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# Melatonin regulates human bone sialoprotein gene transcription

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**Abstract:** Melatonin is produced by the pineal gland and regulates various physiological processes including osteoblast differentiation and bone formation. Bone sialoprotein (BSP) is a mineralized connective tissue-specific protein expressed in the early stage of cementum and bone mineralization. To elucidate the effects of melatonin on human BSP gene expression, we utilized human Saos2 osteoblast-like cells. Melatonin (100 nM) increased the level of BSP mRNA at 3 h, and the level became maximal at 12 and 24 h. We then investigated the melatonin-induced transcriptional activity of luciferase constructs (between -84LUC and -868LUC) including different lengths of the human BSP gene promoter transfected into Saos2 cells. The effects of melatonin abrogated in constructs included 2-bp mutations in the two cAMP response elements (CRE1 and CRE2). The effects of melatonin were suppressed by protein kinase A, tyrosine kinase, ERK1/2 and phosphatidylinositol 3-kinase inhibitors. Gel mobility shift assays showed that melatonin increased the binding of nuclear proteins to CRE1 and CRE2, and antibodies against CRE binding protein 1 (CREB1), phospho-CREB1, c-Fos, c-Jun, JunD and Fra2 disrupted CRE1 and CRE2 protein complex formation. These data indicate that melatonin induces BSP transcription via the CRE1 and CRE2 elements in the human BSP gene promoter. (*J Oral Sci* 56, 67-76, 2014)

Keywords: bone sialoprotein; gene expression; melatonin; osteoblasts; transcription factor.

## Introduction

Melatonin is a neurohormone produced by the pineal gland, which is located in the center of the brain but outside the blood-brain barrier under the control of the light/dark cycle and the suprachiasmatic nucleus. Melatonin production is suppressed by impingement of light on the retina and induced by darkness through the retinohypothalamic tract, suprachiasmatic nuclei and sympathetic nervous system (1-4). Several studies have suggested that melatonin may be synthesized locally in the bone marrow, gastrointestinal tract, testes and lymphocytes (5-7). Furthermore, melatonin regulates various physiological and pathophysiological processes such as control of body temperature, sexual development, inflammation, the immune response and cell proliferation (8-13). Melatonin also regulates bone remodeling, prevents osteoporosis, and induces dentine formation and osseointegration of dental implants (14-18).

Bone sialoprotein (BSP) is a glycosylated, phosphorylated and sulfated protein that can bind to hydroxyapatite through polyglutamic acid sequences, and mediates cell attachment via a RGD motif (19-23). BSP is also expressed in breast, prostate, lung and thyroid cancers and induces the formation of ectopic microcrystals in the tumor tissues (24,25). The BSP gene promoters of rat, mouse and human have been cloned and investigated (26-29). In the human BSP gene promoter, an inverted TATA box (nts -28~-23) (27) and an inverted CCAAT box (nts-54~-50) maintain basal transcription (29,30), and two cAMP response elements (CRE1; -79~-72, CRE2; -674~-667) are also present (31,32). Additionally, a fibroblast growth factor 2 (FGF2) response element (FRE; nts -96~-89) (33,34), three activating protein 1 (AP1) response

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elements (AP1(1); -148~-142, AP1(2); -483~-477 and AP1(3); -797~-791) (31,32), and a homeobox binding site (HOX; -200~-191) (35,36) have been characterized.

In the present study, to determine the mechanism by which melatonin regulates human BSP gene expression, we analyzed the effects of melatonin on the expression of BSP in Saos2 human osteoblast-like cells. We found that melatonin increased the expression of the human BSP gene via the CRE1 and CRE2 elements in the promoter.

## Materials and Methods

### Reagents

Alpha-minimum essential medium ( $\alpha$ -MEM), fetal calf serum (FCS), lipofectamine 2000, penicillin and streptomycin, and TrypLE Express were purchased from Invitrogen (Carlsbad, CA, USA). PGL3-basic luciferase plasmid, pSV- $\beta$ -galactosidase ( $\beta$ -Gal) control plasmid and U0126 (ERK1/2 inhibitor) were obtained from Promega (Madison, WI, USA). Melatonin, the protein kinase A (PKA) inhibitor KT5720, and the tyrosine kinase inhibitor herbimycin A (HA) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). The PKC inhibitor H7 was obtained from Seikagaku Corporation (Tokyo, Japan), and the phosphatidylinositol 3-kinase (PI3K) inhibitor LY249002 was purchased from Calbiochem (San Diego, CA, USA). The PrimeScript RT reagent kit and SYBR Premix Ex Taq™ II were obtained from Takara-bio (Tokyo, Japan), and the ChIP-IT Express Enzymatic Kit was purchased from Active Motif (Carlsbad, CA, USA). Anti-rabbit IgG conjugated with horseradish peroxidase (HRP) and ELC plus Western Blotting Detection Reagents were purchased from GE Healthcare UK Ltd. (Buckinghamshire, UK). All chemicals used were of analytical grade.

