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ARTICLES

Effect of Chlorpromazine on Bone Sialoprotein (BSP) Gene Transcription

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Abstract Bone sialoprotein (BSP), an early marker of osteoblast differentiation. Whereas physical forces may play an important role in the regulation of bone cell function, little is known about how cells are able to sense mechanical loads. Chlorpromazine, a tranquilizing agent for treatments of psychiatric disorders, mimics hypotonic stress and causes membrane deformation. Application of 10 μg/ml of chlorpromazine suppressed BSP mRNA levels after 12 and 24 h in osteoblast-like ROS17/2.8 cells and rat stromal bone marrow cells (SBMC-D8). Chlorpromazine (10 μg/ml) decreased luciferase activity of the construct (pLUC3; −116 to +60 of the rat BSP gene promoter) after 12 h, the effect was inhibited by the tyrosine kinase inhibitor herbimycin A (HA) and MAP kinase kinase inhibitor U0126. Introduction of 2-bp mutation in the pLUC3 construct showed that the chlorpromazine effects were mediated by cAMP response element (CRE) and FGF2 response element (FRE). In gel shift assays, using radiolabeled double-stranded CRE and FRE oligonucleotides, which revealed decreased binding of nuclear proteins from chlorpromazine-stimulated cells. These studies, therefore, show that chlorpromazine suppresses BSP gene transcription through tyrosine and MAP kinases-dependent pathways and that the chlorpromazine effects are mediated by CRE and FRE elements in the proximal promoter of the *BSP* gene. J. Cell. Biochem. 97: 1198–1206, 2006. © 2005 Wiley-Liss, Inc.

Key words: bone sialoprotein; chlorpromazine; gene regulation; hypotonic stress; mineralized tissues; osteoblasts; stromal bone marrow cell; transcription

Chlorpromazine is a phenothiazine derivative and a potent tranquilizing drug for treatment of psychiatric disorders. It is known to occasionally induce toxic reactions such as growth retardation and malformation of bone, caused by slow progress of ossification [Chikuma et al., 1987]. Chlorpromazine decreased alkaline phosphatase (ALP) activity in calvaria in vivo. The effects were more specific for calvaria than for duodenum and liver [Komoda et al., 1985a,b]. Chlorpromazine suppressed ALP activity and collagen synthesis in osteoblast-like MC3T3-E1 cells [Komoda et al., 1985a,b]. Ca and Pi levels in the serum and urine are elevated and ALP activity and vitamin D3 levels are reduced in chlorpromazine-treated rat [Komoda et al., 1988; Oh-ie et al., 2002]. The effect of hypotonic stress is mimicked by chlorpromazine, which is known to cause membrane deformation [Sheetz and Singer, 1974] and mechanical stress to cardiac myocytes [Sadoshima et al., 1996].

Bone sialoprotein (BSP) is a highly sulfated, phosphorylated, and glycosylated protein that is characterized by its ability to bind to hydroxyapatite through polyglutamic acid sequences and to mediate cell attachments through an RGD sequence [Ogata et al., 1995]. The temporospatial deposition of BSP into the extracellular matrix [Chen et al., 1991] and the ability of BSP to nucleate hydroxyapatite crystal formation

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[Hunter and Goldberg, 1993] indicate a potential role for this protein in the initial mineralization of bone, dentin, and cementum. Recent studies have shown that BSP is also expressed by osteotropic cancers, suggesting that BSP might play a role in the pathogenesis of bone metastases [Waltregny et al., 2000]. Regulation of the BSP gene thus appears to be important in the differentiation of osteoblasts, in bone matrix mineralization, and in tumor metastasis. The rat, human, and mouse BSP genes have been cloned and partially characterized [Kerr et al., 1993; Li and Sodek, 1993; Kim et al., 1994; Benson et al., 1999]. These promoters include a functional inverted TATA box (nts −24 to −19) [Li et al., 1995], overlapping vitamin D response elements [Kim et al., 1996], and an inverted CCAAT box (−50 to −46), which is required for basal transcription [Kim and Sodek, 1999; Shimizu and Ogata, 2002]. In addition, a fibroblast growth factor 2 (FGF2) response element (FRE; −92 to −85) [Shimizu-Sasaki et al., 2001; Samoto et al., 2003, Shimizu et al., 2004a, 2005], a cAMP response element (CRE; −75 to −68) [Samoto et al., 2002, 2003], a transforming growth factor-β activation element (−499 to −485) [Ogata et al., 1997; Shimizu et al., 2004b, 2005], a pituitary-specific transcription factor-1 (Pit-1) motif (−111 to −105) that mediates the stimulatory effects of parathyroid hormone [Ogata et al., 2000; Shimizu et al., 2004a], and a homeodomain binding element (−199 to −192) have been characterized [Benson et al., 2000; Shimizu et al., 2004b]. Further upstream, a glucocorticoid response element overlapping an AP-1 site has also been identified [Ogata et al., 1995; Yamauchi et al., 1996].

