Journal of Cellular Biochemistry 86:35–44 (2002)

ARTICLES

Activation of Bone Sialoprotein Gene Transcription by Flavonoids Is Mediated Through an Inverted CCAAT Box in ROS 17/2.8 Cells

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Abstract Bone sialoprotein (BSP) is a major noncollagenous protein of the mineralized bone extracellular matrix that has been implicated in the nucleation of hydroxyapatite. Recent studies have shown that BSP is also expressed by osteotropic cancers suggesting BSP might play a role in the pathogenesis of bone metastases. The present study investigates regulation of BSP transcription in rat osteosarcoma ROS 17/2.8 cells by flavonoids: genistein (an inhibitor of protein tyrosine kinases), daidzein (an inactive compound of genistein), flavone, and flavanone. Genistein, daidzein, and flavone (50 μM) increased steady state levels of BSP mRNA about 1.7-fold at 12 h. From transient transfection assays using various sized BSP promoter-luciferase constructs, genistein increased luciferase activities within 12 h. Constructs including the promoter sequence nucleotides (nts) −116 to −43 (pLUC3) were found to enhance transcriptional activity ~2.6-fold in ROS 17/2.8 cells treated with genistein (50 μM). Daidzein, flavone, and flavanone (50 μM) also increased luciferase activities. In contrast, the tyrosine kinase inhibitors, herbimycin A and lavendustin A, which do not have a flavonoid structure, did not stimulate BSP transcription. Transcriptional stimulation by genistein was almost completely abrogated in a construct that included 2 bp mutations in the inverted CCAAT box. A monoclonal antibody against NF-YA, a CCAAT box-binding transcription factor, inhibited formation of DNA-NF-Y protein complex in gel shift assays formed by nuclear extracts of ROS 17/2.8 cells. These data suggest that the inverted CCAAT box is required for flavonoid-induced BSP expression and that the stimulatory action is dependent on the flavone structure and does not involve an inhibitory action on protein tyrosine kinase. J. Cell. Biochem. 86: 35–44, 2002.

Key words: bone sialoprotein; flavonoid; gene regulation; mineralized tissue; CCAAT box; transcription

Flavonoids, which are found in many plants, including edible fruits and vegetables [Gerritsen et al., 1995; Bai et al., 1998], have been studied extensively for their various pharmacological activities [Manna et al., 1999; Niisato et al., 1999]. In either natural or synthetic forms, flavonoids, are known to exhibit various biological activities including anti-oxidant, anti-

Abbreviations used: ER, estrogen receptor; PTK, protein tyrosine kinases; BSP, bone sialoprotein; FBS, fetal bovine serum; MEM, minimum essential medium; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; OPN, osteopontin; FGF, fibroblast growth factor; FRE, FGF response element; bp, base pair(s); nts, nucleotides; Pit-1, pituitary-specific transcription factor-1; LUC, luciferase; CRE, cAMP response element.

Grant sponsor: Ministry of Education, Science, and Culture of Japan (Grants-in-aid for Scientific Research); Grant numbers: 07771795, 08771738, 09771650, 12671865; Grant sponsor: Nihon University Research Grant (Joint Research Grant for 2001, and General Individual Research Grant for 1997 and 2001); Grant sponsor: Suzuki Memorial Grant of Nihon University School of Dentistry at Matsudo (Joint Research Grant for 1998 and 2000, and General Individual Research Grant for 1998, 1999, and 2000); Grant sponsor: Research for the Frontier Science (The Ministry of Education, Science, Sports, and Culture); Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology to promote 2001-Multidisciplinary Research Project (in 2001–2005).

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Received 24 August 2001; Accepted 7 March 2002
DOI 10.1002/jcb.10194

inflammation, gastroprotective, anti-viral, anti-mutagenic, topoisomerases II inhibition, protein kinase C inhibition, and cytotoxic activities [Akama et al., 1996; Manna et al., 1999]. Since some flavonoid derivatives are known to exhibit anti-proliferative effects against breast cancer cells and can alter binding affinities for the estrogen receptor (ER) [Akama et al., 1996; Shao et al., 2000], they may have anti-cancer activities. For example, genistein has been shown to arrest cell cycle progression at the G_2-M stage in a human gastric cancer cell line HGC-27 [Matsukawa et al., 1993], while flavone inhibits cell growth at G_1 in a A549 lung adenocarcinoma cells [Bai et al., 1998]. Effects on ER binding affinity and estrogenic activity have been reported for the isoflavone derivatives genistein, and daidzein [Cheng et al., 1954]. Genistein, a specific inhibitor of protein tyrosine kinases (PTK) found in soy, has been postulated to be responsible for lowering the rate of breast cancer in Asian woman [Shao et al., 2000]. Recent studies also suggest that genistein may prevent the loss of bone mass in ovariectomized rats [Anderson and Garner, 1998], while a synthetic analogue, ipriflavone, is known to be effective in inhibiting bone resorption in post-menopausal osteoporosis, although it may not directly interact with estrogen receptors [Petilli et al., 1995]. In addition, ipriflavone may affect osteoblasts directly and stimulate osteoblastic cell growth and differentiation [Benvenuti et al., 1991].

