Prostaglandin E2 Stimulates Bone Sialoprotein (BSP) Expression through cAMP and FGF2 Response Elements in the Proximal Promoter of the Rat BSP Gene

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SUMMARY

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Bone sialoprotein (BSP), an early marker of osteoblast differentiation, has been implicated in the nucleation of hydroxyapatite during de novo bone formation. Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) has anabolic effects on proliferation and differentiation of osteoblasts via diverse signal transduction systems. Since PGE<sub>2</sub> increases the proportion of functional osteoblasts in fetal rat calvarial cell cultures, we investigated the regulation of BSP, as an osteoblastic marker, by PGE<sub>2</sub>. Treatment of rat osteosarcoma UMR 106 cells with 3 µM, 300nM and 30 nM PGE<sub>2</sub> increased steady state levels of BSP mRNA about 2.7-, 2.5- and 2.4-fold after 12 h. From transient transfection assays, constructs including the promoter sequence nts −116 to +60 (pLUC3) were found to enhance transcriptional activity 3.8- and 2.2-fold treated with 3 µM and 30 nM PGE<sub>2</sub> for 12 h. 2bp mutations were made in an inverted CCAAT box (between nts -50 and −46), a cAMP response element (CRE; between nts -75 and −68), a fibroblast growth factor 2 (FGF2) response element (FRE; nts -92 to −85) and a pituitary-specific transcription factor-1 motif (Pit-1; between nts −111 and −105) within pLUC3 and pLUC7 constructs. Transcriptional stimulation by PGE<sub>2</sub> was almost completed abrogated in constructs that included 2bp mutations in either the CRE and FRE. In gel shift analyses an increased binding of nuclear extract components to double-stranded oligonucleotide probes containing CRE and FRE was observed following treatment with PGE<sub>2</sub>. These studies show that PGE<sub>2</sub> induces BSP transcription in UMR 106 cells through juxtaposed CRE and FRE elements in the proximal promoter of the BSP gene.

**INTRODUCTION**

Prostaglandins are considered important local factors that modulate bone metabolism through
their effects on osteoblastic cells and osteoclasts (1, 2). Prostaglandin E₂ (PGE₂), a major eicosanoid produced by osteoblasts, is a potent stimulator of bone resorption (3), which can stimulate the formation of osteoclast-like multinuclear cells in mouse bone-marrow cultures (4, 5). The effects of PGE₂ on osteoclastogenesis are, at least in part, mediated by osteogenic cells, which express macrophage-colony stimulating factor (M-CSF) (6) and receptor activator of nuclear factor-κB ligand (RANKL) (7) that promote, and osteoprotegerin (OPG), a decoy receptor for RANKL (8), that suppresses osteoclast formation. PGE₂ has been shown to stimulate RANKL and inhibit OPG production (7, 9) and also increases production of interleukin-6 (IL-6), which can further enhance osteoclastogenesis (10-12). In contrast, studies have revealed that PGE₂ also has bone-forming activity (2, 13). Treatment of male, female and overiectomized mice with PGE₂ increases bone mass in vivo (14), while PGE₂ stimulates collagen and DNA synthesis and induces bone growth in calvarial organ (15) and cell cultures in vitro (16, 17). However, PGE₂ can either stimulate or inhibit cellular growth and differentiation of osteoblastic cells depending on PGE₂ concentration (15, 18, 19).

To explain the diverse effects of PGE₂, the presence of multiple receptors for PGE₂ in osteoblasts was postulated. Recent cloning of four subtypes of the PGE receptor has made it possible to analyze the PGE receptor subtypes (EP1-EP4) on osteoblasts (3, 13). EP1 is coupled to Ca²⁺ mobilization, EP2 and EP4 activate adenylate cyclase, whereas EP3 inhibits adenylate...
cyclase (20-22). An EP1 agonist stimulated cell growth and inhibited alkaline phosphatase activity whereas an EP4 agonist reduced cell growth and increased alkaline phosphatase activity in MC3T3-E1 osteoblast-like cells (23). These studies indicate that osteoblasts express multiple subtypes of the PGE receptor and that each subtype is might be linked to different aspects of PGE$_2$ action. Thus, activation of the EP4 receptor stimulates bone formation and prevents bone loss (24) while bone resorption by lipopolysaccharide (LPS) is impaired in EP4 knockout mice (25). Collectively, these results show that PGE$_2$ has anabolic effects on bone formation.

Bone sialoprotein (BSP) is a highly sulphated, 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 RGD sequence (26-28). The temporo-spatial deposition of BSP into the extracellular matrix (29, 30) and the ability of BSP to nucleate hydroxyapatite crystal formation (31) indicate a potential role for this protein in the initial mineralization of bone, dentin and cementum. Recent studies have shown that BSP is also expressed by osteotropic cancers, suggesting that BSP might play a role in the pathogenesis of bone metastases (32, 33). Thus, regulation of the BSP gene appears to be important in the differentiation of osteoblasts, in bone matrix mineralization and in tumor metastasis. The rat, human and mouse BSP genes have been cloned and partially characterized (34-37). These promoters include a functional inverted TATA element (nts -24 to −19) (38), which overlaps a vitamin D-response element (VDRE) (39) and an inverted CCAAT box (-50 to −46), which is required for basal transcription (40, 41). In addition, a fibroblast growth factor 2 (FGF2) response element (FRE; -92 to −85) (42), a cAMP-response element (CRE; -75 to −68) (43), a
TGF-β activation element (TAE; -499 to -485) (44), a pituitary-specific transcription factor-1 (Pit-1) motif (-111 to -105) that mediates the stimulatory effects of parathyroid hormone (PTH) (45), and a homeodomain binding element (HOX; -199 to -192) (46), have been characterized. Further upstream, a glucocorticoid response element (GRE) overlapping an AP-1 site (27, 47) has also been identified.