To elucidate the effects of chlorpromazine on BSP gene expression and to identify the chlorpromazine response element in the BSP gene promoter, we stimulated osteoblast-like cells with chlorpromazine to mimic hypotonic stress.

METHODS

Materials

α-Minimum essential medium (α-MEM), fetal calf serum (FCS), Lipofectamine, penicillin, and streptomycin, SuperScript one-step RT-PCR with Pratinum Taq and trypsin were obtained from Invitrogen (Carlsbad, CA). The pGL3-basic vector and pSV-β-galactosidase control vector were purchased from Promega Co., (Madison, WI). Chlorpromazine hydrochloride was purchased from Sigma Aldrich (Tokyo, Japan). The protein kinase inhibitors H89 and H7 were from Seikagaku Corporation (Tokyo, Japan), and the tyrosine kinase inhibitor, herbimycin A (HA) and guanidium thiocyanate were purchased from Wako Pure Chemical (Tokyo, Japan).

Cell Culture

The rat clonal cell lines ROS17/2.8 [Ogata et al., 1995] and SBMC-D8 (clonal cells derived from rat stromal bone marrow cell line) [Pitaru et al., 1993] were used in these studies as osteoblastic cell line that synthesizes BSP. The cells were cultured at 37°C in 5% CO_2 air in α-MEM supplemented with 10% FBS. Cells were first grown to confluence in 60 mm tissue culture dishes, then cultured in α-MEM without serum and incubated with or without chlorpromazine (10 μg/ml) for time periods of 3–24 h. RNA was isolated from triplicate cultures and analyzed for the expression of BSP mRNA by Northern hybridization and RT-PCR as described below.

Northern Hybridization

Twenty microgram aliquots of total RNA were fractionated on 1.2% agarose gel and transferred onto a Hybond N+ membrane. Hybridizations were performed at 42°C with ^{32}P-labeled rat BSP, osteopontin (OPN), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe. Following hybridization, membranes were washed four times for 5 min each at 21°C 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 sodium chloride, 1.5 mM trisodium citrate, pH 7.0, 0.1% SDS. The hybridized bands, representing the two polyadenylated forms (1.6 and 2.0 kb) of rat BSP mRNA, were scanned in a Bio-imaging analyzer (BAS2000, Fuji BAS2000). Signals were quantitated by densitometry and normalized to the corresponding values for 18S RNA.

RT-PCR

Following treatments by chlorpromazine, total RNA was extracted from SBMC-D8 cells with guanidium thiocyanate at different times (3, 6, 12 and 24 h after stimulation), and 0.1 μg was used as a template for SuperScript one-step RT-PCR. Primers were synthesized on the basis

of the reported rat cDNA sequences for BSP [Oldberg et al., 1988] and glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Sequences of the primers used for RT-PCR and the expected size of the PCR products were as follows: BSP forward, 5′-CTGCTTTAATCTT-GCTCTG-3′; BSP reverse, 5′-CCATCTCCATTTTCTTCC-3′ (211 bp); GAPDH forward, 5′-CCATGTTTGT-GATGGGTGTG-3′; GAPDH reverse, 5′-GGAT-GCAGGGATGATGTTCT-3′ (264 bp). cDNA synthesis and pre-denaturation were performed for 1 cycle at 50°C, 30 min; 94°C, 2 min, and amplification was carried out for 40 cycles (BSP) and 30 cycles (GAPDH) at 94°C, 30 s; 55°, 30 s; 72°C, 30 s, and final extension at 72°C, 10 min in a 50 μl reaction mixture. After amplification, each reaction mixture was analyzed by 2% agarose gel electrophoresis, and the bands were visualized by ethidium bromide staining.

