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Effects of Quercetin and Quercetin 3-Glucuronide on the Expression of Bone Sialoprotein Gene

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Abstract Quercetin is a typical flavonol-type flavonoid and is present in a variety of vegetables, and their antioxidant effect implies their possible role in the prevention of oxidative stress related chronic diseases. Bone sialoprotein (BSP) is a noncollagenous protein of the extracellular matrix in the mineralized connective tissues that has been implicated in the nucleation of hydroxyapatite crystals. Previously, we reported that isoflavone (genistein) activated BSP gene transcription is mediated through an inverted CCAAT box in the proximal BSP gene promoter. The present study investigates the regulation of BSP transcription in a rat osteoblast-like cell line, ROS 17/2.8 cells, by quercetin and its conjugated metabolite quercetin 3-glucuronide. Quercetin and quercetin 3-glucuronide (5 μM) increased the BSP mRNA levels at 12 h and quercetin upregulated the Cbfa1/Runx2 mRNA expression at 12 h. From transient transfection assays using various sized BSP promoter-luciferase constructs, quercetin increased the luciferase activity of the construct (pLUC3), including the promoter sequence nucleotides −116 to −43. Transcriptional stimulations by quercetin were almost completely abrogated in the constructs that included 2 bp mutations in the inverted CCAAT and FRE elements whereas the CCAAT-protein complex did not change after stimulation by quercetin according to gel shift assays. Quercetin increased the nuclear protein binding to the FRE and 3′-FRE. These data suggest that quercetin and quercetin 3-glucuronide increased the BSP mRNA expression, and that the inverted CCAAT and FRE elements in the promoter of the BSP gene are required for quercetin induced BSP transcription. J. Cell. Biochem. 101: 790–800, 2007.

Key words: bone sialoprotein; flavonol; osteoblast; quercetin; quercetin 3-glucuronide; transcription

Flavonoids are micronutrients that are widely present in food of plant origin. They have been attributed biological properties, such as anti-cancer, anti-oxidant, anti-inflammation, anti-viral, anti-mutagenic, gastroprotective, protein kinase C inhibition, topoisomerases II inhibition, cytotoxic activities and the prevention of age-related pathologies [Akama et al., 1996; Manna et al., 1999; Shimizu and Ogata, 2002].

Quercetin (3,3′,4′,5,7-pentahydroxylflavone) is the major representative of the flavonoid subclass of flavonols commonly found in fruits and vegetables, and is also abundant in broccoli, lettuce, and onion. Flavonoids are present mainly as glycosides, in which hydrogens are substituted to the sugar moiety [Murota et al., 2000, 2002]. Aglycone is released from glycosides in the digestive tract. Although quercetin glycosides are subject to deglycosidation by enterobacteria for absorption in the large intestine, small intestinal cells possess a

Abbreviations used: BSP, bone sialoprotein; FCS, fetal calf serum; MEM, minimum essential medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FGF, fibroblast growth factor; FRE, FGF response element; bp, base pair(s); nts, nucleotides; LUC, luciferase; CRE, cAMP response element; OPN, osteopontin.

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glucoside-hydrolyzing activity, and their glucose transport system is capable of participating in the glucoside absorption in the small intestine. Aglycone in the mucosa is converted into its conjugated metabolites by UDP-glucuronosyltransferase and/or phenol sulfotransferase [Murota and Terao, 2003]. Quercetin 3-glucuronide is one of the major quercetin metabolites in the plasma [Moon et al., 2001]. It is likely to act as an effective antioxidant in blood plasma low-density lipoprotein [Moon et al., 2001] and inhibit angiotensin II-induced increases in AP-1 DNA binding [Yoshizumi et al., 2002]. Quercetin has been reported to inhibit the osteoclastic resorption of the bone in vitro [Wattel et al., 2003]. And also, quercetin suppresses bone resorption by inhibiting the differentiation and activation of osteoclasts. [Woo et al., 2004]. Quercetin stimulates the alkaline phosphatase activity in MG63 human osteoblasts [Prouillet et al., 2004]. These observations suggest that quercetin might inhibit osteoclastogenesis and stimulate osteoblastic activity. However, little is known about the effects of quercetin on the metabolism of osteoblasts.