Since BSP is a marker of osteoblastic differentiation and bone formation we have analyzed the effects of PGE$_2$ on BSP expression in UMR 106 cells. Our studies show that PGE$_2$ increases transcription of the BSP gene through cAMP-dependent protein kinase, tyrosine kinase and MAP kinase pathways, and that the effects are mediated via CRE and FRE transcriptional elements in the proximal promoter of the rat BSP gene.

**EXPERIMENTAL PROCEDURES**

*Materials* - Cell culture media, fetal bovine serum (FBS), Lipofectamine, penicillin and streptomycin, SuperScript one step RT-PCR with Platinum Taq and trypsin were obtained from Invitrogen Corp., CA, USA. DyNAmo SYBR green qPCR Kit and M-MLV reverse transcriptase RNase H$^-$ were from Finnzymes, Espoo, Finland. The pGL2-Promoter, PGL3-Basic, pSV-β-Galactosidase control vector, recombinant Rnasin, random hexamer and MEK inhibitor U0126 were purchased from Promega Co. WI, USA. The protein kinase inhibitors H89 and H7 were from Seikagaku Corporation, Tokyo, Japan, and the tyrosine phosphatase inhibitor, sodium orthovanadate, the tyrosine kinase inhibitor, herbimycin A, and the serine threonine phosphatase inhibitor okadaic acid were purchased from Wako Pure Chemical Industries, Ltd, Tokyo, Japan. Forskolin was from Sigma Chemical Co., St. Louis, USA. PP1 was from Biomol Research Laboratories, Inc, PA, USA. PGE$_2$, prostaglandin E1 alcohol (EP2 and EP4 agonist), 17-phenyl trinor PGE$_2$ (EP1 and EP3 agonist) and butaprost (EP2 agonist) were obtained from
Cayman Chemical, MI, USA. EP3 agonist ONO-AP-324-01 was kindly provided by Ono Pharmaceutical Co., Ltd., Osaka, Japan. All other chemicals were of analytical grade.

**Cell culture** - The rat clonal cell line, UMR 106 cells (generously provided by Dr. T.J. Martin) were cultured at 37°C in 5% CO₂-air in alpha-minimum essential medium (α-MEM) supplemented with 10% FBS and used in these studies as an osteoblastic cell line that synthesizes BSP (27, 48). Rat stromal bone marrow cells (SBMC) (49), were kindly provided by Dr. S. Pitaru (Tel Aviv University, Israel). 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 prostaglandin E₂. RNA was isolated from triplicate cultures and analyzed for expression of BSP mRNA by RT-PCR and Real-time PCR as described below.

**RT-PCR and Real-time PCR** - Following treatment total RNA was extracted from UMR 106 cells with guanidium thiocyanate at different times, as described previously (44), 1 µg used as a template for one-step RT-PCR and cDNA synthesis. cDNA was prepared using random hexamer and M-MLV reverse transcriptase RNase H⁻. Conventional one-step RT-PCR was performed using a SuperScript one-step RT-PCR kit. Primers were synthesized on the basis of the reported rat cDNA sequences for BSP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Sequences of the primers used for PCR were as follow: BSP forward, 5’-CTGCTTTAATCTTGCTCTG-3’; BSP reverse, 5’-CCATCTCCATTTTCTTCC-3’; GAPDH forward, 5’-CCATGTTTGTGATGGGTGTG-3’; GAPDH reverse, 5’-GGATGCAGGGATGATGTTCT-3’. cDNA synthesis and pre-denaturation was performed for 1 cycle at 50 °C, 30min; 94 °C, 2min, and amplification was carried out for 30 (BSP and GAPDH) cycles at 94 °C, 30sec; 55 °C, 30sec; 72 °C, 30sec, and final extension at 94 °C,
10 min 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 expected size of the PCR products for BSP and GAPDH were 211 bp and 264 bp, respectively. Quantitative real-time PCR was performed using the following primer sets: BSP-R-T forward, 5'-TCCTCCTCTGAAACGGTTTCC-3'; BSP-R-T reverse, 5'-CGAACATGCCTCCTCCATT-3'; GAPDH-R-T forward, 5'-AGATGGTGAAGGTCGGTGTC-3'; GAPDH-R-T reverse, 5'-ATTGAACCTTGCCGTGGGTAG-3' using the SYBR Green qPCR Kit in a DNA Engine Opticon 2 continuous fluorescence detection system (MJ Research INC., MA). The expected size of the PCR products for BSP and GAPDH were 73 bp and 167 bp, respectively. The amplification reactions were performed in 20 µl of final volume containing 1 x SYBR Green Master Mix, 0.25 µM primer mixture and 10 ng cDNA. To reduce variability between replicates, PCR premixes, which contain all reagents except for cDNA, were prepared and aliquoted into 0.2 ml thin-wall strip tubes (MJ Research INC., MA). The thermal cycling conditions were 40 cycles of the following protocol; 15 s denaturation at 95 °C; 50 s annealing at 64 °C, followed by 12 s extension at 77 °C. Post-PCR melting curves confirmed the specificity of single-target amplification and ±fold expression of BSP relative to GAPDH was determined in triplicate (50).