Transient Transfection Assays

Exponentially growing ROS17/2.8 and SBMC-D8 cells were used for transfection assays. Twenty-four hours after plating, cells at 50%–70% confluence were transfected using Lipofectamine reagents. The transfection mixture included 1 μg of a luciferase (LUC) plasmid and 2 μg pSV-β-galactositase (β-Gal) vector as an internal control to normalize for individual transfection efficiencies. Two days post-transfection, cells were deprived of serum for 12 h, chlorpromazine (10 μg/ml) was added, and the cells cultured for a further 12 h prior to harvesting. The luciferase assay was performed according to the supplier's protocol using a light reader to measure the luciferase activity. The protein kinase inhibitors 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. U0126 (5 μM) was used to inhibit MAP kinase kinase activity. Oligonucleotide-directed mutagenesis by PCR was utilized to introduce dinucleotide substitutions using the quick-change site directed Mutagenesis Kit (Stratagene, La Jolla, CA). All of the constructs were sequenced as described previously [Shimizu-Sasaki et al., 2001] to verify the fidelity of the mutagenesis.

Gel Mobility Shift Assays

Confluent cells in T-75 flasks incubated for 3–24 h with chlorpromazine (10 μg/ml) in α-MEM without serum were used to prepare nuclear extracts. Nuclear protein was extracted by the method of Dignam et al. [1983]. Double-stranded oligonucleotides corresponding to the inverted CCAAT (nts −61 to −37, 5′-CCGTG-ACCGTG**ATTGG**CTGCTGAGA), the CRE (nts −84 to −59, 5′-CCCACAGCC**TGACGTCG**CA-CCGGCCG), a fibroblast growth factor 2 (FGF2) response element (FRE; nts −98 to −79, 5′-TT-TTCT**GGTGAGAA**CCCACA), and a pituitary specific transcription factor binding site (Pit-1; nts −115 to −96, 5′-CGTG**TTGTAGT**TACG-GATTT) were prepared by Bio-Synthesis, Inc. For gel shift analysis the double-stranded oligonucleotides were end-labeled with [γ-^{32}P] ATP. Nuclear protein extracts (3 μg) were incubated for 20 min at room temperature (RT = 21°C) with radiolabeled double-stranded-oligonucleotide. Following incubation the protein–DNA complexes were resolved by electrophoresis on 5% non-denaturing acrylamide gels run at 150 V. Following electrophoresis, the gels were dried and autoradiograms prepared and analyzed using a bioimaging analyzer.

Statistical Analysis

Triplicate samples were analyzed for each experiment, which was replicated at least three times to ensure consistency of the responses to chlorpromazine. Significant differences between control and chlorpromazine treatments were determined using the Student's *t*-test.

RESULTS

Suppression of BSP mRNA levels in ROS 17/2.8 and SBMC-D8 Cells

To determine the dose-response for chlorpromazine regulation of BSP, confluent ROS 17/2.8 cells were treated with different concentrations of chlorpromazine for 24 h. BSP mRNA levels were determined by Northern blot analysis. Chlorpromazine decreased BSP transcripts at 0.01 ~ 10 μg/ml and had a maximal effect at 10 μg/ml (Fig. 1A). Thus, 10 μg/ml of chlorpromazine was used to determine the time course of BSP mRNA expression (Fig. 1B). Chlorpromazine suppressed BSP mRNA levels at 12 and 24 h. In comparison, OPN and GAPDH mRNA levels did not change after stimulation by chlorpromazine (Fig. 1A,B).

Next, RT-PCR was performed using total RNA extracted from the SBMC-D8 cells. BSP mRNA levels were decreased in a dose-dependent manner (0.01 ~ 10 μg/ml) by chlorpromazine for 12 h. A 24 h time-course revealed a time-dependent decrease in BSP mRNA levels

