelial cells [Resnick et al., 1993; Khachigian et al., 1995]. Mechanical induction of c-*fos* expression in UMR 106 cells is mediated by multiple response elements (SRE, SSRE, and CRE) [Peake and El Haj, 2003]. Fluid shear stress-induced cyclooxygenase-2 expression is mediated by C/EBP β, CRE binding protein, and AP-1 in osteoblastic MC3T3-E1 cells [Ogasawara et al., 2001]. In this study, we identified the SMF-responsive

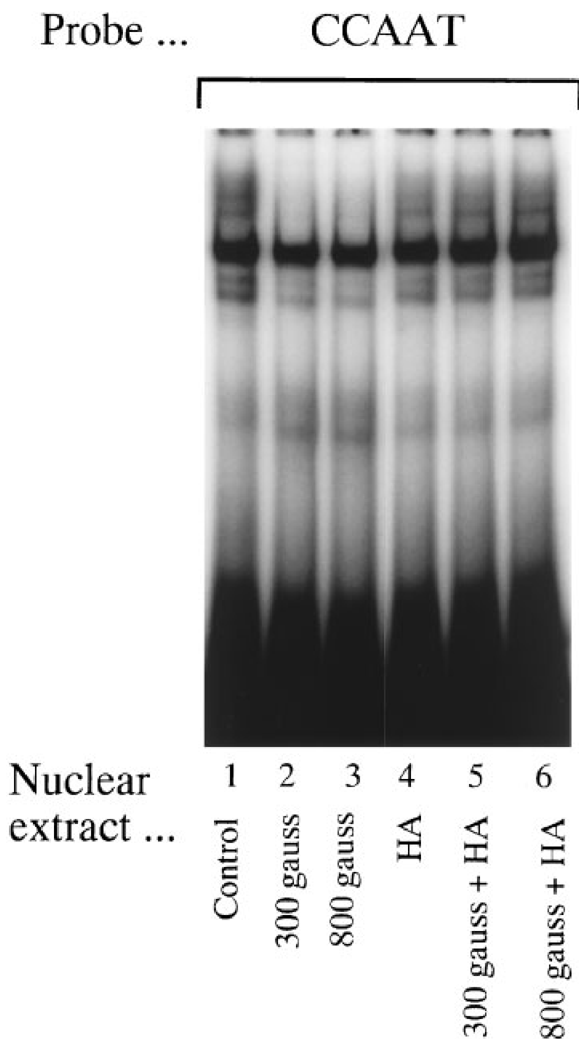

Fig. 9. Gel mobility shift assays using inverted CCAAT oligonucleotides. Radiolabeled double-stranded CCAAT (−61 CCGTGACCGTG**ATTGG**CTGCTGAGA −37) oligonucleotide was incubated for 20 min at 21°C with nuclear protein extracts (3 μg) obtained from UMR 106 cells incubated without (**lane 1**) and with 300 Gauss SMF (**lane 2**), 800 Gauss (**lane 3**), HA (**lane 4**), 300 Gauss + HA (**lane 5**), 800 Gauss + HA (**lane 6**) SMF for 24 h. DNA–protein complexes were separated on 5% polyacrylamide gel in low-ionic-strength tris-borate buffer, dried under vacuum, and exposed to an imaging plate for quantitation using an imaging analyzer.

elements FRE and Pit-1 in the proximal promoter of the rat *BSP* gene. The nucleotide sequence of FRE (CT**GGT<u>GAGAA</u>**CC) is quite similar to that of SSRE. While PTH rapidly inhibits the Pit-1 binding to Pit-1 motif within 7 min in ROS 17/2.8 cells [Ogata et al., 2000], SMF stimulates BSP transcription and inhibits formation of the Pit-1–DNA protein complex at 24 h, suggesting that SMF regulates BSP transcription indirectly. The ability of the tyrosine kinase inhibitor HA (Figs. 7 and 8) to block the SMF effects on the FRE and Pit-1 binding proteins implicated tyrosine phosphorylation as an early event in the signaling pathway of SMF.

In this study, we have shown that SMF increases BSP expression through a tyrosine kinase signaling pathway, and that FRE and Pit-1 elements in the BSP promoter have been identified as the target of SMF-mediated regulation of BSP gene transcription.

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