**Transient transfection assays** - Exponentially growing UMR 106 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 (45) and 2 µg pSV-β-galactosidase (β-gal) vector as an internal transfection control. Two days post-transfection, cells were deprived of serum for 12 h, and 3 µM or 30 nM PGE2, or 3 µM of the respective EP agonists were added for 12 h prior to harvesting. The
luciferase assay was performed according to the supplier’s protocol (picaGene, Toyo Inki, Japan) using a Luminescence reader BLR20 (Aloka) to measure the luciferase activity. The protein kinase inhibitor H89 (5 µM) and H7 (5 µM) were used to inhibit protein kinase A and C. Herbimycin A (1 µM) and PP1 (10 µM) were used for tyrosine kinase and src tyrosine kinase inhibition respectively (42, 51). U0126 (5 µM) was used to inhibit MAP kinase kinase (MEK) activity (52). Sodium orthovanadate (50 µM) and okadaic acid (50 nM) were used for tyrosine phosphatase and serine threonine phosphatase inhibition, respectively (53, 54). Forskolin (1 µM) was used for activation of adenylate cyclase (45). Oligonucleotide-directed mutagenesis by PCR was utilized to introduce dinucleotide substitutions using the Quikchange Site-directed Mutagenesis Kit (Stratagene, CA, USA). All constructs were sequenced as described previously (42) to verify the fidelity of the mutagenesis.

Gel mobility shift assays - Confluent UMR 106 cells in T-75 flasks incubated for 6 and 12 h with 3 µM or 30 nM PGE2 in α-MEM without serum were used to prepare nuclear extracts. Nuclear protein was extracted by the method of Dignam et al. (55) with the addition of extra proteinase inhibitors (the extraction buffer was 0.42 M NaCl, 1.5 mM MgCl2, 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). Protein concentration was determined by the Bradford assay (56). Double-stranded oligonucleotides encompassing the inverted CCAAT (nts -61 to -37, 5'-CCGTGACCGTGATTGGCTGCTGAGA); cAMP response element (CRE; nts -84 to -59, 5'-CCCACAGCCGTGACGTACCGGCCG), and FGF2 response element (FRE; nts -98 to -79, 5'-TTTTCTGGTGAGAACCCACACAGAGCTGAGAGCTGAGAGCTGAGAGCTGAGAGCTGAGAGCTGAG) were prepared by Bio-Synthesis, Inc., TX, USA; while consensus CRE (5'-AGAGATTGCGCTGACGTACGAGCTGAGAGCTGAGAGCTGAGAGCTGAGAGCTGAGAGCTGAGAGCTGAG) was purchased from Promega. For gel shift
analysis the double-stranded-oligonucleotides were end-labeled with \( \gamma^{32}\text{P} \)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-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% non-denaturing acrylamide gels (38:2 acrylamide/bis acrylamide) run at 150 V at RT. For competition experiments unlabelled oligonucleotides for the CRE, mutation CRE (mCRE; 5’-CCCACAGCCeGACGeCGCACC GGCCG), FRE and mutated FRE (mFRE; 5’-TTTTCTGGcaAGAACCACA) were used at 20- and 40-fold molar excess. After electrophoresis, the gels were dried and autoradiograms prepared and analyzed using an image analyzer. Supershift experiments were performed with 1-2 µl of antibodies (Santa Cruz Biotechnology, CA) against CRE binding protein (CREB-1; sc-58), c-Jun (sc-44), c-Fos (sc-253), Pit-1 (sc-442), Oct-1 (sc-232), NFkB p65 (sc-109), NFkB p50 (sc-7178) and phospho-CREB (Upstate biotechnology; 06-519) separately to each gel shift reaction. Extracts were incubated for 5 h at 4 °C with the appropriate antibody before electrophoresis was performed under the same conditions as described above.

\[Ca^{2+}\] Determination - Confluent cells were preincubated with 2 µM fura-2/AM in α-MEM for 30 min at 37°C. After they were loaded with fura-2/AM, cells were detached from tissue culture flasks with the trypsin-EDTA solution, washed twice and suspended in fresh α-MEM. Just before \( [Ca^{2+}]_i \) determination, cells were washed again, and resuspended in Krebs-Ringer-Hepes solution [120 mM NaCl, 5 mM KCl, 1 mM MgSO4, 0.96 mM NaH2PO4, 0.2% glucose, 0.1% bovine serum albumin, 20 mM HEPES (pH 7.4), and 1 mM CaCl2. The fluorescence of
fura-2-loaded cells was measured with a CAF-110 spectrofluorometer (Nihon Bunkou, Tokyo, Japan) with excitation at 340 nm and 380 nm and emission at 500 nm. [Ca$^{2+}$]$_i$ was calculated from the measurement of the ratio of fluorescence intensities (57, 58). All experiments were performed 3 times with different cell batches.