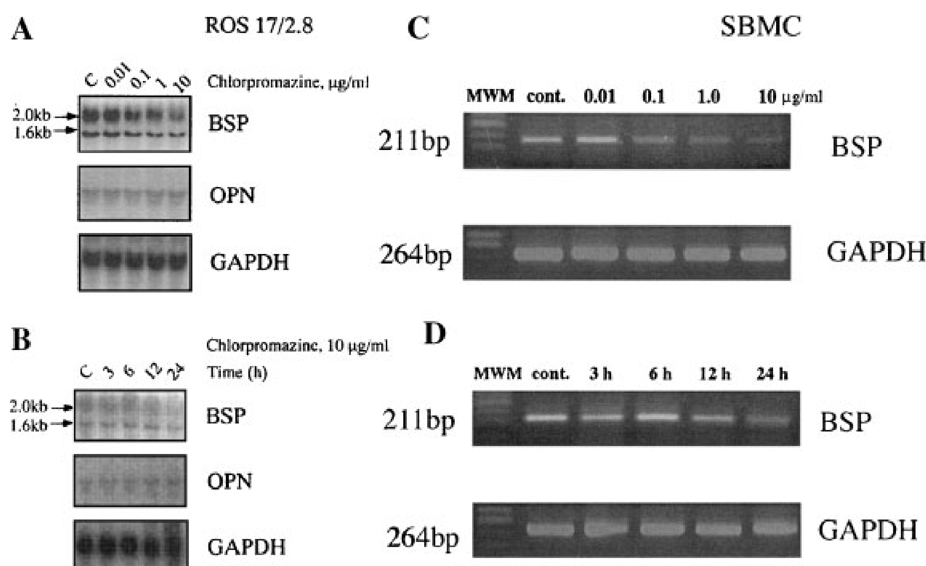


Fig. 1. Effects of chlorpromazine on BSP mRNA levels in ROS17/2.8 and SBMC-D8 cells. **A**: Dose-response effect of chlorpromazine on BSP mRNA levels in the osteoblastic cell line ROS17/2.8 treated for 24 h. At 0.01 ~ 10 μg/ml, chlorpromazine decreased BSP mRNA with a maximal effect at 10 μg/ml. In addition, results of hybridization analyses for osteopontin (OPN) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on the same samples are shown for comparison. **B**: Twenty-four hour time-course revealed a decrease in BSP mRNA at 24 h after the administration of 10 μg/ml chlorpromazine to ROS17/2.8 cells. Total RNA was isolated from triplicate cultures harvested after incubation times of 3, 6, 12, and 24 h, and used for Northern hybridization analysis using ^{32}P-labeled BSP, OPN, and GAPDH cDNA probes. **C**: Dose-response effect of chlorpromazine on BSP mRNA levels in SBMC-D8 cells treated for 12 h. At 0.01 ~ 10 μg/ml, chlorpromazine decreased BSP mRNA levels with a maximal effect at 10 μg/ml. Lower figure shows GAPDH mRNA levels. **D**: Twenty-four hour time-course revealed a decrease in BSP mRNA at 24 h after the administration of 10 μg/ml chlorpromazine to SBMC cells. Total RNA was isolated from triplicate cultures harvested after incubation times of 3, 6, 12, and 24 h, and mRNA levels in the cells were analyzed by RT-PCR.

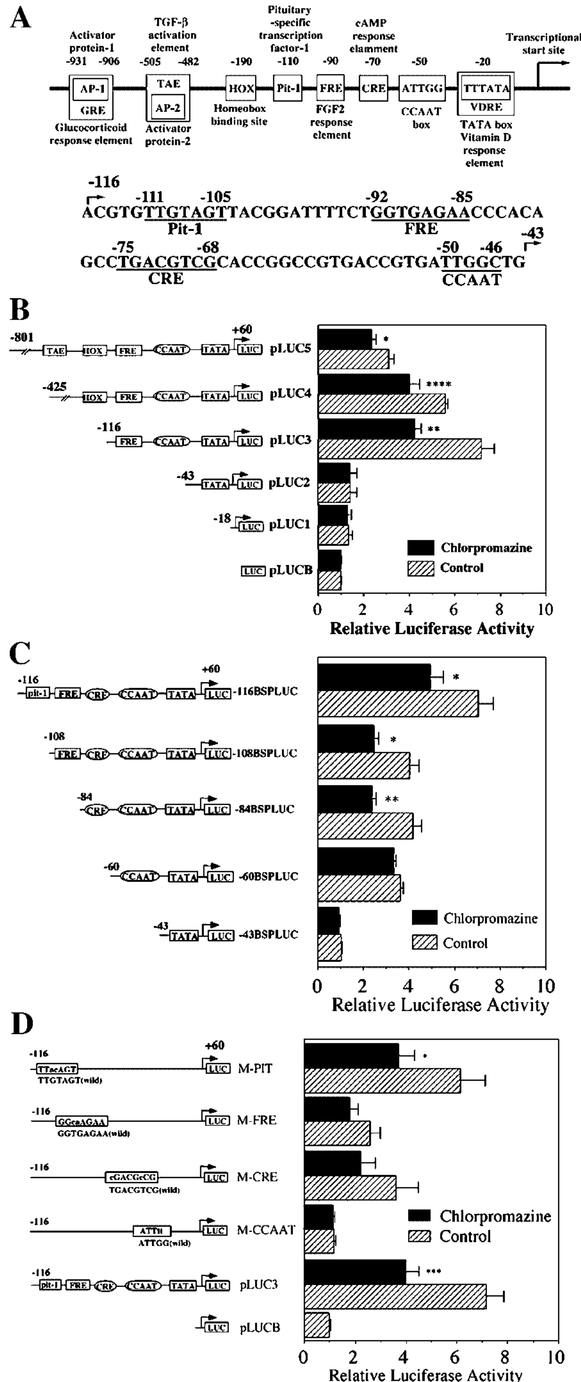
after stimulation by 10 μg/ml chlorpromazine (Fig. 1C,D).

