Transcriptional Regulation of Bone Sialoprotein Gene by Melatonin and Interleukin-11

(メラトニンとインターロイキン 11 による骨シアロタンパク質の転写の調節)

日本大学大学院松戸歯学研究科歯学専攻

松村　浩禎

（指導：小方　頼昌　教授）

Contents

Preface

This article is based on a main reference paper, "Melatonin regulates human bone sialoprotein gene transcription" in the Journal of Oral Science, and a reference paper, "Transcriptional regulation of bone sialoprotein gene by interleukin-11" in Gene.

Abstract

Bone sialoprotein (BSP) is a mineralized tissue-specific protein that is highly expressed during the initial mineralization of bone. Interleukin-11 (IL-11) is a stromal cell-derived cytokine that belongs to the interleukin-6 family of cytokines. Melatonin is produced by pineal gland and regulates a variety of physiological processes including osteoblast differentiation and bone formation.

IL-11 (20 ng/ml) increased BSP mRNA and protein levels at 12 h in osteoblast-like ROS17/2.8 cells. In a transient transfection assay, IL-11 (20 ng/ml) increased luciferase activity of the construct (-116 to +60) in ROS17/2.8 cells and rat bone marrow stromal cells (RBMC). Gel shift analyses showed that IL-11 (20 ng/ml) increased nuclear protein binding to cAMP response (CRE), fibroblast growth factor 2 (FGF2) response element (FRE) and homeodomain protein binding site (HOX). These results demonstrate that IL-11 stimulates BSP transcription by targeting CRE, FRE and HOX sites in the rat BSP gene promoter.

Melatonin (100 nM) increased BSP mRNA levels at 3 h and reached maximal at 12 and 24 h in osteoblast-like Saos2 cells, and increased Runx2 mRNA at 6 h and Osterix mRNA levels at 12 and 24 h. Melatonin (100 nM) induced BSP protein expression at 3 h and reached maximal at 6 and 12 h. Transient transfection analyses were performed using chimeric constructs of the human BSP gene promoter linked to a luciferase reporter gene. Treatment of Saos2 cells with melatonin (100 nM, 12 h) increased luciferase activities of all constructs between -84LUC to -868LUC which are including the human BSP gene promoter. Effects of melatonin were abrogated in constructs included 2 bp mutation in the two CRE (CRE1 and CRE2). Luciferase activities induced by melatonin were blocked by PKA inhibitor KT5720,

tyrosine kinase inhibitor HA, ERK 1/2 inhibitor U0126 and PI3 kinase inhibitor LY294002. Gel mobility shift analyses showed that melatonin increased nuclear proteins binding to CRE1 and CRE2. CRE binding protein 1 (CREB1), phospho-CREB1, c-Fos, c-Jun, JunD and Fra2 antibodies disrupted the formation of CRE1 and CRE2 proteins complexes. These data demonstrate that melatonin stimulates BSP gene transcription via CRE1 and CRE2 elements in the human BSP gene promoter.

These studies indicate IL-11 and melatonin induce BSP gene and protein expressions. BSP is a multifunctional protein that has a critical role in the early onset of mineralization. Also, BSP expression could be important for periodontal regeneration. Therefore, IL-11 and melatonin might be helpful in the treatment of periodontitis.

1. Introduction

Interleukin-11 (IL-11) is a stromal cell-derived cytokine that belongs to the interleukin-6 family of cytokines (1, 2). Other members of this cytokine family are oncostatin M, cardiotrophin-1 and leukemia inhibitory factor. IL-11 has many biological activities and has roles in hematopoiesis, immune responses, the nervous system and bone metabolism (3-6). IL-11 enhances the growth of early progenitors and promotes megakaryocytopoiesis and erythropoiesis (7). In healthy animals, IL-11 administration stimulates megakaryocyte maturation and increases peripheral platelets. IL-11 accelerates the recovery of peripheral neutrophil, erythrocyte and platelet counts in mice that have undergone cytoablative treatment (7). IL-11 and its family members are secreted from osteoblasts in response to factors which induce bone resorption (8-11). IL-11 induces bone resorption by enhancing osteoclast formation and osteoblast-mediated type I collagen degradation (12, 13). IL-11 increased alkaline phosphatase (ALP) activities, which are a marker of osteoblasts (14); therefore, it is possible that IL-11 may have an important role in osteogenesis. Overexpression of the human IL-11 gene in transgenic mice resulted in the stimulation of bone formation to increase the cortical thickness and strength of the long bones, possibly through enhancing bone morphogenetic protein (BMP) actions in bone (15); however, little is

known about the role of IL-11 in osteogenesis, osteoblast differentiation and bone formation.

Melatonin is produced by pineal gland, a small endocrine gland located in the center of the brain but outside the blood-brain barrier under the control of the suprachiasmatic nucleus and the light/dark cycle. Production of melatonin by pineal gland is inhibited by light to the retina and permitted by darkness through the retinohypothalamic tract, suprachiasmatic nuclei and sympathic nervous system (16-19). Apart from the pineal gland, several studies suggest that melatonin may also be synthesized locally in the bone marrow, gastrointestinal tract, testes and lymphocytes (20-22). Further, melatonin regulates a variety of physiological and pathophysiological processes including regulation of body temperature, sexual development, inflammation, immune system and cell proliferation (23-28). Melatonin also regulates bone remolding, prevents osteoporosis, and induces osseointegration of dental implant and dentine formation (29-33).

Bone sialoprotein (BSP) is a sulfated, phosphorylated and glycosylated protein that mediates cell attachment through a RGD motif, and has an ability to bind to hydroxyapatite through polyglutamic acid sequences (34-38). BSP is also expressed in prostate, lung, breast and thyroid cancers and to be associated with the ectopic microcrystals formation in the tumor tissues (39, 40). The rat, mouse and human BSP gene promoters have been cloned and partially characterized (41-44). Rat and human BSP have an inverted TATA box (-24 to -19) (45) and an inverted CCAAT box (-50 to -46), which is required for basal transcription (46). In addition, a cAMP response element (CRE; -75 to -68) (47), a fibroblast growth factor 2 (FGF2) response element (FRE; -92 to -85) (48), a runt-related transcription factor 2 (Runx2) binding site (49), a pituitary-specific transcription factor-1 (pit-1) motif (-111 to -105) (50), a homeodomain protein binding site (HOX; -199 to -192) (51), and a glucocorticoid response element (GRE) overlapping an AP1 site (52) have also been characterized in the rat BSP promoter. Thus, two cAMP response element (CRE1; -79 to -72 and CRE2; -674 to -667) (53, 54), a FRE (-96 to -89) (47, 48), three activator protein 1 (AP1) response element (AP1(1); -148 to -142, AP1(2); -483 to -477 and AP1(3); -797 to -791) (53, 54), and a HOX (-200 to -191) (51, 55) have been characterized in the human

BSP promoter.

To determine the mechanism of BSP gene regulation by IL-11 or melatonin, we have analyzed the effects of IL-11 or melatonin on the expression of rat or human BSP gene in osteoblast-like cells.

2. Materials and Methods

2.1. Materials

Alpha-minimum essential medium (α-MEM), fetal calf serum (FCS), lipofectamine 2000, penicillin and streptomycin, TrypLE™ Express was obtained from Invitrogen (Carlsbad, CA). PGL3-basic, pSV-β-galactosidase (β-Gal) control vector and ERK1/2 inhibitor U0126 were purchased from Promega (Madison, WI). Protein kinase C (PKC) inhibitor H7 was from Seikagaku Corporation (Tokyo, Japan). PKA inhibitor KT5720, tyrosine kinase inhibitor herbimycin A (HA) and melatonin were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Human recombinant IL-11 was purchased from Wako Pure Chemical Industries (Tokyo, Japan). LY249002 (phosphatidylinositol 3-kinase (PI3K) inhibitor) was from Calbiochem (San Diego, CA). EXScript RT reagent kit and SYBR Premix Ex Taq were purchased from Takara (Tokyo, Japan). The ChIP-IT™ Express Enzymatic kit was purchased from Active Motif (Carlsbad, CA). Anti-rabbit IgG conjugated with HRP and ELC plus Western Blotting Detection Reagents were purchased from GE Healthcare UK Ltd. (Buckinghamshire, England). All chemicals used were of analytical grade.

2.2. Cell Culture

Rat osteoblast-like ROS17/2.8 cells (34), rat stromal bone marrow cells (RBMC) (51) and human osteosarcoma cell line (56) Saos2 cells were cultured at 37°C in 5% CO_2, 95% air in α-MEM supplemented with 10% FCS. These cells were first grown to confluence in 60 mm tissue culture dishes in α-MEM medium containing 10% FCS, then cultured in α-MEM without serum for 12 h and incubated with or without IL-11 or melatonin for dose dependency and time dependency in 0, 3, 6, 12 and 24 h.

2.3. Northern hybridization

Aliquots (20 μg) of total RNA were fractionated in a 1.2% agarose gel and transferred onto a Hybond-N+ membrane. Hybridizations were performed at 42°C with ^{32}P-labeled human BSP 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 and 0.1% SDS, pH 7.0. The hybridized band, representing the rat BSP mRNA and human BSP mRNA, was scanned using a Bio-imaging analyzer (Fuji BAS 2500, GE Healthcare, Tokyo; Japan).

2.4 Western blot

For Western blotting, cell lysates from Saos2 cells were separated on 10% SDS-PAGE and transferred onto a membrane. The membrane was then incubated for 3 h with anti-BSP polyclonal antibody (LF-100, provided by Dr. Larry W. Fisher) and anti-α tubulin monoclonal antibody (sc-5286; Santa Cruz Biotechnology, CA). Anti-rabbit and mouse IgG conjugated with HRP was used as the secondary antibodies. Immunoreactivities were detected by ELC plus Western Blotting Detection Reagents.

2.5. 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. Human BSP, human Runx2, human Osterix, human GAPDH (Table 1); 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 2x SYBR Premix EX Taq (12.5 μl), 0.2 M forward and reverse primers (0.1 μl) and 50 ng cDNA (5 μl) for BSP, Runx2 and Osterix and 10 ng cDNA (1 μl) for GAPDH. To reduce variability between replicates, PCR premixes, which contain all reagents except for cDNA, were prepared and aliquoted into 0.2 ml Hi-8-tubes. The thermal cycling conditions were 10 s at 95°C

and 40 cycles of 5 s at 95℃ and 30 s at 60℃. Post-PCR melting curves confirmed the specificity of single-target amplification, and the expressions of BSP, Runx2 and Osterix relative to GAPDH were determined in triplicate.

2.6. Transient Transfection assays

Exponentially growing ROS17/2.8, RBMC and Saos2 cells were used for transfection assays. Twenty four hour after plating, the cells at 40-60% confluence were transfected using a Lipofectamine 2000. The transfection mixture included 1 μg of a luciferase (LUC) construct and 2 μg of β-Gal vector as an internal transfection control. Various sized rat BSP promoter ligated to a luciferase reporter gene (pLUC1 ~ pLUC6, pLUC1 ; -18 to +60, pLUC2 ; -43 to +60, pLUC3 ; -116 to +60, pLUC4 ; -425 to +60, pLUC5 ; -801 to +60, pLUC6 ; -938 to +60) were transiently transfected into ROS17/2.8 and RBMC cells. The human BSP promoter sequences were cloned into the Bgl II site of the multiple cloning site of pGL3-promoter luciferase plasmid (-43 LUC ; -43 to +60, -60 LUC ; -84 to +60, -116 LUC ; -116 to +60, -184 LUC ; -184 to +60, -211 LUC ; -211 to +60, -428 LUC ; -428 to +60, -868 LUC ; -868 to +60) (44). Human BSP promoter regions ligated to luciferase reporter genes were transiently transfected into Saos2 cells. Two days post transfection, the cells were deprived of serum for 12 h, IL-11 (20 ng/ml) or melatonin (100 nM) were added for 12 h prior to harvesting. The luciferase assays were performed according to the supplier's protocol (PicaGene, Toyo Inki, Japan) using a Luminescence reader (Acuu FLEX Lumi 400 ; Aloka) to measure the luciferase activities. KT5720 (100 nM) and H7 (5 μM) were used to inhibit protein kinase A and C. HA (1 μM) and U0126 (5 μM) were used to inhibit tyrosine kinase and ERK1/2. LY294002 (10 μM) and N-acetylcysteine (NAC, 20 mM) were used to inhibit PI3-K and antioxidant. Oligonucleotide-directed mutagenesis by PCR was utilized to introduce the dinucleotide substitutions using the Quikchange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) All constructs were sequenced previously to verify the fidelity of the mutagenesis (Table 2).