**Statistical Analysis** - Triplicate samples were analyzed for each experiment, and experiments replicated to ensure consistency of the responses to PGE$_2$. Significant differences between control and PGE$_2$ treatment were determined using Student’s $t$-test.

**RESULTS**

**Stimulation of BSP mRNA Expression in UMR 106 Cells** – BSP gene expression was investigated at 6 and 12 h after PGE$_2$ stimulation by conventional (Fig. 1A) and real-time PCR (Fig. 1B). When osteoblastic UMR106 cells were exposed to 3 µM, 300nM and 30 nM PGE$_2$, expression of BSP mRNA was increased 2.3-, 2.0- and 2.2-fold at 6 h and 2.7-, 2.5- and 2.4-fold at 12 h, respectively as shown by conventional RT-PCR (Fig. 1A). To further confirm the PGE$_2$ effects on BSP transactivation, we applied real-time PCR to examine the mRNA expression level of BSP. As Fig. 1B shows, PGE$_2$ (3 µM, 300nM and 30 nM) can induce the mRNA expression of BSP.

**Transient Transfection Analysis of Rat BSP Promoter Constructs** - To determine the site of PGE$_2$-regulated transcription in the 5’-flanking region of the BSP gene, various sized promoter constructs ligated to a luciferase reporter gene were transiently transfected into UMR 106 cells and their transcriptional activity determined in the presence of PGE$_2$. With the construct pLUC3, encompassing BSP promoter nucleotides -116 to +60, transcription was increased 3.8-
fold with 3 µM PGE₂ and 2.2-fold with 30 nM PGE₂ after 12 h treatment (Fig. 2A and B). PGE₂ also increased transcription of pLUC4 (-425--+60), pLUC5 (-801--+60), pLUC6 (-938--+60) and pLUC7 (-1149--+60). In shorter constructs (pLUC1; -18 to +60, pLUC2; -43 to +60), luciferase activities were not increased by PGE₂ (data not shown). When transcriptional activity in response to 30 nM PGE₂ was analyzed in normal rat stromal bone marrow cells (SBMC) (49) the transcriptional activity of pLUC3 was increased 2-fold (Fig. 2C). Within the DNA sequence that is unique to pLUC3 (between nts -116--43), an inverted CCAAT box (ATTGG; between nts -50 and -46), CRE (between nts -75 and -68), FRE (between nts -92 and -85) and Pit-1 motif (between nts -111 and -105) are present (Fig. 3).

Since PGE₂ signaling activities are mediated by different protein kinases, we investigated the effects of the PKC inhibitor H7 (5 µM), the PKA inhibitor H89 (5 µM), the tyrosine kinase inhibitor herbimycin A (HA; 1 µM), the Src kinase inhibitor PP1 (10 µM), and the MEK inhibitor U0126 (5 µM), on PGE₂-mediated transcription to determine the signaling pathway. Whereas PGE₂-induced pLUC3 promoter activation was inhibited by H89, HA, PP1 and U0126, no effect was observed for H7 (Fig. 4), indicating an involvement of cAMP-dependent protein kinase, tyrosin kinase (Src) and MAP kinases in mediating the effects on BSP transcription. To assay for the responsiveness of the BSP promoter to serine-threonine phosphorylation, tyrosine phosphorylation or elevated intracellular cAMP level we used the serine-threonine phosphatase inhibitor okadaic acid, tyrosine phosphatase inhibitor sodium orthovanadate, and also forskolin, which is known to stimulate an adenylate cyclase. Vanadate (50 µM) and forskolin (1 µM) stimulated pLUC3 promoter activity ~1.6 and ~1.7-fold respectively, whereas okadaic acid (50 nM) was without effect (Fig. 5). Simultaneous
stimulation with vanadate (50 µM) and PGE2 (3 µM) up-regulated pLUC3 promoter activity synergistically. However, a combination of forskolin and PGE2 increased pLUC3 transcription to the same level observed for PGE2 stimulation (Fig. 5).

To determine which PGE2 receptor subtype transduced the PGE2 effects on BSP transcription, the following receptor agonists were used in the transcription assays: 3 µM 17-phenyl trinor PGE2 (for EP1 and EP3), ONO-AP-324-01 (for EP3), butaprost (for EP2) and prostaglandin E1 alcohol (for EP2 and EP4) (Fig. 6). Butaprost and prostaglandin E1 alcohol stimulated pLUC3 promoter activity to a similar extent as PGE2, whereas no significant increase in transcription was observed with either 17-phenyl trinor and ONO-AP-324-01, indicating that PGE2 activates BSP transcription by a mechanism involving cAMP stimulation through EP2 and EP4 receptors.