Transient Transfection Analysis of Rat BSP Promoter Constructs

To determine the site of chlorpromazine-regulated transcription in the 5′-flanking region of the *BSP* gene, various sizes of promoter construct ligated to a luciferase reporter gene were transiently transfected into ROS17/2.8 and SBMC-D8 cells, and their transcriptional activity was determined in the presence of chlorpromazine (10 μg/ml, 12 h). The constructs used, pLUC1-pLUC5, and their responsiveness to chlorpromazine are shown in Figure 2 (B ~ D). The transcriptional activity of pLUC3, which encompasses nucleotides −116 to +60, was reduced ~0.58-fold in ROS 17/2.8 (Fig. 2B) and ~0.41-fold in SBMC-D8 cells (data not shown), and almost the same level of decrease was observed in constructs pLUC4 (−425 to +60) and pLUC5 (−801 to +60). However, deletion of the sequence between −116 and −43 abolished the chlorpromazine-mediated reduction (pLUC2 and pLUC1) (Fig. 2B). Within the DNA sequence that is unique to pLUC3 (nts −116 to −43) is an inverted CCAAT box (nts −50 to −46), a CRE (nts −75 to −68), a FGF2 response element (FRE; nts −92 to −85), and a Pit-1 (nts −111 to −105) motif (Fig. 2A). To more closely define the regulatory element between nts −116 and −43 that is utilized by chlorpromazine, we prepared a series of 5′-deletion constructs. −84BSPLUC and longer constructs were suppressed by chlorpromazine (Fig. 2C). Next, we introduced mutations in the possible response elements encoded within nts −116 to +60 of pLUC3 as shown in Figure 2D. Whereas mutations in the Pit-1 had little effect on chlorpromazine and mutation of the CCAAT box essentially abolished basal expression, mutations of the FRE and the CRE significantly reduced the chlorpromazine effects in the transcriptional activities (Fig. 2D). These results suggest that the FRE and the CRE are required as functional *cis*-elements for regulation of BSP transcription by chlorpromazine.

Since protein kinases mediate chlorpromazine signaling, we also investigated the effects of the protein kinase C inhibitor H7, the protein

kinase A inhibitor H89, the tyrosine kinase inhibitor HA, and the MAP kinase kinase inhibitor U0126 on chlorpromazine-mediated transcription. Whereas chlorpromazine suppression of pLUC3 promoter activity was inhibited by HA and U0126, no effects were observed for PKC and PKA, indicating the involvement of tyrosine kinase and MAP kinase in the signaling pathways (Fig. 3).



Gel Mobility Shift Assays

To determine how the nuclear proteins that bind to the inverted CCAAT, CRE, FRE, and Pit-1 elements might be modulated by chlorpromazine, double-stranded oligonucleotides were end-labeled and incubated with equal amounts (3 μg) of nuclear proteins extracted from confluent ROS17/2.8 cells that were either untreated (control) or treated with chlorpromazine (10 μg/ml). When inverted CCAAT, CRE, FRE, and Pit-1 in the BSP promoter were used as probes, the formation of CCAAT- and Pit-1–protein complexes did not change after stimulation with chlorpromazine. CRE– and FRE–protein complexes were downregulated at 6, 12, and 24 h after stimulation with chlorpromazine (Fig. 4A). That these DNA–protein complexes represent specific interactions was confirmed by competition experiments in which a 40-fold molar excess of CCAAT, CRE, and FRE double-stranded oligonucleotides reduced by the

