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Aggregatibacter actinomycetemcomitans Lipopolysaccharide Regulates Bone Sialoprotein Gene Transcription

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ABSTRACT

Aggregatibacter actinomycetemcomitans (*A. actinomycetemcomitans*) is believed to be associated with aggressive periodontitis characterized by a rapid bone loss. *A. actinomycetemcomitans* lipopolysaccharide (LPS) has a similar structure to *Escherichia coli* LPS, and they are Toll-like receptor 4 agonists. Bone sialoprotein (BSP) is an early marker of osteoblast differentiation. To investigate the effects of *A. actinomycetemcomitans* LPS on bone formation, we targeted BSP as a marker for osteogenic differentiation and bone formation. BSP mRNA levels were decreased by 0.1 μg/ml and increased by 0.01 μg/ml *A. actinomycetemcomitans* LPS at 6 h in osteoblast-like ROS17/2.8 cells. In transient transfection analyses, 0.1 μg/ml decreased and 0.01 μg/ml *A. actinomycetemcomitans* LPS increased luciferase activities of the construct (−116 to +60). Introduction of 2 bp mutations to the constructs showed that the effects of *A. actinomycetemcomitans* LPS were mediated by a cAMP response element (CRE), a FGF2 response element (FRE), and a homeodomain protein-binding site (HOX). Tyrosine kinase, ERK1/2, and PI3-kinase/Akt participated in the effects of both 0.1 and 0.01 μg/ml *A. actinomycetemcomitans* LPS. The results of gel shift showed that 0.1 μg/ml decreased while 0.01 μg/ml *A. actinomycetemcomitans* LPS increased CRE-, FRE-, and HOX-binding protein complexes formation at 6 h, and revealed that 0.01 μg/ml *A. actinomycetemcomitans* LPS induced BSP transcription through CREB1, JunD, Fra2, c-Fos, Runx2, Dlx5, and Smad1 targeting those response elements. These studies therefore indicated that 0.1 μg/ml suppressed and 0.01 μg/ml *A. actinomycetemcomitans* LPS increased BSP gene transcription mediated through CRE, FRE, and HOX elements in the rat BSP gene promoter. J. Cell. Biochem. 113: 2822–2834, 2012.

KEY WORDS: *AGGREGATIBACTER ACTINOMYCETEMCOMITANS*; BONE SIALOPROTEIN; LIPOPOLYSACCHARIDE; OSTEOBLASTS; TRANSCRIPTION

Abbreviations: AP1, activator protein 1; α-MEM, α-minimum essential medium; bp, base pair(s); BSP, bone sialoprotein; CRE, cAMP response element; Dlx5, distalless 5; FCS, fetal calf serum; FGF2, fibroblast growth factor 2; FRE, FGF response element; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; IL-1β, interleukin-1β, IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; LUC, luciferase; nts, nucleotides; PKA, cAMP dependent protein kinase; PKC, protein kinase C; Runx2, runt homeodomain protein 2.

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Periodontitis is an oral infectious disease that may result in tooth loss. It is caused by Gram-negative anaerobic bacteria including *Porphyromonas gingivalis* (*P. gingivalis*), which is associated with chromic periodontitis, and *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*), which is associated with aggressive periodontitis characterized by a rapid alveolar bone loss [Listgarten et al., 1995; Genco et al., 1998; Rogers et al., 2007]. *A. actinomycetemcomitans* produces a variety of periodontal-tissue-disintegrating products, including collagenase, fibroblast inhibitory factor, bone-resorption-inducing toxin, leukotoxin, and lipopolysaccharide (LPS) [Kiji et al., 2007]. LPS as a major component of the outer membrane of Gram-negative bacteria is considered to be a major factor in the pathogenesis of periodontitis. It acts as a microbe-associated molecular pattern recognized through pattern-recognition receptors on resident immune and non-immune cells within the periodontium [Akira and Hemmi, 2003]. This immune response involves recruitment of inflammatory cells, generation of prostanoids and cytokines, elaboration of lytic enzymes, and osteoclast activation. Within periodontal tissues, activated monocytes, macrophages, and fibroblasts produce proinflammatory cytokines, such as interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α). These cytokines stimulate matrix metalloproteinases which destroy tissues through degradation of extracellular matrix. Moreover, IL-1β, IL-6, and TNF-α induce bone resorption [Genco et al., 1998; Kadono et al., 1999]. Although the general structure of LPS is shared by all Gram-negative bacteria, the LPS molecules obtained from different bacteria have structural and functional differences [Netea et al., 2002]. The structure of *A. actinomycetemcomitans* LPS is known to resemble that of *Escherichia coli* (*E. coli*) LPS, while the structure of *P. gingivalis* LPS is different from that of *E. coli* [Mayrand and Holt, 1988; Masoud et al., 1991]. In all Gram-negative bacteria, the shape of the lipid A component determines the bioactivity of LPS [Netea et al., 2002], and a receptor that recognize the conserved lipid A of LPS would thus be able to detect the presence of any Gram-negative bacterium [Medzhitov and Janeway, 1997]. Lipid A with a conical shape (e.g., from *E. coli*) induces cytokine production through Toll-like receptor (TLR) 4, and *E. coli* LPS and *A. actinomycetemcomitans* LPS are known to be TLR4 agonists [Lee et al., 2006]. Whereas more cylindrical lipid A (e.g., from *P. gingivalis*) induces the expression of a different set of cytokines through TLR2 [Netea et al., 2002], and *P. gingivalis* LPS has been found to have an agonistic effect on TLR2 [Hirschfeld et al., 2001] and antagonist for TLR4 [Yoshimura et al., 2002]. LPS is known to induce not only inflammatory responses but also bone resorption by enhancing osteoclastogenesis via osteoblast-mediated activities [Jiang et al., 2002; Nemoto et al., 2006]; however, the effects of *A. actinomycetemcomitans* LPS on bone metabolism are not well understood.

Bone sialoprotein (BSP) is a highly sulfated, phosphorylated, and glycosylated protein that is characterized by its ability to bind to hydroxyapatite through polyglutamic acid sequences and to mediate cell attachment through an Arg-Gly-Asp (RGD) sequence. The expression of BSP is essentially restricted in mineralized connective tissues, and BSP mRNA is produced at high levels at the onset of bone, dentin, and cementum formation [Ganss et al., 1999; Ogata, 2008]. BSP deficiency impairs bone growth and mineralization, concomitant with markedly reduced bone formation, and impairs osteoclastogenesis and mineral resorption [Malaval et al., 2008; Boudiffa et al., 2010]. The temporo-spatial deposition of BSP to nucleate hydroxyapatite crystal formation indicates a potential role for BSP in the initial mineralization of bone [Chen et al., 1992; Hunter and Goldberg, 1993]. BSP is also expressed in osteotropic cancers such as breast, lung, thyroid, and prostate cancers. Thus, regulation of BSP gene expression is important in the differentiation of osteoblasts, bone matrix mineralization, and tumor metastasis [Ogata, 2008; Waltregny et al., 2000]. Human, mouse, and rat BSP genes have been cloned and partially characterized. These BSP promoters include a highly conserved region that extends upstream from the transcription start site to nts -370 [Li and Sodek, 1993; Kim et al., 1994; Benson et al., 1999]. This region includes a functional inverted TATA box [Li et al., 1995] overlapping with the vitamin D response element [Kim et al., 1996], an inverted CCAAT box which is required for basal promoter transcription activity [Kim and Sodek, 1999; Shimizu and Ogata, 2002]. In addition, a cAMP response element (CRE) [Samoto et al., 2003; Araki et al., 2009; Mezawa et al., 2009; Li et al., 2010; Yang et al., 2010], a fibroblast growth factor (FGF) 2 response element (FRE) [Shimizu-Sasaki et al., 2001; Samoto et al., 2003; Nakayama et al., 2006; Shimizu et al., 2006; Li et al., 2010; Wang et al., 2010; Yang et al., 2010], a pituitary-specific transcription factor-1 (Pit-1) motif [Ogata et al., 2000], and a homeodomain protein-binding site (HOX) [Benson et al., 2000; Nakayama et al., 2006] have been characterized. Further upstream, a transforming growth factor-β (TGF-β) activation element (TAE) [Ogata et al., 1997] and a glucocorticoid response element (GRE) overlapping an AP1 site [Ogata et al., 1995; Yamauchi et al., 1996; Takai et al., 2008] have also been identified.