To determine the regulatory element(s) between nts -116 and -43 that is utilized by PGE2, a series of 5’ deletion constructs were prepared. Transcription by constructs -116BSPLUC and -108BSPLUC was increased by PGE2 but no increase was seen with -84BSPLUC. These results indicated that the element responding to PGE2 was present between nts -108 and -85 in the BSP promoter (Fig. 7). Next we introduced mutations in the possible response elements encoded within nts -116 to +60 of pLUC3. In addition, we examined whether these sites function in the large promoter construct (nts -1149 to +60; pLUC7), as shown in Fig. 8A and B. Whereas mutations in the Pit-1 had little effect on PGE2 stimulation and mutation of the CCAAT box essentially abolished basal expression, mutations of the CRE and especially the FRE significantly reduced the PGE2 effects on the transcriptional activities (Fig. 8A, B).
Furthermore, when both CRE and FRE sites were mutated, PGE$_2$ induced luciferase activity was completely abolished (Fig. 8A, B). These results suggest that the FRE and possibly the CRE are required as functional cis elements for up-regulation of BSP transcription by PGE$_2$.

*Gel Mobility Shift Assays* - To identify nuclear proteins whose binding to the CRE and FRE elements might be modulated by PGE$_2$, double-stranded oligonucleotides were end-labeled and incubated with equal amounts (3 µg) of nuclear proteins extracted from confluent UMR 106 cells that were either not treated (control) or treated with 30 nM PGE$_2$ for 6 and 12 h. When the CRE and FRE were used as probes, the formation of FRE-protein complexes, and slowly-migrating CRE-protein complexes, were increased by PGE$_2$ (Fig. 9, lanes 1-3, lanes 4-6). That these DNA-protein complexes represent specific interactions was demonstrated by competition experiments in which 20- and 40-fold molar excess of CRE and consensus CRE (Fig. 10, lanes 3, 4, 7 and 8), and FRE ds-oligonucleotides (Fig. 11, lanes 3, 4) reduced the amount of complex formation dose-dependently. In contrast, mutated CRE, FRE and inverted CCAAT (Fig. 10, lanes 5, 6, 9-12), and mutated FRE, CRE, consensus CRE and inverted CCAAT oligonucleotides (Fig. 11, lanes 5-12) did not compete with CRE-protein and FRE-protein complexes formation. To verify that the PGE$_2$ was operating through CRE and FRE, we also used gel mobility shift analyses to evaluate the potential effects of PGE$_2$ on the nearby inverted CCAAT and consensus CRE sites. When we used the inverted CCAAT sequence as a probe, the CCAAT-NF-Y protein complex (40, 41, 59) did not change after PGE$_2$ stimulation (Fig. 12, lanes 1-3). In comparison, CRE binding was increased by PGE$_2$ (Fig. 12, lanes 4-6). Notably, a stronger shift was obtained with the consensus CRE compared to the CRE in the
proximal promoter of the BSP gene.

To further characterize the proteins in the complexes formed with the CRE and FRE, we used antibodies for several transcription factors. The addition of antibody to CREB disrupted the formation of the CRE DNA-protein complexes (Fig. 13, lane 4), while incubation of nuclear extracts with anti-phospho-CREB antibody produced a visible supershift complex (Fig. 13, lane 5). FRE–nuclear protein complex was not disrupted or supershifted by antibodies to CREB, c-Jun, c-Fos, Pit-1, Oct-1, NFκB p65 and NFκB p50 (data not shown).

**DISCUSSION**

Prostaglandins are among the most potent regulators of bone cell function (4, 17). Extensive studies have demonstrated that PGE2 has both anabolic and catabolic effects on osteoblastic cells (13, 18). While the effects on bone resorption are indirect, involving the expression of cytokines such as RANKL, which promote osteoclast formation (7), prostaglandins can directly stimulate osteoblastic cells to differentiate and form bone (2, 13). The expression of BSP, which is essentially specific to mineralized tissues and is expressed by newly-formed osteoblasts co-incident with mineralization, provides a valuable marker for osteogenic differentiation and bone formation (28). Our studies show that PGE2, consistent with its promoting osteogenesis, increases expression of BSP by activation of EP2 and EP4 receptors in UMR 106 cells. Transduction of the PGE2 signaling is mediated by cyclic AMP-dependent protein kinase A, src tyrosine kinase, and MAP kinase which target nuclear proteins that bind to CRE and FRE elements in the proximal promoter of the BSP gene.

Prostaglandins, acting through different cell surface receptors on osteoblastic cells stimulate bone remodeling by promoting both anabolic and catabolic responses, the relative responses
being dependent on the target cell population and the concentration of PGE2. In bone marrow
cells, which are targets for the anabolic actions of PGE2 (60), PGE2 can stimulate both
phospholipase C and adelylate cyclase pathways in osteoblasts (2, 10). The stimulation of
phospholipase C results in the breakdown of phospholipid to form diacylglycerol, which
activates protein kinase C (PKC) (61), and inositol phosphates, which cause the release of
intracellular concentration of free calcium ([Ca^{2+}]_{i}) (58). Although 3 µM PGE2 evoked an
increase in [Ca^{2+}]_{i} in UMR106 cells, 30 nM and lower concentrations of PGE2 could not
induce [Ca^{2+}]_{i} (data not shown), suggesting that PGE2 stimulation of BSP transcription is
independent of Ca^{2+}-signaling. In contrast, stimulation of BSP transcription appears to utilize the
cAMP-dependent protein tyrosine kinase pathway since transcription is inhibited by herbimycin
A, and stimulated by vanadate and forskolin. Moreover, BSP transcription is mediated by EP2
and EP4 receptors (Fig. 6), through which cAMP production is stimulated (21). That
transcription is suppressed by src inhibitors to src kinase and MAP kinase (Fig. 4) further
implicates these enzymes in the signalling pathway.