2.7. **Gel mobility shift assa**y

Confluent ROS 17/2.8 and Saos2 cells in T-75 flasks incubated for 3, 6 and 12 h with IL-11 (20

ng/ml) or melatonin (100 nM) in α-MEM without serum were used to prepare the nuclear extracts. Double-stranded oligonucleotides encompassing the inverted CCAAT, CRE, FRE, Pit-1 and HOX in the rat BSP gene promoter and the inverted CCAAT, CRE1, CRE2 and FRE sequences in the human BSP gene promoter were prepared (Table 3). 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 with 0.1 pM radiolabeled double-stranded oligonucleotide in buffer containing 50 mM KCl, 0.5 mM EDTA, 10 mM Tris–HCl (pH 7.9), 1 mM dithiothreitol, 0.04% Nonidet P-40, 5% glycerol and 1 μg of poly(dI-dC). Following incubation, the protein-DNA complexes were resolved by electrophoresis on 5% non-denaturing acrylamide gels run at 200 V at room temperature. After electrophoresis, the gels were dried and autoradiograms were prepared and analyzed using an image analyzer. For competition experiments, 40 times molar unlabeled oligonucleotides for CRE1, CRE2, CCAAT, mutation CRE1 (mCRE1; TGACA**ga**A) and mutation CRE2 (mCRE2; TGACC**ga**A) were used. Supershift experiments were performed using anti-CREB1 (p43; Rockland), phospho-CREB1 (Ser133; Upstate), Dlx5 (AB5728; Chemicon), Runx2 (PC287; Calbiochem), c-Fos (sc-253), c-Jun (sc-44), JunD (sc-74), Fra2 (sc-604), Msx2 (sc-15396) and Smad1 (sc-7965) (Santa Cruz Biotechnology) antibodies. Antibodies were added to each reaction mixture and incubated for 4 h at 4°C before electrophoresis was performed under the same conditions as described above.

2.8. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were carried out using a Chip-IT^{TM} Express Enzymatic kit (Active Motif) according to the manufacturer's protocol. Briefly, Saos2 cells were grown to confluence in 100 mm dishes and cultured in α-MEM without serum for 12 h and incubated with or without melatonin (100 nM) for 6 h. Saos2 cells were fixed for 10 min with 1% formaldehyde and then chromatin was prepared. After washed by PBS, cell pellets were homogenized by dounce homogenizer and centrifuged to pellet the nuclei. The nuclei pellet was digested by the enzymatic shearing cocktail (200 U/ml) to shear the chromatin at 37°C for 5 min and the reaction was stopped with the addition of cold EDTA. The equivalent of 6.3 μg of DNA

(sheared chromatin) was used as starting material (input) in each ChIP reaction with 2 μg of the appropriate antibody (CREB1, phospho-CREB1, c-Fos, c-Jun, JunD, Fra2 and rabbit IgG was used as control) and protein G magnetic beads at 4°C overnight. The tube was placed on a magnetic stand to pellet beads on the tube side and, after washing the beads. Chromatin was eluted from the beads by elution buffer and reversed cross link buffer, and then the samples were treated with proteinase K for 1 h at 37°C. The purified DNA was subjected to PCR amplification (1 cycle at 94°C; 5 min, and amplification was carried out for 30 cycles at 94°C; 30 s, 55°C; 30 s, 72°C; 30 s, and final extension at 72°C; 10 min) for the CRE1 and CRE2 site within the human BSP promoter using CRE1ChIP(1), CRE1ChIP(2), CRE2ChIP(3), CRE2ChIP(4) primers (Table 4). The PCR products were separated on 2% agarose gels and visualized with ultraviolet light. All ChIP assays were repeated at least three times and with triplicate samples for each antibody used in ChIP reactions.

2.9. Statistical analysis

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

3. Results

3.1. Effects of IL-11 on BSP expression

3.1.1. Effects of IL-11 on BSP mRNA

To study the regulation of BSP transcription by IL-11, we performed Northern hybridization analysis of total RNA extracted from osteoblastic ROS17/2.8 cells. First, the dose-response relation of BSP induction by IL-11 was established by treating ROS17/2.8 cells with different concentrations of IL-11 for 12 h. IL-11 increased BSP mRNA levels at 1, 5, 20 and 100 ng/ml and had a maximal effect at 20 ng/ml in these four concentrations (Fig. 1A). Thus, 20 ng/ml IL-11 was used to determine the time courses of BSP mRNA expression. IL-11 (20 ng/ml) induced BSP mRNA levels at 3 h and reached maximal at 12

h (Fig. 1B).

3.1.2. Transient transcription analyses of rat BSP promoter constructs

To determine the site of IL-11-regulated transcription in the 5'-flanking region of the BSP gene, various-sized promoter constructs ligated to a luciferase reporter gene were transiently transfected into ROS17/2.8 and RBMC cells and their transcriptional activities determined in the presence or absence of IL-11. The transcriptional activity of pLUC3, which encompasses nucleotides -116 to +60, was increased after 12 h treatment with 20 ng/ml IL-11 in ROS17/2.8 and RBMC cells (Fig. 2A, B). IL-11 (20 ng/ml) also increased BSP transcription of pLUC4 (-425 to +60), pLUC5 (-801 to +60) and pLUC6 (-938 to +60). In shorter constructs (pLUC1, -18 to +60; pLUC2, -43 to +60), luciferase activities were not influenced by IL-11 (Fig. 2A, B).

Within the DNA sequence that is unique in these regions, an inverted CCAAT box (ATTGG; nts -50 to -46), a CRE (nts -75 to -68), a Runx2 (nts -84 to -79), a FRE (nts -92 to -85), a Pit-1 (nts -111 to -105), another Runx2 (nts -184 to -179) and a HOX (nts -194 to -185) are present (Fig. 3). After introducing 2 bp mutations into the putative response elements within pLUC3 and pLUC4, the basal activities of M-CCAAT, M-CRE, M-FRE and M-PIT in pLUC3, and M-FRE and M-HOX in pLUC4 were lower than the basal transcriptional activities of pLUC3 and pLUC4. Transcriptional induction by IL-11 (20 ng/ml) was partially inhibited in the M-CRE and M-FRE (pLUC3), and M-FRE and M-HOX (pLUC4) constructs (Fig. 4). To confirm the functional elements, we also performed double mutation analyses. Double mutation in CRE and FRE (M-CRE/FRE in pLUC3) partially abolished, whereas FRE and HOX (M-FRE/HOX in pLUC4) completely abolished the effect of IL-11 (Fig. 4). The results suggested that double mutations of M-FRE/HOX in pLUC4 had synergistically effect, but in M-CRE/FRE in pLUC3.

3.1.3. Gel mobility shift assay

To identify nuclear proteins that bind to the CCAAT, CRE, FRE, Pit-1 and HOX elements and mediate IL-11 effects on transcription, 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 not

treated (control) or treated with 20 ng/ml IL-11 for 3, 6 and 12 h. When we used the inverted CCAAT and Pit-1 as probes, the DNA-protein complexes did not change after stimulation by IL-11 (Fig. 5, lanes 1-4, 13-16). With nuclear extracts from confluent control cultures of ROS17/2.8 cells, shifts of CRE, FRE and HOX-protein complexes were evident (Fig. 5, lanes 5, 9 and 17). After stimulation by 20 ng/ml IL-11 (3-12 h), CRE- and FRE-protein complexes were increased at 12 h (Fig. 5, lanes 5-8, 9-12), and HOX-protein complexes were increased at 3 h and reached maximal at 12 h (Fig. 5, lanes 17-20).

3.2. Effect of melatonin on BSP expression

3.2.1. Effect of BSP, Runx2 and Osterix mRNA and BSP protein levels by melatonin

To study the regulation of BSP expression by melatonin, we used Saos2 cells to express BSP mRNA constitutively. Firstly, the dose-response relationship of BSP induction by melatonin was established by treating the Saso2 cells with different concentrations of melatonin for 12 h. Melatonin induced BSP mRNA level maximal at 100 nM (Fig. 6B). When 100 nM melatonin was used to determine the time course of expression of BSP mRNA, melatonin (100 nM) increased BSP mRNA level at 3 h and reached maximal at 12 and 24 h (Fig. 6A). Results of real-time PCR showed that treatment with melatonin (100 nM) increased Runx2 mRNA levels at 6 h and Osterix mRNA levels at 12 and 24 h (Fig. 6B). Melatonin (100 nM) induced BSP protein expression at 3 h and reached maximal at 6 and 12 h. Tubulin was used as a loading control (Fig. 6C).

3.2.2. Transient transcription analyses of human BSP promoter constructs

To determine the site of melatonin-regulated transcription in the 5'-flanking region of the human BSP gene, -43 to +60 (-43LUC), -60 to +60 (-60LUC), -84 to +60 (-84LUC), -116 to +60 (-116LUC), -184 to +60 (-184LUC), -211 to +60 (-211LUC), -428 to +60 (-428LUC) and -868 to +60 (-868LUC) human BSP promoter regions ligated to luciferase reporter genes were transiently transfected into Saos2 cells and the transcriptional activities determined in the presence or absence of melatonin. Melatonin (100 nM) increased luciferase activities all of the constructs from (-84LUC to -868LUC) (Fig. 7). Including within the DNA sequence that is unique in -60LUC to -868LUC, is an inverted CCAAT box (ATTGG; between,

-54 and -50), a CRE1 (-79 to -72), a FRE (-96 to -89), a HOX (-200 to -191) and CRE2 (-674 to -667) in the human BSP gene promoter (Fig. 8). Next, we introduced 2 bp mutations in CCAAT, CRE1, FRE and CRE2 elements targeted by melatonin within the -184LUC and -868LUC constructs. When mutation was made in the CRE1, melatonin-induced -184LUC (-184mCRE1) and -868LUC (-868mCRE1) activities were partially inhibited. However, the transcriptional activities of mutation CCAAT (-184mCCAAT) and mutation FRE (-184mFRE) constructs were induced by melatonin almost same as wild -184LUC (Fig. 9). Next we introduced 2 bp mutation into the CRE2, melatonin-induced luciferase activity in -868mCRE2 was partially abolished (Fig. 9). When mutations were made in the pairs of CRE1 and CRE2 in -868LUC (-868mCRE1/mCRE2), the effect of melatonin on the luciferase activity was almost totally abrogated (Fig. 9). These results indicated that CRE1 and CRE2 act as functional response elements for melatonin regulation of BSP gene transcription. Melatonin-induced BSP transcription (-184LUC and -868LUC) was inhibited by KT5720, HA, U0126 and LY294002, and was not inhibited by H7, indicating an involvement of PKA tyrosine kinase, ERK1/2 and PI3K in the signaling pathways (Fig. 10).

3.2.3. Gel mobility shift assays

To identify nuclear proteins that bind to CCAAT, CRE1, CRE2 and FRE, double-stranded oligonucleotides were ^{32}P-labeled and incubated with nuclear proteins (3 μg) extracted from Saos2 cells which were stimulated by 100 nM melatonin for 3, 6 and 12 h. When we used the inverted CCAAT as a probe, the DNA-protein complex (NF-Y) (30) did not change after stimulation with melatonin (Fig. 11, lanes 1-4). With nuclear extracts from control cultures of Saos2 cells, shifts of CRE1 and CRE2-protein complexes were found (Fig. 11, lanes 5 and 9). After stimulation with 100 nM melatonin (3-12 h), CRE1- and CRE2-protein complexes were increased at 3 h and reached maximal at 6 h (Fig. 11, lanes 6, 7, 10 and 11). Melatonin could not induce FRE-protein complex formation (Fig. 11, lanes 13-16). That these DNA-protein complexes represent specific interactions were confirmed by competition with 40-fold molar excesses CRE1 and CRE2 to reduce CRE1- and CRE2-protein complexes formation (Fig. 12,

lanes 3 and 9). On the other hand, mCRE1, mCRE2 and inverted CCAAT could not compete with CRE1- and CRE2-protein complexes formation (Fig. 12, lanes 4, 6, 10 and 12). CRE1- and CRE2-protein complexes partially competed with 40-fold molar excess CRE2 and CRE1 (Fig. 12, lanes 5 and 11). To identify which proteins in the DNA-complexes bind to CRE1 and CRE2, we used antibodies for several transcription factors. CRE1- and CRE2-protein complexes were almost completely disappeared by phospho-CREB1 antibodies (Fig. 13, lanes 5 and 14). CREB1, c-Fos, c-Jun, JunD and Fra2 antibodies partially disrupted CRE1- and CRE2-protein complexes formation, whereas the addition of c-Fos antibody almost completely abolished the CRE2-protein complex formation (Fig. 13, lanes 4, 6-9, 13, 15-18).

3.2.4. ChIP assays

Next we examined several transcription factors which bind with CRE1 and CRE2 by gel mobility shift assays can interact directly with human BSP gene promoter and how melatonin regulates these transcription factors. We did ChIP assays to examine the in vivo association of these transcription factors with CRE1 and CRE2 in Saos2 cells. Confluent Saos2 cells were treated with 100 nM melatonin for 6 h to induce BSP expression and cross-linked with formaldehyde. After enzymatic shearing, soluble chromatins were immunoprecipitated with either antibodies or control IgG. The PCR bands amplified and corresponding to DNA-protein complexes immunoprecipitated with antibodies revealed that CREB1, phospho-CREB, c-Jun, c-Fos, JunD and Fra2 interacted with a chromatin fragment containing the CRE1 and CRE2 that were increased in Saos2 cells after stimulation by melatonin (Fig. 14).

4. Discussion

4.1. Regulation of BSP gene transcription by IL-11

Results of this study show that IL-11 increases BSP transcription in osteoblast-like ROS 17/2.8 cells by targeting CRE, FRE and HOX elements in the rat BSP gene promoter. IL-11 (20 ng/ml) induced BSP mRNA levels at 3 and 12 h in ROS17/2.8 cells (Fig. 1). When we used rat stromal bone marrow cells

(RBMC), IL-11 also increased BSP transcription (Fig. 2B); therefore, IL-11 increases BSP transcription not only in transformed ROS17/2.8 cells but also in normal osteoprogenitors (RBMC). These two kinds of cells may distinctively respond to IL-11, because of residing in different stages of cell maturation. From transient transfection analyses, we initially located the IL-11 response region to the proximal promoter (-116 to +60) of the rat BSP gene (Fig. 2), which encompasses inverted CCAAT, CRE, Runx2, FRE and Pit-1 motifs (Fig. 3). The specific response elements between -116 to +60 form the transcriptional start site are CRE, Runx2 and FRE. Transcriptional regulation by IL-11 was abrogated when CRE and FRE or FRE and HOX were double mutated in pLUC3 or pLUC4 (Fig. 4). The involvement of CRE, FRE and HOX elements is further supported by gel shift assays in which nuclear proteins that formed complexes with CRE, FRE and HOX elements were increased by IL-11 (20 ng/ml) in ROS17/2.8 cells (Fig. 5).