### Cell culture

Human osteosarcoma-derived Saos2 cells (37) were cultured at 37°C in 5% CO<sub>2</sub> and 95% air in  $\alpha$ -MEM medium containing 10% FCS. The cells were grown to confluence in 60-mm cell culture dishes in  $\alpha$ -MEM including 10% FCS, then cultured for 12 h in  $\alpha$ -MEM without FCS, and stimulated with melatonin (100 nM). Total RNA was purified from triplicate cultures at 3, 6, 12, and 24 h after stimulation and analyzed for genes that were stimulated by melatonin.

### Northern blotting

Aliquots (20  $\mu$ g) of total RNA were resolved in a 1.2% agarose gel and transferred to a Hybond-N+ membrane. Hybridizations were performed at 42°C with <sup>32</sup>P-labeled human BSP and a glyceraldehyde-3-phosphate dehydro-

genase (GAPDH) cDNA probe. After hybridization, the membranes were washed four times for 10 min each at 21°C in 300 mM sodium chloride and 30 mM trisodium citrate, pH 7.0, containing 0.1% SDS. The membranes were then washed two times for 20 min at 55°C in 15 mM sodium chloride, 1.5 mM trisodium citrate and 0.1% SDS, pH 7.0. The hybridized band, representing human BSP mRNA, was scanned using a Bio-imaging analyzer (Fuji BAS 2500, Tokyo, Japan).

### Western blotting

For Western blotting, cell lysates of Saos2 cells were separated in 10% SDS-PAGE and transferred to 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- $\alpha$  tubulin monoclonal antibody (sc-5286; Santa Cruz Biotechnology, Paso Robles, CA, USA). Anti-rabbit and mouse IgG conjugated with HRP were used as the secondary antibodies. Immunoreactivities were detected using ELC plus Western Blotting Detection Reagents.

### Real-time PCR

Total RNA (1  $\mu$ g) was used as a template for cDNA synthesis. cDNA was prepared using the PrimeScript RT reagent kit. Quantitative real-time PCR was performed using the following primer sets: BSP forward, 5'-CTG-GCACAGGGTATACAGGGTTAG-3'; BSP reverse, 5'-ACTGGTGCCGTTTATGCCTTG-3'; Runx2 forward, 5'-ATGTGTGTTTGTTCAGCAGCA-3'; Runx2 reverse, 5'-TCCCTAAAGTCACTCGGTATGTGTA-3'; Osterix forward, 5'-GCCATTCTGGGCTTGGGTATC-3'; Osterix reverse, 5'-GAAGCCGGAGTGCAGGTATCA-3'; GAPDH forward, 5'-GCACCGTCAAGGCTGAGAAC-3'; GAPDH reverse, 5'-ATGGTGGTGAAGACGCCAGT-3', using the SYBR Premix Ex Taq II in a TP800 thermal cycler dice real-time system (Takara-Bio, Tokyo, Japan). The amplification reactions were performed in a total volume of 25  $\mu$ L 2x SYBR Premix Ex Taq II (12.5  $\mu$ L), 10  $\mu$ M forward and reverse primers and 50 ng cDNA for BSP, Runx2 and Osterix and 10 ng cDNA for GAPDH. To reduce variability between replicates, PCR premixes containing all reagents except for cDNA were prepared and aliquoted into 0.2 mL PCR tubes. The conditions for thermal cycling were 10 s at 95°C, and 40 cycles of 5 s at 95°C and 30 s at 60°C. 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.

### Luciferase assays

Exponentially growing Saos2 cells were used for transient transfection assays. Twenty-four hours after plating, cells at 60-80% confluence were transfected using Lipofectamine 2000. The transfection mixture included 1 µg of a luciferase (LUC) plasmid and 2 µg β-Gal plasmid as a transfection control. β-Gal activity was determined separately to normalize the values. Human BSP gene promoter fragments of different lengths were cloned into the BglII site of the multi-cloning site of the pGL3basic luciferase plasmid. Two days after transfection, the cells were cultured in α-MEM lacking serum for 12 h, and then stimulated with 100 nM melatonin for 12 h prior to harvest. The luciferase activities were measured in accordance with the supplier's protocol using a luminescence reader (AcuuFlex Lumi 400; Aloka, Tokyo, Japan). The tyrosine kinase inhibitor herbimycin A (HA; 1 µM), the PKA inhibitor KT5702 (100 nM), the PKC inhibitor H7 (5 µM), the ERK1/2 inhibitor U0126 (5 µM), and the PI3K inhibitor LY249002 (10 µM) were used for protein kinase inhibition. Oligonucleotide-directed mutagenesis by PCR was utilized to introduce dinucleotide substitutions using a Quikchange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). All constructs were sequenced as described previously to verify the fidelity of the mutagenesis.