We previously reported that *E. coli* LPS (1 μg/ml) suppressed BSP gene expression, and that a high concentration of *P. gingivalis* LPS (0.1 μg/ml) decreased and a low concentration of *P. gingivalis* LPS (0.01 μg/ml) increased BSP transcription mediated through CRE and FRE in the proximal promoter of the rat BSP gene [Kato et al., 2006; Li et al., 2010]. To determine the molecular mechanism of *A. actinomycetemcomitans* LPS regulation of BSP gene transcription, we analyzed the effects of *A. actinomycetemcomitans* LPS on the expression of BSP in osteoblast-like ROS17/2.8 cells. These studies have revealed that a high concentration of *A. actinomycetemcomitans* LPS decreased and a low concentration of *A. actinomycetemcomitans* LPS increased BSP transcription. In addition, *A. actinomycetemcomitans* LPS possessed properties differing from *P. gingivalis* LPS on BSP gene transcription mediated through different signaling pathways and targeting different response elements in the rat BSP gene promoter.

METHODS

MATERIALS

Alpha-minimum essential medium (α-MEM), fetal calf serum (FCS), penicillin, streptomycin, and lipofectamine were obtained from Invitrogen (Carlsbad, CA). PGL3-basic vector, pSV-β-galactosidase (β-Gal) control vector and ERK1/2 inhibitor U0126 were purchased from Promega Co., (Madison, WI). Phosphoinositide 3-kinase (PI3K) inhibitor LY294002 was purchased from Calbiochem (San Diego,

CA). LPS (L2630, *E. coli* 0111: B4), *N*-acetylcysteine (NAC) was purchased from Sigma-Aldrich Japan (Tokyo, Japan). The protein kinase C (PKC) inhibitor H7 and PKA inhibitor H89 were purchased from Seikagaku Corporation (Tokyo, Japan), and the tyrosine kinase inhibitor, herbimycin A (HA) and guanidium thiocyanate were purchased from Wako Pure Chemical Industries (Tokyo, Japan). EXScript RT reagent Kit and SYBR Premix Ex Taq were purchased from TaKaRa (Tokyo, Japan). Anti-rabbit IgG, anti-mouse IgG conjugated with HRP and ECL Plus Western Blotting Detection Reagent were purchased from GE Healthcare Japan (Tokyo, Japan).

PURIFICATION OF LPS

A. actinomycetemcomitans Y4 and *P. gingivalis* 381 were cultured in Todd–Hewitt broth supplemented with 1% yeast extract and GAM broth supplemented with vitamin K3 (5 μg/ml) and hemin (5 μg/ml) at 37°C under anaerobic conditions (10% H_2, 10% CO_2, 80% N_2) for 3 days. The cells were then sedimented by centrifugation, washed with pyrogen-free water three times and freeze-dried. Crude LPS was extracted according to a slight modification of the hot phenol–water procedure [Westphal and Jann, 1965]. Briefly, lyophilized organisms were suspended in 110 ml pyrogen-free water and 88 ml phenol. The mixture was stirred vigorously at 68°C for 20 min, cooled on ice, and then separated by centrifugation at 7,000 g for 20 min. The aqueous phase was removed and insoluble precipitates were re-extracted with 110 ml water twice. The combined aqueous phase was dialyzed against distilled water at 4°C, neutralized with NaOH, and lyophilized. Crude LPS was ultracentrifuged and digested with nuclease P1 (Yamasa Shoyu, Choshi, Japan) and pronase (Boehringer Mannheim GmbH, Mannheim, Germany) [Koga et al., 1985]. Briefly, crude LPS was suspended in 100 ml pyrogen-free water and ultracentrifuged at 100,000 g for 3 h. The pellet was suspended in 10 ml of 10 mM Tris buffer (pH 7.4) containing 0.1 mM $ZnCl_2$ and 400 mg nuclease P1. This suspension was incubated at 37°C for 16 h and dialyzed against distilled water at 4°C. The dialyzate was ultracentrifuged twice, and then the pellet was washed with pyrogen-free water and lyophilized. Freeze-dried LPS was suspended in 10 ml of 0.1M borate buffer (pH 7.4) containing 2 mM $CaCl_2$ and 1 mg pronase. The mixture was incubated at 37°C for 24 h, and heated at 100°C for 5 min, followed by centrifugation at 5,000 g for 10 min. The supernatant was dialyzed against distilled water, neutralized with NaOH, and lyophilized.

CELL CULTURE

Rat osteosarcoma-derived osteoblast-like ROS 17/2.8 cells and rat stromal bone marrow cells (RBMC) [Nakayama et al., 2006; Jheon et al., 2009] were used in this study. Cells were cultured at 37°C in 5% CO_2 95% air in α-MEM containing 10% FCS. Cells were grown to confluence in 60 mm tissue culture dishes, then changed to α-MEM without serum for 12 h, and incubated with or without *A. actinomycetemcomitans* LPS for a dose-response or for time periods extending over 3–24 h. Total RNA was isolated from triplicate cultures and analyzed for the expressions of BSP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA by northern blot or real-time PCR as described below.

REAL-TIME PCR

Total RNA (1 μg) was used as a template for cDNA performed with an ExScript RT reagent kit (TaKaRa). Quantitative real-time PCR was performed using the following primer sets: BSP forward, 5′-AGACCACAGCTGACGCTGGA-3′; BSP reverse, 5′-CCGTTGAC-GACCTGCTCATT-3′; GAPDH Forward, 5′- GACAACTTTGG-CATCGTGGA-3′; GAPDH Reverse, 5′-ATGCAGGGATGATGTTCT-GG-3′; using SYBR Premix Ex Taq in a TP800 thermal cycler dice real time system (TaKaRa). The amplification reactions were performed in 25 μl of final reaction mixture containing: 2× SYBR Premix EX Taq (12.5 μl); 50 μM (0.1 μl) forward and reverse primers (final concentration was 0.2 μM); 25 ng (2.5 μl) cDNA for BSP and 10 ng (1.0 μl) cDNA for GAPDH. To reduce variability between replicates, PCR premixes which contained all reagents except for cDNA, were prepared and aliquoted into 0.2 ml Hi-8-tubes (TaKaRa). The thermal cycling condition was 10 s at 95°C and 40 cycles of 5 s, 95°C and 30 s, 60°C. Post-PCR melting curves confirmed the specificity of single-target amplification. The initial amount of RNA was quantified using a standard curve, and fold expressions of BSP relative to GAPDH were determined in quadruplicate.

NORTHERN HYBRIDIZATION

Total RNA from ROS17/2.8 cells treated with or without *A. actinomycetemcomitans* LPS (0.1 μg/ml and 0.01 μg/ml, 3–12 h) was extracted with guanidium thiocyanate and, following purification, 25 μg aliquots of RNA were fractionated on a 1.2% agarose gel and transferred onto a Hybond N+ membrane. Hybridizations were performed at 42°C with ^{32}P-labeled rat BSP and GAPDH cDNA probes. Following hybridization, membranes were washed four times for 5 min each at 21°C in 300 mM sodium chloride, 30 mM trisodium citrate pH 7.0 containing 0.1% SDS. This was followed by two 20 min washes at 55°C in 15 mM sodium chloride, 1.5 mM trisodium citrate pH 7.0, 0.1% SDS. The hybridized bands, representing rat BSP mRNA (two polyadenylated forms: 1.6 and 2.0 kilobases) and GAPDH mRNA were scanned in a Bio-imaging analyzer (BAS 2500; Fuji, Tokyo, Japan).

WESTERN BLOT

For Western blot analyses, cell lysates from ROS17/2.8 cells were separated on 10% SDS–PAGE and transferred onto a Hybond-P membrane. The membrane was then incubated for 3 h with anti-human BSP polyclonal antibody (LF-100 provided by Dr. Larry W. Fisher) and anti-α-tubulin monoclonal antibody (sc-5286) (Santa Cruz Biotechnology, Inc.). Anti-rabbit IgG or anti-mouse IgG conjugated with HRP was used as the secondary antibodies. Immunoreactivities were detected by ECL Plus Western Blotting Detection Reagents.

TRANSIENT TRANSFECTION ASSAYS

Exponentially growing ROS 17/2.8 cells and RBMC were used for transfection assays. Twenty-four hours after plating, cells at 50–70% confluence were transfected using a lipofectamine reagent. The transfection mixture included 0.5 μg luciferase (LUC) construct (pLUC1, −18∼+60; pLUC2, −43∼+60; pLUC3, −116∼+60; pLUC4, −425∼+60; pLUC5, −801∼+60) and 2 μg β-Gal vector as

an internal control. Two days post-transfection, cells were deprived of FCS for 12 h, *A. actinomycetemcomitans* LPS (0.1 or 0.01 μg/ml) was added for a further 12 h prior to harvesting. The luciferase assays were performed according to the supplier's protocol (picaGene, Toyo Inki Tokyo, Japan) using a luminescence reader (Acuu FLEX Lumi 400; Aloka, Tokyo, Japan) to measure the luciferase activities. The tyrosine kinase inhibitor herbimycin A (HA; 1 μM), the PKC inhibitor H7 (5 μM), the PKA inhibitor H89 (5 μM), the ERK1/2 inhibitor U0126 (5 μM), and the PI3K inhibitor LY249002 (10 μM) were used for protein kinases inhibition and an antioxidant *N*-acetylcystein (NAC; 20 mM) was used as a free radical scavenger. 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 as described previously to verify the fidelity of the mutagenesis.