Bone sialoprotein (BSP) is a mineralized tissue-specific 34-kDa protein that is heavily glycosylated, phosphorylated, and sulfated [Oldberg et al., 1988; 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., 1991, 1992]. BSP is also expressed in breast, lung, thyroid, and prostate cancers [Bellahcene 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 crystals and to mediate cell attachment through cell-surface integrins [Bellahcene et al., 1994; Waltregny et al., 2000; Shimizu-Sasaki et al., 2001]. Thus, regulation of the BSP gene is potentially important in the differentiation of osteoblasts, in bone matrix mineralization, and in tumor metastasis. Since flavonoids influence these processes, we have analyzed their effects on BSP expression. These studies show that flavonoids have structure-dependent stimulatory effects on the expression of BSP in ROS 17/2.8 cells and exert their effects via an inverted CCAAT box in the BSP promoter.

MATERIALS AND METHODS

Materials

Cell culture media, fetal bovine serum (FBS), LipofectACE, penicillin, streptomycin, SuperScript one step RT-PCR with Platinum Taq, and trypsin were obtained from GIBCO BRL Life Technologies (Tokyo, Japan). The pSV-β-Galactosidase control vector was purchased from Promega Co. (Madison, WI). Genistein, flavone, herbimycin A, and guanidium thiocyanate were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Flavanone was purchased from extrasynthese (Genay, France). Daidzein was purchased from Fujikko Co. Ltd. (Tokyo, Japan). Lavendustin A was purchased from Calbiochem (San Diego, CA).

Cell Cultures

The rat clonal cell lines, ROS 17/2.8, was used in these studies as an osteoblastic cell line that synthesizes BSP [Majeska et al., 1980; Shimizu-Sasaki et al., 2001]. Cells were first grown to confluence in 60 mm tissue culture dishes in α-MEM medium containing 10% FBS, then cultured in α-MEM without serum and incubated with genistein, daidzein, and flavone (50 μM) for 12 h. RNA was isolated from triplicate cultures and analyzed for the expression of BSP mRNA by RT-PCR and Northern hybridization as described below.

RT-PCR

Total RNA (1 μg) was extracted with guanidium thiocyanate described previously [Ogata et al., 1997] and used as a template for cDNA synthesis. cDNA was prepared by use of a SuperScript one step RT-PCR kit according to the supplier's protocol. Primers were synthesized on the basis of the reported rat cDNA sequence for BSP, osteopontin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Sequences of the primers used for PCR were as follows: BSP forward, 5′-CTGCTTTAATCTTGCTCTG-3′; BSP reverse, 5′-CCATCTCCATTTTCTTCC-3′; osteopontin forward, 5′-GTTTGCCTTT-GCCTGTTC-3′; osteopontin reverse, 5′-ATC-

GTCGTCGTCATCATC-3′; GAPDH forward, 5′-CCATGTTTGTGATGGGTGTG-3′; GAPDH reverse, 5′-GGATGCAGGGATGATGTTCT-3′. Amplification was carried out for 30 (GAPDH)-35 (BSP and osteopontin) cycles under saturation, each at 94°C, 30 sec; 55°C, 30 sec; 72°C, 30 sec in a 50 μl reaction mixture. After amplification, 10 μl of each reaction mixture was analyzed by 2% agarose gel electrophoresis, and the bands were then visualized by ethidium bromide staining. The PCR products for BSP, osteopontin, and GAPDH were 211, 261, and 264 bps, respectively.

Northern Hybridization

Twenty microgram aliquots of total RNA were fractionated on 1.2% agarose gel and transferred onto a Hybond XL membrane, as described previously [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 in a Bio-imaging analyzer (BAS2000, Fuji BAS 2000).

Transient Transfection Assays

Exponentially growing ROS 17/2.8 cells were used for transfection assays. Twenty-four hours after plating, cells at 50–70% confluence were transfected using a LipofectACE reagent. The transfection mixture 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, cells were deprived of serum for 12 h, each test compound was added, and the cells cultured a further 12 h prior to harvesting. The luciferase assay was performed according to the supplier's protocol (picaGene, Toyo Inki, Tokyo, Japan) using a Luminescence reader BLR201 (Aloka) to measure the luciferase activity.