### Gel mobility shift analysis

Confluent Saos2 cells incubated for 3, 6, and 12 h with melatonin (100 nM) in α-MEM without serum were used to prepare the nuclear extracts. Double-stranded oligonucleotides encompassing the inverted CCAAT, CRE1, CRE2 and FRE sequences in the human BSP promoter were prepared. For gel mobility shift analysis, the double-stranded oligonucleotides were end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP and T4 polynucleotide kinase. Nuclear proteins (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). After incubation, the DNA-protein complexes were separated by electrophoresis in 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. The double-stranded oligonucleotide sequences were: CCAAT (nts, -64 to -41, 5'-CGTGACAGTGATTGGCTGTTGGAA-3'), CRE1 (nts, -89 to -63, 5'-ATCCACGTTCTGACATCACCTTGCTCG-3'), CRE2 (nts, -680 to -658, 5'-ATCAGCTGACCTCACATGCACGA-3') and FRE

(nts, -107 to -83, 5'-ATCCTTTTCTGGTGAGAATC-CACGA-3'). Forty times molar unlabeled CRE1, CRE2, CCAAT, mutation CRE1 (mCRE1; TGACAgA) and mutation CRE2 (mCRE2; TGACCgaA) oligonucleotides were used for competition gel shift assays. Super-shift analyses were performed using antibodies against CREB1 (p43; Rockland), phospho-CREB1 (Ser133; Upstate), c-Fos (sc-253), c-Jun (sc-44), JunD (sc-74), and Fra2 (sc-604) (Santa Cruz Biotechnology). The antibodies were added to each reaction mixture and incubated for 3-5 h at 4°C before electrophoresis under the same conditions as those described above.

### Chromatin immunoprecipitation (ChIP) assay

A Chip-IT Express Enzymatic Kit was used for the ChIP assay. In brief, Saos2 cells were grown in 100-mm dishes until confluence, cultured in α-MEM without serum for 12 h, and incubated with or without melatonin (100 nM) for 6 h. The cells were fixed with 1% formaldehyde for 10 min and then chromatin was prepared in accordance with the manufacturer's protocol. After being washed with PBS, cell pellets were homogenized in a dounce homogenizer and centrifuged to pellet the nuclei. The nuclear pellet was digested using an enzymatic shearing cocktail (200 U/mL) to shear the chromatin at 37°C for 5 min, and the reaction was stopped by addition of cold EDTA. The sheared chromatin (6.3 µg DNA) was used as the starting material (input) in each ChIP reaction with 2 µg of the appropriate antibody (those against CREB1, phospho-CREB1, c-Fos, c-Jun, JunD and Fra2, rabbit IgG being used as the control) and protein G magnetic beads overnight at 4°C. The tube was placed on a magnetic stand to pellet the beads, which were then washed. The chromatin was then eluted from the beads using elution buffer and reverse cross-link buffer, and then the samples were incubated with proteinase K at 37°C for 1 h. The purified DNA was used for PCR amplification (1 cycle at 94°C for 5 min, and then amplification was carried out for 30 cycles at 94°C; 30 s, 55°C; 30 s, 72°C; 30 s, with a final extension at 72°C for 10 min) of the CRE1 and CRE2 sites within the human BSP promoter using CRE1ChIP(1); 5'-CCTCTTGGCTCTAGAATCACGTTTC-3', CRE1ChIP(2); 5'-CCCTCTCACTCACTCATTCACTTGC-3', CRE2ChIP(3); 5'-CAATGGATCACTCCTGCTAGCTCTAG-3', CRE2ChIP(4); 5'-CCACTGAGAGGCAGATTTTATATTTTG-3' primers. The PCR products were fractionated 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 the ChIP reactions.