Bone sialoprotein (BSP) is a mineralized connective tissue-specific protein that is glycosylated, phosphorylated, and sulfated [Ogata et al., 1995]. Studies on the developmental expression of BSP have shown that BSP mRNA is expressed at high levels at the onset of bone, dentin, and cementum formation [Chen et al., 1992]. BSP is also expressed in breast, lung, thyroid, and prostate cancers [Bellahcène et al., 1994; Waltregny et al., 2000]. Thus, it has been suggested that BSP may be involved in the osteotropism of the metastatic cancer cells through its ability to bind to hydroxyapatite and to mediate cell attachment through cell-surface integrins [Hunter and Goldberg, 1993; Ganss et al., 1999] Thus, regulation of the BSP gene is potentially important in bone matrix mineralization, in the differentiation of osteoblasts and in tumor metastasis. To study the transcriptional regulation of BSP, rat, human, and mouse BSP gene promoters were characterized [Kerr et al., 1993; Li and Sodek, 1993; Kim et al., 1994, Benson et al., 1999]. These promoters have an inverted TATA (−24 to −19) [Li et al., 1995] and an inverted CCAAT box (−50 to −46), which is required for basal transcription [Kim and Sodek, 1999; Shimizu and Ogata, 2002]. In addition, a cAMP response element (CRE; −75 to −68) [Samoto et al., 2002, 2003], a fibroblast growth factor 2 response element (FRE; −92 to −85) [Shimizu-Sasaki et al., 2001; Samoto et al., 2003; Shimizu et al., 2004a, 2005, 2006; Nakayama et al., 2006], a pituitary-specific transcription factor-1, Pit-1 motif (−111 to −105) that mediates the stimulatory effects of parathyroid hormone [Ogata et al., 2000; Shimizu et al., 2004a, 2006], and a homeodomain binding element (HOX; −199 to −192) [Benson et al., 2000; Shimizu et al., 2004b, Nakayama et al., 2006], a transforming growth factor-β (TGF-β) activation element (−499 to −485) [Ogata et al., 1997; Shimizu et al., 2004b, 2005], and a glucocorticoid response element (−920 to −906), overlapping an AP-1 site (−921 to −915) [Ogata et al., 1995; Yamauchi et al., 1996] have also been characterized.

The aim of the present study was to evaluate the ability of quercetin and its metabolites quercetin 3-glucuronide on the transcription of BSP in ROS17/2.8 cells. In this study, we showed that quercetin regulates the BSP expression mediated through inverted CCAAT and FRE elements in the rat BSP gene promoter.

METHODS

Materials

Alpha-minimum essential medium (α-MEM), fetal calf serum (FCS), lipofectamine, penicillin and streptomycin, and Trypsin were obtained from Invitrogen (Carlsbad, CA). The pGL3-basic vector and pSV-β-galactosidase (β-Gal) control vector were purchased from Promega Co., (Madison, WI). Quercetin and guanidium thiocyanate were purchased from Wako Pure Chemical (Tokyo, Japan). EXScript RT reagent Kit and SYBR Premix Ex Taq were purchased from Takara (Tokyo, Japan). Quercetin 3-glucuronide was chemically synthesized using Koenigs–Knorr reaction as previously described [Moon et al., 2001] and characterized by electrospray ionization mass spectrometry.

Cell Culture

Rat clonal osteoblast-like ROS 17/2.8 cells were cultured in α-MEM containing 10% FCS. Cells were grown to confluence in 60 mm tissue culture dishes and then cultured in α-MEM without serum and incubated with or without quercetin (5 μM) or quercetin 3-glucuronide (5 μM) for time periods extending from 3 to 24 h.

RNA was isolated from triplicate cultures at various time intervals and analyzed for the expression of BSP mRNA by Real-time-PCR and Northern hybridization as described below.

Real-Time PCR

Following treatment, total RNA was extracted from ROS 17/2.8 cells with guanidium thiocyanate at different time points, as described previously [Ogata et al., 1997]. One microgram was used as a template for cDNA synthesis. cDNA was prepared using EXScript RT reagent Kit. Quantitative real-time PCR was performed using the following primer sets: BSP forward, 5′-AGACCACAGCTGACGCTGGA-3′; BSP reverse, 5′-CCGTTGACGACCTGCTCATT-3′; Runx2 forward, 5′-CAAGTGGCCAGGTTCAACGA-3′; Runx2 reverse, 5′-TGTGAAGACCGTTATGGTCAAAGTG-3′; Osterix forward, 5′-GCAAGGCTTCGCATCTGA-3′; Osterix reverse, 5′-CTTGGAGCAGAGCAGACAGG-3′; GAPDH forward, 5′-GACAACTTTGGCATCGTGGA-3′; GAPDH reverse, 5′-ATGCAGGGATGATGTTCTGG-3′ using the SYBR Premix Ex Taq in a TP800 thermal cycler dice real time system (Takara). The amplification reactions were performed in 25 μl of final volume containing 2× SYBR Premix EX Taq (12.5 μl), 0.2 μM forward and reverse primers (0.1 μl) and 25 ng cDNA (2.5 μl). 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 (Takara). The thermal cycling condition was 10 s at 95°C, 40 cycles of 5 s, 95°C, and 30 s, 60°C. Post-PCR melting curves confirmed the specificity of single-target amplification, and fold expressions of BSP, Runx2, and Osterix relative to GAPDH were determined in triplicate.

Northern Hybridization

Aliquots (20 μg) of total RNA were fractionated on 1.2% agarose gel and transferred onto a hybond-N+ membrane as previously described [Ogata et al., 1997]. Hybridizations were performed at 42°C with either ^{32}P-labeled rat BSP or rat glyceraldehyde-3-phosphate dehydrogenase (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 the two polyadenylated forms (1.6 and 2.0 kb) of rat BSP mRNA, were scanned using a Bio-imaging analyzer (BAS2000, Fuji BAS2000).