Gel Mobility Shift Assays

Confluent ROS 17/2.8 cells in T-75 flasks incubated for 12 h with genistein, daidzein, herbymycin A, and flavone in α-MEM without serum were used to prepare nuclear extracts as we have described previously [Ogata et al., 1997, 2000; Shimizu-Sasaki et al., 2001]. Double-stranded oligonucleotides encompassing the inverted CCAAT (nts −61 to −37, 5′-CCGTGACCGTG**ATTGG**CTGCTGAGA) in the BSP promoter were prepared by Bio-Synthesis, Inc. (Lewisville, TX). For gel shift analysis, the double-stranded-oligonucleotides were end-labeled with [γ-32P]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-stranded-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% nondenaturing acrylamide gels (38:2 acrylamide/bis acrylamide) run at 150 V at RT. Following electrophoresis, the gels were dried and autoradiograms prepared and analyzed using an image analyzer. Supershift assays were performed using an anti-NF-YA monoclonal antibody (Pharmingen, SanDiego, CA). Antibody was added to the reaction mixture and incubated for 3 h at 4°C before electrophoresis was performed under the same conditions as described above.

Statistical Analysis

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

RESULTS

Stimulation of BSP mRNA Expression in ROS 17/2.8 Cells

The structures of genistein, daidzein, flavone, and flavanone are compared in Figure 1. Genistein and daidzein are composed of isoflavone. Flavanone is a reduction product of flavone. All these compounds are flavonoids. In contrast, herbimycin A and lavandustin A (structures not shown), lack the flavone group and, therefore, are not considered to be flavonoids. Genistein is an inhibitor of PTK, while daidzein is an inactive form of genistein. Herbimycin A and

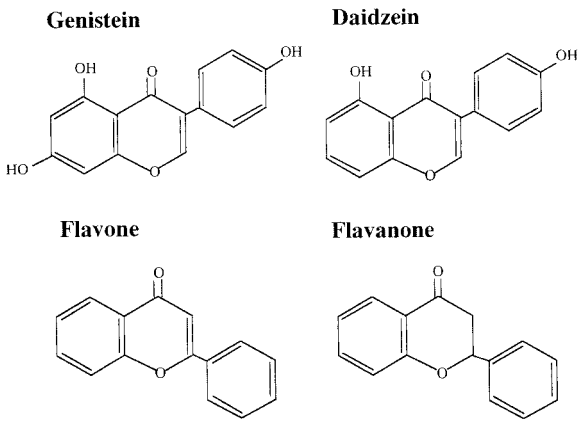

Fig. 1. Structure formulas of genistein, daidzein, flavone, and flavanone. Genistein and daidzein contain isoflavone. Flavone and flavanone contain flavone structure.

lavandustin A are also inhibitors of PTK. When osteoblastic ROS 17/2.8 cells were exposed to 50 μM genistein, daidzein or flavone for 12 h, expression of BSP mRNA was increased 1.6-, 1.7-, 1.7-fold and osteopontin (OPN) mRNA was increased 1.4-, 1.9-, 1.6-fold, respectively by RT-PCR (Fig. 2A). Results of Northern hybridization showed that the treatments with 50 μM genistein, daidzein, and flavone for 12 h increased BSP mRNA expression 1.7-, 2.3-, 1.7-fold, respectively (Fig. 2B).

That these effects are specific, is indicated by the lack of an effect on the mRNA levels for GAPDH. Because the effects of genistein likely involve transcription factor interaction in the promoter region of the rat BSP gene, subsequent studies were directed at transcriptional regulation using chimeric constructs prepared from the rat BSP gene promoter.

Transient Transfection Analysis of Rat BSP Promoter Constructs

Transient transfection of chimeric constructs encompassing different regions of the rat BSP promoter ligated to a luciferase reporter gene (pLUC3–7), were performed in ROS 17/2.8 cells. Results of transfection assays (Fig. 3A) indicated an ~2.6-fold increase in transcription after 12 h treatment by 50 μM genistein using the construct, pLUC3, which encompasses nucleotides −116 to +60, as well as in longer constructs. In shorter constructs (pLUC1; −18 to +60, pLUC2; −43 to +60), luciferase activities were not increased by genistein (data not shown). Included within the DNA sequence