BSP has been characterized as a unique marker of osteogenic differentiation that can regulate
the formation of mineral crystals (28). In this study, we have identified response elements in the
BSP gene promoter that mediate the PGE2 action on BSP transcription. In UMR106 cells,
PGE2 (3 µM and 30 nM) stimulated BSP promoter activity (pLUC3) ~3.8- and 2.2-fold (Fig. 2A and
B) comparable to the increase in BSP mRNA levels of ~2.7- and 2.4-fold by conventional RT-
PCR (Fig. 1A), and ~3.6- and 3.7-fold by Real-time PCR (Fig. 1B). PGE2 also induced BSP
transcription in stromal bone marrow cells (Fig. 2C) indicating that the increased BSP
expression occurs in normal osteoprogenitor cells and is not a specific feature of transformed UMR106 cells. From transient transfection assays we initially located the PGE$_2$-responsive region to the proximal promoter (nts -116 and -43; Fig. 2) of the BSP gene, which encompasses an inverted CCAAT box (nts -50 and -46), a putative CRE (nts -75 and -68), a FGF2 response element (FRE; nts –92 and –85) and a Pit-1 (nts -111 and -105) motifs (Fig. 3). The results of luciferase analyses using fine 5’-deletion constructs between nts -116 to -43 in the BSP promoter show that the PGE$_2$ effects are targeted to a region encompassed by nts -108 and -43 (Fig. 7). Whereas mutation of the Pit-1 element was without effect, mutation of the CCAAT element resulted in the loss of basal transcriptional activity, as reported previously (40, 43). As a consequence the involvement of the inverted CCAAT was difficult to ascertain. However, the lack of PGE$_2$-induced transcription with constructs –84BSPLUC and –60BSPLUC (Fig. 7) indicate that the CCAAT is not a target of PGE$_2$ regulation. In comparison, mutations in the CRE and FRE sites suggests that they are required for the induction of BSP expression by the PGE$_2$. The involvement of the FRE and CRE elements is further supported by EMSA analyses in which proteins from nuclear extracts formed complexes with the FRE and CRE elements that were increased by PGE$_2$ (Fig. 9). However, while the luciferase assays show a much reduced PGE$_2$-stimulated transcription when the individual CRE and FRE sites are mutated and the combined mutations show total abrogation, the formation of CRE-nuclear factor complexes is weak compared to results obtained with a consensus CRE (Fig. 9 and 12). Moreover, there is no significant increase in transcription with the –84BSPLUC construct (Fig. 7) which omits the FRE but retains the CRE element. In comparison, the FRE clearly shows increased binding of the nuclear protein in response to PGE$_2$. Thus, our studies suggest that transcriptional activation
is mediated by the juxtaposed FRE and CRE elements, with the FRE being the predominant target of the PGE₂ effects.

Although the CRE element binding of nuclear protein was not strong, the binding protein was, nevertheless up-regulated by PGE₂ (Fig. 9) and could be identified as CRE- binding protein (CREB) by antibody interference (Fig. 13). Moreover, phosphorylation of the CREB was induced by PGE₂ (Fig. 13). While PKA signaling does not affect CREB binding to its cognate CRE element it can direct phosphorylation of CREB, which is required for transcriptional activation (62, 63). In comparison, the nuclear factor binding to the FRE element, which is regulated by tyrosine kinase, has yet to be characterized and is the focus of current studies because of its potential role in regulating basal and FGF2-induced transcription of BSP in osteoblasts (42), as well as mediating the PGE₂ effects.

The molecular pathways of PGE₂-regulation of BSP gene transcription identified in these studies. PGE₂ acting through EP2 and EP4 prostaglandin receptors on osteoblastic cells activates signaling pathways involving cAMP generation and tyrosine phosphorylation (protein tyrosine kinase), which activate MAP kinase to phosphorylate CREB and thereby transactivate BSP transcription through the CRE. Whether this same pathway also activates the FRE response through the same or a linked pathway, or whether there is a concerted action on the CRE and FRE through a single pathway is difficult to discern at this time as the FRE-binding nuclear protein is yet to be characterized.

In conclusion, our study has identified CRE and FRE elements in the rat BSP proximal promoter that mediate BSP transcription induced by PGE₂ and that the PGE₂ increases the nuclear protein binding activities of CRE and FRE, and enhances CREB phosphorylation. Since
Fig. 1

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<tr>
<th></th>
<th>3μM PGE&lt;sub&gt;2&lt;/sub&gt;</th>
<th>300nM PGE&lt;sub&gt;2&lt;/sub&gt;</th>
<th>30nM PGE&lt;sub&gt;2&lt;/sub&gt;</th>
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<tr>
<td>Relative Fold Compared to GAPDH</td>
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<tr>
<td>Control</td>
<td>6h</td>
<td>12h</td>
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Fig. 1
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BSP is expressed by differentiated osteoblasts, and PGE$_2$ is a crucial factor for bone metabolism, it is conceivable that these two response elements may contribute to the cell-specific expression of the BSP gene during the formation of the mineralized extracellular matrix of bone.