Transient Transfection Assays

Exponentially growing ROS 17/2.8 cells were used for the transfection assays. Twenty-four hours after plating, cells at 40–60% confluence were transfected using a lipofectamine reagent. Transfection included 1 μg of a luciferase (LUC) construct [Ogata et al., 1995] and 2 μg pSV-β-galactosidase (β-gal) vector as an internal control. Two days post-transfection, the cells were deprived of serum for 12 h, quercetin (5 μM) or quercetin 3-glucuronide (5 μM) was added, and the cells were cultured for a further 12 h prior to harvesting. The LUC assay was performed according to the supplier's protocol using a luminescence reader (Acuu Flex Lumi 400; Aloka, Tokyo, Japan) to measure the LUC activity.

Gel Mobility Shift Assays

Confluent ROS 17/2.8 cells in T-75 flasks incubated for 3, 6, and 12 h with 5 μM quercetin in α-MEM without serum were used to prepare the nuclear extracts. Nuclear protein was extracted according to the method of Dignam et al. [1983] with the addition of extra proteinase inhibitors (the extraction buffer was 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol (DTT), 25% (v/v) glycerol, 0.5 mM phenylmethyl-sulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 μg/ml aprotinin, pH 7.9). Double-stranded oligonucleotides encompassing the inverted CCAAT (nts, −61 to −37, 5′-CCGTGACCGTGATTGGCTGCTGAGA), CRE (nts, −84 to −59, 5′-CCCACAGCCTGACGTCGCACCGGCCG) FRE (nts, −98 to −79, 5′-TTTTCTGGTGAGAACCCACA), and HOX (nts, −204 to −179, 5′-TCCTCAGCCTTCAATTAAATCCCACA) in the BSP promoter were prepared by Bio-Synthesis, Inc. (Lewisville, TX). For gel shift analysis, the double-standard oligonucleotides were end-labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase. Nuclear protein extracts (3 μg) were incubated for 20 min at room temperature (RT, 21°C) with 0.1 pM radiolabeled double-standard oligonucleotide in buffer containing 50 mM KCl, 0.5 mM EDTA, 10 mM Tris-HCl (pH 7.9), 1 mM DTT, 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% non-denaturing acrylamide gels (38:2 acrylamide/bis acrylamide) run at 150 V at RT. After electrophoresis, the gels were dried and autoradiograms were prepared and analyzed using an image analyzer.

Statistical Analysis

Triplicate samples were analyzed for each experiment, and the experiments were replicated to ensure consistency of the responses to quercetin or quercetin 3-glucuronide. Significant differences between the control and quercetin treatments were determined using Student's *t*-test.

RESULTS

Stimulation of BSP mRNA Expression in ROS17/2.8 Cells

The structures of quercetin, genistein, and flavone are compared in Figure 1. Quercetin is a flavonol and genistein is an isoflavone. All these compounds are flavonoids. To study the regulation of BSP expression by quercetin, we performed Northern hybridization analysis of total RNA extracted from osteoblastic ROS 17/2.8 cells. First, a dose response relation for the quercetin induction of BSP was established by treating the ROS 17/2.8 cells with different concentrations of quercetin for 12 h. Quercetin increased the BSP mRNA levels at 0.05∼50 μM and had a maximal effect at 5 μM (Fig. 2A). Thus, 5 μM quercetin was used to determine the time course of BSP mRNA expression by Northern hybridization (Fig. 2B). Quercetin upregulated the BSP mRNA accumulation at 12 h; whereas, no effect on GAPDH mRNA was observed. Next, we used the conjugated metabolite quercetin 3-glucuronide, because quercetin 3-glucuronide is one of the major quercetin metabolites in the plasma. Quercetin 3-glucuronide stimulated BSP mRNA levels at 12 h (Fig. 2C). Results of real-time P CR showed that the treatment with 5 μM quercetin increased the BSP mRNA levels at 12 h (Fig. 2D). The Cbfa1/Runx2 mRNA level was raised at 12 h. The osterix mRNA level did not change after stimulation by quercetin (Fig. 2D).

Transient Transfection Analysis of Rat BSP Promoter Constructs

Since the effects of quercetin likely involve transcription factor interaction in the promoter region of the rat BSP gene, subsequent studies were directed at transcriptional regulation utilizing chimeric constructs prepared from the promoter from the rat BSP gene. Transient transfection of chimeric constructs encompassing different regions of the rat BSP gene promoter ligated to a LUC reporter gene (pLUC1∼pLUC6, pLUC1; −18 to +60, pLUC2; −43 to +60, pLUC3; −116 to +60, pLUC4; −425 to +60, pLUC5; −801 to +60, and pLUC6; −938 to +60) were performed in ROS 17/2.8 cells. The results of LUC assays (Fig. 3A) indicated an increase in transcription after 12 h treatment with 5 μM quercetin using the constructs, pLUC3, which encompasses nucleotides −116 to +60, as well as in longer constructs. In shorter constructs (pLUC1; −18 to +60, PLUC2; −43 to +60), the LUC activities were