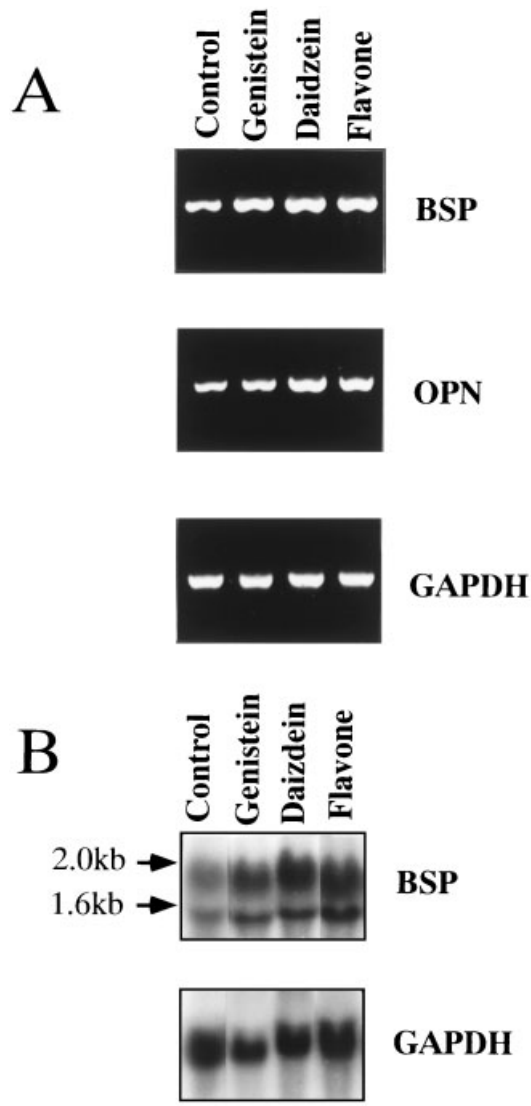

Fig. 2. Effects of genistein, daidzein, and flavone on BSP expression in ROS 17/2.8 cells. **A**: Cells were treated with genistein, daidzein, and flavone (50 μM) for 12 h. Then, total RNA was extracted, and expression of BSP, OPN, and GAPDH mRNA in the cells was analyzed by RT-PCR. Results of a representative RT-PCR analysis for Control and flavonoids-treated cells are shown. **B**: Northern hybridization analysis of flavonoids effect on BSP mRNA expression. Total RNA was isolated from triplicate cultures harvested after 12 h and used for Northern hybridization analysis.

that is unique in this region (pLUC3; between nts −116 to −43), is an inverted CCAAT box (ATTGG; between nts −50 and −46), a putative cAMP response element (CRE; between nts −75 and −68), a fibroblast growth factor 2 response element (FRE, between nts −92 and −85), and a pituitary-specific transcription factor-1 (Pit-1) motif (between nts −111 and −105), which is

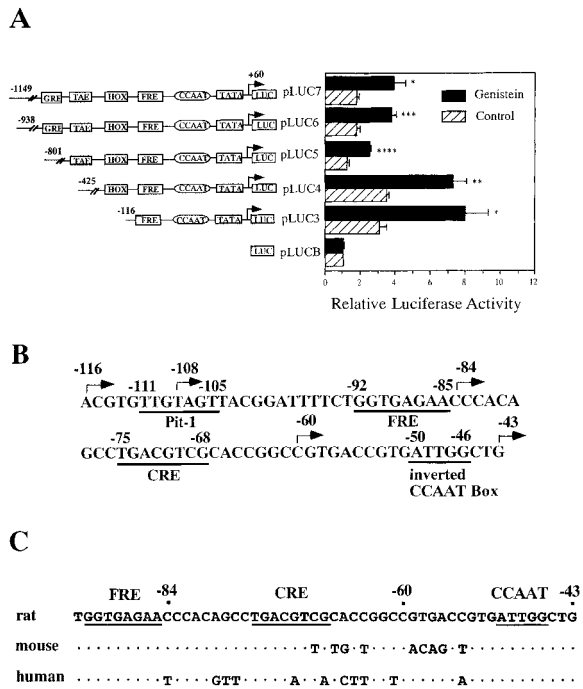

Fig. 3. **A**: Genistein upregulates BSP promoter activity. Transient transfections of ROS 17/2.8 cells, in the presence or absence of genistein (50 μM) for 12 h, were used to determine transcriptional activity of chimeric constructs that included various regions of the BSP promoter ligated to a luciferase reporter gene. The results of transcriptional activity obtained from three separate transfections with constructs; pLUC basic (pLUCB) and pLUC3 to pLUC7 have been combined and the values expressed with standard errors. Significant differences from control: *($P<0.1$); **($P<0.05$); ***($P<0.02$); ****($P<0.01$). **B**: The nucleotide sequences of the rat BSP gene proximal promoter is shown from nts −116 to −43. The inverted CCAAT box, cAMP response element (CRE), FGF response element (FRE), and pituitary-specific transcription factor-1 binding site (Pit-1) are present. **C**: Comparison of rat, mouse, and human DNA sequences from nucleotide −93 to −43. The rat sequence is shown on the top line and the mouse and human sequence below. DNA sequences that are identical between the species are shown by a dot.