Fig. 2. Chlorpromazine downregulates BSP promoter activity. **A**: Regulatory elements in the proximal rat BSP promoter. Upper panel: The inverted TATA and CCAAT boxes, FGF2 response element (FRE), pituitary-specific transcription factor-1 (Pit-1) motif, homeodomain binding site (HOX), inverted repeater (IR), TGF-β activation element (TAE) overlapping with AP-2 and glucocorticoid response element GRE overlapping the AP-1, a vitamin D response element (VDRE) that overlaps the inverted TATA box are present within a proximal promoter. Lower panel: The nucleotide sequence of proximal promoter region of the rat BSP gene is shown from nucleotides −116 to −43. The inverted CCAAT box, CRE, Pit-1, and FGF response element (FRE) are present. **B**: Transient transfection of ROS 17/2.8 cells in the presence or absence of chlorpromazine (10 μg/ml) for 12 h was used to determine the transcriptional activities of chimeric constructs that included various regions of the BSP promoter ligated to a luciferase reporter gene. With the construct pLUC3 encompassing BSP promoter nucleotides −116 to +60, transcription was decreased with chlorpromazine (10 μg/ml). **C**: Fine 5′-deletion mapping of the nts −116 to −43 elements in the BSP promoter. A series of rat BSP promoter 5′-deletion constructs were analyzed for relative promoter activity after transfection into SBMC-D8 cells and were examined for reduction in the presence of chlorpromazine (10 μg/ml) for 12 h. The results demonstrated that a chlorpromazine response region exists between −60 and −116. **D**: Site mutation analysis of luciferase activities in response to chlorpromazine. Dinucleotide substitutions were made within the context of homologous −116 to +60 (pLUC3) BSP promoter fragments. M-CCAAT (ATTtt), M-CRE (cGACGcCG), and M-FRE (GGcaAGAA), M-PIT (TTacAGT) were analyzed for relative promoter activity after transfection into SBMC-D8 cells and examined for induction in the presence of chlorpromazine for 12 h. Significant differences compared to controls are shown at the following probability levels: *($P < 0.1$); **($P < 0.05$); ***($P < 0.02$).

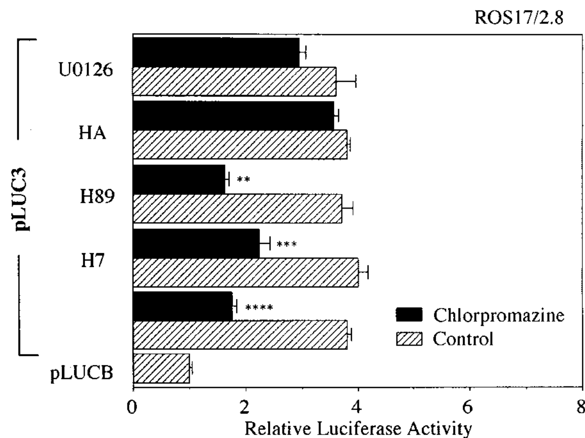


Fig. 3. Effects of kinase inhibitors on transcriptional activation by chlorpromazine. Transient transfection analysis of pLUC3 in the presence or absence of chlorpromazine (10 μg/ml) for 12 h in ROS17/2.8 cells is shown together with the effects of the PKC inhibitor (H7, 5 μM), PKA inhibitor (H89, 5 μM), tyrosine kinase inhibitor (HA, 1 μM), and MAP kinase kinase inhibitor (U0126, 5 μM). Significant differences compared to controls are shown at the following probability levels: **($P < 0.05$); ***($P < 0.02$); ****($P < 0.01$).

amount of CCAAT–, CRE–, and FRE–protein complexes formation in ROS17.2.8 cells (Fig. 4C). When gel shift assays were analyzed using nuclear extracts from SBMC-D8 cells, formation of CRE– and FRE–protein complexes was decreased by chlorpromazine. However, the binding activities of CCAAT and Pit-1-binding proteins did not change after stimulation by chlorpromazine (Fig. 4B).

DISCUSSION

These studies have shown for the first time that chlorpromazine suppresses expression of BSP gene in osteoblastic ROS 17/2.8 and rat stromal bone marrow SBMC-D8 cells. Transduction of the chlorpromazine signaling is mediated through tyrosine and MAP kinases,