### Statistical analysis

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

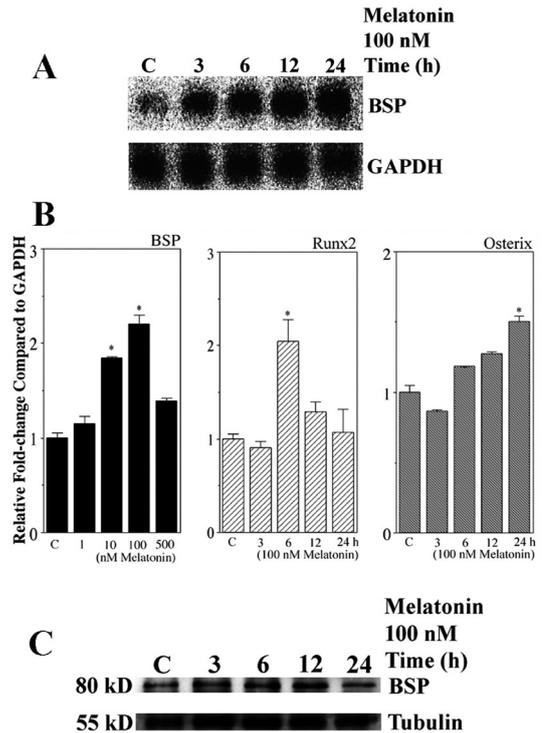
## Results

### Regulation of the mRNA and BSP protein levels of BSP, Runx2 and Osterix by melatonin

To investigate the effects of melatonin on BSP transcription, we used Saos2 cells to express BSP mRNA constitutively. Firstly, the dose-response effect of melatonin on the level of BSP mRNA was studied by treating the cells with different concentrations of melatonin for 12 h. The level of melatonin-induced BSP mRNA became maximal at 100 nM (Fig. 1B). When 100 nM melatonin was used to determine the time course of BSP mRNA expression, induction of BSP mRNA was evident at 3 h and became maximal at 12 h and 24 h (Fig. 1A). The results of real-time PCR indicated that melatonin (100 nM) increased the levels of mRNA for Runx2 and Osterix at 6 h and 24 h, respectively (Fig. 1B). Melatonin (100 nM) induced BSP protein at 3 h and this became maximal at 6 and 12 h. Tubulin was used as a loading control (Fig. 1C).

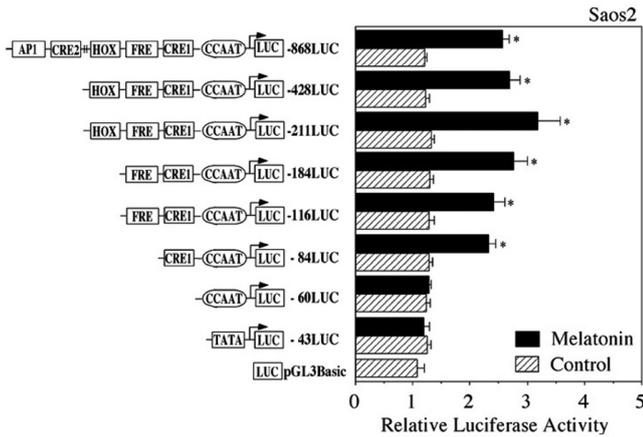
### Luciferase assays for human BSP promoter constructs

To elucidate the site of melatonin-regulated transcription in the 5'-flanking region of the BSP gene, various regions of the human BSP promoter were ligated to luciferase reporter plasmids to create the constructs -43LUC (-43~+60), -60LUC (-60~+60), -84LUC (-84~+60), -116LUC (-116~+60), -184LUC (-184~+60), -211LUC (-211~+60), -428LUC (-428~+60) and -868LUC (-868~+60). These were then transiently transfected into Saos2 cells, and the resulting transcriptional activities were determined in the absence and presence of melatonin (100 nM). Melatonin increased the luciferase activities of all the constructs from -84LUC to -868LUC (Fig. 2). The sequence -60LUC to -868LUC of the human BSP gene promoter contains an inverted CCAAT box (ATTGG; between -54~-50), and also CRE1 (-79~-72), FRE (-96~-89), HOX (-200~-191) and CRE2 (-674~-667) elements (Fig. 3). Next, we introduced 2-bp mutations within the -184LUC and -868LUC constructs in the CCAAT, CRE1, FRE and CRE2 elements targeted by melatonin. When we stimulated the mutant CRE1 (-184mCRE1 and -868mCRE1) and CRE2 (-868mCRE2) constructs with melatonin (100 nM), the induced -184mCRE1,

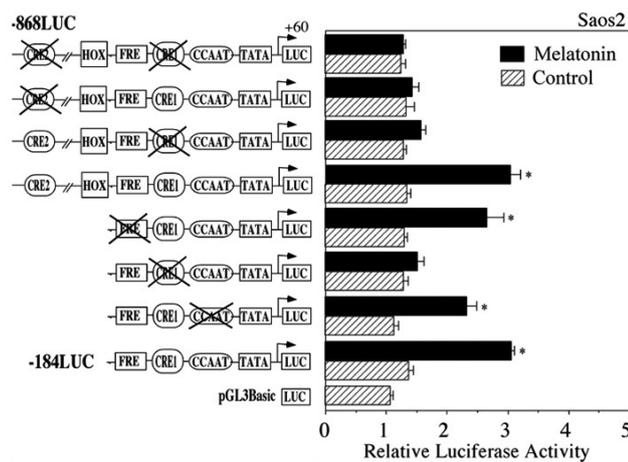


**Fig. 1** Effects of melatonin on levels of BSP, Runx2 and Osterix mRNA and BSP protein in Saos2 cells. Saos2 cells were treated with or without 100 nM melatonin for 3, 6, 12, and 24 h. Total RNA was extracted and the expression of BSP mRNA was measured by Northern blotting (A). Levels of Runx2 and Osterix mRNA were measured by real-time PCR (B). Dose-response effects of melatonin were also measured by real-time PCR (B). The amounts of mRNA for BSP, Runx2 and Osterix relative to those of GAPDH were calculated. Quantitative analyses of triplicate data sets are shown with standard errors. Significant differences from control: \* $P < 0.01$ . Levels of BSP and tubulin protein in Saos2 cells were determined in the absence (Control) or presence of melatonin (100 nM) for 3, 6, 12, and 24 h. Cell lysates were then prepared and the expression of BSP and tubulin was analyzed by Western blotting using relevant antibodies. A representative result is shown (C).