the target of parathyroid hormone stimulation, are present (Fig. 3B). To determine whether the stimulatory effects of genistein on BSP transcription are dependent on its inhibitory action of tyrosine phosphorylation, we used daidzein (an inactive form of genistein), and other non-flavonoid PTK inhibitors, herbimycin A and lavendustin A. Whereas, daidzein stimulated pLUC3 promoter activity ~1.8-fold, neither herbimycin A nor lavendustin A stimulated pLUC3 activity (Fig. 4). To test whether the structure of flavonoids is important for the effects of genistein, we used flavone and flavanone in transient transfection assays using

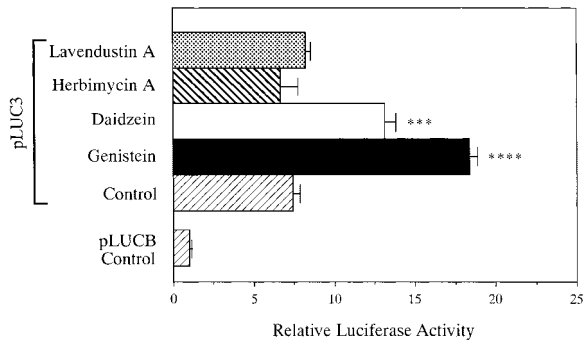

Fig. 4. Stimulatory effect of daidzein on BSP transcription is not mediated through PTK inhibitory activity. PLUC3 (−116 to +60) luciferase construct was analyzed for relative promoter activity after transfection into ROS17/2.8 cells and examined for induction in the presence of daidzein, herbimycin A, and lavandustin A (50 μM) for 12 h. The results of transcriptional activity obtained from three separate transfections have been combined and the values expressed with standard errors. Significant differences from control: ***($P<0.02$); ****($P<0.01$).

pLUC3. Flavone and flavanone (50 μM) stimulated pLUC3 activity 2.4- and 1.9-fold, respectively (Fig. 5). To determine more precisely the target site in the BSP promoter through which the flavonoid effects were being mediated, we prepared a series of 5′ deletion constructs between nts −116 and −43. Using these constructs in transient transfection assays we found that the genistein response was mediated by a region between nts −60 and −43 of the promoter sequence (Fig. 6). The functional site

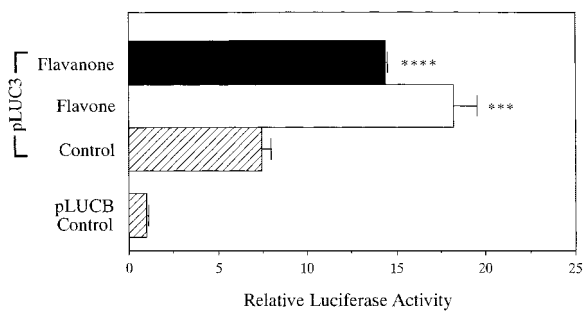

Fig. 5. Flavone and flavanone stimulate BSP promoter activity. Transient transfections of ROS 17/2.8 cells, in the presence or absence of flavone and flavanone (50 μM) for 12 h, were used to determine transcriptional activity of pLUC3 construct that included −116 to +60 of the rat BSP gene promoter. The results of transcriptional activity were obtained from three separate transfections with constructs; pLUCB and pLUC3 have been combined and the values expressed with standard errors. Significant differences from control: ***($P<0.02$); ****($P<0.01$).

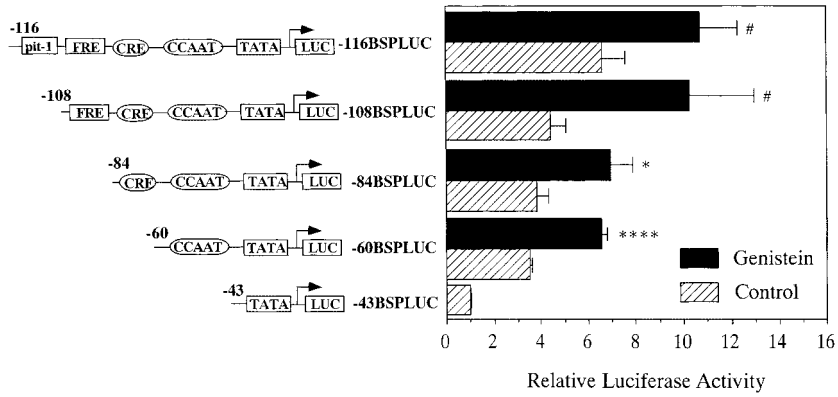

Fig. 6. Fine 5′-deletion mapping of the nts −116 to −43 element in the BSP promoter. A series of rat BSP promoter 5′ deletion constructs were analyzed for relative promoter activity after transfection into ROS17/2.8 cells and examined for induction in the presence of genistein (50 μM). The results of transcriptional activity obtained from three separate transfections with constructs; −43 BSPLUC (−43 to +60), −60 BSPLUC (−60 to +60), −84 BSPLUC (−84 to +60), −108 BSPLUC (−108 to +60), and −116 BSPLUC (−116 to +60) have been combined and the values expressed with standard errors. Significant differences from control: $^{\#}(P<0.2)$; $^{*}(P<0.1)$; $^{****}(P<0.01)$.