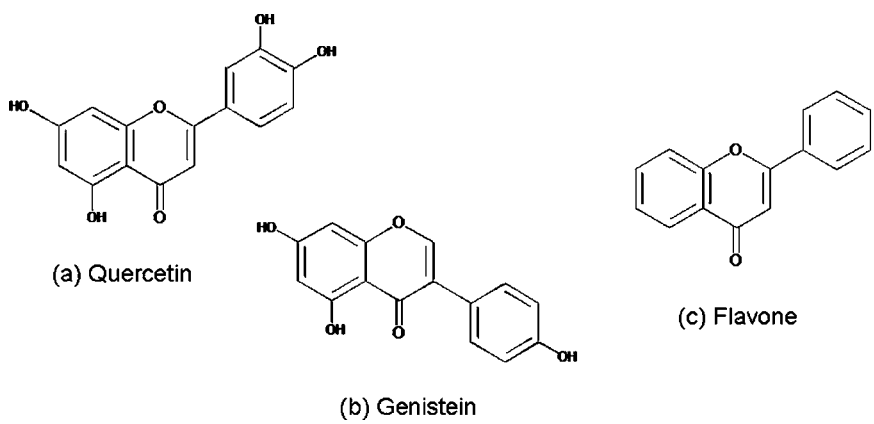

Fig. 1. Structural formulae of quercetin, genistein, and flavone. Quercetin and genistein contain flavonol and isoflavone structures. Flavone contains a flavone structure.

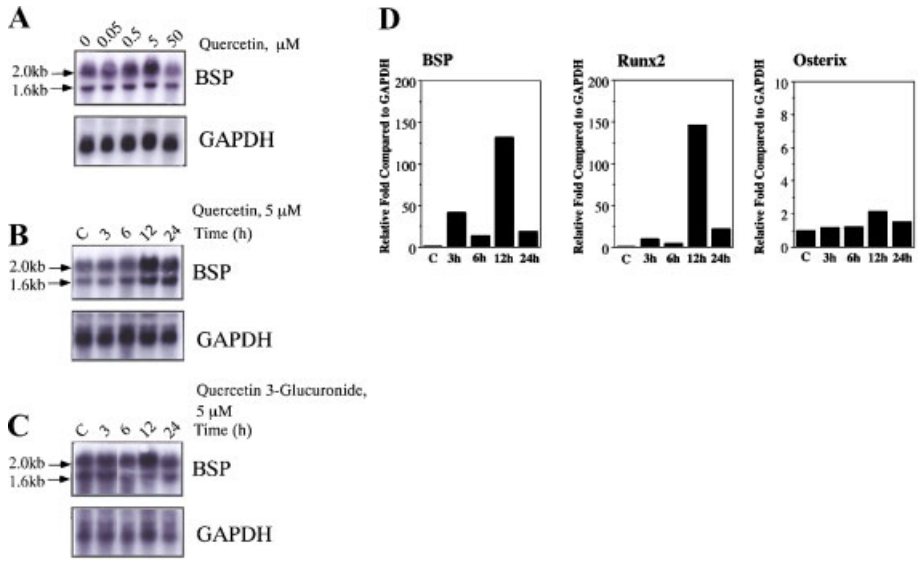

Fig. 2. Effects of quercetin and quercetin 3-glucuronide on BSP mRNA expression in ROS 17/2.8 cells. **A**: Dose-response effect of quercetin on BSP mRNA levels for 12 h. At 0.05–50 μM, quercetin increased BSP mRNA with a maximal effect at 5 μM. **B**, **C**: A twenty-four hours time course revealed an increase in BSP mRNA following stimulation with 5 μM quercetin (B) and quercetin 3-glucurinide (C) at 12 h in ROS17/2.8 cells. **D**: Relative gene expression for BSP, Cbfa1/Runx2, and Osterix generated from real-time PCR of ROS17/2.8 cells treated with 5 μM quercetin. The expression of GAPDH was also examined as control. The relative amounts of mRNA of BSP, Runx2, and Osterix to GAPDH were calculated. The experiments were performed in triplicate for each datapoint. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

not increased by quercetin. When we used the quercetin metabolite for LUC assays in ROS 17/2.8 cells, quercetin 3-glucuronide increased the transcriptional activity (pLUC3∼6; Fig. 4). Included within the DNA sequence that is unique in this region (pLUC3; nts −116 to −43), is an inverted CCAAT box (ATTGG; nts −50 and −46), a CRE (nts −75 and −68), a FGF2 response element (FRE; nts −92 and −85), and a pituitary-specific transcription factor-1 motif (Pit-1; nts −111 and −105), which is the target of parathyroid hormone stimulation, are present (Fig. 5). To determine more precisely the target sites in the BSP promoter through which the quercetin effect was being mediated, we prepared a series of 5′ deletion constructs between nts −280 and −43. The LUC activities of promoter constructs −60, −84, −108, −116, −280 BSPLUC were increased by quercetin (5 μM, 12 h), but no increase was seen in the constructs −43 BSPLUC (Fig. 3B). Next, we introduced 2 bp mutations in the putative response elements targeted by quercetin within nts −116 to −43 of pLUC 3 (M-CCAAT, M-CRE, M-FRE, M-Pit). Whereas mutations in the CRE (M-CRE) and Pit-1 (M-Pit) had no effect on the quercetin stimulation, mutation of the CCAAT (M-CCAAT) and FRE (M-FRE) significantly reduced the quercetin effect on the transcriptional activity. These results suggest that the inverted CCAAT and FRE are required as functional *cis*-elements for the upregulation of BSP transcription by quercetin (Fig. 6).