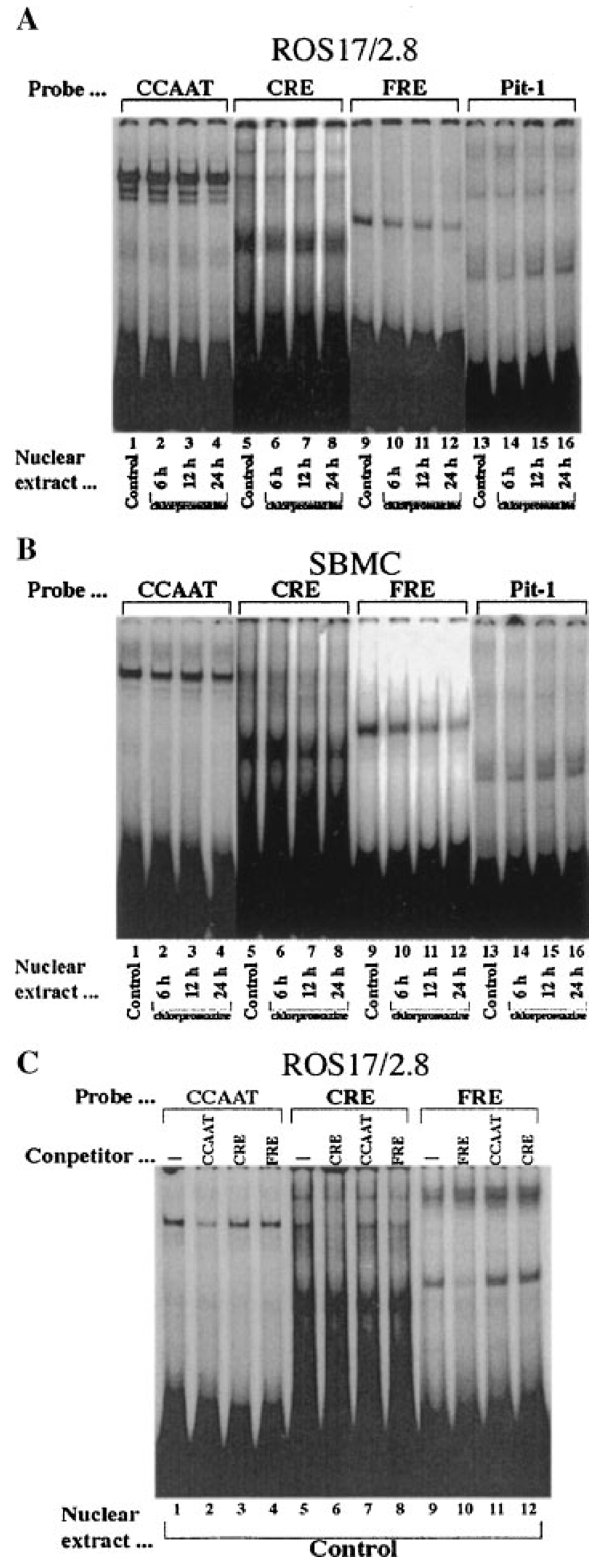


Fig. 4. Chlorpromazine downregulates nuclear proteins from ROS 17/2.8 and SBMC-D8 cells that recognize CRE and FRE. **A**,**B**: Radiolabeled double-stranded inverted CCAAT (-61 5′-CCGTGACCGTG**ATTGG**CTGCTGAGA-37), CRE (−84 5′-CC-CACAGCC**TGACGTCG**CACCGGCCG−59), FRE (−98 TTTT-CT**GGTGAGAA**CCCACA−79), and Pit-1 (−115 CGTG**TT-GTAGT**TACGGATTT−96) oligonucleotides were incubated for 20 min at 21°C with nuclear protein extracts (3 μg) obtained from ROS17/2.8 (A) and SBMC-D8 cells (B) and incubated without (lanes 1, 5, 9, 13) and with chlorpromazine 6 h (lanes 2, 6, 10, 14), 12 h (lanes 3, 7, 11, 15), and 24 h (lanes 4, 8, 12, 16). 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. **C**: Specific binding of nuclear proteins to CCAAT, CRE, and FRE. Radiolabeled double-stranded inverted CCAAT, CRE, and FRE oligonucleotides were incubated for 20 min at 21°C with nuclear protein extracts (3 μg) obtained from confluent ROS 17/2.8 cells. Competition reactions were performed using a 40-fold molar excess of unlabeled CCAAT, CRE, and FRE.

which target nuclear proteins that bind to CRE and FRE elements in the BSP promoter.

Mechanical stress plays a crucial role for maintaining bone mass and strength [Klein-Nulend et al., 1997]. Reduced mechanical loading caused by long-term bed rest, immobilization and microgravity conditions in space induce significant bone loss and mineral changes [Bikle et al., 1987; Vico et al., 1987]. Human and animal studies have indicated that impaired bone formation and osteoporosis significantly contribute to unloading conditions [Morey and Baylink, 1978; Inoue et al., 2000]. It is thus important to elucidate the mechanism of bone formation and remodeling induced by mechanical loading and to establish an effective treatment for osteoporosis caused by reduced mechanical stress.