was then determined by preparing 2-bp mutations in the inverted CCAAT, CRE, and FRE elements that are present within pLUC3 construct. Three constructs (M-CCAAT, M-CRE, and M-FRE) had lower basal activities than pLUC3 (Fig. 7), while transcriptional stimulation by genistein (50 μM) was almost completely abrogated in the M-CCAAT construct. Conversely, mutations in CRE (M-CRE) and FRE (M-FRE) increased the genistein-induced luciferase activities ∼1.9 and ∼1.7-fold, respectively, of the value for the control (Fig. 7).

Gel Mobility Shift Assays

Nuclear factor-Y (NF-Y) is a principal nuclear factor that bound to the inverted CCAAT box [Tezuka et al., 1996; Kim and Sodek, 1999]. One mechanism whereby flavonoids could increase transcription would be to increase binding of NF-Y to the CCAAT sequence. To test this possibility, ROS 17/2.8 cells were treated for 12 h with 50 μM genistein, daidzein, flavone or flavanone; nuclear extracts were prepared for assessment of binding activity by gel shift assay.

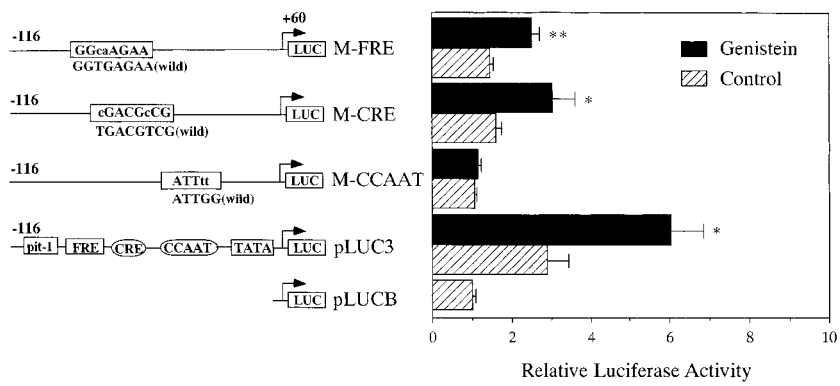

Fig. 7. Inverted CCAAT box (ATTGG motif) at nts −50 to −46 in the rat BSP promoter is necessary for induction by genistein. Dinucleotide substitutions were made within context of the homologous −116 to +60 BSP promoter fragment (pLUC3). M-CCAAT (ATT**tt**), M-CRE (**c**GACG**c**CG), M-FRE (GG**ca**AGAA) constructs were analyzed for relative promoter activity after transfection into ROS17/2.8 cells and examined for induction in the presence of genistein (50 μM) for 12 h. The results of transcriptional activity were obtained from three separate transfections with constructs; pLUCB, pLUC3 and M-CCAAT, M-CRE, M-FRE were combined and the values expressed with standard errors. Significant differences from relative luciferase activity of pLUC3: $^{*}(P<0.1)$; $^{**}(P<0,05)$.

When the inverted CCAAT was used as a probe, the DNA-NF-Y protein complex was unaffected after stimulation by genistein, daidzein, flavone or flavanone (Fig. 8, lanes 2–4). That the DNA–protein complex represents a specific interaction was indicated by competition experiments in which an excess of CCAAT reduced the amount of complex formed in a dose-dependent manner (20-, 40-, 100-fold molar excess) (Fig. 8, lanes 6–8). In contrast, the CCAAT mutation (mCCAAT; 20-, 40-, 100-fold molar excess) (Fig. 8, lanes 9–11) did not compete with complex formation. To further characterize the protein in the complex formed with the inverted CCAAT box, nuclear extracts were preincubated with antibody to NF-YA which blocked complex formation (Fig. 8, lanes 13–15) [Mantovani et al., 1992].

DISCUSSION

These studies have shown that flavonoids increase expression of the BSP gene in osteoblastic ROS 17/2.8 cells through an inverted CCAAT box in the BSP gene promoter. While flavonoids increased BSP mRNA level in ROS 17/2.8 cells, they suppressed BSP expression in MC3T3E1 and UMR106 cells [E. Shimizu and Y. Ogata, unpublished results]. These opposite results may be caused by different characteristics of these cell lines, since MC3T3E1 and UMR106 cells possess much younger differentiation stage than ROS 17/2.8 cells [Ogata et al., 1995].