-868mCRE1 and -868mCRE2 activities were partially reduced. On the other hand, the luciferase activities of the mutant CCAAT and FRE (-184mCCAAT and -184mFRE) constructs were increased by melatonin to almost the same level as that of wild-type -184LUC (Fig. 4). When mutations were introduced on both sides of CRE1 and CRE2 in -868LUC (-868mCRE1/mCRE2), the melatonin-induced luciferase activity was almost completely abrogated (Fig. 4). These results suggested that CRE1 and CRE2 act as functional response elements through which melatonin regulates BSP gene transcription. Melatonin-induced luciferase activities (-184LUC and -868LUC) were inhibited by KT5720, HA, U0126 and LY294002, but not inhibited by H7, suggesting involvement of PKA, tyrosine phosphorylation, ERK1/2



**Fig. 2** Melatonin upregulates human BSP promoter activity in Saos2 cells. Transient transfection of Saos2 cells in the presence or absence of melatonin (100 nM) for 12 h was performed to determine the transcriptional activities of chimeric constructs that included various regions of the human BSP promoter ligated to a luciferase reporter gene. The transcriptional activities determined 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: \*  $P < 0.01$ .

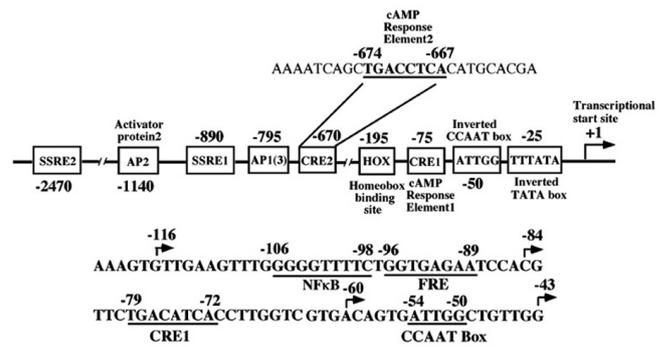


**Fig. 4** Site mutation analysis of luciferase activities. Dinucleotide substitutions were made within the context of the homologous -184 to +60 (-184LUC) and -868 to +60 (-868LUC) BSP promoter fragment. -184mCRE1 and -868mCRE1 (TGACAgA), -868mCRE2 (TGACCgA), -184mCCAAT (ATTtt), -184mFRE (GGcaAGAA) 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 data for transcriptional activity obtained from four separate transfections with constructs were combined, and the values are expressed with standard errors. Significant differences from control: \*  $P < 0.01$ .

and PI3K in the signaling pathways (Fig. 5).

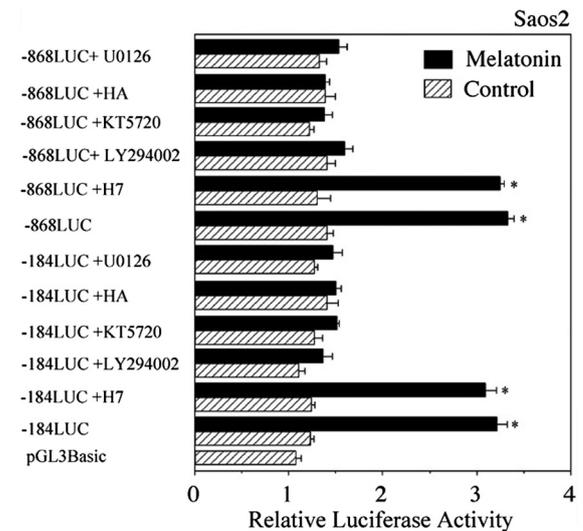
### Gel mobility shift analysis

To identify the nuclear proteins that bind to CCAAT,



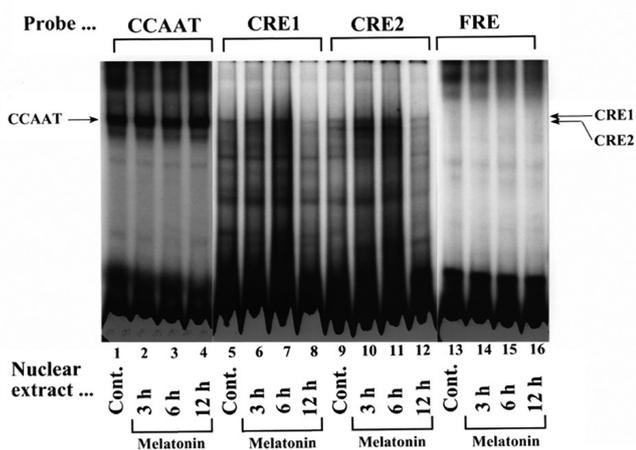
**Fig. 3** Regulatory elements in the proximal human BSP promoter. Upper panel: The positions of the inverted TATA and CCAAT boxes, CRE1, HOX, CRE2, AP1(3), shear stress response element 1 (SSRE1), AP2 and SSRE2 are shown in the proximal promoter region of the human BSP gene. The numbering of nucleotides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

Lower panel: Nucleotide sequences of the human BSP promoter encompassing an inverted CCAAT box, CRE1, FRE and NFkB are shown from -121 to -43.