GEL MOBILITY SHIFT ASSAYS

Confluent ROS 17/2.8 cells in T-75 flasks incubated for 3, 6, and 12 h with *A. actinomycetemcomitans* LPS (0.1 or 0.01 μg/ml) in α-MEM without FCS were used to prepare nuclear extracts, with the addition of extra proteinase inhibitors (the extraction buffer was 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol, 25% (v/v) glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A, 1 μg/ml aprotinin, pH 7.9). Double-stranded oligonucleotides encompassing the inverted CCAAT (nts, −61 to −37, 5′-CCGTGA-CCGTG**ATTGG**CTGCTGAGA), CRE (nts, −84 to −59, 5′-CCCA-CAGCC**TGACGTCG**CACCGGCCG), FRE (nts, −98 to −79, 5′-TTTTC-TGGTGAGAACCCACA), Pit-1 (nts, −115 to −96, 5′-CGTG**TTGT-AGT**TACGGATTT) and HOX (nts, −204 to −179, 5′-TCCTCAGCCTT-CAATTAAATCCCACA) in the rat BSP promoter were prepared by Bio-Synthesis, Inc (Lewisville, TX). For gel shift analysis, the double stranded-oligonucleotides were end-labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase. Nuclear protein extracts (3 μg) were incubated for 20 min at room temperature (21°C) 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 poly (dI-dC). Following incubation, the protein-DNA complexes were resolved by electrophoresis on 5% nondenaturing acrylamide gels (38:2 acrylamide/bis acrylamide) run at 200 V at room temperature. Following electrophoresis, the gels were dried and autoradiograms prepared and analyzed using an image analyzer. For competition experiments, 40× molar unlabeled oligonucleotides for the inverted CCAAT, CRE, mutation-CRE (mCRE), FRE, mutation-FRE (mFRE), HOX, and mutation-HOX (mHOX) were used. Supershift experiments were performed using anti-CREB1 (p43; Rockland), JunD (sc-74), Fra2 (sc-604), c-Fos (sc-253), c-Jun (sc-44), Smad1 (sc-7965) (Santa Cruz Biotechnology), Runx2 (M06; Abnova), and Dlx5 (AB5728; Chemicon) 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.

CHROMATIN IMMUNOPRECIPITATION (ChiP) ASSAY

ChIP assays were carried out using a ChIP-IT™ Express Enzymatic kit (Active Motif) according to the manufacturer's protocol. ROS17/

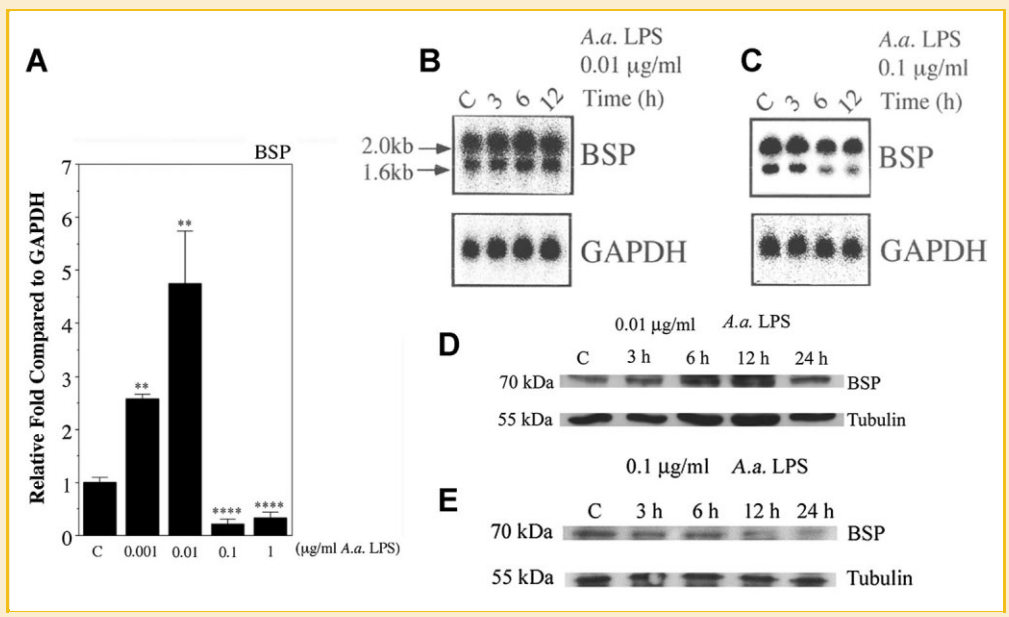

Fig. 1. Effects of *A. actinomycetemcomitans* LPS on BSP mRNA and protein levels in ROS17/2.8 cells. A: Dose response effects of *A. actinomycetemcomitans* LPS on BSP mRNA levels in ROS17/2.8 cells (real-time PCR). B, C: Time course effects of 0.01 μg/ml (B) and 0.1 μg/ml (C) *A. actinomycetemcomitans* LPS on BSP and GAPDH expressions. Total RNA was extracted with guanidium thiocyanate and Northern hybridizations (B, C) were performed with rat BSP and GAPDH cDNA probes. D, E: Time course effects of 0.01 μg/ml (D) and 0.1 μg/ml (E) *A. actinomycetemcomitans* LPS on BSP and tubulin protein levels. The expression of BSP protein was analyzed by western blotting using anti-BSP antibody. α-tubulin antibody was used as a loading control. The experiments were performed in triplicate (quadruplicate for real-time PCR) for each data point. Results of representative hybridization and western blot analysis of control and *A. actinomycetemcomitans* LPS treated cells are shown. $^{**}(P < 0.05)$; $^{****}(P < 0.01)$.

2.8 cells were grown to confluence in 100 mm dishes (six dishes for each chromatin preparation, approximately 4.5×10^7 cells) and cultured in serum-free α-MEM for 12 h before treatment with (six dishes) or without (six dishes) *A. actinomycetemcomitans* LPS (0.01 μg/ml) for 6 h. The cells were fixed for 10 min at RT using 1% formaldehyde and then chromatin was prepared using the ChIP-IT Express Enzymatic Kit protocol. 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, JunD, Fra2, c-Jun, c-Fos, Runx2, Dlx5, Smad1, and rabbit IgG were used as control) and protein G magnetic beads at 4°C overnight. Place tube on magnetic stand to pellet beads on the tube side and wash the beads extensively. Elute chromatin from the beads by elution buffer and reverse 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, 94°C for 5 min; amplification was carried out for 30 cycles, denature 94°C for 30 s, anneal 58°C for 30 s, and extend 72°C for 30 s; final extension at 72°C for 10 min) for the CRE and FRE sites within the rat BSP promoter using forward; 5′-GCCTCTCACCCATTCACTCGCTCTTGC-3′, reverse; 5′-CGACCTCTT-GGCAGCAGGCGCGCCTTTC-3′ primers. For the HOX sites, using forward; 5′-CCGTAACTACAACACGTTTTCAAGAC-3′, reverse; 5′-