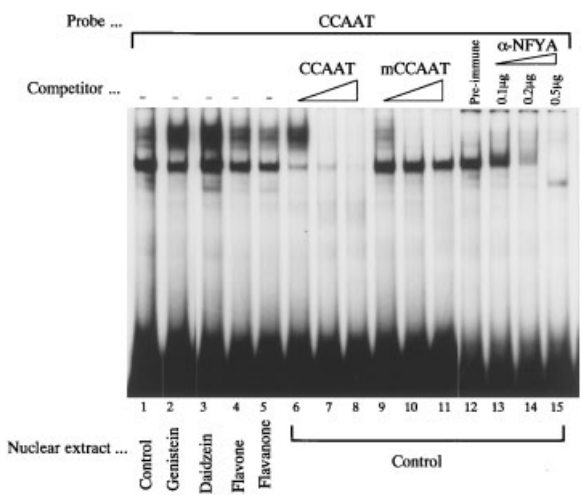

Fig. 8. Specific binding of a nuclear protein to the inverted CCAAT. Radiolabeled double-stranded CCAAT oligonucleotide (−61 CCGTGACCGTG**ATTGG**CTGCTGAGA −37) were incubated with nuclear protein extracts (3 μg) obtained from ROS 17/2.8 cells incubated without (**lane 1**) or with genistein (50 μM, **lane 2**), daidzein (50 μM, **lane 3**), flavone (50 μM, **lane 4**), and flavanone (50 μM, **lane 5**) for 12 h. Competition reactions were performed using a 20-, 40-, and 100-fold molar excess of unlabeled CCAAT (**lanes 6–8**) and mutation CCAAT (CCGTGACCGTG**ATTtt**CTGCTGAGA; **lanes 9–11**). Nuclear extract from ROS 17/2.8 cells (3 μg) was used to bind to CCAAT probe with pre-immune serum (**lane 12**) and 0.1, 0.2, and 0.5 μg of monoclonal antibody (α-NF-YA) that recognizes the A subunit of NF-Y (**lanes 13–15**).

Flavonoids are widely found in vegetables and fruits [Gerritsen et al., 1995; Bai et al., 1998]. Many types of flavonoids prevent or inhibit cancer development in vivo and inhibit cell growth in vitro [Matsukawa et al., 1993; Akama et al., 1996; Manna et al., 1999; Shao et al., 2000]. Many derivatives of flavone, which has the basic flavonoid structure, have been reported to have strong anti-cancer effects in human breast cancer cells. Thus, flavone is a potent inhibitor of proliferation of H-29 human colon carcinoma cells. It restores apoptosis sensitivity and differentiation, and changes gene expression in the H-29 cells [Wenzel et al., 2000]. Quercetin (another flavonoid) exhibits anti-tumor properties, possibly through its ability to stimulate the immune system, induce apoptosis, alter the mitotic cycle, scavenge free radicals, modify gene expression, and block angiogenesis [Hayashi et al., 2000]. Genistein, an isoflavone, exhibits anti-proliferative activity in the presence of estrogen in MCF-7 estrogen-responsive human breast cancer cell line [Shao et al., 2000] and abolishes the estradiol stimulatory effect on erk-2 activity and cell proliferation in human colon carcinoma-derived Caco-2 cells [Domenico et al., 1996]. These observations suggest that the effect of genistein on BSP transcription likely involves estrogenic activity mediated through estrogen receptors. There are two estrogen receptors: the classical estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) [Kuiper et al., 1997]. Both receptors are expressed in bone tissue, primary cultured osteoblasts, and in ROS 17/2.8 cells. In ROS 17/2.8 cells, expression of ERβ is higher than the ERα [Onoe et al., 1997]. ER binding affinity and estrogenic activity have been reported for the isoflavone derivatives, genistein, and daidzein [Cheng et al., 1954], although they bind to the ERα with much lower affinities than 17β-estradiol [Miksicek, 1994]. Since genistein has a higher affinity for ERβ than for ERα [Onoe et al., 1997], these

data suggest that in osteoblasts flavonoids act mainly through the ERβ. However, 17β-estradiol (10 nM) does not upregulate BSP promoter (pLUC3) activity (data not shown) and there is no estrogen response element in the proximal promoter of the BSP gene (Fig. 3B), suggesting that the regulation of BSP transcription by flavonoids is independent of their estrogenic activity.