IL-11 induces osteoblast differentiation and acts synergistically with BMP-2 in C3H10T1/2 cells (57). IL-11 also acts synergistically with BMP-2 to accelerate bone formation in a rat ectopic model (58). IL-11 stimulated transcription of the target gene for BMP via STAT3, leading to osteoblastic differentiation in the presence of BMP-2, but inhibited adipogenesis in bone marrow stromal cells (15). IL-11 enhanced ALP activity and Runx2, osteocalcin and BSP gene expression in the presence of ascorbic acid in human periodontal ligament cells (59).

FRE (–92 to -85; GGTGAGAA) in the rat BSP gene promoter is juxtaposed to a putative Runx2 site (-84 to -79; CCCACA) (48, 60). Another putative Runx2 site (-184 to -179; CCCACA) is juxtaposed to a HOX site (-194 to -185; TCAATTAAAT) (Fig. 3), and HOX oligonucleotides compete with the FRE-protein complex (data not show). Thus, FRE and HOX binding transcription factors might interact. We have characterized a region of the rat BSP gene promoter that is required for IL-11 mediated transcription. This region contains CRE, FRE and HOX, which are required for the IL-11 response.

4.2. Regulation of BSP gene transcription by melatonin

Melatonin is synthesized and secreted by the pineal grand and other organs. It has a variety of

physiological actions such as control of circadian rhythm, sexual development, inflammation and hormone secretion (18, 24, 28, 26, 31). Melatonin promotes osteoblast differentiation and bone formation (61, 62). Melatonin may play a role in protecting the oral cavity from tissue damage (63, 64). The increase in salivary melatonin secretion may keep a healthy condition of periodontal tissue (65). In this study, we have shown that melatonin induced BSP gene transcription by targeting CRE1 and CRE2 in the human BSP gene promoter. BSP constitutes from 8 to 12% of the total non-collagenous proteins in bone and cementum. BSP is a potential nucleator of hydroxyapatite and is a specific marker of osteoblast and cementoblast differentiation (35, 36). The results in this study showed that melatonin increased BSP transcription mediated through two CRE via cAMP, tyrosine kinase, ERK1/2 and PI3K pathways in Saos2 human osteoblast-like cells. Melatonin induced BSP, Runx2 and Osterix mRNA and BSP protein levels (Fig. 6). Runx2 and Osterix are crucial transcription factors for osteoblast differentiation. Runx2 and Osterix knockout mice have no bone due to stop of osteoblast differentiation (60, 66). Melatonin increased BSP mRNA levels at 3 h and it increased the mRNA levels gradually till 12 and 24 h (Fig. 6A). On the other hand, melatonin increased Runx2 mRNA levels at 6 h and Osterix mRNA levels at 24 h (Fig. 6B). The results suggest that most important transcription factor for BSP gene induction is distinct from Runx2 and Osterix. From transient transfection assays, BSP promoter activities between -84LUC to -868LUC were increased by melatonin at 12 h in Saos2 cells (Fig. 7). Moreover, the results of luciferase assays using single- and double-mutation constructs in CRE1 and CRE2 elements suggested that they are essential for the induction of BSP transcription by melatonin (Fig. 9). However, melatonin could increase transcriptional activities of mutation CCAAT (-184mCCAAT) and mutation FRE (-184mFRE) constructs almost same as wild construct (-184LUC), suggesting that inverted CCAAT and FRE were not target of melatonin. The involvement of two CRE elements is further confirmed by gel mobility shift assays in which nuclear proteins from Saos2 cells formed complexes with CRE1 and CRE2 that were increased by melatonin at 3 and 6 h (Fig. 11). CRE1- and CRE2-protein complexes, showing specific bindings, were demonstrated by competition gel shift, whereas CRE1- and CRE2 binding proteins

partially competed by a 40-fold molar excess of CRE2 and CRE1, suggesting that the components of CRE1 and CRE2 binding proteins are not completely same (Fig. 12). The results of gel shift assays using antibodies for several transcription factors indicated that CRE1 and CRE2 interacted with CREB1, phospho-CREB1, c-Fos, c-Jun, JunD and Fra2 (Fig. 13), and these transcription factors interacted with a chromatin fragment containing the CRE1 and CRE2 were increased after stimulation by melatonin (Fig. 14). CREB1 and activating transcription factor (ATF)/CREB family members can bind to CRE as homodimers, but some of these proteins can bind as heterodimers, both within the ATF/CREB family and with members of the AP1 transcription factors family (67). AP1 transcription factor family is composed of Jun family members (c-Jun, JunB, and JunD) that can form homo- or heterodimers among themselves and bind to AP1 consensus sequence (TGACTCA). Jun proteins also dimerize with Fos family members (c-Fos, FosB, Fra1 and Fra2). Most AP1 are present at low levels in cells but are rapidly induced and activated in response to specific stimuli (68). JunD and Fra2 are able to bind the CRE1 in the human BSP proximal promoter as heterodimers and/or with CREB1 and regulate BSP protein expression in breast cancer cells as well as osteoblastic cells (55, 69). Inhibition of AP1 activity may block proliferation, migration, invasion and metastasis of tumor cells (70).

Previously we have reported that PKA, tyrosine kinase, ERK1/2 and PI3K regulate BSP gene transcription (53-55, 71). Activation of PKA may induce CREB1 phosphorylation and phospho-CREB1 can bind to CRE firmly, thereby induce gene transcription (72). HA, U0126 and LY294002 inhibited the increased binding activities of nuclear proteins to FRE and HOX elements in the rat BSP gene promoter (51). In our previous studies FGF2 stimulated BSP gene transcription via a tyrosine kinase and ERK1/2 pathways (48), PTH activated cAMP and phospholipase C through a PKA and tyrosine kinase pathways (53), and PGE2 induced BSP transcription via PKA, tyrosine kinase and ERK1/2, which target nuclear proteins that bind to CRE and FRE element in the rat BSP gene promoter (47). LY294002 is an inhibitor of PI3K/Akt, which is one of the critical pathways for the differentiation of skeletal component cells, such as chondrocytes, osteoblasts, myoblasts and adypocytes (73). Further, Runx2-induced osteoblast

differentiation is inhibited by the PI3K/Akt (74), suggesting that Runx2 and PI3K/Akt may be important signaling molecules for BSP transcription. We have identified CRE1 and CRE2 in the human BSP gene promoter that mediate BSP transcription induced by melatonin and shown that the melatonin increases the nuclear protein binding to CRE1 and CRE2, which may involve CREB1, phospho-CREB1, c-Fos, c-Jun, JunD and Fra2 transcription factors. We have also shown that melatonin increases human BSP transcription through a PKA, tyrosine kinase, ERK1/2 and PI3K signaling pathways.

The results in this study demonstrate that IL-11 and melatonin increase BSP transcription and protein expression. BSP is expressed in differentiated osteoblasts, IL-11 and melatonin is an important factor for bone metabolism, it is presumable that response elements contribute to these two factors can contribute to the tissue-specific expression of BSP gene during the bone and cementum formation.

5. References

1. Paul SR, Bennett F, Calvetti JA, Kelleher K, Wood CR, O'Hara RM Jr, Leary AC, Sibley B, Clark SC, Williams DA. 1990. Molecular cloning of a cDNA encoding interleukin 11, a stromal cell-derived lymphopoietic and hematopoietic cytokine. Proc Natl Acad Sci USA 87: 7512-7516.
2. Yin TG, Schendel P, Yang YC. 1992. Enhancement of in vitro and in vivo antigen-specific antibody responses by interleukin 11. J Exp Med 175: 211-216.
3. Du XX, Williams DA. 1994. Interleukin-11: a multifunctional growth factor derived from the hematopoietic microenvironment. Blood 83: 2023-2030.
4. Du XX, Williams DA. 1997. Interleukin-11; review of molecular, cell biology, and clinical use. Blood 89: 3897-3908.
5. Musashi M, Yang YC, Paul SR, Clark SC, Sudo T, Ogawa M. 1991. Direct and synergistic effects of interleukin 11 on murine hemopoiesis in culture. Proc Natl Acad Sci USA 88: 765-769.
6. Mehler MF, Rozental R, Dougherty M, Spray DC, Kessler JA. 1993. Cytokine regulation of neuronal differentiation of hippocampal progenitor cells. Nature 362: 62-65.

7. Teramura M, Kobayashi S, Yoshinaga K, Iwabe K, Mizoguchi H. 1996. Effect of interleukin 11 on normal and pathological thrombopoiesis. Cancer Chemother Pharmacol 38 (suppl): S99-S102.
8. Löwik CW, van der Pluijm G, Bloys H, Hoekman K, Bijvoet OL, Aarden LA, Papapoulos SE. 1989. Parathyroid hormone (PTH) and PTH-like protein (PLP) stimulate interleukin-6 production by osteogenic cells: a possible role of interleukin-6 in osteoclastogenesis. Biochem Biophys Res Commun 162: 1546-1552.
9. Allan EH, Hilton DJ, Brown MA, Evely RS, Yumita S, Metcalf D, Gough NM, Ng KW, Nicola NA, Martin TJ. 1990. Osteoblasts display receptors for and responses to leukemia-inhibitory factor. J Cell Physiol 14: 110-119.
10. Reid LR, Lowe C, Cornish J, Skinner SJ, Hilton DJ, Willson TA, Gearing DP, Martin TJ. 1990. Leukemia inhibitory factor: a novel bone-active cytokine. Endocrinology 126: 1416-1420.
11. Littlewood AJ, Russell J, Harvey GR, Hughes DE, Russell RG, Gowen M. 1991. The modulation of the expression of IL-6 and its receptor in human osteoblasts in vitro. Endocrinology 129: 1513-1520.
12. Girasole G, Passeri G, Jilka RL, Manolagas SC. 1994. Interleukin-11: a new cytokine critical for osteoclast development. J Clin Invest 93: 1516-1524.
13. Hill PA, Tumber A, Papaioannou S, Meikle MC. 1998. The cellular actions of interleukin-11 on bone resorption in vitro. Endocrinology 139: 1564-1572.
14. Gimble JM, Wanker F, Wang CS, Bass H, Wu X, Kelly K, Yancopoulos GD, Hill MR. 1994. Regulation of bone marrow stromal cell differentiation by cytokines whose receptors share the gp130 protein. J Cell Biochem 54: 122-133.
15. Takeuchi Y, Watanabe S, Ishii G, Takeda S, Nakayama K, Fukumoto S, Kaneta Y, Inoue D, Matsumoto T, Harigaya K, Fujita T. 2002. Interleukin-11 as a stimulatory factor for bone formation prevents bone loss with advancing age in mice. J Biol Chem 277: 49011-49018.
16. Karasek M, Winczyk K. 2006. Melatonin in humans. J Physiol Pharmacol 57: 19-39.

17. Srinivasan V, Spence WD, Pandi-Perumal SR, Zakharia R, Bhatnagar KP, Brzezinski A. 2009. Melatonin and human reproduction: Shedding light on the darkness hormone. Gynecol Endocrinol 25: 779-785.
18. Zawilska JB, Skene DJ, Arendt J. 2009. Physiology and pharmacology of melatonin in relation to biological rhythms. Pharmacol Rep 61: 383-410.
19. Stehle JH, Saade A, Rawashdeh O, Ackermann K, Jilg A, Sebesteny T, Maronde E. 2011. A survey of molecular details in the human pineal gland in the light of phylogeny, structure, function and chronobiological diseases. J Pineal Res 51: 17-43.
20. Dubocovich ML, Markowska M. 2005. Functional MT1 and MT2 melatonin receptors in mammals. Endocrine 27: 101-110.
21. Tan DX, Manchester LC, Reiter RJ, Qi WB, Zhang M, Weintraub ST, Cabrera J, Sainz RM, Mayo JC. 1990. Identification of highly elevated levels of melatonin in bone marrow: Its origin and significance. Biochim Biophys Acta 1472: 206-214.
22. Conti A, Conconi S, Hertens E, Skwarlo-Sonta K, Markowska M, Maestroni JM. 2000. Evidence for melatonin synthesis in mouse and human bone marrow cells. J Pineal Res 28: 193-202.
23. Dollins AB, Zhdanova IV, Wurtman RJ, Lynch HJ, Deng MH. 1994. Effect of inducing nocturnal serum melatonin concentrations in daytime on sleep, mood, body temperature, and performance. Proc Natl Acad Sci USA 91: 1824-1828.
24. Esquifino AI, Villanua MA, Agrasal C. 1987. Effect of neonatal melatonin administration on sexual development in the rat. J Steroid Biochem. 27: 1089-1093.
25. Radogna F, Diederich M, Ghibelli L. 2010. Melatonin: A pleiotropic molecule regulating inflammation. Biochem Pharmacol 80: 1844-1852.
26. Krause DN, Barrios VE, Duckles SP. 1995. Melatonin receptors mediate potentiation of contractile responses to adrenergic nerve stimulation in rat caudal artery. Eur J Pharmacol 276: 207-213.
27. Cos S, Sánchez-Barceló EJ. 1995. Melatonin inhibition of MCF-7 human breast-cancer cells

growth: influence of cell proliferation rate. Cancer Lett 93: 207-212.