Gel Mobility Shift Assays

To identify nuclear proteins that bind to the inverted CCAAT and FRE, double-standard oligonucleotides were end-labeled and incubated with nuclear proteins (3 μg) extracted from confluent ROS 17/2.8 cells that were either not treated (control) or treated with 5 μM quercetin for 3, 6, and 12 h. When we used the inverted CCAAT sequence as a probe, the DNA-NF-Y protein complex [Kim and Sodek, 1999; Shimizu and Ogata, 2002] did not change after quercetin treatment (Fig. 7; lanes 1∼4). With nuclear extracts from confluent control cultures of ROS 17/2.8 cells, shifts of CRE-protein complex, FRE-protein complex, and 3′-FRE-protein complex were evident (Fig. 7; lanes 5, 9, 13). CRE binding proteins did not change after stimulation by quercetin (Fig. 7; lanes 5∼8). When the FRE and 3′-FRE were used as probes, the formation of FRE-protein complexes upregulated at 12 h (Fig. 7; lanes 9∼12), and rapidly migrating 3′-FRE-protein complexes

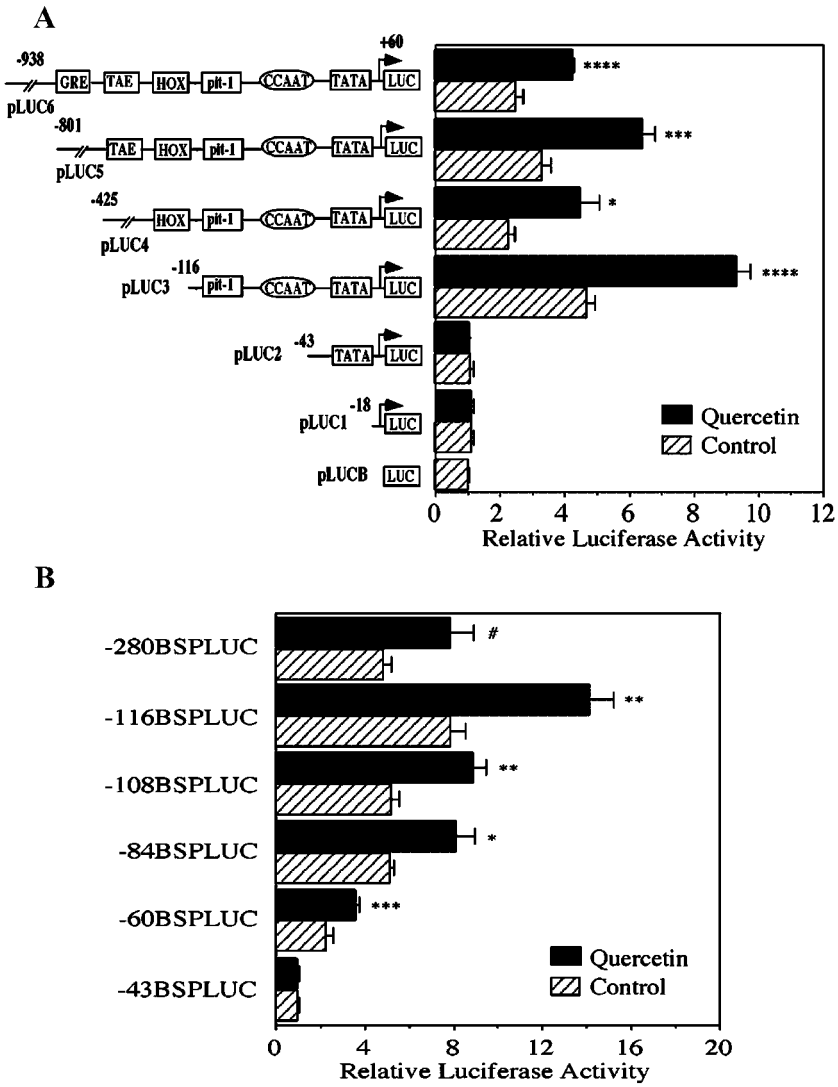

Fig. 3. Quercetin upregulated BSP promoter activities in ROS17/2.8 cells. **A**: Transient transfections of ROS17/2.8 cells in the presence or absence of quercetin (5 μM) for 12 h were used to determine the transcriptional activity of chimeric constructs that included various regions of the BSP promoter ligated to a LUC reporter gene. The results of transcriptional activity obtained from three separate transfections with constructs, pLUC basic (pLUCB) and pLUC1 to pLUC6, have been combined. **B**: Fine 5′ deletion mapping of the nt −280 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 induction in the presence or absence of quercetin (5 μM) for 12 h. The results of transcriptional activity obtained from three separate transfections with constructs, −43BSPLUC (−43 to +60), −60BSPLUC (−60 to +60), −84BSPLUC (−84 to +60), −108BSPLUC (−108 to +60), −116BSPLUC (−116 to +60), and −280BSPLUC (−280 to +60), have been combined. $^{\#}P < 0.2$; $^{*}P < 0.1$; $^{**}P < 0.05$; $^{***}P < 0.02$; $^{****}P < 0.01$.

were increased at 3 h by quercetin (Fig. 7; lanes 13∼16).