The results of luciferase analyses using fine 5′-deletion constructs between nts −116 to −43 in the BSP promoter show that the target for flavonoids is present within nts −60 and −43 (Fig. 6). Within this region of the promoter, the only recognizable consensus sequence is an inverted CCAAT box (nts −50 and −46). Dinucleotide substitutions of the inverted CCAAT box (M-CCAAT; Fig. 7) showed almost complete abrogation of genistein induced BSP transcription. Also, double nucleotide mutations in the CCAAT oligonucleotide produced in mCCAAT (corresponding to mutation in M-CCAAT in pLUC3 construct) eliminated its ability to compete for complex formation (Fig. 8, lanes 9–11). These results suggest that the flavonoids upregulate BSP gene expression via the inverted CCAAT box. Notably, the CCAAT sequence is conserved in the rat, mouse, and human BSP promoters [Kerr et al., 1993; Li and Sodek, 1993; Kim et al., 1994; Benson et al., 1999] and is also present at a similar location in the OPN promoter [Tezuka et al., 1996], indicative of a potentially important role in transcriptional regulation of the BSP (Fig. 3C) and the OPN genes. Transcription of the BSP and OPN genes is stimulated by v-Src through an inverted CCAAT box [Tezuka et al., 1996; Kim and Sodek, 1999], which is bound with high affinity by an ubiquitous trimeric complex, NF-Y transcription factor. This complex is composed of three conserved subunits, NF-YA, NF-YB, and NF-YC [Li et al., 1992; Caretti et al., 1999], all of which are required for DNA binding. It is essential for expression of the class II genes of the major histocompatibility complex (MHC) and is likely involved in the regulation of albumin, α1(I), and α2(I)collagen genes [Maity et al., 1992; Caretti et al., 1999]. NF-Y is also required for cyclin B1 transcription, and that the switch-off of cyclin B1 expression in terminally differentiated skeletal muscle cells depends upon the loss of a functional NF-Y complex [Manni et al., 2001]. That the promoters of several cell cycle regulatory genes such as cyclin A, cyclin B1, cyclin B2, cdk1, and cdc25C contain CCAAT boxes suggests NF-Y is a key regulator of cell cycle [Manni et al., 2001]. However, the mechanism of NF-Y mediated gene transcription regulation is not well understood. In gel shift assays, we could not see any differences in the DNA-NF-Y protein complex formed with the inverted CCAAT sequence obtained from nuclear extracts of cells treated or not with flavonoids (Fig. 8). Therefore, it is likely that flavonoid treatment results in modifications of NF-Y that influence its transactivation properties, but these changes do not affect the binding of the DNA-NF-Y protein complex to the CCAAT element.

Ipriflavone prevents bone loss in several experimental models of osteoporosis [Yamazaki et al., 1986; Aloysio et al., 1997]. Studies have shown that it can induce the differentiation of human bone marrow stromal osteoprogenitor cells and stimulate the expression of α1(I)collagen, BSP and decorin transcription in differentiated human osteoblasts [Cheng et al., 1994]. Further, genistein improves bone mass in the ovariectomized rat [Anderson et al., 1998]. Since NF-Y mediates cell-cycle-dependent enzymes [Manni et al., 2001], and the transcription of BSP, NF-Y may be a key regulator of the anti-proliferative effects of flavonoids and their effects on bone formation.

PTK inhibitors herbimycin A and lavendustin A, which do not contain a structure flavonoids, had no stimulatory effect on BSP transcription indicating that the stimulatory effect of flavonoids on BSP expression is dependent on the flavonoid structure. Signaling pathways underlying flavonoid effects on the BSP transcription are unclear but are thought to be linked to Src or MAP kinases. Fibroblast growth factor 2 (FGF2) stimulates BSP transcription via Src and MAP kinases in the signaling pathway [Shimizu-Sasaki et al., 2001]. Thus, when we use genistein before treatment of FGF2 in luciferase assays, the combination of genistein and FGF2 synergistically upregulated pLUC3 promoter activity (data not shown). The results suggest Src tyrosine kinase or MAP kinase are involved in transmitting the flavonoid effects in the cell. Flavonoids upregulate BSP and OPN transcription and also stimulate α1(I) collagen and decorin expression [Cheng et al., 1994]. These effects are mediated by NF-Y transcription factor, suggesting NF-Y is an important mediator in the regulation of bone metabolism.

In summary, we have shown that an inverted CCAAT box exists in the rat BSP proximal promoter through which the stimulatory effects of flavonoids on BSP gene transcription are mediated. Moreover, the CCAAT binding NF-Y transcription factor appears to be a key regulator of the flavonoid activity on BSP transcription.

ACKNOWLEDGMENTS

We thank Dr. Jaro Sodek for critical reading of the manuscript and helpful discussions; Dr. Y. Marunaka and Dr. N. Niisato for advice with experiments; and Dr. M. Yamazaki, Dr. S. Furuyama, and Dr. H. Sugiya for their encouragement and support.

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