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**FOOTNOTES**

1. The abbreviations used are: PGE₂, prostaglandin E₂; BSP, bone sialoprotein; bp, base pair(s); CRE, cyclic AMP response element; CREB, cAMP response element binding protein; LUC, luciferase; FRE, FGF2 response element; nts, nucleotides; MAP kinase, mitogen activated protein kinase; MEK, MAP kinase kinase; Pit-1, pituitary-specific transcription factor-1; PKC, protein kinase C; PKA cAMP-dependent protein kinase.
FIGURE LEGENDS

FIG. 1. **Effect of PGE$_2$ on BSP mRNA levels.** A, Conventional RT-PCR for BSP gene expression in UMR 106 cells treated with 3 µM, 300nM and 30 nM PGE$_2$ for 6 and 12 h. Total RNA was extracted, and the expression of BSP and GAPDH mRNAs in the cells were analyzed by one-step RT-PCR. Results were quantitated by densitometry and normalized to the expression of GAPDH. B, Relative gene expression for BSP generated from Real-time PCR of UMR 106 cells treated with 3 µM, 300nM and 30 nM PGE$_2$. The expression of GAPDH was also examined as control. The relative amounts of mRNA of BSP to GAPDH were calculated. Experiments were performed in triplicates for each data point and standard errors are shown in vertical lines. Significant differences compared to controls are shown at the following probability levels: #P<0.2; *P<0.1; **P<0.05; ****P<0.01.

FIG. 2. **PGE$_2$ up-regulates BSP promoter activity in UMR-106 cells.** A, B, Transient transfections of UMR 106 cells in the presence or absence of PGE$_2$ (A; 3 µM and B; 30nM) 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 compared to controls are shown at the following probability levels: #P<0.2; *P<0.1; **P<0.05; ***P<0.02; ****P<0.01.

C, Transient transfections of rat stromal bone marrow cells (SBMC) in the presence or absence
of PGE$_2$ (30nM) 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 have been combined and the values expressed with standard errors. Significant difference compared with control.

at the *$P<0.1$ level is shown.

FIG. 3. Regulatory elements in the proximal rat BSP promoter. The positions of the inverted TATA and CCAAT boxes, a cAMP response element (CRE), an FGF response element (FRE), a pituitary-specific transcription factor-1 (Pit-1), a homeobox-binding site (HOX), a TGF-β activation element (TAE) overlapping with AP2, glucocorticoid response elements (GRE) overlapping the AP1, and a vitamin D response element (VDRE) that overlaps the inverted TATA box are shown in the proximal promoter region of the rat BSP gene. The numbering of nucleotides is relative to the transcription start site (+1).

FIG. 4. Effect of kinase inhibitors on transcriptional activation by PGE$_2$. Transient transfection analysis of pLUC3 in the presence or absence of PGE$_2$ (3 µM) for 12 h in UMR 106 cells is shown together with the effects of the PKC inhibitor (H7, 5 µM), PKA inhibitor (H89, 5 µM), tyrosine kinase inhibitor (herbimycin A; HA, 1 µM), src kinase inhibitor (PP1, 10 µM) and MEK inhibitor (U0126, 5 µM). The results obtained from three separate transfections were combined and the values expressed with standard errors. Significant differences compared to controls are shown at the following probability levels: **$P<0.05$; ****$P<0.01$. 
FIG. 5. **PGE₂ and tyrosine phosphatase inhibitor (sodium orthovanadate) synergistically up-regulate BSP transcription.** pLUC3 was analyzed for relative promoter activity after transfection into UMR 106 cells and examined for induction in the presence of okadaic acid (50 nM), sodium orthovanadate (50 µM), forskolin (1 µM) and simultaneous stimulation of each reagent with PGE₂ (3 µM). The results of transcriptional activity, obtained from three separate transfections with constructs pLUCB and pLUC3, were combined and the values expressed with standard errors. Significant differences in the relative luciferase activities obtained with pLUC3 are indicated at the following probability levels: *P<0.1; **P<0.05; ***P<0.02.

FIG. 6. **Effect of PGE agonists on the BSP promoter activity.** Transient transfection analysis of pLUC3 in the presence or absence of 3 µM PGE₂, 17-phenyl trinor PGE₂ (EP1 and EP3 agonist), ONO-AP-324-01 (EP3 agonist), butaprost (EP2 agonist) and prostaglandin E1 alcohol (EP2 and EP4 agonist) for 12 h in UMR 106 cells is shown. The results obtained from three separate transfections were combined and the values expressed with standard errors. Significant difference compared to controls is shown at the **P<0.05 level.

FIG. 7. **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 UMR 106 cells and examined for induction in the presence of PGE₂ (3 µM) for 12 h. The results of transcriptional activity obtained from four separate transfections with constructs; -43 BSPLUC (-43 to +60), -60 BSPLUC (-60 to +60), -84 BSPLUC (-84 to +60), -108BSPLUC (-108 to +60), -116 BSPLUC (-116 to +60) have been combined and the values expressed with standard errors. Significant differences compared to controls are shown at the
following probability levels: *$P<0.1$; ***$P<0.02$.