DISCUSSION

Quercetin is one of the most common flavonols [Murota et al., 2000]. It binds to estrogen receptors and influences the development of cell lines from several hormone-dependent cancers [Wattel et al., 2003]. Little is known about the action of quercetin on the skeleton. Quercetin is believed to play an important role in preventing bone loss by affecting osteoclastogenesis and regulating many systemic and local factors, including hormones and cytokines [Woo et al., 2004]. Flavonols, such as quercetin and kaempferol, decreased osteoclastic bone resorption by directly targeting the mature osteoclast by a mechanism involving at least in part via the estrogen receptor [Rassi et al., 2004; Wattel

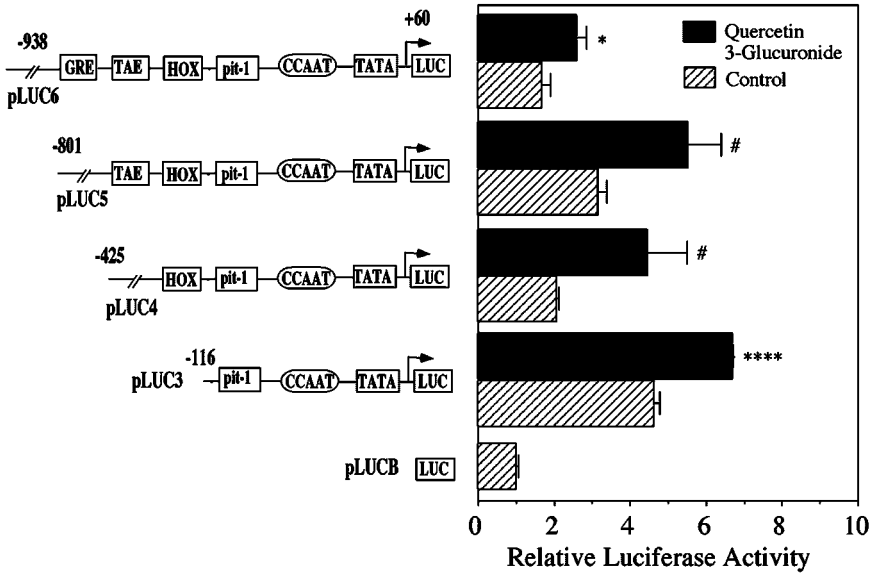

Fig. 4. Quercetin 3-glucuronide increased the BSP transcription in ROS17/2.8 cells. Transient transfections of ROS17/2.8 cells in the presence or absence of quercetin 3-glucuronide (5 μM) for 12 h were used to determine the transcriptional activity of chimeric constructs that included various regions of the BSP promoter ligated to a luciferase reporter gene. The results of transcriptional activity obtained from three separate transfections with constructs, pLUC basic (pLUCB) and pLUC3 to pLUC6, have been combined. $^{\#}P < 0.2$; $^{*}P < 0.1$; $^{****}P < 0.01$.