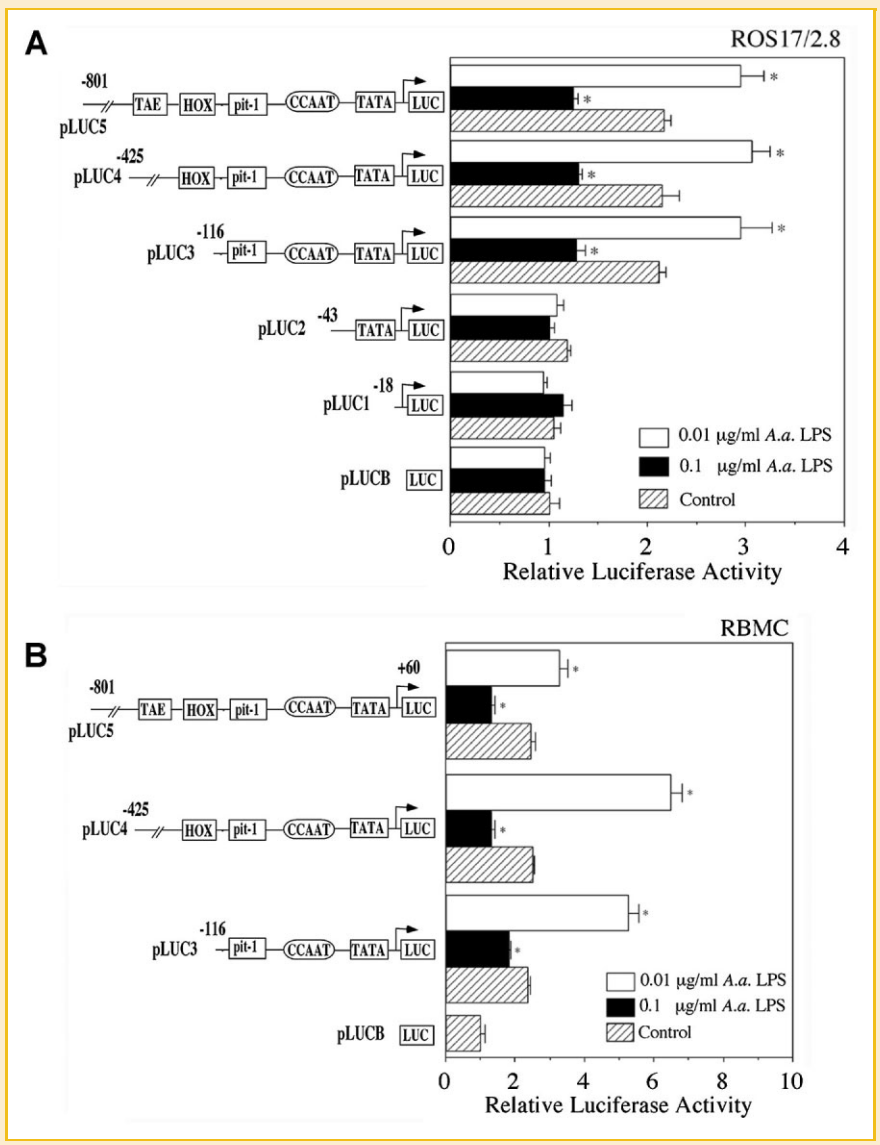

Fig. 2. *A. actinomycetemcomitans* LPS regulates BSP promoter activities in ROS 17/2.8 cells and RBMC. Transient transfections of ROS 17/2.8 cells (A) and RBMC (B) in the presence or absence of 0.1 and 0.01 μg/ml *A. actinomycetemcomitans* LPS for 12 h were used to determine transcriptional activities of chimeric constructs that included various regions of the rat BSP promoter ligated to a luciferase reporter gene. The results of transcriptional activity obtained from four separate transfections with constructs, pLUC basic (pLUCB) and pLUC1–pLUC5, have been combined, and the values are expressed with standard errors *($P < 0.01$).

GTTTAAATGCTTAAGTCGTTTGCC-3′ primers and annealing temperature was 52°C. 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.

STATISTICAL ANALYSIS

Triplicate or quadruplicate samples were analyzed for each experiment, and experiments were replicated to ensure the consistency of the responses to LPS. Significant differences between the control and LPS treatments were determined using unpaired Student's *t*-test (Fig. 1) and one-way analysis of variance (ANOVA).

RESULTS

EFFECT OF *A. ACTINOMYCETEMCOMITANS* LPS ON BSP mRNA AND PROTEIN LEVELS IN ROS 17/2.8 OSTEOBLAST-LIKE CELLS

A. actinomycetemcomitans is a periodontopathic bacteria that causes rapid bone and tooth loss by aggressive periodontitis. *A. actinomycetemcomitans* LPS could be a major factor in the pathogenesis of aggressive periodontitis [Schreiner et al., 2003; Nemoto et al., 2006; Rogers et al., 2007]. Therefore, we used *A. actinomycetemcomitans* LPS to study the effect of LPS on BSP expression which is an early differentiation marker of osteoblasts [Ogata, 2008]. Firstly, the dose-response effect of *A. actinomycetemcomitans* LPS on BSP expression was established by treating the ROS 17/2.8 cells with different concentrations (0.001, 0.01, 0.1, 1 μg/ml) of *A. actinomycetemcomitans* LPS for 12 h. Real-time PCR results revealed that BSP mRNA levels were increased by 0.001 and 0.01 μg/ml *A. actinomycetemcomitans* LPS, while BSP mRNA levels were decreased by 0.1 and 1 μg/ml *A. actinomycetemcomitans* LPS (Fig. 1A). Then 0.01 and 0.1 μg/ml *A. actinomycetemcomitans* LPS were used to determine the time-course effects of LPS on BSP mRNA levels. 0.1 μg/ml *A. actinomycetemcomitans* LPS suppressed BSP mRNA levels at 6 and 12 h (Fig. 1C), while 0.01 μg/ml *A. actinomycetemcomitans* LPS increased BSP mRNA levels at 6 and 12 h (Fig. 1B). *A. actinomycetemcomitans* LPS (0.01 μg/ml) induced BSP protein expression at 3 h and reached maximal at 12 h. *A. actinomycetemcomitans* LPS (0.1 μg/ml) reduced BSP protein levels time dependent manner in ROS 17/2.8 cells. Tubulin was used as a loading control (Fig. 1D,E).

A. ACTINOMYCETEMCOMITANS LPS REGULATED RAT BSP GENE TRANSCRIPTION

To determine how *A. actinomycetemcomitans* LPS regulates BSP transcription, transient transfection analyses were performed using chimeric constructs encompassing different regions of the rat BSP gene promoter ligated to a luciferase reporter gene (pLUC1∼5) transfected into ROS 17/2.8 cells and RBMC. The transcriptional activities of BSP promoter constructs pLUC3 (nts −116∼+60), pLUC4 (nts −425∼+60), and pLUC5 (nts −801∼+60) were decreased by 0.1 μg/ml *A. actinomycetemcomitans* LPS, whereas increased by 0.01 μg/ml *A. actinomycetemcomitans* LPS in ROS 17/2.8 and RBMC cells (Fig. 2A,B). In shorter constructs (pLUC1, −18 to +60; pLUC2, −43 to +60), luciferase activities were not influenced by high and low concentrations of *A. actinomycetemcomitans* LPS (Fig. 2A). In our previous study, 0.1 μg/ml *P. gingivalis* LPS

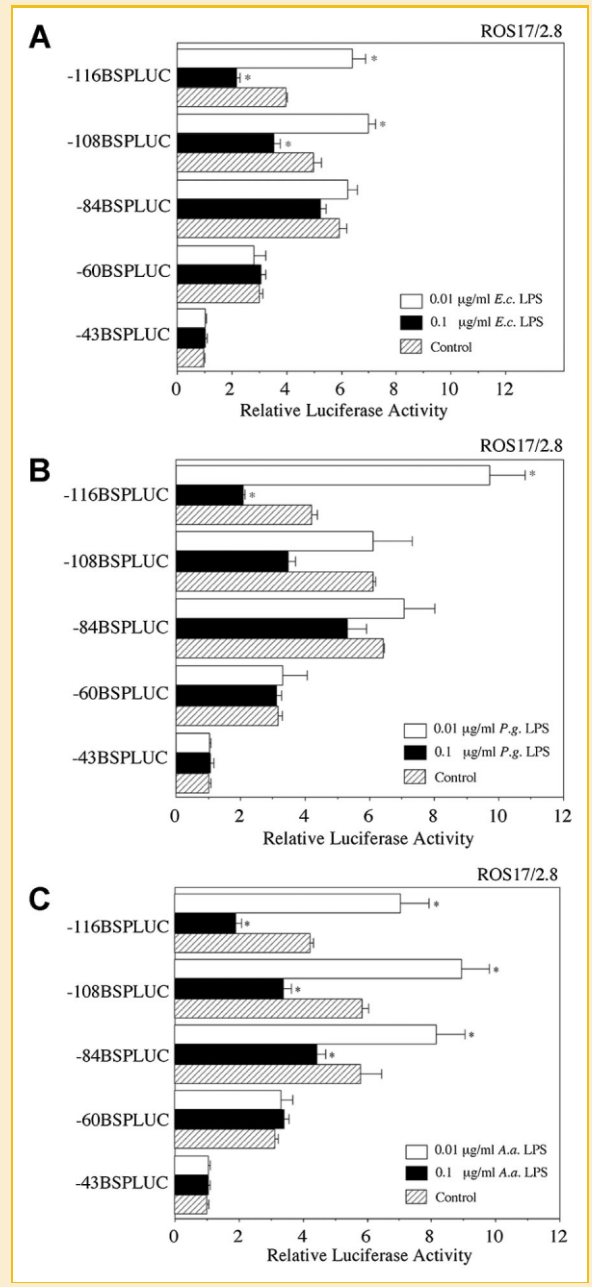

Fig. 3. Fine 5′ deletion mapping of the nts −116 to −43 elements in the BSP promoter. A series of rat BSP promoter 5′ deletion constructs were analyzed for relative promoter activity after transfection into ROS17/2.8 cells and examined for regulation in the presence or absence of 0.1 and 0.01 μg/ml *E.coli* LPS (A), *P. gingivalis* (B), and *A. actinomycetemcomitans* LPS (C) for 12 h. The results of transcriptional activities obtained from four separate transfections with constructs, −43BSPLUC (−43 to +60), −60BSPLUC (−60 to +60), −84BSPLUC (−84 to +60), −108BSPLUC (−108 to +60) and −116BSPLUC (−116 to +60), have been combined and the values are expressed with standard errors *($P < 0.01$).