Mechanical stress on bone may lead to direct effects on the cell membrane consisting of either folding or stretching. The folding process can be stimulated by applying hyperosmotic stress. On the other hand, the stretching can be stimulated by hypotonic stress. A hyperosmotic challenge leads to an initial cell shrinkage resulting in a coupled regulation of cellular volume and an intracellular pH increase due to activation of Na^{+}–H^{+} exchanger [Hoffman and Simonsen, 1989; Dascalu et al., 1992]. The intracellular pH response induced by hyperosmotic stress was abolished by two calmodulin inhibitors, W7 and chlorpromazine, and by the actin polymerization inhibitor cytochalasin B. These results suggest the involvement of calmodulin and actin in the transduction process induced by hyperosmotic stress [Dascalu et al., 1992]. Hypotonic cells welling rapidly activates tyrosine kinases and activation of tyrosine kinases induces ERK activation and c-*fos* induction in cardiac myocytes [Sadoshima et al., 1996]. The mechanical stretch (linear strain) activates phospholipase C and PKC, but the hypotonic stress (radial strain) does not [Sadoshima and Izumo, 1993b]. Chlorpromazine distributes preferentially into the lipid portions at the cytoplasmic half of the bilayer, causing that half to expand relative to the exterior half, and thereby inducing membrane deformation [Sheetz and Singer, 1974]. Hypotonic cells welling induced c-*fos* expression, increase in tyrosine phosphorylation and activation of ERKs all mimicked by chlorpromazine [Sadoshima et al., 1996]. Chlorpromazine decreased ALP activity in calvaria in vivo [Komoda et al., 1985a,b]. Chlorpromazine suppressed ALP activity and collagen synthesis in MC3T3-E1 cells [Komoda et al., 1985a,b]. These suggest that the chlorpromazine effects on BSP expression is relevant to the hypotonic stress effects on BSP and bone.

Fluid shear stress-induced cyclooxygenase-2 (COX-2) expression is mediated by C/EBPβ, CRE, and AP1 elements in MC3T3-E1 cells [Ogasawara et al., 2001]. Intermittent hydrostatic compression promotes OPN mRNA expression in MC3T3-E1 cells [Klein-Nulend et al., 1997]. Static magnetic field-induced BSP expression is mediated through FRE and Pit-1 elements in UMR106 osteoblast like cells [Shimizu et al., 2004a]. In this study, chlorpromazine suppressed BSP gene expression through CRE and FRE elements. Different effects of static magnetic field and chlorpromazine on the osteoblast cell membrane might have caused the discrepancy of two mechanical stresses on BSP transcription. The results indicate that the effect of the static magnetic field is similar to that of hyperosmotic stress and chlorpromazine exhibits hypotonic stress on the osteoblast cell membrane.

In ROS 17/2.8 and SBMC-D8 cells, chlorpromazine decreased BSP promoter activities (Fig. 2), which is comparable with the decreases in BSP mRNA levels (Fig. 1). The results of luciferase assays using 5′-deletion constructs of BSP promoter show that the chlorpromazine effects are targeted to a region between nts −116 and −60 (Fig. 2C). Mutation in the CRE and FRE suggests that they are required for the reduction of BSP transcription by chlorpromazine (Fig. 2D). The involvement of CRE and FRE is further supported by gel shift assays. Since the CRE– and FRE–protein complexes were reduced by chlorpromazine (Fig. 4A,B). In addition, tyrosine kinase inhibitor HA and MAP kinase kinase inhibitor U0126 abolished the effect of chlorpromazine (Fig. 3). We previously identified FRE in the proximal promoter of the BSP gene that mediates both constitutive and FGF2-induced BSP transcription via tyrosine kinase and MAP kinases [Shimizu-Sasaki et al., 2001]. Prostaglandin E_2 induces BSP transcription through CRE and FRE in the BSP promoter via cAMP, tyrosine, and MAP kinases [Samoto et al., 2003].

Mechanical stretch activates c-*fos* expression in cardiac myocytes, which is mediated through a serum response element (SRE; CCATAT-TAGG) in c-*fos* promoter [Sadoshima and

Izumo, 1993a]. Platelet-derived growth factor (PDGF) B chain promoter contains a fluid shear-stress-responsive element (SSRE; GAG-ACC). 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 CREs) [Peake and El Haj, 2003]. In this study, we identified the chlorpromazine responsive elements are CRE and FRE, and the nucleotide sequence of FRE (CT**GGTGAGAA**CC) is quite similar to that of SSRE.

In this study, we have shown that chlorpromazine suppresses BSP expression through tyrosine kinase and MAP kinase-signaling pathways, and CRE and FRE elements in the BSP promoter have been identified as the target of chlorpromazine-mediated regulation of BSP gene transcription.

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