28. Roth JA, Rabin R, Agnello K. 1997. Melatonin suppression of PC12 cell growth and death. Brain Res 768: 63-70.
29. Cardinali DP, Ladizesky MG, Boggio V, Cutrera RA, Mautalen C. 2003. Melatonin effects on bone: Experimental facts and clinical perspectives. J Pineal Res 34: 81-87.
30. Kotlarczyk MP, Lassila HC, O'Neil CK, D'Amico F, Enderby LT, Witt-Enderby PA, Balk JL. 2012. Melatonin osteoporosis prevention study (MOPS): A randomized, double-blind, placebo-controlled study examining the effects of melatonin on bone health and quality of life in perimenopausal women. J Pineal Res 52: 414-426.
31. Ladizesky MG, Boggio V, Albornoz LE, Castrillon PO, Mautalen C, Cardinali DP. 2003. Melatonin increases oestradiol-induced bone formation in ovariectomized rats. J Pineal Res 34: 143-151.
32. Cutando A, Gomez-Moreno G, Arana C, Munoz F, Lopez-Pena M, Stephenson J, Reiter RJ. 2008. Melatonin stimulates osteointegration of dental implants. J Pineal Res 45: 174-179.
33. Liu J, Zhou H, Fan W, Dong W, Fu S, He H, Huang F. 2013. Melatonin influences proliferation and differentiation of rat dental papilla cells in vitro and dentine formation in vivo by altering mitochondrial activity. J Pineal Res 54: 170-178.
34. Ogata Y, Yamauchi M, Kim RH, Li JJ, Freedman LP, Sodek J. 1995. Glucocorticoid regulation of bone sialoprotein (BSP) gene expression. Identification of a glucocorticoid response element in the bone sialoprotein gene promoter. Eur J Biochem 230: 183-192.
35. Ganss B, Kim RH, Sodek J. 1999. Bone sialoprotein. Crit Rev Oral Biol Med 10: 79-98.
36. Ogata Y. 2008. Bone sialoprotein and its transcriptional regulatory mechanism. J Periodontal Res 43: 127-135.
37. Hunter GK, Goldberg HA. 1993. Nucleation of hydroxyapatite by bone sialoprotein. Proc Natl Acad Sci USA 90: 8562-8565.

38. Chen J, Shapiro HS, Sodek J. 1992. Development expression of bone sialoprotein mRNA in rat mineralized connective tissues. J Bone Miner Res 7: 987-997.

39. Bellahcène A, Merville MP, Castronovo V. 1994. Expression of bone sialoprotein, a bone matrix protein, in human breast cancer. Cancer Res 54: 2823-2826.

40. Waltregny D, Bellahcène A, de Leval X, Florkin B, Weidle U, Castronovo V. 2000. Increased expression of bone sialoprotein in bone metastases compared with visceral metastases in human breast and prostate cancers. J Bone Miner Res 15: 834-843.

41. Li JJ, Sodek J. 1993. Cloning and characterization of the rat bone sialoprotein gene promoter. Biochem J 289: 625-629.

42. Benson MD, Aubin JE, Xiao G, Thomas PE, Franceschi RT. 1999. Cloning of a 2.5 kb murine bone sialoprotein promoter fragment and functional analysis of putative Osf2 binding sites. J Bone Miner Res 14: 396-405.

43. Kim RH, Shapiro HS, Li JJ, Wrana JL, Sodek J. 1994. Characterization of the human bone sialoprotein (BSP) gene and its promoter sequence. Matrix Biol 14: 31-40.

44. Kiyoshima T, Yamauchi M, Wong C, Jheon A, Ganss B, Sodek J. 2002. An L1 element disrupts human bone sialoprotein promoter: lack of tissue-specific regulation by distalless5 (Dlx5) and runt homeodomain protein2 (Runx2)/core binding factor a1 (Cbfa1) elements. Gene 299: 205-217.

45. Li JJ, Kim RH, Sodek J. 1995. An inverted TATA box directs downstream transcription of the bone sialoprotein gene. Biochem J 310: 33-40.

46. Kim RH, Sodek J. 1999. Transcription of the bone sialoprotein gene is stimulated by v-Src acting through an inverted CCAAT box. Cancer Res 59: 565-571.

47. Samoto H, Shimizu E, Matsuda-Honjyo Y, Saito R, Nakao S, Yamazaki M, Furuyama S, Sugiya H, Sodek J, Ogata Y. 2003. Prostaglandin E2 stimulates bone sialoprotein (BSP) expression through cAMP and fibroblast growth factor 2 response elements in the proximal promoter of the rat BSP gene. J Biol Chem 278: 28659-28667.

48. Shimizu-Sasaki E, Yamazaki M, Furuyama S, Sugiya H, Sodek J, Ogata Y. 2001. Identification of a novel response element in the rat bone sialoprotein (BSP) gene promoter that mediates constitutive and fibroblast growth factor 2-induced expression of BSP. J Biol Chem 276: 5459-5466.

49. Shimizu E, Nakayama Y, Nakajima Y, Kato N, Takai H, Kim DS, Arai M, Saito R, Sodek J, Ogata Y. 2006. Fibroblast growth factor 2 and cyclic AMP synergistically regulate bone sialoprotein gene expression. Bone 39: 42-52.

50. Ogata Y, Nakao S, Kim RH, Li JJ, Furuyama S, Sugiya H, Sodek J. 2000. Parathyroid hormone regulation of bone sialoprotein (BSP) gene transcription is mediated through a pituitary-specific transcription factor-1 (Pit-1) motif in the rat BSP gene promoter. Matrix Biol 19: 395-407.

51. Nakayama Y, Nakajima Y, Kato N, Takai H, Kim DS, Arai M, Mezawa M, Araki S, Sodek J, Ogata Y. 2006. Insulin-like growth factor-I increases bone sialoprotein (BSP) expression through fibroblast growth factor-2 response element and homeodomain protein-binding site in the proximal promoter of the BSP gene. J Cell Physiol 208: 326-335.

52. Ogata Y, Niisato N, Furuyama S, Cheifetz S, Kim RH, Sugiya H, Sodek J. 1997. Transforming growth factor-beta 1 regulation of bone sialoprotein gene transcription: identification of a TGF-beta activation element in the rat BSP gene promoter. J Cell Biochem 65: 501-512.

53. Araki S, Mezawa M, Sasaki Y, Yang L, Li Z, Takai H, Nakayama Y, Ogata Y. 2009. Parathyroid hormone regulation of the human bone sialoprotein gene transcription is mediated through two cAMP response elements. J Cell Biochem 106: 618-625.

54. Mezawa M, Araki S, Takai H, Sasaki Y, Wang S, Li X, Kim DS, Nakayama Y, Ogata Y. 2009. Regulation of human bone sialoprotein gene transcription by platelet-derived growth factor-BB. Gene 435: 80-87.

55. Li Z, Sasaki Y, Mezawa M, Wang S, Li X, Yang L, Wang Z, Zhou L, Araki S, Matsumura H, Takai H, Ogata Y. 2001. cAMP and fibroblast growth factor 2 regulate bone sialoprotein gene expression in human prostate cancer cells. Gene 471: 1-12.

56. Nakayama Y, Kato N, Nakajima Y, Shimizu E, Ogata Y. 2004. Effect of TNF-alpha on human osteosarcoma cell line Saos2--TNF-alpha regulation of bone sialoprotein gene expression in Saos2 osteoblast-like cells. Cell Biol Int 28: 653-660.

57. Suga K, Saitoh M, Fukushima S, Takahashi K, Nara H, Yasuda S, Miyata K. 2001. Interleukin-11 induces osteoblast differentiation and acts synergistically with bone morphogenetic protein-2 in C3H10T1/2 cells. J Interferon Cytokine Res 21: 695-670.

58. Suga K, Saitoh M, Kokubo S, Fukushima S, Kaku S, Yasuda S, Miyata K. 2003. Interleukin-11 acts synergistically with bone morphogenetic protein-2 to accelerate bone formation in a rat ectopic model. J Interferon Cytokine Res 23: 203-207.

59. Leon ER, Iwasaki K, Komaki M, Kojima T, Ishikawa I. 2007. Osteogenic effect of interleukin-11 and synergism with ascorbic acid in human periodontal ligament cells. J Periodontal Res 42: 527-535.

60. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. 1997. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89: 755-764.

61. Roth JA, Kim BG, Lin WL, Cho MI. 1999. Melatonin promotes osteoblast differentiation and bone formation. J Biol Chem 274: 22041-22047.

62. Park KH, Kang JW, Lee EM, Kim JS, Rhee YH, Kim M. 2011. Melatonin promotes osteoblastic differentiation through the BMP/ERK/Wnt signaling pathways. J Pineal Res 51: 187-194.

63. Cutando A, Gómez-Moreno G, Arana C, Acu-a-Castroviejo C, Reiter RJ. 2007. Melatonin: potential functions in the oral cavity. J Periodontol 78: 1094-1102.

64. Gómez-Moreno G, Guardia J, Ferrera MJ, Cutando A, Reiter RJ. 2010. Melatonin in diseases of the oral cavity. Oral Dis 16: 242-247.

65. Cutando A, Gómez-Moreno G, Villalba J, Ferrera MJ, Escames G, Acu-a-Castroviejo D. 2003.

Relationship between salivary melatonin levels and periodontal status in diabetic patients. J Pineal Res 35: 239-244.

66. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B. 2002. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108: 17-29.
67. Sassone-Corsi P. 1995. Transcription factors responsive to cAMP. Annu Rev Cell Dev Biol 11: 355-377.
68. Sharma SC, Richards JS. 2000. Regulation of AP1 (Jun/Fos) factor expression and activation in ovarian granulosa cells. Relation of JunD and Fra2 to terminal differentiation. J Biol Chem 275: 33718-33728.
69. Detry C, Lamour V, Castronovo V, Bellahcène A. 2008. CREB-1 and AP-1 transcription factors JunD and Fra-2 regulate bone sialoprotein gene expression in human breast cancer cells. Bone 42: 422-431.
70. Ozanne BW, Spence HJ, McGarry LC, Hennigan RF. 2007. Transcription factors control invasion: AP-1 the first among equals. Oncogene 26: 1-10.
71. Wang Z, Li X, Li Z, Yang L, Sasaki Y, Wang S, Zhou L, Araki S, Mezawa M, Takai H, Ogata Y. 2010. Effects of inorganic polyphosphate on bone sialoprotein gene expression. Gene 452: 79-86.
72. Zhang R, Edwards JR, Ko SY, Dong S, Liu H, Oyajobi BO, Papasian C, Deng HW, Zhao M. Transcriptional regulation of BMP2 expression by the PTH-CREB signaling pathway in osteoblasts. PLoS One 6. e20780.
73. Ghosh-Choudhury N, Abboud SL, Nishimura R, Celeste A, Mahimainathan L, Choudhury GG. 2002. Requirement of BMP-2-induced phosphatidylinositol 3-kinase and Akt serine/threonine kinase in osteoblast differentiation and Smad-dependent BMP-2 gene transcription. J Biol Chem 277: 33361-33368.
74. Fujita T, Azuma Y, Fukuyama R, Hattori Y, Yoshida C, Koida M, Ogita K, Komori T. 2004. Runx2

induces osteoblast and chondrocyte differentiation and enhances their migration by coupling with PI3K-Akt signaling. J Cell Biol 166: 85-95.