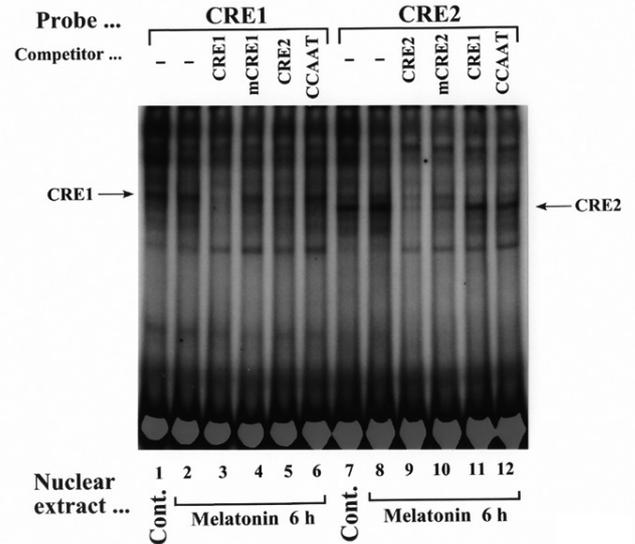
**Fig. 5** Effects of kinase inhibitors on transcriptional activation by melatonin. Transient transfection analysis of -184LUC and -868LUC in the presence or absence of melatonin (100 nM) in Saos2 cells is shown together with the effects of the PKC inhibitor (H7, 5  $\mu$ M), PI3K inhibitor (LY294002,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

CRE1, FRE and CRE2, double-stranded oligonucleotides were labeled with  $^{32}$ P and incubated with nuclear proteins (3  $\mu$ g) extracted from Saos2 cells, which were then incubated with or without 100 nM melatonin for 3, 6, and 12 h.



**Fig. 6** Melatonin upregulates nuclear proteins that recognize CRE1 and CRE2. Radiolabeled double-stranded CCAAT (-64 CGTGACAGTGATTGGCTGTTGGAA -41), CRE1 (-89 ATCCACGTTCTGACATCACCTTGGTCG -63), CRE2 (-680 ATCAGCTGACCTCACATGCACGA -658), and FRE (-111 GTTTGGGGGTTTTCTGGTGAGAATCCACGTTCTG -78) oligonucleotides were incubated with nuclear protein extracts (3  $\mu$ g) obtained from Saos2 cells that were unstimulated (lanes 1, 5, 9, and 13) or stimulated with melatonin for 3 h (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.

When we used the CCAAT element as a probe, the DNA-protein (NF-Y) complex (30) did not change after stimulation with melatonin (Fig. 6, lanes 1-4). Shifts of CRE1 and CRE2-protein complexes with nuclear extracts from control Saos2 cells were found (Fig. 6, lanes 5 and 9). CRE1- and CRE2-protein complexes were increased after stimulation with melatonin (100 nM) at 3 h, and became maximal at 6 h (Fig. 6, lanes 6, 7, 10, and 11). Melatonin was unable to induce FRE-protein complex formation (Fig. 6, lanes 13-16). Specific bindings of the CRE1- and CRE2-protein complexes was confirmed by competition gel shift assay with a 40-fold molar excess of non-isotope-labeled CRE1 and CRE2 to reduce the formation of CRE1- and CRE2-protein complexes (Fig. 7, lanes 3 and 9). On the other hand, mCRE1, mCRE2 and inverted CCAAT did not compete with CRE1- and CRE2-protein complex formation (Fig. 7, lanes 4, 6, 10, and 12). A 40-fold molar excess of non-isotope-labeled CRE2 and CRE1 partially competed with CRE1- and CRE2-protein complex formation (Fig. 7, lanes 5 and 11). To identify which transcription factors in the nuclear proteins from Saos2 cells were able to bind to CRE1 and CRE2, we used several antibodies. CRE1- and CRE2-protein complexes disappeared almost completely when