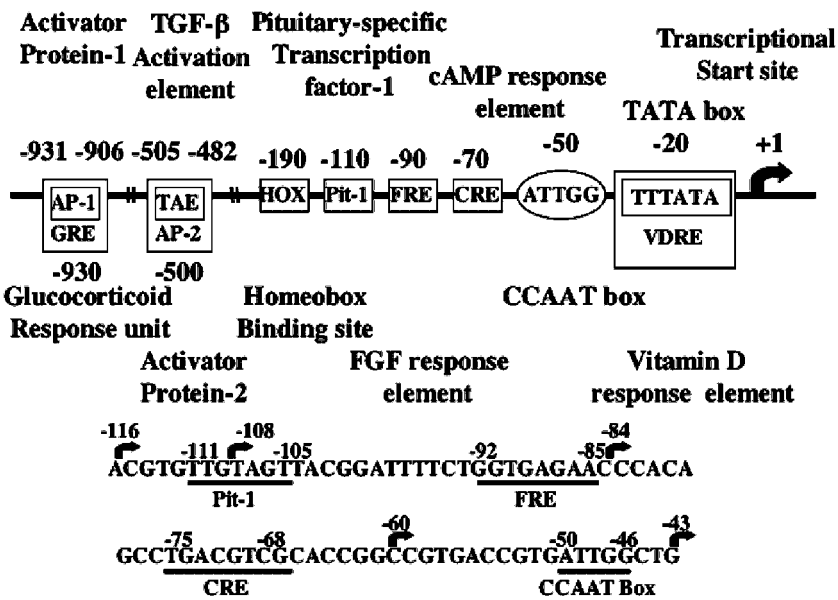

Fig. 5. Regulatory elements in the proximal rat BSP gene promoter. The position of the inverted TATA and CCAAT boxes, cAMP response element (CRE), FGF2 response element (FRE), Pit-1, homeobox-binding site (HOX), transforming growth factor-β activation element (TAE) overlapping with AP-2, glucocorticoid response element (GRE) overlapping with AP-1, and vitamin D response element (VDRE) overlaps with the inverted TATA box are shown in the proximal promoter region of the rat BSP gene. The numbering of the nucleotides is relative to the transcription start sites (+1). The nucleotide sequences of the proximal promoter region of the rat BSP gene are shown from nucleotides −116 to −43. An inverted CCAAT box (nts −50∼−46), a CRE (nts −75∼−68), a FRE (nts −92∼−85), and a Pit-1 (nts −111∼−105) are present.

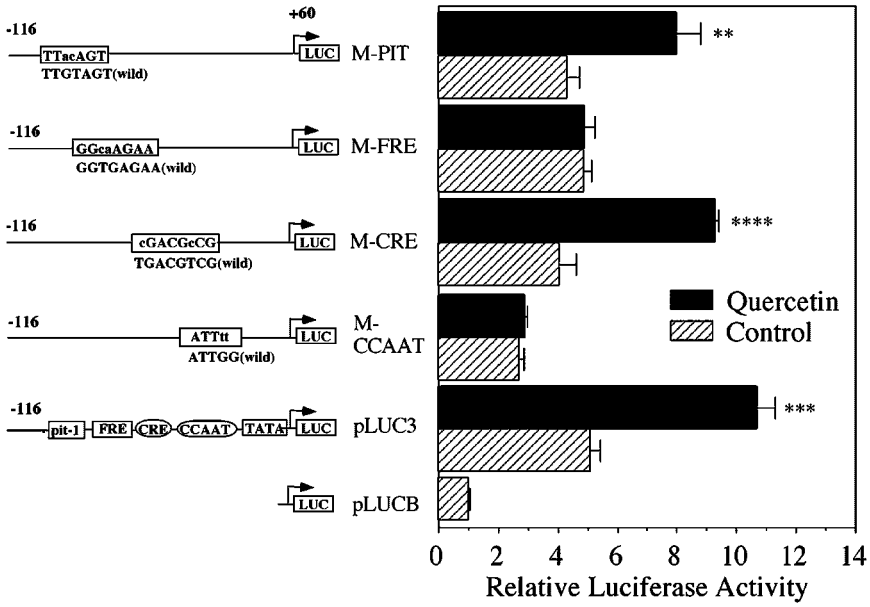

Fig. 6. Site mutation analysis of luciferase activities in response to quercetin. Dinucleotide substitutions were made within context of the homologous −116 to +60 (pLUC3) BSP promoter fragments. M-CCAAT (ATTtt), M-CRE (cGACGcCG), M-FRE (GGcaAGAA), and M-PIT (TTacAGT) were analyzed for relative promoter activity after transfection into ROS17/2.8 cells and examined for induction in the presence or absence of quercetin (5 μM) for 12 h. The results of transcriptional activity obtained from three separate transfections with constructs were combined. $^{**}P < 0.05$; $^{***}P < 0.02$; $^{****}P < 0.01$.

et al., 2004]. Quercetin and kaempferol have a stimulatory effect on the alkaline phosphatase activity and can rapidly activate ERK in osteoblasts via an estrogen receptor dependent pathway [Prouillet et al., 2004].

In this study, we show that quercetin and the quercetin-conjugated metabolite (quercetin 3-glucuronide) increases the BSP transcription in osteoblast-like cells by targeting inverted CCAAT and FRE elements in the proximal BSP gene promoter. This is the first report that quercetin metabolite (quercetin 3-glucuronide) has a positive effect on bone metabolism. Quercetin and quercetin 3-glucuronide