Table 1 Primers used for real-time PCR

Primer		Sequence
Human BSP	Forward	5’-CTGGCACAGGGTATACAGGGTTAG-3’
	Reverse	5’-ACTGGTGCCGTTTATGCCTTG-3’
Human Runx2	Forward	5’-ATGTGTGTTTGTTTCAGCAGCA-3’
	Reverse	5’-TCCCTAAAGTCACTCGGTATGTGTA-3’
Human Osterix	Forward	5’-GCCAT TCTGGGCTTGGGTATC-3’
	Reverse	5’-GAAGCCGGAGTGCAGGTATCA-3’
Human GAPDH	Forward	5’-GCACCGTCAAGGCTGAGAAC-3’
	Reverse	5’- ATGGTGGTGAAGACGCCAGT -3’

Table 2 Mutation plasmids used for transient transfection assays

Plasmids	Station	Sequence
Mutation rat CCAAT (M-CCAAT)	-50 ~ -46	ATT**tt**
		ATTGG(wild type)
Mutation rat CRE (M-CRE)	-75 ~ -68	**c**GACG**c**CG
		TGACGTCG(wild type)
Mutation rat FRE (M-FRE)	-92 ~ -85	GG**ca**AGAA
		GGTGAGAA(wild type)
Mutation rat Pit-1 (M-PIT)	-111 ~ -105	TT**ac**AGT
		TTGTAGT(wild type)
Mutation rat HOX (M-HOX)	-194 ~ -185	**cc**TTAAA
		AATTAAA(wild type)
Mutation human CRE1 (mCRE1)	-79 ~ -72	TGACA**ga**A
		TGACATCA(wild type)
Mutation human CRE2 (mCRE2)	-674 ~ -667	TGACC**ga**A
		TGACCTCA(wild type)
Mutation human FRE (mFRE)	-96 ~ -89	GG**ca**AGAA
		GGTGAGAA(wild type)

Table 3 Oligonucleotides used for gel mobility shift assays

Oligonucleotide	Station	Sequence
Rat CCAAT	-61 ~ -37	5’-CCGTGACCGTG**ATTGG**CTGCTGAGA-3’
Rat CRE	-84 ~ -59	5’-CCCACAGCC**TGACGTCG**CACCGGCCG-3’
Rat FRE	-98 ~ -79	5’-TTTTCT**GGTGAGAA**CCCACA-3’
Rat Pit-1	-115 ~ -96	5’- CGTG**TTGTAGT**TACGGATTT-3’
Rat HOX	-204 ~ -179	5’- TCCTCAGCCTT**CAATTAAA**TCCCACA-3’
Human CCAAT	-64 ~ -41	5’-CGTGACAGTG**ATTGG**CTGTTGGAA-3’
Human CRE1	-89 ~ -63	5’-ATCCACGTTC**TGACATCA**CCTTGGTCG-3’
Human FRE	-102 ~ -83	5’-TTTTCT**GGTGAGAA**TCCACGA-3’
Human CRE2	-680 ~ -658	5’-ATCAGC**TGACCTCA**CATGCACGA-3’

Table 4 Primer sequence for PCR (ChIP assays)

	Primer	Sequence
CRE1 primer	CRE1ChIP(1)	5’-CCTCTTGGCTCTAGAATCACGTTTC-3’
	CRE1ChIP(2)	5’-CCCTCTCACTCACTCATTCACTTGC-3’
CRE2 primer	CRE2ChIP(3)	5’-CAATGGATCACTCCTGCTAGCTCTAG-3’
	CRE2ChIP(4)	5’-CCACTGAGAGGCAGATTTTATATTTTG-3’

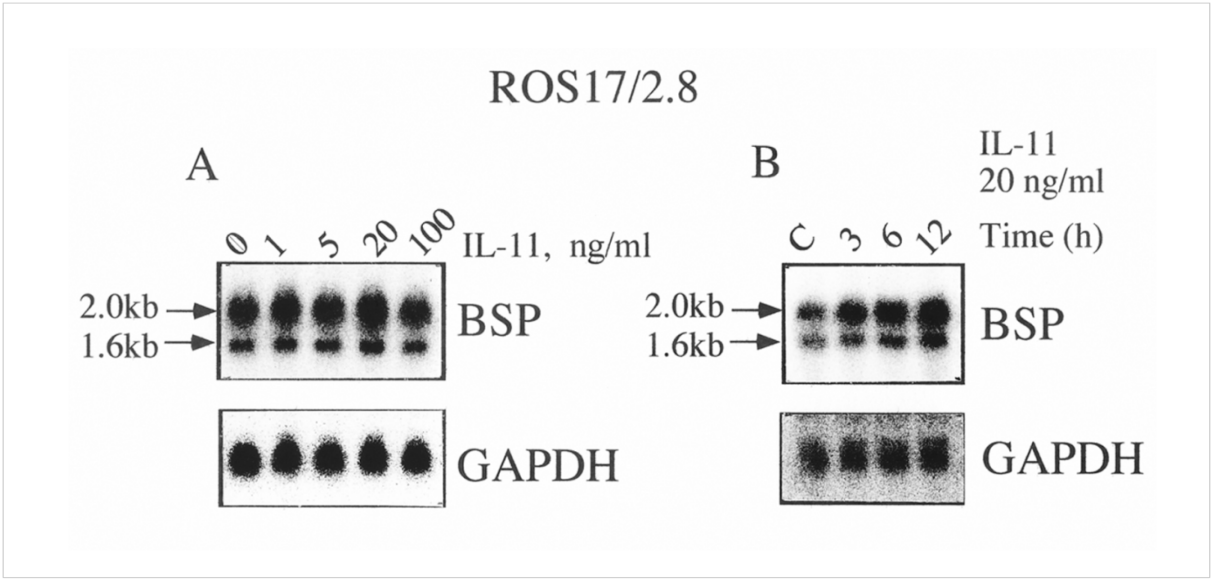

Fig. 1 Effects of IL-11 on BSP mRNA levels in ROS17/2.8 cells.

A. Dose-response effect of IL-11 on BSP mRNA levels in ROS17/2.8 cells treated for 12 h. IL-11 increased BSP mRNA levels at 1, 5, 20 and 100 ng/ml and had a maximal effect at 20 ng/ml. **B**. ROS17/2.8 cells were treated with or without IL-11 (20 ng/ml) for 3, 6 and 12 h. induced BSP mRNA levels were increased at 3 h and reached maximal at 12 h. Northern hybridization was performed with either BSP or GAPDH.

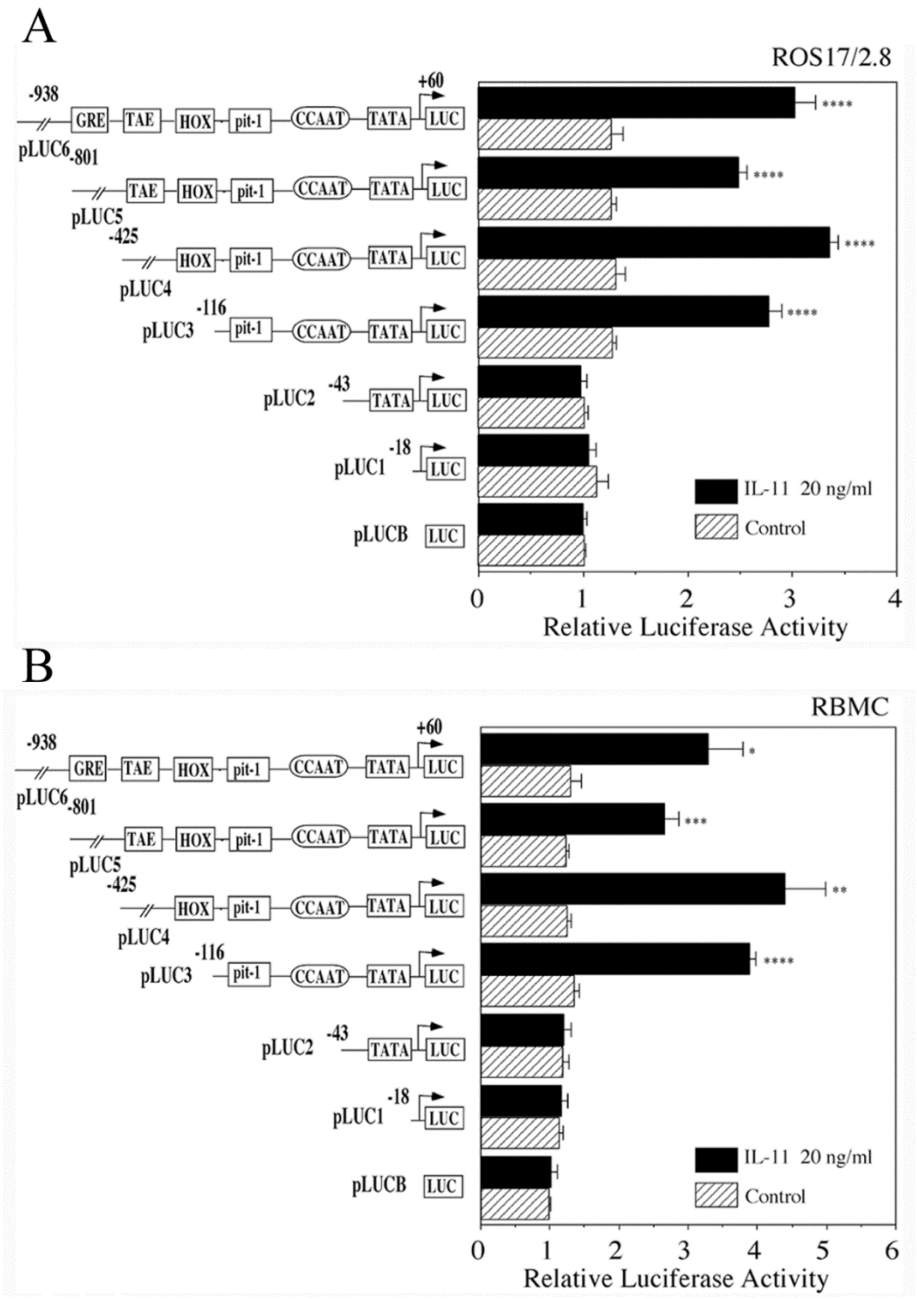

Fig. 2 IL-11 up-regulates rat BSP promoter activities.

The transcriptional activities of pLUC3 (-116 to +60), pLUC4 (-425 to +60), pLUC5 (-801 to +60) and pLUC6 (-938 to +60) were increased after 12 h treatment with 20 ng/ml IL-11 in ROS17/2.8(A) and RBMC (B) cells. The results of transcriptional activities obtained from four separate transfections with constructs, pLUC basic (pLUCB) and pLUC1 to pLUC6, have been combined and values expressed with standard errors. *($P < 0.1$), **($P < 0.05$), ***($P < 0.02$), ****($P < 0.01$).

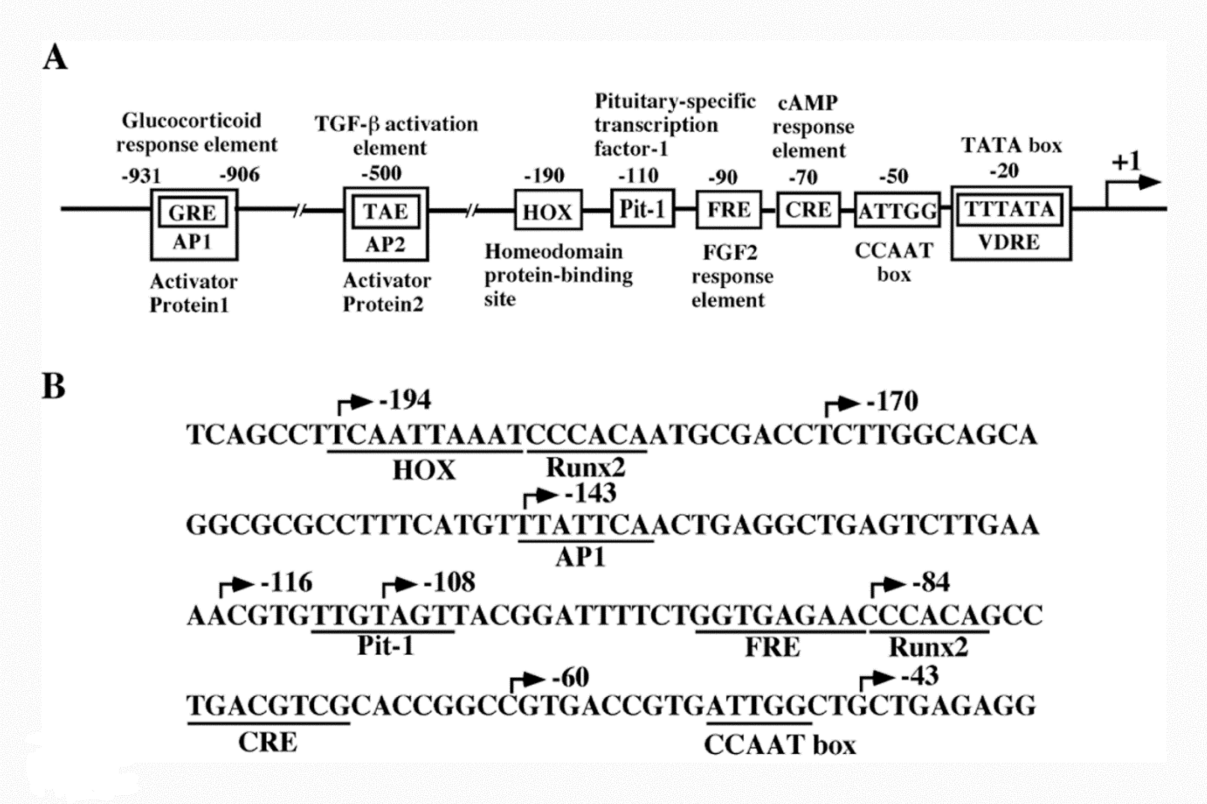

Fig. 3 Regulatory element in the proximal promoter of rat BSP gene.

A. The positions of inverted TATA and CCAAT boxes, vitamin D response element (VDRE) that overlaps the inverted TATA box, CRE, FRE, Pit-1, HOX, TGF-β activation element (TAE) overlaps activator protein 2 (AP2), and GRE overlapping AP1 are shown. **B**. The nucleotide sequence of the rat BSP gene proximal promoter is shown from -194 to -43. An inverted CCAAT box, CRE, Runx2, FRE, Pit-1, AP1, another Runx2 and HOX are present.