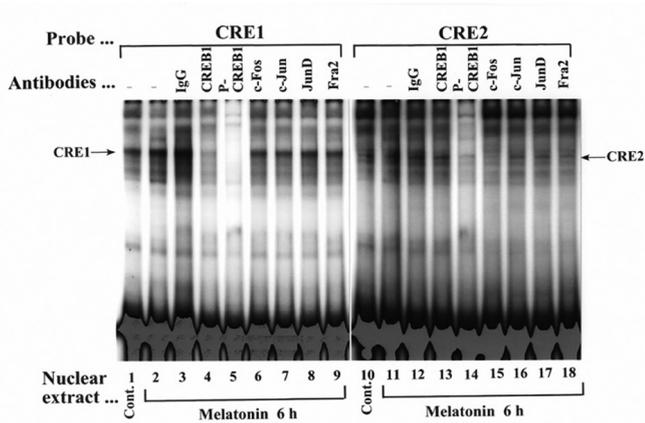
**Fig. 7** Specific binding of nuclear proteins to CRE1 and CRE2. Competition reactions were performed using a 40-fold molar excess of unlabeled CRE1 (lanes 3 and 11), mCRE1 (lane 4), CRE2 (lanes 5 and 9), mCRE2 (lane 10) and CCAAT (lanes 6 and 12) oligonucleotides. DNA-protein complexes were separated 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.

phospho-CREB1 antibodies were added (Fig. 8, lanes 5 and 14). Antibodies against CREB1, c-Fos, c-Jun, JunD and Fra2 partially inhibited the formation of CRE1- and CRE2-protein complexes (Fig. 8, lanes 4, 6-9, 13, and 15-18).

#### ChIP assays

Next we examined several transcription factors that were shown by gel mobility shift assays to bind to CRE1 and CRE2 and thus interact directly with the human BSP gene promoter.

We performed ChIP assays to determine the *in vivo* association of these transcription factors with CRE1 and CRE2 in Saos2 cells. Confluent Saos2 cells were treated with melatonin (100 nM) for 6 h to stimulate BSP transcription, and then cross-linked with formaldehyde. After enzymatic digestion, soluble chromatin was immunoprecipitated with either control IgG or antibodies against transcription factors. The precipitated DNA-protein complexes were purified, and the PCR bands were amplified using CRE1 and CRE2 primers. This revealed that CREB1, phospho-CREB, c-Fos, c-Jun, JunD and Fra2 interacted with a chromatin fragment containing CRE1 and CRE2, which was increased in Saos2 cells after

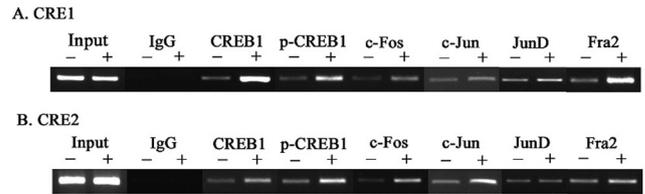


**Fig. 8** Specific binding of nuclear proteins to CRE1 and CRE2. Radiolabeled double-stranded CRE1 and CRE2 were incubated with nuclear protein extracts (3  $\mu$ g) obtained from Saos2 cells that were untreated or treated with melatonin (100 nM) for 6 h. Supershift experiments were performed with 0.4  $\mu$ g antibodies added separately to each gel shift reaction.

stimulation with melatonin (Fig. 9).

## Discussion

Melatonin is synthesized and secreted by the pineal gland and other organs. It has a variety of physiological actions such as control of circadian rhythm, sexual development, inflammation and hormone secretion (3,8,10,13,15). Melatonin promotes osteoblast differentiation and bone formation (38,39), and can protect the oral cavity from tissue damage (40,41). If the level of melatonin secreted into saliva increases, it may help to maintain periodontal tissue in a healthy condition (42). In the present study, we have shown that melatonin increased BSP transcription by targeting CRE1 and CRE2 in the human BSP gene promoter. BSP is a non-collagenous protein that is abundant in bone and cementum. It is produced by osteoblasts and cementoblasts at an early differentiation stage, and can induce nucleation of hydroxyapatite crystals (20,21). Here we showed that melatonin induced BSP transcription mediated through the CRE1 and CRE2 elements via the PKA tyrosine kinase, ERK1/2 and PI3K pathways in Saos2 human osteoblast-like cells. Melatonin increased the levels of BSP, Runx2 and Osterix mRNA and BSP protein (Fig. 1). Runx2 and Osterix are transcription factors crucial for osteoblast differentiation; Runx2- and Osterix-knockout mice have no bone due to cessation of osteoblast differentiation (43,44). Melatonin increased the levels of BSP mRNA at 3 h, and the level gradually increased thereafter until 12 and 24 h (Fig. 1A). On the other hand, melatonin increased the levels of mRNA for Runx2 and Osterix at 6 h and 24 h, respectively (Fig. 1B). These results suggest the importance of a transcrip-