decreased pLUC3, pLUC4, and pLUC5 luciferase activities, whereas 0.01 μg/ml *P. gingivalis* LPS increased only pLUC3 activity [Li et al., 2010]. These results suggest that *A. actinomycetemcomitans* LPS regulates BSP transcription in a very different way than *P. gingivalis* LPS. Next, we compared the actions of LPS from *E. coli*, *P. gingivalis* together with *A. actinomycetemcomitans* LPS on BSP transcription (Fig. 3A–C). Using a series of 5′-deletion constructs between nts −116 and −43, we found that 0.1 μg/ml decreased and 0.01 μg/ml *P. gingivalis* LPS increased only −116BSPLUC (−116 to +60) activity (Fig. 3B). High and low concentrations of *E. coli* LPS decreased and increased luciferase activities of −108 and −116BSPLUC (Fig. 3A), and high and low concentrations of *A. actinomycetemcomitans* LPS decreased and increased luciferase activities of −84, −108, and −116BSPLUC (Fig. 3C). 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 FGF2 response element (FRE; nts −92 to −85), a pituitary-specific transcription factor-1 motif (Pit-1; nts −111 to −105), another Runx2 (nts −184 to −179) and a HOX (nts −194 to −185) are present (Fig. 4). To determine the signaling pathways mediating the effects of *A. actinomycetemcomitans* LPS, we used several protein kinase inhibitors and antioxidant on *A. actinomycetemcomitans* LPS that regulated BSP transcription. The tyrosine kinase inhibitor HA, ERK1/2 inhibitor U0126, PI3K inhibitor LY24900 and antioxidant NAC completely abolished the effects of high and low concentrations of *A. actinomycetemcomitans* LPS (0.1 and 0.01 μg/ml) on BSP transcription. On the other hand, the PKC inhibitor H7 and the PKA inhibitor H89 did not inhibit the effects of *A. actinomycetemcomitans* LPS on BSP transcription (Fig. 5A). To confirm the functional elements, we performed mutation analyses. After introducing 2 bp mutations in the putative response elements within pLUC3 and pLUC4, the basal activities of single and double mutations were lower than the basal transcriptional activities of pLUC3 and pLUC4. The transcriptional suppression by 0.1 μg/ml *A. actinomycetemcomitans* LPS and the stimulatory effects of 0.01 μg/ml *A. actinomycetemcomitans* LPS were abrogated in the M-CRE and M-FRE of pLUC3, and M-FRE in the pLUC4 single mutation constructs; however, M-HOX did not abrogate transcriptional suppression by 0.1 μg/ml *A. actinomycetemcomitans* LPS. Double mutation in CRE and FRE (M-CRE/FRE) of pLUC3, FRE, and HOX (M-FRE/HOX) of pLUC4 completely abolished the effects of 0.1 and 0.01 μg/ml *A. actinomycetemcomitans* LPS (Fig. 5B).

GEL MOBILITY SHIFT ASSAYS

To identify nuclear proteins that bind to the CCAAT, CRE, FRE, Pit-1, and HOX elements and mediate the *A. actinomycetemcomitans* LPS effects on transcription, double-stranded oligonucleotides were end-labeled and incubated with equal amounts (3 μg) of nuclear proteins extracted from confluent ROS 17/2.8 cells that were either not treated (control) and treated with 0.1 or 0.01 μg/ml *A. actinomycetemcomitans* LPS 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 *A. actinomycetemcomitans* LPS (0.1 and 0.01 μg/ml) (Fig. 6A,B, lanes 1–4, 13–16). 0.1 μg/ml *A. actinomycetemcomitans* LPS decreased CRE and FRE binding protein complexes from 3 h (Fig. 6A, lanes 5–8, 9–12), reduced HOX-protein formation at 12 h (Fig. 6A, lanes 17–20). On the other hand, 0.01 μg/ml *A. actinomycetemcomitans* LPS-induced CRE, FRE, and HOX binding protein formations significantly in a time-dependent manner, reaching the maximal levels at 12 h (Fig. 6B, lanes 5–8, 9–12, 17–20). From mutation luciferase assays and gel shift

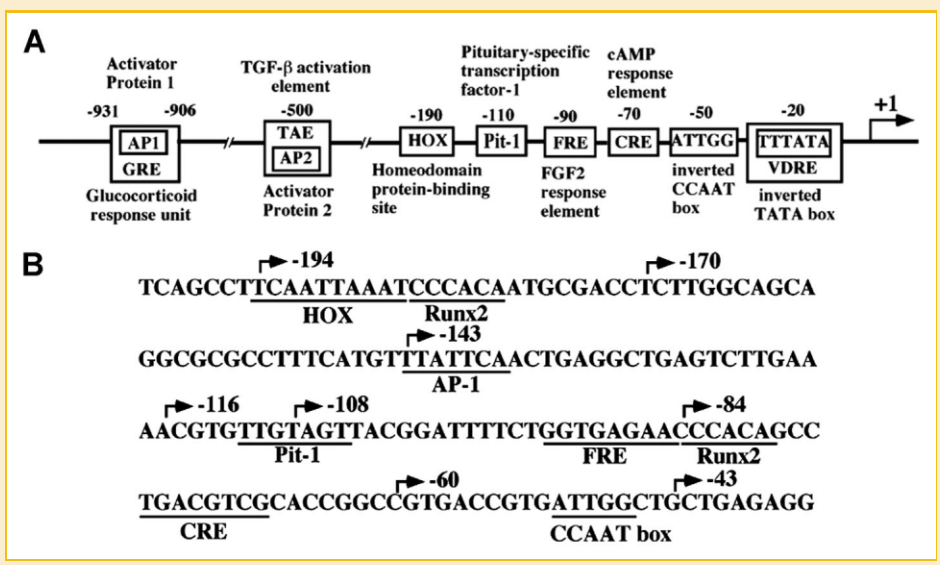

Fig. 4. Regulatory elements in the proximal promoter of rat BSP gene. A: The positions of inverted TATA and CCAAT boxes, vitamin D response element (VDRE) that overlaps the inverted TATA box, a CRE (cAMP response element), FRE (FGF2 response element), Pit-1 (mediates the stimulatory effects of parathyroid hormone, PTH), HOX (homeodomain protein-binding site), TAE (TGF-β activation element), and GRE (glucocorticoid response element) overlapping with AP1 are shown. B: The nucleotide sequence of the rat BSP gene proximal promoter is shown from −201 to −35. An inverted CCAAT box, CRE, Runx2, FRE, Pit-1, AP1, another Runx2, and HOX are present.