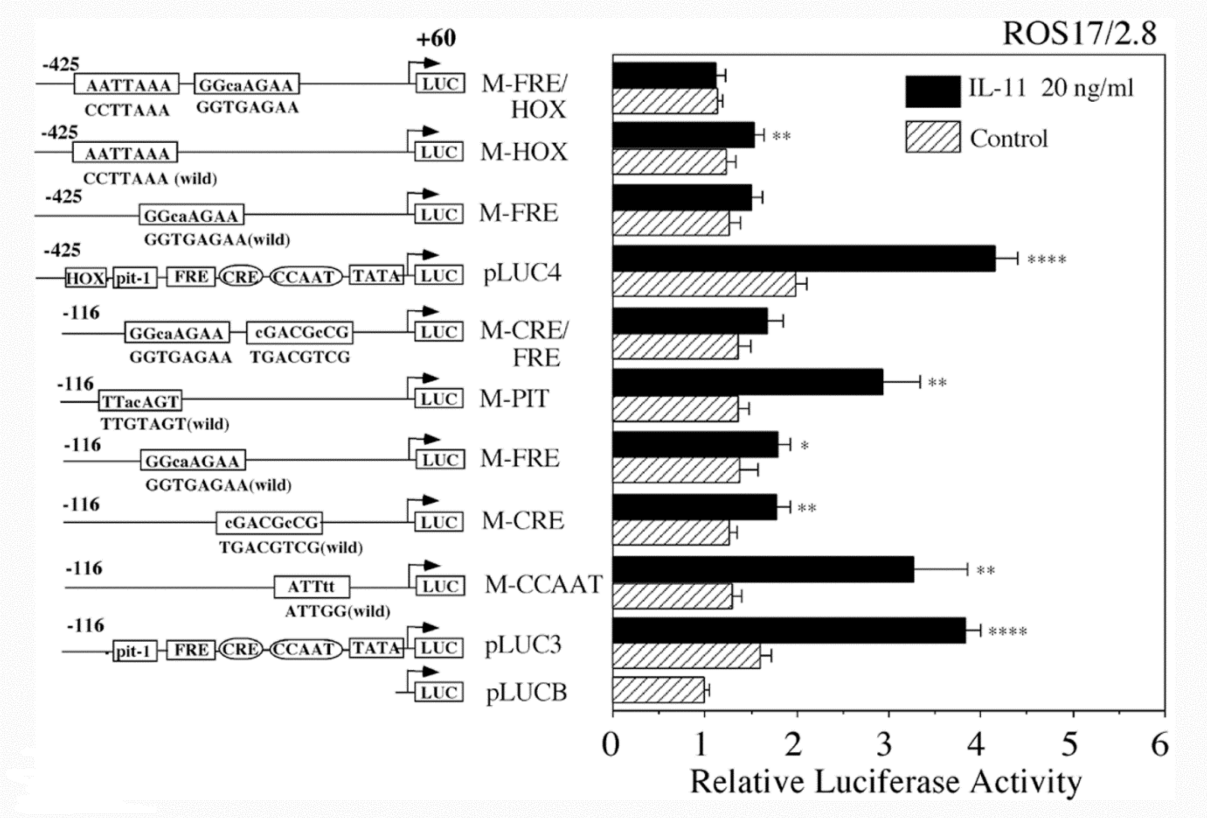

Fig. 4 Site- specific mutation analysis of luciferase activities.

After introducing 2 bp mutations, transcriptional induction by IL-11 (20 ng/ml) was partially inhibited in the M-CRE and M-FRE (pLUC3), and M-FRE and M-HOX (pLUC4) constructs. Double mutation in CRE and FRE (M-CRE/FRE in pLUC3) partially abolished, whereas FRE and HOX (M-FRE/HOX in pLUC4) completely abolished the effect of IL-11. The results of transcriptional activities obtained from four separate transfections with constructs were combined and the values expressed with standard errors. Significant differences from control : *($P < 0.1$), **($P < 0.05$), ***($P < 0.01$).

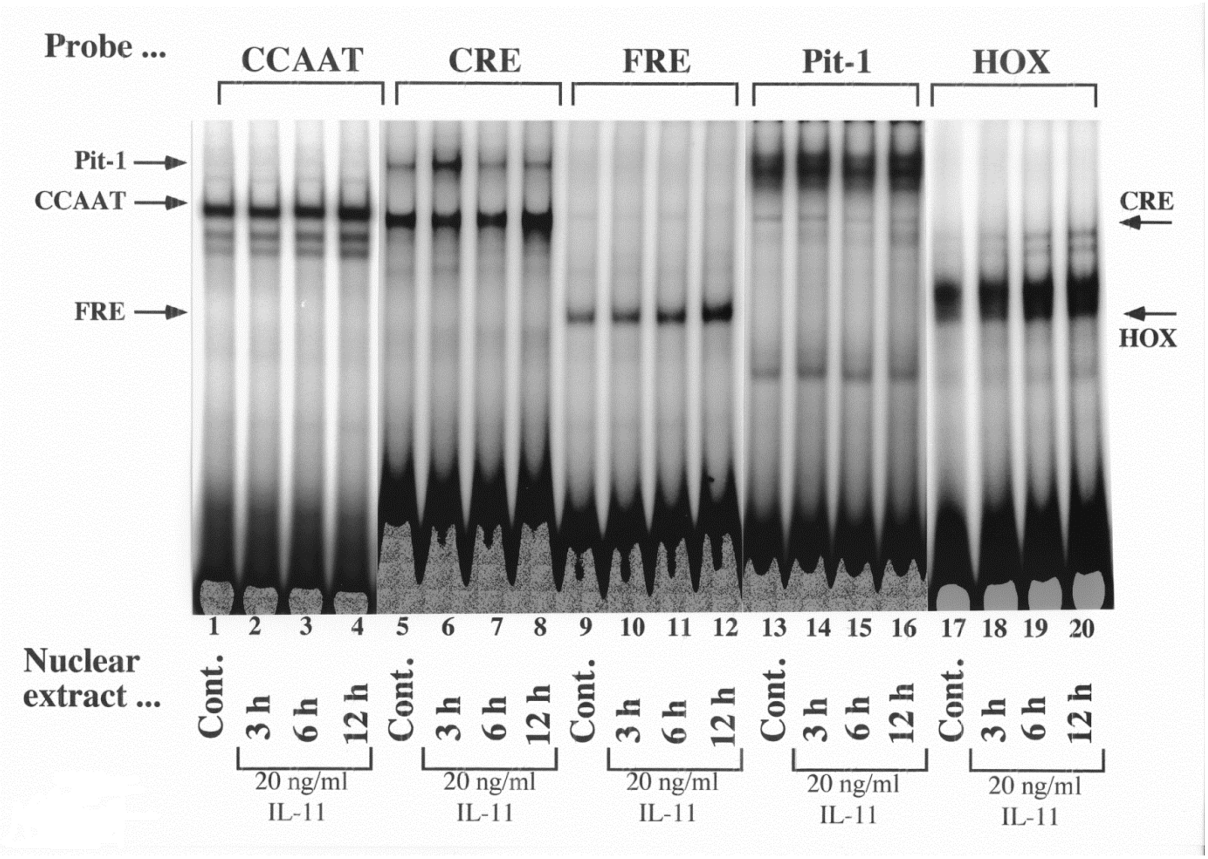

Fig. 5 Gel mobility shift assays using inverted CCAAT, CRE, FRE, Pit-1, and HOX.

After stimulation by 20 ng/ml IL-11 (3, 6 and 12 h), CRE- and FRE-protein complexes were increased at 12 h (lanes 5-8, 9-12), and HOX-protein complexes were increased at 3 h and reached maximal at 12 h (lanes 17-20). CCAAT- and Pit-1-protein complexes did not change after stimulation by IL-11 (lane 1-4, 13-16). DNA-protein complexes were separated on 5% polyacrylamide gel in low-ionic-strength Tris-boraate buffer, dried under a vacuum, and exposed to an imaging plate for quantitation using an imaging analyzer.

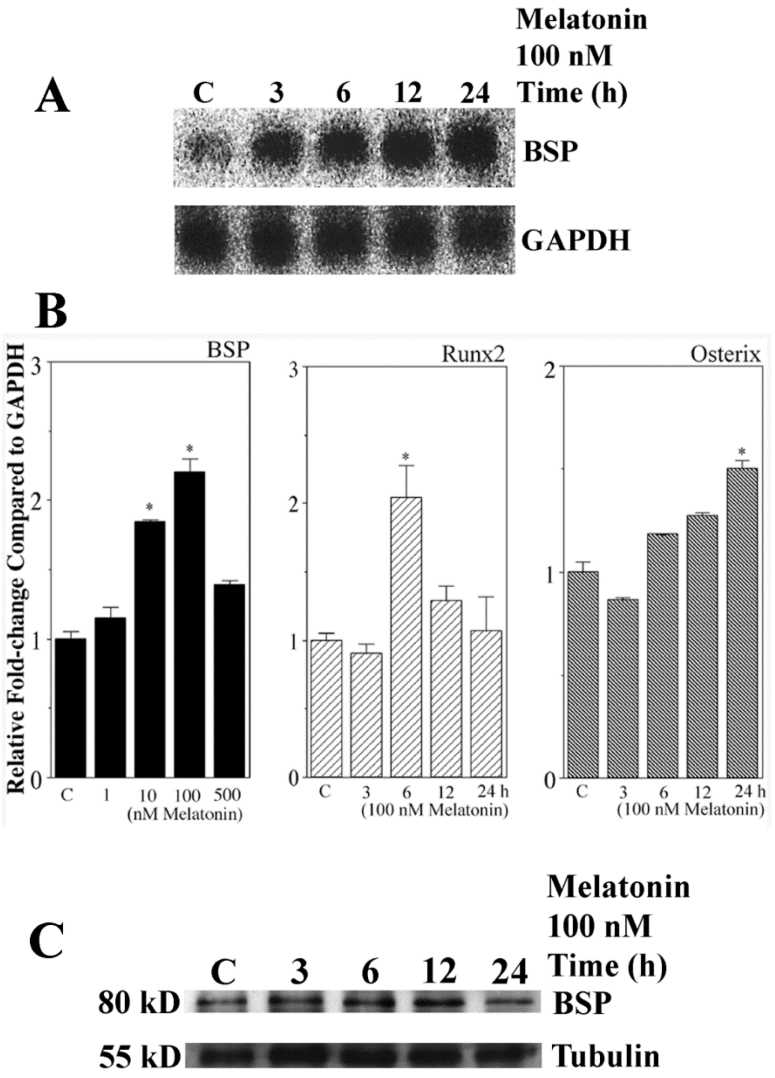

Fig. 6 Effects of melatonin on BSP, Runx2 and Osterix mRNA and BSP protein levels in Saoa2 cells.

A. Saos2 cells were treated with or without melatonin (100 nM) for 3, 6, 12, and 24 h. Total RNA was extracted and the expressions of BSP mRNA was measured by Northern blot . **B**. Runx2 and Osterix mRNA levels were measured by real-time PCR. Dose response effects of melatonin also measured by real-time PCR. The relative amounts of mRNA of BSP, Runx2 and Osterix to GAPDH were calculated. Quantitative analyses of the triplicate data sets are shown with standard errors. Significant differences from control: *($P<0.01$). **C**. BSP and tubulin protein levels in Saos2 cells were treated without (Control) or with melatonin (100 nM) for 3, 6, 12 and 24 h, then cell lysates were prepared and the expressions of BSP and tubulin were analyzed by Western blot using antibodies. A representative result was shown .

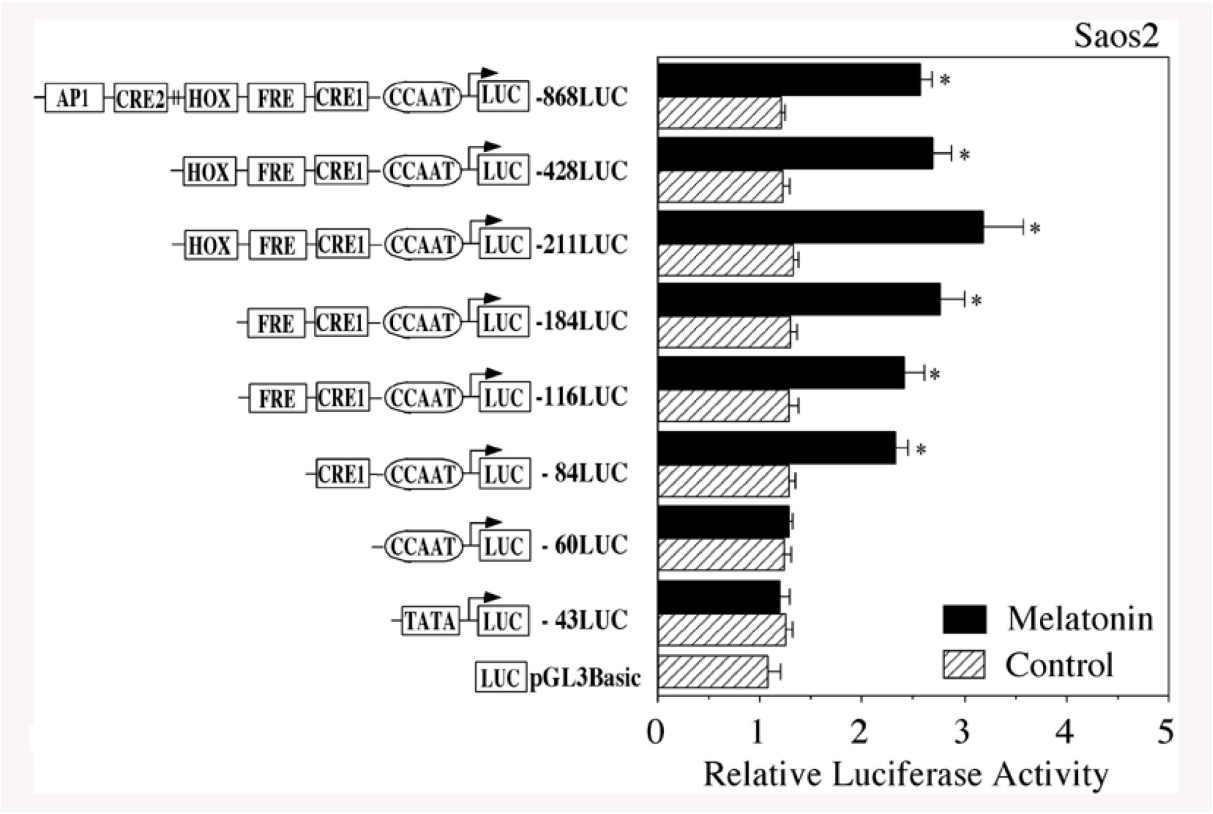

Fig. 7 Melatonin up-regulate human BSP promoter activity in Saos2 cells.