**Fig. 9** ChIP analyses of CREB1, phospho-CREB1, c-Fos, c-Jun, JunD and Fra2 binding to the CRE1 and CRE2 sites in the human BSP promoter in Saos2 cells. Saos2 cells (100 mm plates) were untreated (C) or treated with melatonin (100 nM) for 6 h, then cross-linked with formaldehyde for ChIP analysis. Three independent IP reactions were carried out using relevant antibodies (CREB1, phospho-CREB1, c-Fos, c-Jun, JunD, and Fra2), and rabbit IgG was used as a control. Agarose gels of the PCR products obtained with ChIP DNAs using the human BSP promoter primers were stained with ethidium bromide. Input DNA was also used as a control in PCR analysis.

tion factor other than Runx2 and Osterix for BSP gene induction. The expression of BSP protein induced by melatonin was lower at 24 h than at 12 h. The half-life of BSP mRNA is about 16 h (45), but no data for the half-life of BSP protein are available. Further studies of this issue are needed.

Luciferase assays using Saos2 cells showed that melatonin induced BSP promoter activity between -84LUC and -868LUC at 12 h (Fig. 2). Furthermore, the results obtained using single- and double-mutation constructs in the CRE1 and CRE2 elements suggested that these elements are essential for induction of BSP transcription by melatonin (Fig. 4). However, the transcriptional activities induced by melatonin using mutant CCAAT (-184mCCAAT) and FRE (-184mFRE) constructs were almost the same as that induced using the wild-type construct (-184LUC), suggesting that the inverted CCAAT and FRE elements were not targeted by melatonin. The involvement of CRE1 and CRE2 was further confirmed by gel mobility shift assays in which nuclear proteins from Saos2 cells formed complexes with two CRE elements that were reactive with melatonin at 3 and 6 h (Fig. 6). Specific binding of the CRE1- and CRE2-protein complexes was confirmed by competition gel shift assays, whereas the CRE1- and CRE2-binding proteins were unable to completely compete with a 40-fold molar excess of CRE2 and CRE1, suggesting that the constituents of the CRE1- and CRE2-binding proteins are not same (Fig. 7). Supershift assays using antibodies against several transcription factors indicated that CRE1 and CRE2 interacted with CREB1, phospho-CREB1, c-Fos, c-Jun, JunD and Fra2 (Fig. 8), and that

the complexes formed by these transcription factors with chromatin fragments containing CRE1 and CRE2 were increased by melatonin stimulation (Fig. 9). CREB1 and activating transcription factor (ATF)/CREB family members are able to 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 factor family (46). The AP1 transcription factor family comprises members of the Jun family (c-Jun, JunB, and JunD) that can form homo- or heterodimers among themselves and bind to the AP1 consensus sequence (TGACTCA). Jun proteins also dimerize with Fos family members (c-Fos, FosB, Fra1 and Fra2). Most AP1 members are present at low levels in cells, but are rapidly induced and activated by specific stimuli (47). JunD and Fra2 can bind to CRE1 in the human BSP proximal promoter as heterodimers with CREB1 and regulate BSP expression in both breast cancer cells and osteoblast-like cells (36,48). Inhibition of AP1 activity may block the proliferation, migration, invasion and metastasis of tumor cells (49).

We have reported that PKA, tyrosine kinase, ERK1/2 and PI3K regulate BSP gene transcription (31,32,36,50). Activation of PKA may induce CREB1 phosphorylation, and phospho-CREB1 can bind firmly to CRE, thereby inducing gene transcription (51). HA, U0126 and LY294002 have been shown to inhibit the increased binding activities of transcription factors to the FRE and HOX elements in the rat BSP gene promoter (35). In our previous study, we showed that FGF2 stimulated BSP gene transcription via the tyrosine kinase and ERK1/2 pathways (33), PTH activated cAMP and phospholipase C through the PKA and tyrosine kinase pathways (31), and PGE2 increased BSP transcription via PKA, tyrosine kinase and ERK1/2, which target nuclear proteins binding to CRE and FRE in the rat BSP gene promoter (34). PI3K/Akt is one of the critical pathways for differentiation of skeletal component cells, such as chondrocytes, osteoblasts, myoblasts and adipocytes (52). Furthermore, Runx2-induced osteoblast differentiation is inhibited by PI3K/Akt (53), suggesting that Runx2 and PI3K/Akt could be important signaling molecules for BSP transcription.

In conclusion, we have demonstrated that CRE1 and CRE2 in the human BSP gene promoter mediate the transcription of BSP induced by melatonin, and that melatonin induces binding of nuclear proteins to CRE1 and CRE2, with the possible involvement of CREB1, phospho-CREB1, c-Fos, c-Jun, JunD and Fra2 as transcription factors. We have also demonstrated that melatonin increases human BSP gene transcription

through the PKA, tyrosine kinase, ERK1/2 and PI3K signaling pathways. BSP is expressed in osteoblasts at an early stage of differentiation, and melatonin plays an important role in bone metabolism. It is assumed that the CRE1 and CRE2 elements may contribute to tissue-specific expression of the BSP gene during the formation of bone and cementum.

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