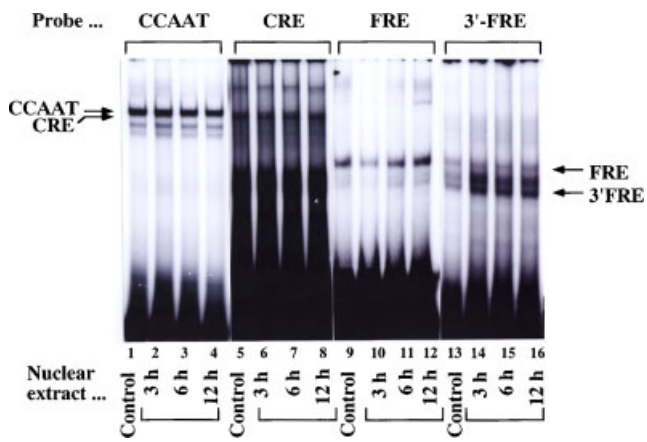

Fig. 7. Gel mobility shift assays using inverted CCAAT, CRE, FRE, and 3′-FRE. Radiolabeled double-stranded inverted CCAAT (−61 CCGTGACCGTGATTGGCTGCTGAGA −37), CRE (−84 CCCACAGAATGACGTCGCACCGGCCG −59), FRE (−98 TTTTCTGGTGAGAACCCACA −79), and 3′-FRE (−95 TCTGGTGAGAACCCACAGCCTGA −73) oligonucleotides were incubated for 20 min at 21°C with nuclear protein extracts (3 μg) obtained from ROS17/2.8 cells incubated without (**lanes 1**, **5**, **9**, **13**) or with quercetin at 5 μM for 3 h (**lanes 2**, **6**, **10**, and **14**), and 6 h (**lanes 3**, **7**, **11**, **15**) and 12 h (**lanes 4**, **8**, **12**, **16**). DNA-protein complexes were separated on 5% polyacrylamide gel in low-ionic-strength Tris-borate buffer, dried under vacuum, and exposed to an imaging plate for quantitation using an imaging analyzer. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

increased the BSP mRNA levels at 12 h in ROS17/2.8 cells (Fig. 2B,C). Furthermore, quercetin upregulated the mRNA expression of Cbfa1/Runx2 maximally at 12 h (Fig. 2D). From transient transfection assays we initially located the quercetin and quercetin 3-glucuronide responsive region to the proximal promoter (pLUC3; nts −116 to −43) of the BSP gene (Fig. 3A and 4), which encompasses an inverted CCAAT box (nts −50 to −46), a CRE (nts −75 to −68), a FRE (nucleotides −92 to −85), and a Pit-1 (nts −111 to −105) motif. Moreover, the results of LUC analyses using fine 5′ deletion constructs between nts −280 to −43 in the BSP promoter show that the quercetin effects are targeted to a region encompassed by nts −60 and −43 (Fig. 3B). Within this region of the promoter, the only recognizable consensus sequence is an inverted CCAAT box (Fig. 5). Dinucleotide substitutions of the inverted CCAAT box (M-CCAAT) and FRE (M-FRE) showed abrogation of the quercetin induced BSP transcription (Fig. 6). The results indicated that CCAAT and FRE elements are required for the induction of BSP transcription by quercetin. The inverted CCAAT sequence is conserved in the rat, mouse, and human BSP gene promoter [Li and Sodek, 1993; Kim et al., 1994; Benson et al., 1999] and present at a similar location in the osteopontin (OPN) promoter [Tezuka et al., 1996]. Transcription of the BSP and OPN genes is induced by v-Src through an inverted CCAAT box [Tezuka et al., 1996; Kim and Sodek, 1999], which is bound with NF-Y transcription factor [Shimizu and Ogata, 2002]. It is essential for the expression of the class II genes of the major histocompatibility complex and is involved in the regulation of albumin, α1(I) and α2(I) collagen genes [Maity et al., 1992; Caretti et al., 1999]. The promoters of several cell cycle regulatory genes, such as cyclin A, cyclin B1, cyclin B2, cdk1, and cdc25C, contain CCAAT boxes, suggesting that NF-Y is a key regulator of the cell cycle [Manni et al., 2001]. Results of gel shift assays showed CCAAT-NF-Y protein complex did not change after stimulation by quercetin (Fig. 7). Therefore, it is likely that quercetin treatment results in modifications of NF-Y that influence its transactivation properties, but these changes do not affect the binding activities of NF-Y. The involvement of the FRE element is further supported by gel shift analyses, in which the nuclear proteins that formed complex with FRE were increased by quercetin (Fig. 7). In our previous paper [Shimizu et al., 2006; Nakayama et al., 2006], we showed the possible binding proteins in FRE are Runx2, Smad1, and Dlx5. The binding activities of nuclear proteins to 3′-FRE were also increased by quercetin (Fig. 7). We previously identified the FRE (GGTGAGAA) in the rat BSP gene promoter which is juxtaposed to a putative Cbfa1/Runx2 binding site (CCCACA) [Shimizu-Sasaki et al., 2001; Nakayama et al., 2006]. 3′-FRE has a Cbfa1/Runx2 motif in the middle of the sequence and migrates more rapidly than the FRE-protein complexes. Whereas the formation of FRE-protein complexes was upregulated at 12 h, the 3′-FRE-protein complexes that might have contained Runx2 transcription factor were increased by quercetin at 3 h. These results confirmed the results of real-time PCR, in which quercetin increased the BSP and Runx2 mRNA levels at 3 h, and the mRNA levels were raised maximal at 12 h.

In conclusion, we have shown the inverted CCAAT and FRE elements in the rat BSP proximal promoter through which the stimulatory effects of quercetin on BSP gene transcription are mediated. Moreover, the CCAAT binding NF-Y and 3′-FRE binding Cbfa1/Runx2 transcription factors appear to be key regulators of the quercetin effects on BSP transcription and bone metabolism.

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