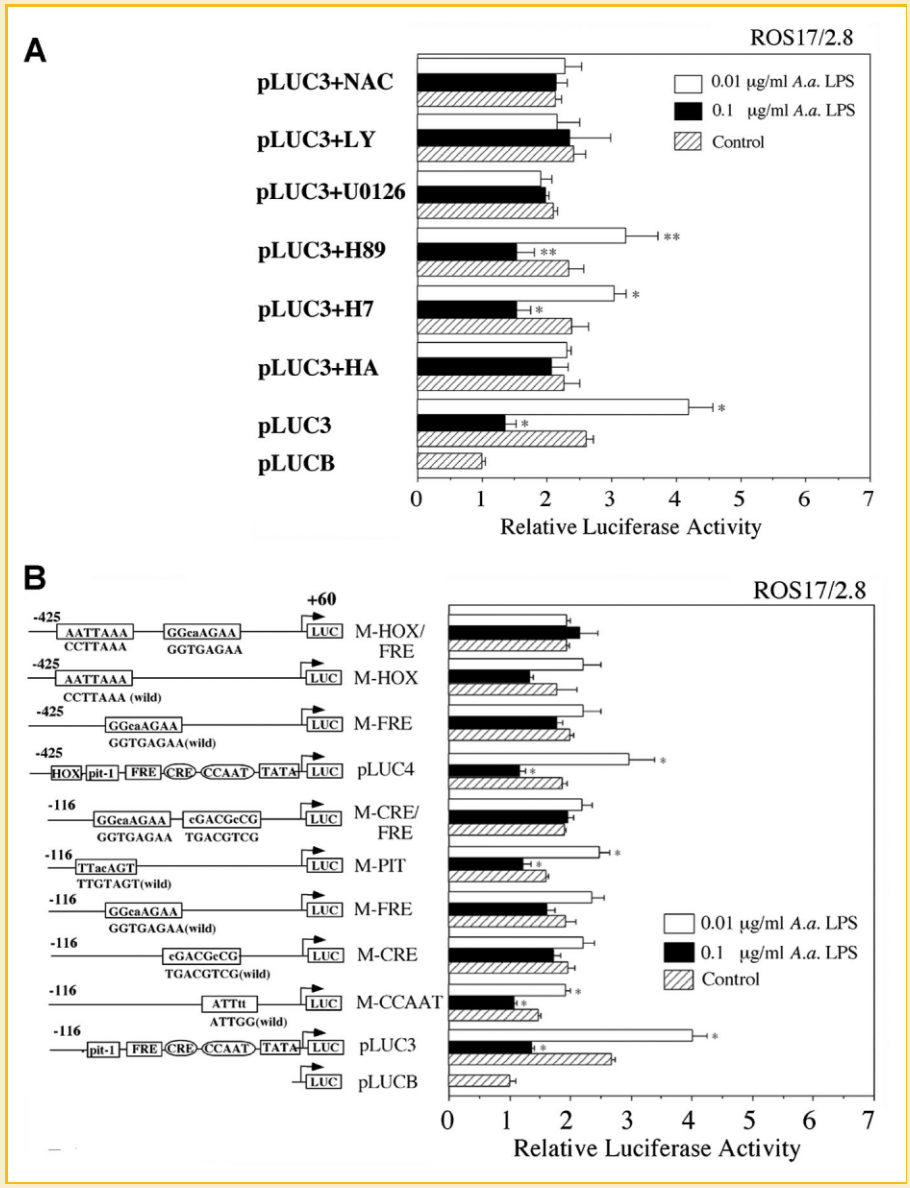

Fig. 5. Effects of kinase inhibitors and antioxidant on transcriptional regulation by *A. actinomycetemcomitans* LPS and site mutation analyses of luciferase activities. A: Transient transfection analysis of pLUC3 in the presence or absence of 0.1 and 0.01 μg/ml *A. actinomycetemcomitans* LPS for 12 h in ROS17/2.8 cells is shown together with the effects of HA (1 μM), H7 (5 μM), H89 (5 μM), U0126 (5 μM), LY294002 (10 μM), and NAC (20 mM). B: Dinucleotide substitutions were made within contexts of the pLUC3 and pLUC4 BSP promoter fragments. mCCAAT (ATTtt), mCRE (cGACGcCG), mFRE (GGcaAGAA), mPit-1 (TTacAGT), mHOX (aaTTAAA), mCRE/FRE, and mFRE/HOX constructs were analyzed for relative promoter activity after transfection into ROS17/2.8 cells, and examined for regulation after treatment with 0.1 and 0.01 μg/ml *A. actinomycetemcomitans* LPS for 12 h. The results of transcriptional activity obtained from four separate transfections with constructs were combined and the values expressed with standard errors $^{*}(P < 0.01)$; $^{**}(P < 0.05)$.

analyses, HOX could be involved in the regulatory effects of *A. actinomycetemcomitans* LPS on BSP transcription (Figs. 5 and 6). That these DNA-protein complexes represent specific interactions was confirmed by competition experiments in which a 40-fold molar excess of CRE, FRE, and HOX double-stranded oligonucleotides reduced, and mutated CRE (mCRE), mFRE, and mHOX did not reduce CRE-, FRE-, and HOX-protein complex formation (Fig. 7, lanes 3, 4, 9, 10, 15, and 16). A 40-fold molar excess of FRE and inverted CCAAT double-stranded oligonucleotides did not reduce CRE-protein complex formation (Fig. 7, lanes 5 and 6). Inverted CCAAT did not reduce FRE-protein complex formation (Fig. 7, lane 12). FRE and inverted CCAAT did not reduce HOX-protein complex formation (Fig. 7, lanes 17 and 18). Notably, a 40-fold molar excess of HOX double-stranded oligonucleotide was able to compete for FRE-protein complex formation (Fig. 7, lane 11). To further characterize the proteins in the complexes formed with CRE, FRE, and HOX, we used antibodies to several transcription factors. CREB1, JunD, Fra2, and c-Fos antibodies disrupted the formation of CRE-protein complexes (Fig. 8, lanes 4–7). Runx2, Dlx5, and Smad1 antibodies disrupted FRE-protein complex formation (Fig. 8, lanes

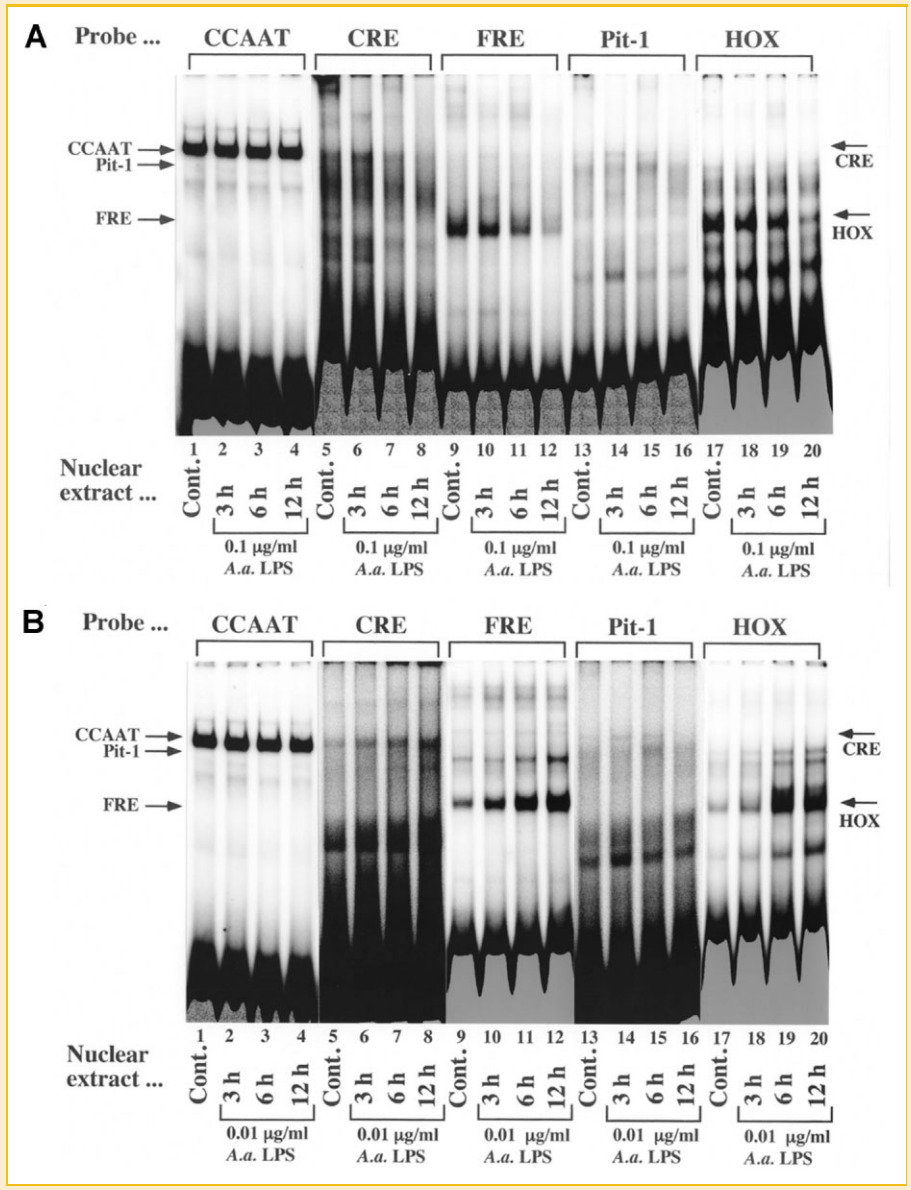

Fig. 6. Gel mobility shift assays using inverted CCAAT, CRE, FRE, Pit-1 and HOX. Radiolabeled double-stranded inverted CCAAT (−61 CCGTGACCGTG**ATTGG**CTGCTGAGA −79), CRE (−84 CCCACAAGCC**TGACGTCG**CACCGGCCG −59), FRE (−98 TTTTCT**GGTGAGAA**CCCACA −79), Pit-1 (−115 CGTG**TTGTAGT**TACGGATTT −96), and HOX (−204 TCCTCAGCCT**TCAATTAAAT**CCCACA −179) oligonucleotides were incubated with nuclear protein extracts (3 μg) obtained from ROS17/2.8 cells stimulated without (lanes 1, 5, 9, 13, and 17), with 0.1 μg/ml (A) or 0.01 μg/ml (B) *A. actinomycetemcomitans* LPS for 3 h (lanes 2, 6, 10, 14, and 18), 6 h (lanes 3, 7, 11, 15, and 19) and 12 h (lanes 4, 8, 12, 16, and 20). DNA-protein complexes were separated on 5% polyacrylamide gel in low-ionic-strength Tris-borate buffer, dried in a vacuum and exposed to an imaging plate for quantitation using an image analyzer.