Transient transfections of Saos2 cells in the presence or absence of melatonin (100 nM) for 12 h were used to determine transcriptional activities of chimeric constructs that included various regions of the human BSP promoter ligated to a luciferase reporter gene. The results of transcriptional activities obtained from quadruplicate transfections with constructs, pGL3-basic and -43LUC to -868LUC, have been combined, and the values are expressed with standard errors. Significant differences from control: * ($P<0.01$).

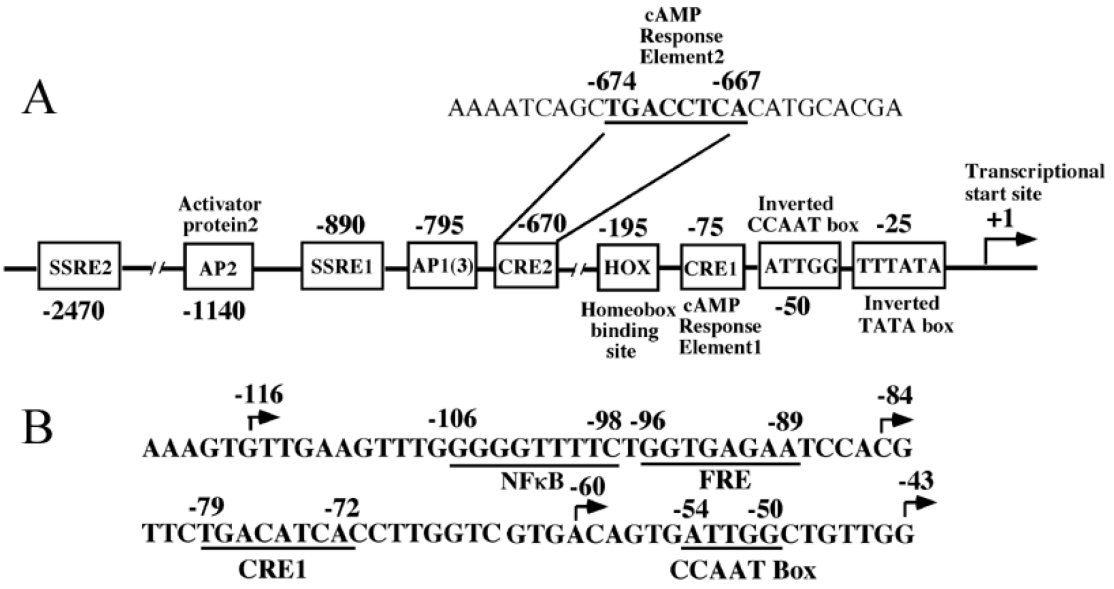

Fig. 8 Regulatory elements in the proximal human BSP promoter.

A. The positions of the inverted TATA and CCAAT boxes, CRE1, HOX, CRE2, AP1(3), shear stress response element 1 (SSRE1), AP2 and SSRE2 are shown in the proximal promoter region of the human BSP gene. The numbering of nucleotides is relative to the transcription start site (+1). The nucleotide sequences of CRE2 in the human BSP gene promoter are shown from -674 to -667 (**TGACCTCA**).

B. Nucleotide sequences of the human BSP gene promoter encompassing an inverted CCAAT box, CRE1, FRE and NFκB are shown from -121 to -43.

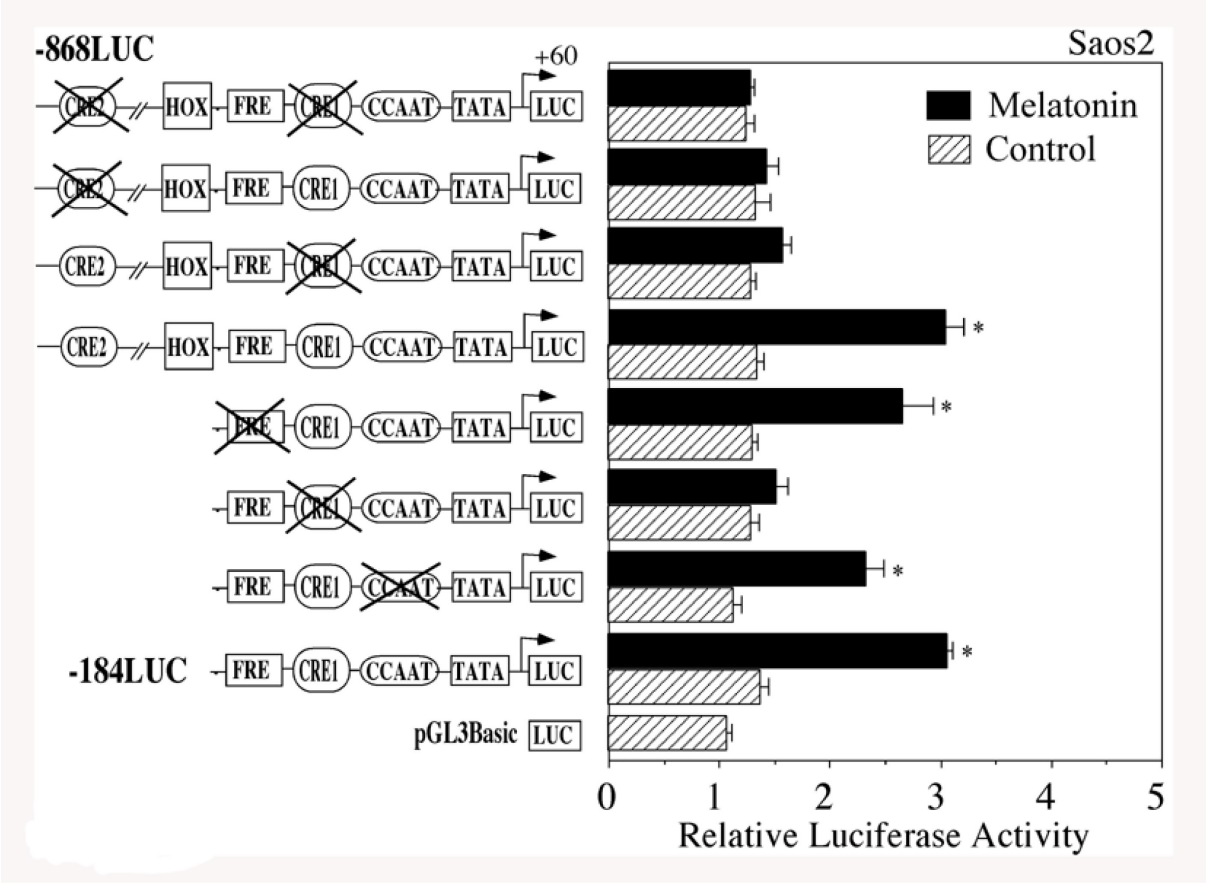

Fig. 9 Site mutation analysis of luciferase activities.

Dinucleotide substitutions were made within the context of the homologous -184 to +60 (-184LUC) and -868 to +60 (-868LUC) BSP promoter fragment. -184mCRE1 and -868mCRE1 (TGACA**ga**A), -868mCRE2 (TGACC**ga**A), -184mCCAAT (ATT**tt**), -184mFRE (GG**ca**AGAA) and -868mCRE1/mCRE2 constructs were analyzed for relative promoter activity after transfection into Saos2 cells and examined for induction after treatment with melatonin (100 nM) for 12 h. The results of transcriptional activity obtained from four separate transfections with constructs were combined and the values expressed with standard errors. Significant differences from control: * ($P<0.01$).

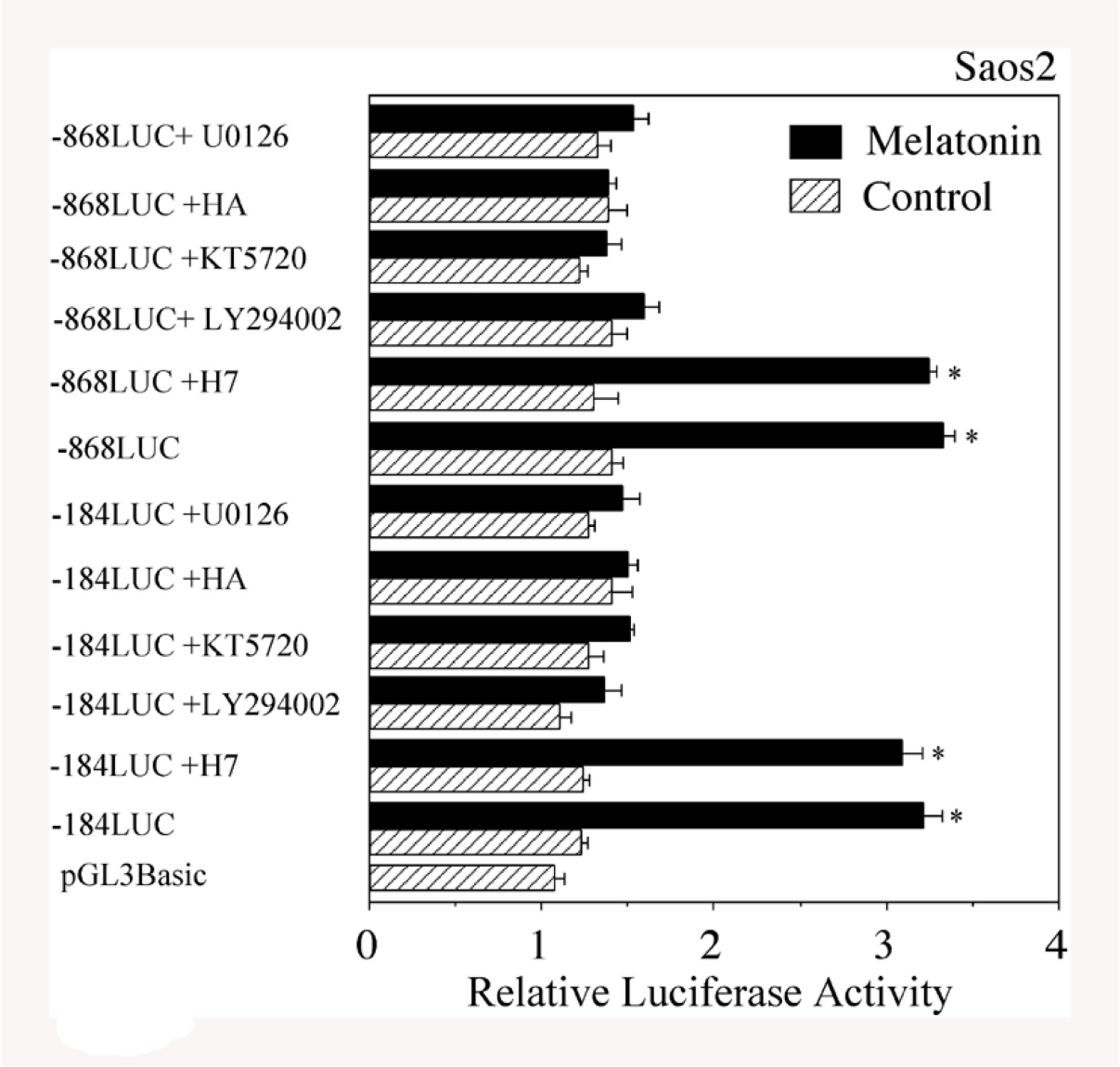

Fig. 10 Effect of kinase inhibitors on transcriptional activation by melatonin.

Transient transfection analysis of -184LUC and -868LUC in the presence or absence of melatonin (100 nM) in Saos2 cells is shown together with the effects of the PKC inhibitor (H7, 5 μM), PI3K inhibitor (LY294002, 10 μM), PKA inhibitor (KT5720, 100 nM), tyrosine kinase inhibitor (HA, 1 μM) and ERK1/2 inhibitor (U0126, 5 μM). The results of transcriptional activity obtained from four separate transfections with constructs were combined and the values expressed with standard errors. Significant differences from control: * ($P<0.01$).