FIG. 8. **Site mutation analysis of luciferase activities in response to PGE$_2$.** Dinucleotide substitutions were made within context of the homologous -116 to +60 (pLUC3) and -1149 to +60 (pLUC7) BSP promoter fragments. M-CCAAT (ATTtt), M-CRE (eGACGeCG), M-FRE (GGcaAGAA), M-PIT (TTacAGT) and double-mutated constructs (M-CRE and M-FRE) were analyzed for relative promoter activity after transfection into UMR 106 cells and examined for induction in the presence of PGE$_2$ (A; 3 µM, B; 30 nM) for 12 h. The results of transcriptional activity obtained from three separate transfections with constructs were combined and the values expressed with standard errors. Significant differences compared to controls are shown at the following probability levels: #$P<0.2$; *$P<0.1$; **$P<0.05$; ****$P<0.01$.

FIG. 9. **PGE$_2$ up-regulates a nuclear protein that recognizes the CRE and FRE.** Radiolabeled double-stranded CRE (-84 CCCACAGGCTGACGTCGCCCCAGCCG -59) and FRE oligonucleotides (-98 TTTTCTGGTGAGAACCCACA -79) were incubated for 20 min at 21°C with nuclear protein extracts (3 µg) obtained from UMR 106 cells incubated without (lanes 1 and 4) or with PGE$_2$ at 30 nM for 6 h (lanes 2 and 5) and 12 h (lanes 3 and 6). 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 image analyzer.

FIG. 10. **Specific binding of nuclear proteins to the CRE.** Radiolabeled double-stranded CRE was incubated for 20 min at 21°C with nuclear protein extracts (3 µg) obtained from UMR 106
cells stimulated in the absence (control; lane 1) or presence (lanes 2–12) of PGE₂ (30 nM) for 12 h. Competition reactions were performed using a 20- and 40-fold molar excess of unlabelled CRE (-84 CCCACAGCCTGACGTCGCACCGGCGGCG –59; lanes 3 and 4), mutation CRE (m-CRE; CCCACAGCCcGACGcCGACCGGCGGCG; lanes 5 and 6), consensus CRE (AGAGATTCGCTGAGGAGTGAGAGCTAG; lanes 7 and 8), FRE (-98 TTTTCTGGTGAGAACCACA -79; lanes 9 and 10) and inverted CCAAT (-61 CCGTGACCGTGATTGGCTGAGA -37; lanes 11 and 12). DNA-protein complexes were separated on polyacrylamide gel in low-ionic-strength Tris-borate buffer, dried under vacuum and exposed to an imaging plate for quantitation using an imaging analyzer.

FIG. 11. Specific binding of nuclear proteins to the FRE. Radiolabeled double-stranded FRE was incubated for 20 min at 21°C with nuclear protein extracts (3 µg) obtained from UMR 106 cells stimulated in the absence (control; lane 1) or presence (lanes 2–12) of PGE₂ (30 nM) for 12 h. Competition reactions were performed using a 20- and 40-fold molar excess of unlabelled FRE (-98 TTTTCTGGTGAGAACCACA -79; lanes 3 and 4), mutation FRE (m-FRE; TTTTCTGGcaAGAACCACA; lanes 5 and 6), CRE (-84 CCCACAGCCTGACGTCGCACCGGCGGCG –59; lanes 7 and 8), consensus CRE (AGAGATTCGCTGAGGAGTGAGAGCTAG; lanes 9 and 10) and inverted CCAAT (-61 CCGTGACCGTGATTGGCTGAGA -37; lanes 11 and 12). 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.

FIG. 12. Comparison of CCAAT and consensus CRE DNA-protein complexes. Radiolabeled
double-stranded CCAAT oligonucleotide (-61 CCGTGACCGT GT TGGCTGCTGAGA –37) and consensus CRE (5\’-AGAGATTGCCTGACG TGAGAGCTAG) were incubated with nuclear protein extracts (3 µg) obtained from UMR 106 cells incubated without (lanes 1 and 4) or with PGE2 at 30 nM for 6 h (lanes 2 and 5) and 12 h (lanes 3 and 6). 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.

CONT, nuclear extract from control confluent cells.

FIG. 13. Specific binding of nuclear proteins to the CRE. Radiolabeled double-stranded CRE oligonucleotide (–84 CCCACAGCCTGACGTCGCACGGCCG –59) was incubated with nuclear protein extracts (3 µg) obtained from UMR106 cells incubated without (lane 1) or with PGE2 (30 nM, lanes 2~5) for 12 h. Supershift experiments were performed with 1-2 µl of antibodies against CREB-1 (lane 4) and phospho-CREB (lane 5) added separately to each gel shift reaction.
Prostaglandin E2 stimulates bone sialoprotein (BSP) expression through cAMP and FGF2 response elements in the proximal promoter of the rat BSP gene
Hiroshi Samoto, Emi Shimizu, Yuko Matsuda-Honjyo, Ryoichiro Saito, Sumi Nakao, Muneyoshi Yamazaki, Shunsuke Furuyama, Hiroshi Sugiyama, Jaro Sodek and Yorimasa Ogata

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