11–13). Runx2 and Dlx5 antibodies disrupted HOX-protein complex formation (Fig. 8, lanes 17 and 18).

CHIP ASSAY

We next examined whether transcription factors are able to interact directly with BSP gene promoter and how *A. actinomycetemcomitans* LPS influences these transcription factors interaction with the CRE, FRE, and HOX. To clarify this issue further, we used ChIP assays to examine the in vivo association of these transcription factors with response elements in ROS17/2.8 cells. For this experiment, cells were treated with 0.01 μg/ml *A. actinomycetemcomitans* LPS 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, JunD, Fra2, c-Jun, and c-Fos interacted with a chromatin fragment containing the CRE, and Runx2, Dlx5, and Smad1 interacted with the FRE and HOX sites. The bindings of CREB1, JunD, Fra2, and c-Fos to the CRE, and Runx2 and Dlx5

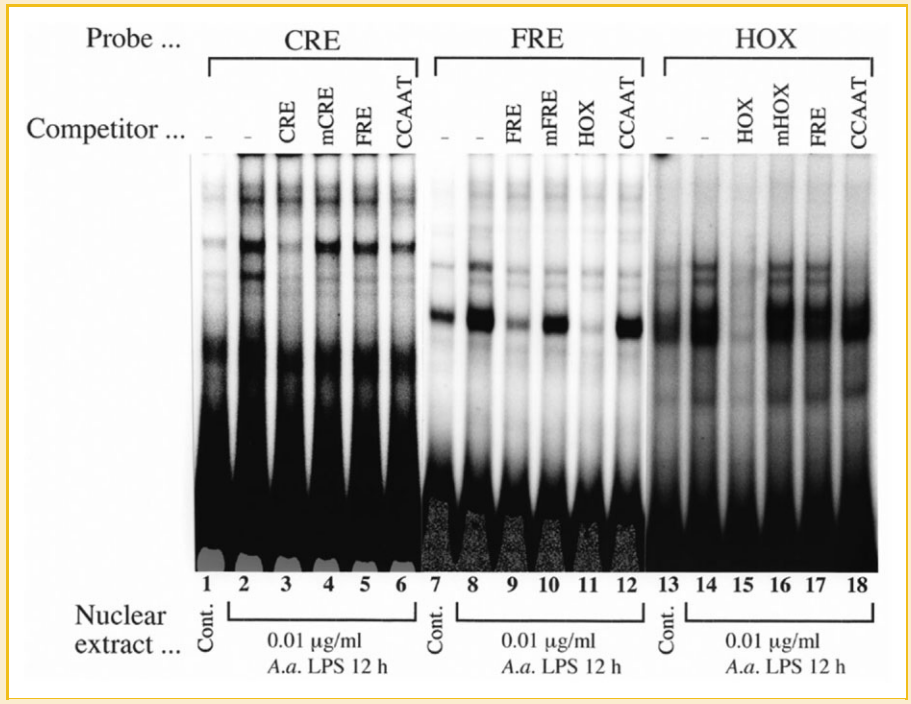

Fig. 7. Specific binding of nuclear proteins to CRE, FRE, and HOX. A: Radiolabeled double-stranded CRE (−84 CCCACAAGCC**TGACGTCG**CACCGGCCG −59), FRE (−98 TTTTCT**GGTGAGAA**CCCACA −79) and HOX (−204 TCCTCAGCCT**TCAATTAAAT**CCCACA −179) oligonucleotides were incubated with nuclear protein extracts (3 μg) obtained from ROS17/2.8 cells treated without (lanes 1, 7, and 13) or with 0.01 μg/ml *A. actinomycetemcomitans* LPS for 12 h (lanes 2–6, 8–12, and 15–18). Competition reactions were performed using a 40-fold molar excess of unlabeled CCAAT (lanes 6, 12, and 18), CRE (lane 3), mCRE (lane 4), FRE (lanes 5, 9, and 17), mFRE (lane 10), HOX (lanes 11 and 15), mHOX (lane 16) oligonucleotides.

to the FRE and HOX were increased by 0.01 μg/ml *A. actinomycetemcomitans* LPS (Fig. 9A–C). The binding of Smad1 to the FRE was increased, in contrast the binding of Smad1 to the HOX was not induced by the stimulation of *A. actinomycetemcomitans* LPS (Fig. 9B,C).

DISCUSSION

LPS is a major component of the outer membrane of Gram-negative periodontopathic bacteria (*A. actinomycetemcomitans, P. gingivalis*, etc.) is considered to be a major factor in the pathogenesis of periodontitis [Yoshimura et al., 2002; Nemoto et al., 2006]. BSP deficiency impairs bone growth and mineralization, concomitant with markedly reduced bone formation, and impairs osteoclastogenesis and mineral resorption [Malaval et al., 2008; Boudiffa et al., 2010]. We wish to know the effects of periodontopathic bacteria-derived LPS (especially *A. actinomycetemcomitans* LPS) on bone metabolism. Therefore, we used BSP as a valuable marker for osteogenic differentiation and bone formation.

In this study, we demonstrated that 0.1 μg/ml suppressed and 0.01 μg/ml *A. actinomycetemcomitans* LPS enhanced BSP gene transcription by targeting CRE, FRE, and HOX elements in the rat BSP gene promoter. Furthermore, 0.1 μg/ml decreased and 0.01 μg/ml *A. actinomycetemcomitans* LPS increased BSP protein levels, indicating *A. actinomycetemcomitans* LPS regulates BSP gene expression as well as protein expression (Fig. 1D,E). High and low concentrations of *P. gingivalis* LPS increased only -116BSPLUC. High and low concentrations of *E. coli* LPS decreased and increased luciferase activities of −108 and −116BSPLUC, and *A. actinomycetemcomitans* LPS decreased and increased luciferase activities of −84, −108, and −116BSPLUC (Fig. 3A–C). These results suggest that *A. actinomycetemcomitans* LPS regulates BSP transcription differently from *E. coli* and *P. gingivalis* LPS [Kato et al., 2006; Li et al., 2010]. We previously reported that *E. coli* LPS (1 μg/ml) suppressed BSP gene transcription through PKA and tyrosine kinase-dependent pathways and that LPS effects are mediated through CRE and FRE elements in the rat BSP gene promoter [Kato et al., 2006]. Whereas, 10-fold lower concentrations (0.1 μg/ml) of periodontopathic bacteria (*A. actinomycetemcomitans* and *P. gingivalis*) derived LPS could suppress BSP transcription (Figs. 1 and 2) [Li et al., 2010]. We previously reported, 0.1 μg/ml *P. gingivalis* LPS decreased and 0.01 μg/ml *P. gingivalis* LPS increased BSP gene transcription mediated through CRE and FRE elements in the rat BSP gene promoter [Li et al., 2010]. In this study, we found similar findings of BSP gene transcription regulated by *A. actinomycetemcomitans* LPS (Figs. 1 and 2); however, the regulatory mechanisms are very different. At first, 0.1 μg/ml *P. gingivalis* LPS decreased luciferase activities of three BSP promoter constructs (pLUC3; nts −116∼+60, pLUC4; nts −425∼+60 and pLUC5; nts −801∼+60), whereas 0.01 μg/ml *P. gingivalis* LPS increased only pLUC3