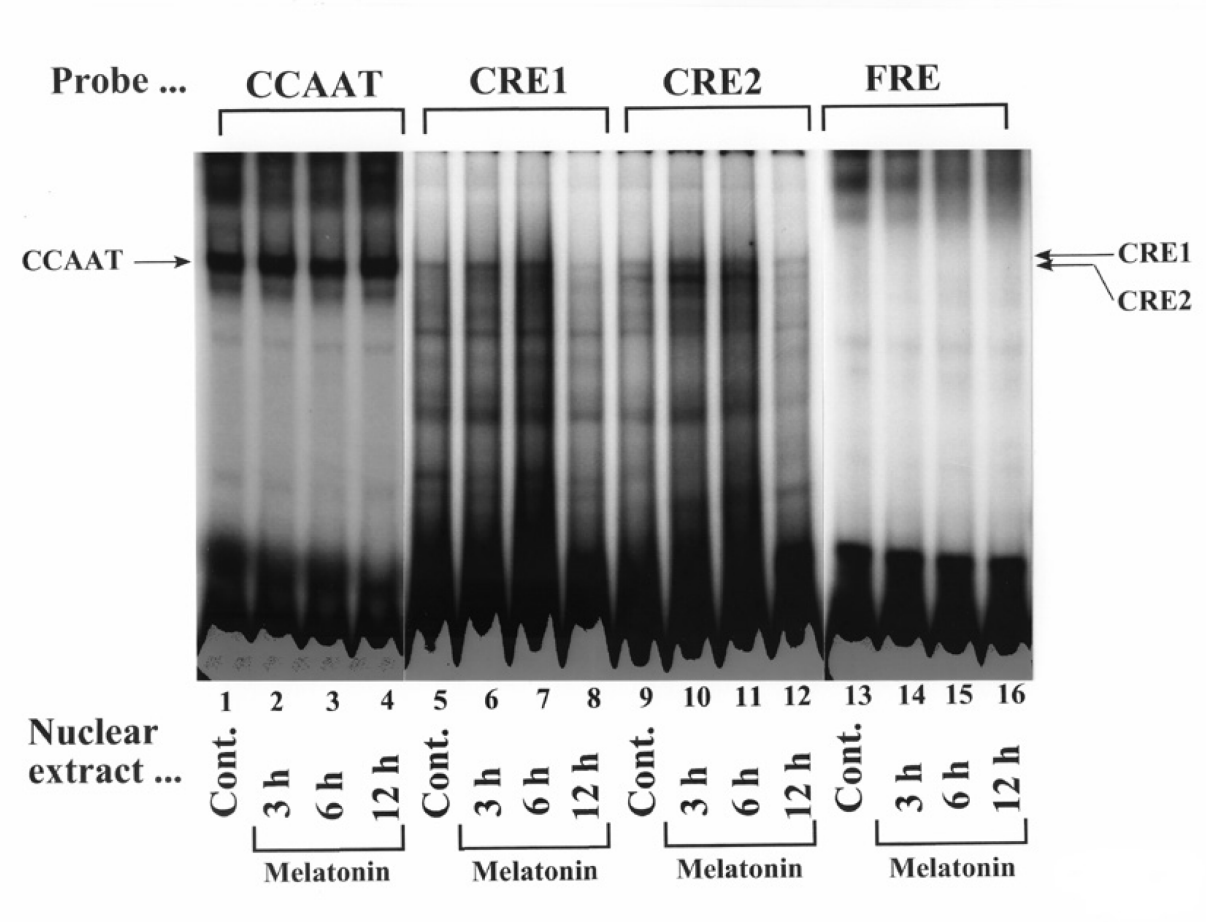

Fig. 11 Melatonin up-regulates nuclear proteins that recognize CRE1 and CRE2.

Radiolabeled double-stranded CCAAT, CRE1, CRE2, FRE oligonucleotides were incubated with nuclear protein extracts (3 μg) obtained from Saos2 cells stimulated without (lanes 1, 5, 9, and 13) or with melatonin for 3h (lanes 2, 6, 10, and 14), 6 h (lanes 3, 7, 11, and 15) and 12 h (lanes 4, 8, 12, and 16). DNA-protein complexes were separated on 5% polyacrylamide gel in low-ionic-strength Tris-borate buffer, dried under a vacuum and exposed to an imaging plate for quantitation using an image analyzer.

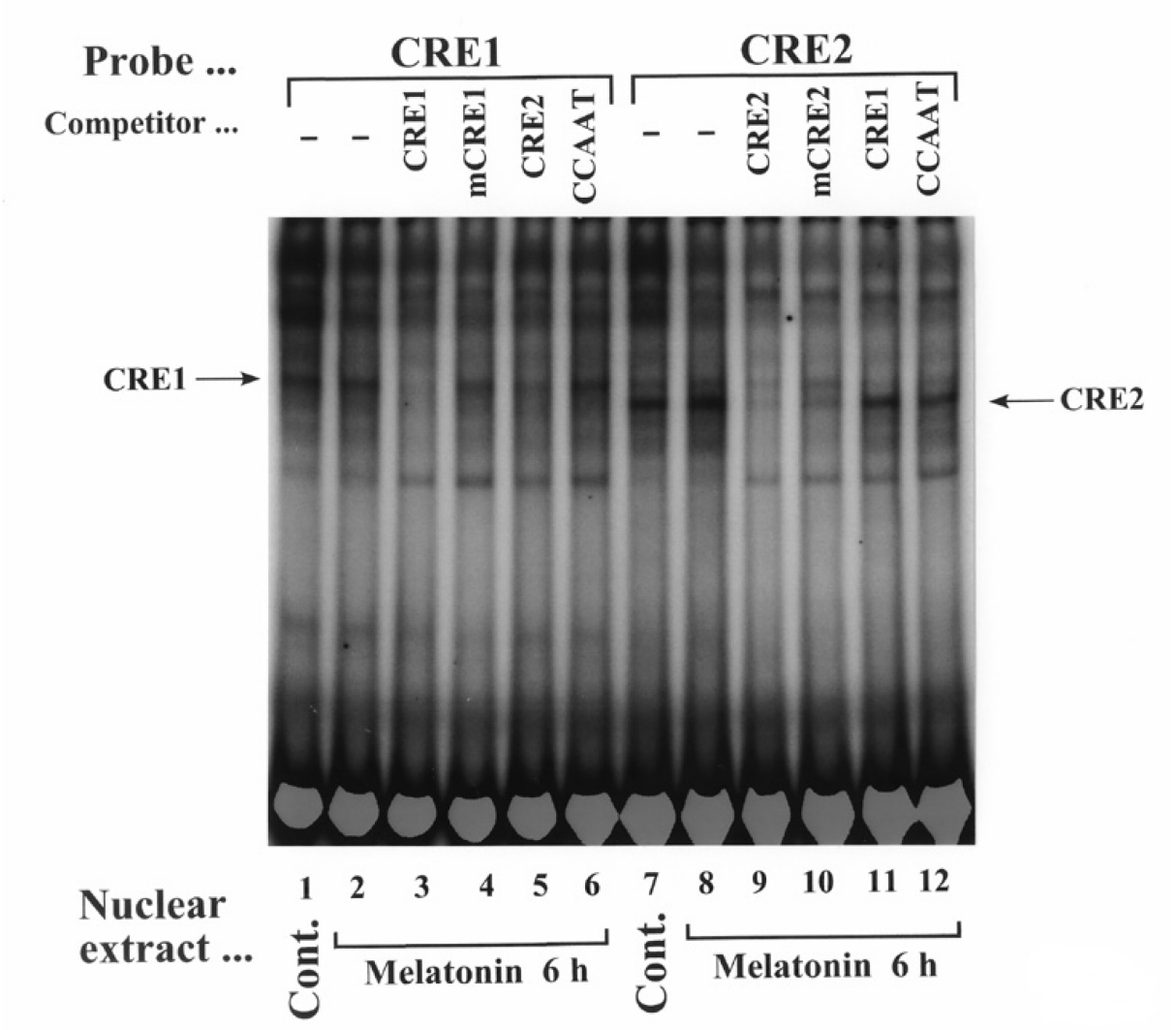

Fig. 12 Specific binding of nuclear proteins to CRE1 and CRE2.

Competition reactions were performed using a 40-fold molar excess of unlabeled CRE1 (lanes 3 and 11), mCRE1 (lane 4), CRE2 (lanes 5 and 9), mCRE2 (lane 10) and CCAAT (lanes 6 and 12) oligonucleotides. DNA-protein complexes were separated on 5% polyacrylamide gel in low-ionic-strength Tris-borate buffer, dried under a vacuum and exposed to an imaging plate for quantitation using an image analyzer.

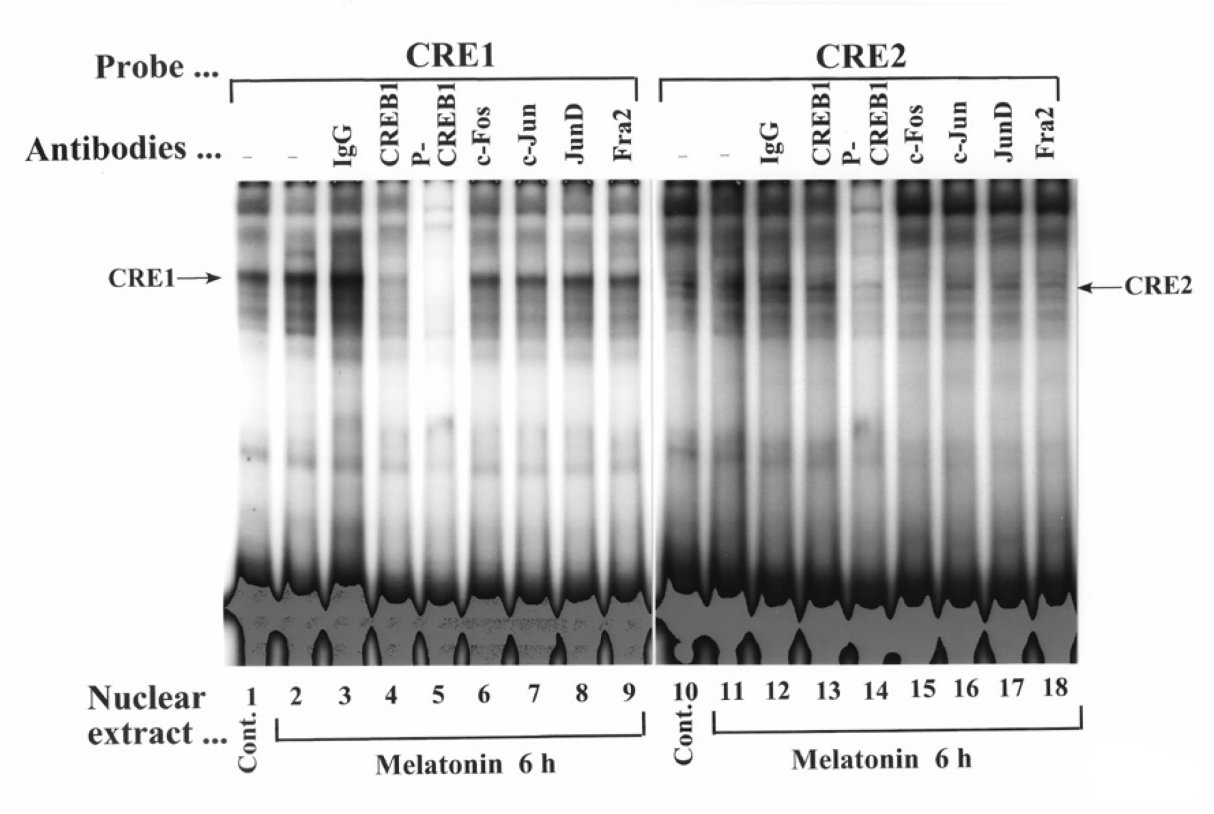

Fig. 13 Specific binding of nuclear proteins to CRE1 and CRE2.

Radiolabeled double-stranded CRE1 and CRE2 were incubated with nuclear protein extracts (3 μg) obtained from Saos2 cells stimulated without or with melatonin (100 nM) for 6 h. Supershift experiments were performed with 0.4 μg antibodies added separately to each gel shift reaction.

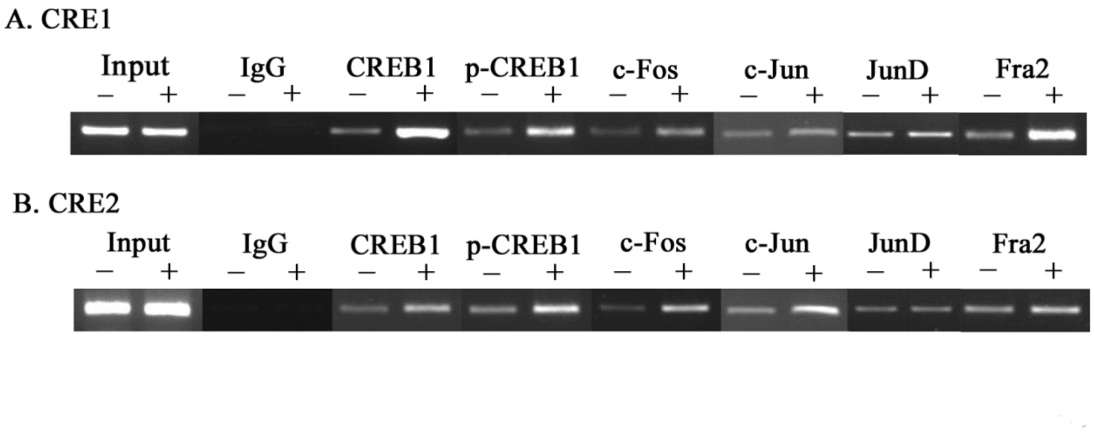

Fig. 14 ChIP analyses of CREB1, phospho-CREB1, c-Fos, c-Jun, JunD and Fra2 binding to CRE1 and CER2 sites in the human BSP promoter in Saos2 cells.

Saos2 cells (100 mm plates) were treated without (C), with melatonin (100 nM) for 6 h, before cells were cross-linked with formaldehyde for ChIP analysis. Three independent IP reactions were carried out with antibodies (CREB1, phospho-CREB1, c-Fos, c-Jun, JunD and Fra2) and control reactions with rabbit IgG. Ethidium bromide stained agarose gels of the PCR products obtained with ChIP DNAs using the human BSP promoter primers. Input DNA was also used as control in PCR analysis.