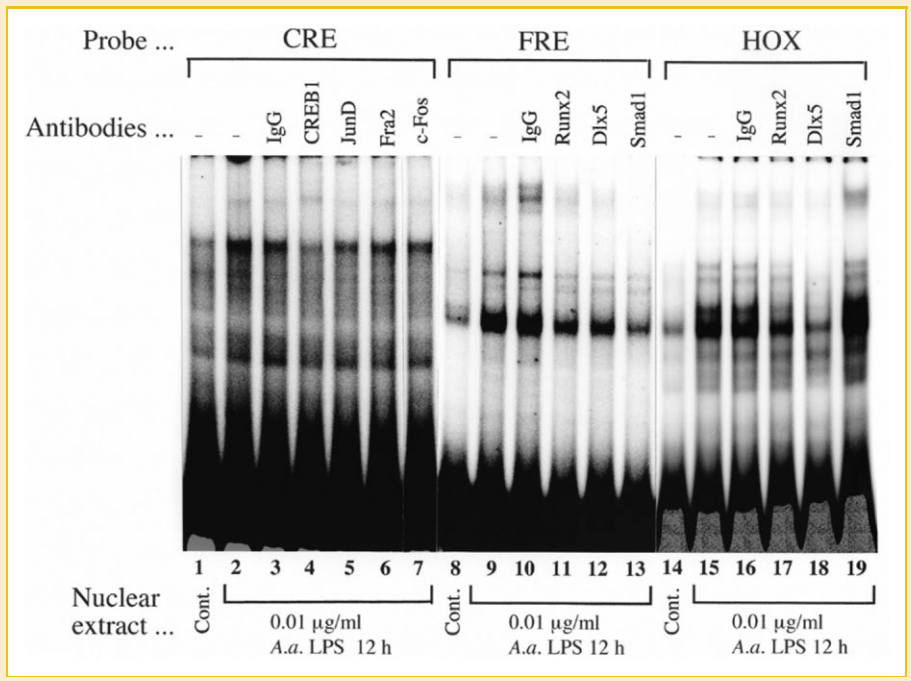

Fig. 8. Specific binding of nuclear proteins to CRE, FRE, and HOX. Radiolabeled double-stranded CRE, FRE, and HOX were incubated with nuclear protein extracts (3 μg) obtained from ROS17/2.8 cells stimulated without (lanes 1, 8, and 14) or with 0.01 μg/ml *A. actinomycetemcomitans* LPS for 12 h (A: Lanes 2–7, 9–13, and 15–19). Supershift experiments were performed with 0.4 μg antibodies against IgG (lanes 3, 10, and 16), CREB1 (lane 4), JunD (lane 5), Fra2 (lane 6), c-Fos (lane 7), Runx2 (lanes 11 and 17), Dlx5 (A: Lanes 12 and 18), Smad1 (lanes 13 and 19), added separately to each gel shift reaction.

luciferase activity [Li et al., 2010]. On the other hand, the transcriptional activities of three BSP promoter constructs (pLUC3, pLUC4, and pLUC5) were decreased by 0.1 μg/ml and increased by 0.01 μg/ml *A. actinomycetemcomitans* LPS in ROS17/2.8 cells (Fig. 2A). Secondly, the tyrosine kinase inhibitor, ERK1/2 inhibitor, and antioxidant completely inhibited the effects of high and low concentrations of *P. gingivalis* LPS (0.1 and 0.01 μg/ml) on BSP transcription, whereas the PKA and the PI3K inhibitors abolished only the effect of 0.01 μg/ml *P. gingivalis* LPS on BSP transcription [Li et al., 2010]. In contrast, the tyrosine kinase, ERK1/2 and PI3K inhibitors and the antioxidant completely abolished the effects of high and low concentrations of *A. actinomycetemcomitans* LPS (0.1 and 0.01 μg/ml) on BSP transcription. PKC and PKA inhibitors did not inhibit the effects of *A. actinomycetemcomitans* LPS on BSP transcription (Fig. 5A). Thirdly, from mutation luciferase assays and gel shift analyses, HOX could be involved in the regulatory effects of *A. actinomycetemcomitans* LPS on BSP transcription (Figs. 5–8). An antioxidant NAC abolished the effects of *A. actinomycetemcomitans* LPS (Fig. 5A). NAC is an inhibitor of reactive oxygen species production considered as second messengers in the inflammatory response [Peng et al., 2005; Wang et al., 2007]; therefore, the effects of *A. actinomycetemcomitans* LPS on BSP transcription might be mediated through reactive oxygen species. Transcriptional regulation by *A. actinomycetemcomitans* LPS was abrogated completely by CRE/FRE and FRE/HOX double mutation in the BSP promoter (Fig. 5B). The involvement of these elements is further supported by gel shift analyses in which nuclear proteins formed complexes with the CRE, FRE, and HOX elements that were decreased by 0.1 μg/ml and increased by 0.01 μg/ml *A. actinomycetemcomitans* LPS in a time-dependent manner (Fig. 6). These findings are different from the effects of *P. gingivalis* LPS on BSP transcription, which regulates BSP gene transcription mediated

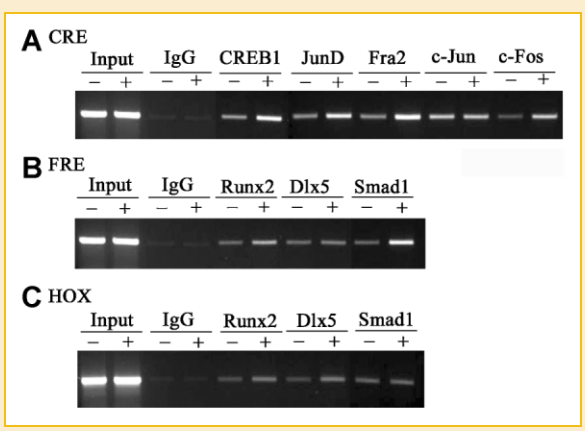

Fig. 9. ChIP analyses of transcription factors binding to CRE, FRE, and HOX sites in the rat BSP promoter in ROS17/2.8 cells. The PCR bands amplified and corresponding to DNA-protein complexes immunoprecipitated with antibodies. The bindings of CREB1, JunD, Fra2, and c-Fos to the CRE (A), and Runx2 and Dlx5 to the FRE (B) and HOX (C) were increased by 0.01 μg/ml *A. actinomycetemcomitans* LPS. Smad1 binding to the FRE was increased, in contrast the binding of Smad1 to the HOX was not induced by the stimulation of *A. actinomycetemcomitans* LPS.

through CRE and FRE; therefore, the mechanisms of *A. actinomycetemcomitans* LPS regulation of BSP transcription are more complicated and mediated through multiple response elements in the rat BSP promoter. Results of the supershift assay (Fig. 8) and ChIP assays (Fig. 9) showed that 0.01 μg/ml *A. actinomycetemcomitans* LPS induced BSP transcription through CREB1, JunD, Fra2, and c-Fos targeting to CRE, through Runx2, Dlx5, and Smad1 targeting to FRE and HOX in the rat BSP gene promoter. Although CREB1 binding to CRE were decreased and increased by the effects of high and low concentrations of *A. actinomycetemcomitans* LPS (Fig. 6A,B), PKA signaling did not affect BSP transcription regulated by *A. actinomycetemcomitans* LPS (Fig. 5A). The results suggest that tyrosine kinase, ERK 1/2, or PI3K might regulate CREB1 binding to CRE induced by *A. actinomycetemcomitans* LPS. CREB1, ATF/CREB, and AP1 family transcription factors can bind to CRE as homodimers or heterodimers [Sassone-Corsi, 1995], and we previously reported that CREB and AP1 family transcription factors (c-Fos, JunD, and Fra2) interacted with CRE in the rat BSP gene promoter [Yang et al., 2010]. We had identified a FRE in the rat BSP gene promoter that is juxtaposed to a putative Runx2 binding site (−84 to −79; CCCACA) [Komori et al., 1997; Shimizu-Sasaki et al., 2001]. Another putative Runx2 site (−184 to −179; CCCACA) is juxtaposed to a HOX site (Fig. 4), and HOX oligonucleotides compete with the FRE-protein complex (Fig. 7). Thus, FRE and HOX binding transcription factors might interact each other. Dlx and Msx families are known to play important roles in the patterning and formation of skeletal structures during embryogenesis and are supposed to act as upstream regulators of Runx2, a key regulator of osteogenesis [Hassan et al., 2006; Samee et al., 2007]. Dlx5 can form co-regulatory complexes with Runx2 that alter the transcription activity of Runx2 [Samee et al., 2007]. All of these findings suggest a close relationship among Dlx families and Runx2. We previously reported that FRE and HOX binding proteins were Runx2, Dlx5, and Smad1, and anti-Smad 1 antibody co-precipitated Runx2 and Smad1 [Nakayama et al., 2006]. Dlx5 binds to the HOX site in the mouse BSP gene promoter [Benson et al., 2000]. Therefore, it is conceivable that Runx2, Dlx5, and Smad 1 may interact with FRE and HOX.

In summary, we have shown that 0.1 μg/ml *A. actinomycetemcomitans* LPS suppressed and 0.01 μg/ml *A. actinomycetemcomitans* LPS enhanced BSP transcription via different signaling pathways mediated through CRE, FRE, and HOX elements in the rat BSP gene promoter, and could involve CREB1, JunD, Fra2, c-Fos, Runx2, Dlx5, and Smad1 transcription factors in *A. actinomycetemcomitans* LPS regulated BSP gene transcription.

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