Prostaglandin E₂ Stimulates Bone Sialoprotein (BSP) Expression through cAMP and Fibroblast Growth Factor 2 Response Elements in the Proximal Promoter of the Rat BSP Gene*

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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 E₂ (PGE₂) has anabolic effects on proliferation and differentiation of osteoblasts via diverse signal transduction systems. Because PGE₂ increases the proportion of functional osteoblasts in fetal rat calvarial cell cultures, we investigated the regulation of BSP, as an osteoblastic marker, by PGE₂. Treatment of rat osteosarcoma UMR 106 cells with 3 μM, 300 nM, and 30 nM PGE₂ increased the steady state levels of BSP mRNA about 2.7-, 2.5-, and 2.4-fold after 12 h. From transient transfection assays, the constructs including the promoter sequence of nucleotides (nt) −116 to +60 (pLUC3) were found to enhance transcriptional activity 3.8- and 2.2-fold treated with 3 μM and 30 nM PGE₂ for 12 h. 2-bp mutations were made in an inverted CCAAT box (between nt −50 and −46), a cAMP response element (CRE; between nt −75 and −68), a fibroblast growth factor 2 response element (FRE; nt −92 to −85), and a pituitary-specific transcription factor-1 motif (between nt −111 and −105) within pLUC3 and pLUC7 constructs. Transcriptional stimulation by PGE₂ was almost completely abrogated in constructs that included 2-bp 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 PGE₂. These studies show that PGE₂ induces BSP transcription in UMR 106 cells through juxtaposed CRE and FRE elements in the proximal promoter of the BSP gene.

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) that 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 (6) and receptor activator of nuclear factor κB ligand (RANKL) (7) that promote, and osteoprotegerin, a decoy receptor for RANKL (8), that suppresses osteoclast formation. PGE₂ has been shown to stimulate RANKL and inhibit osteoprotegerin production (7, 9) and also increases production of interleukin-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), whereas 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 reduced cell growth and increased alkaline phosphatase activity, whereas an EP₄ agonist reduced cell growth and increased alkaline phosphatase activity, whereas an EP₄ agonist reduced cell growth and increased alkaline phosphatase activity.

Received for publication, January 21, 2003, and in revised form, May 16, 2003

This paper is available on line at http://www.jbc.org

The abbreviations used are: PGE₂, prostaglandin E₂; BSP, bone sialoprotein; CRE, cyclic AMP response element; CREB, cAMP response element-binding protein; LUC, luciferase; FRE, FGF2 response element; nt, nucleotide(s); MAP, mitogen-activated protein; Pit-1, pituitary-specific transcription factor-1; FGF2, fibroblast growth factor 2; RANKL, receptor activator of nuclear factor κB ligand; RT, reverse transcription; α-MEM, α-minimum essential medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
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 temporospatial deposition of BSP into the extraacellular 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 (nt -24 to -19) (38), which overlaps a vitamin D response element (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 (CRE; -92 to -85) (42), a Camp response element (CRE; -75 to -68) (43), a transforming growth factor-β activation element (499 to -485) (44), a pituitary-specific transcription factor-1 (Pit-1) motif (-111 to -105) that mediates the stimulatory effects of parathyroid hormone (45), and a homeodomain binding element (−199 to −192) (46) have been characterized. Further upstream, a glucocorticoid response element overlapping an AP-1 site (27, 47) has also been identified. Because BSP is a marker of osteoblastic differentiation and bone formation, we have analyzed the effects of PGE2 on BSP expression in UMR 106 cells. Our studies show that PGE2 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, LipofectAMINE, penicillin and streptomycin, SuperScript one-step RT-PCR with Platinum DNA, and trypsin were obtained from Invitrogen. DynAmino SYBR green qPCR Kit and Moloney murine leukemia virus reverse transcriptase RNase H − . Conventional one-step RT-PCR was performed using a SuperScript one-step RT-PCR kit. The 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 follows: BSP forward, 5'-CCTGGTTTAATCTGCTCCTG-3'; BSP reverse, 5'-CATGTCCCATTCTTCCT-3'; GAPDH forward, 5'-CACATTGGTGGATGTG-3'; and GAPDH reverse, 5'-GGATGCGCTGATACGTCTC-3'. CDNA synthesis and pre-denaturation was performed for 1 cycle at 50 °C for 30 min and 94 °C for 2 min, and amplification was carried out for 30 (BSP and GAPDH) cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and final extension was 94 °C for 10 min in a 50-μl reaction mixture. After amplification, 10 μl of each reaction mixture was analyzed by 2% (w/v) agarose gel electrophoresis, the bands were visualized by ethidium bromide staining. The expected size of the PCR products for BSP and GAPDH were 211 and 264 bp, respectively. Quantitative real time PCR was performed using the following primer sets: BSP-R forward, 5'-TCTCCTCTGAAACGGTTTC-3'; BSP-R reverse, 5'-CAACTATGGCCATCTCATTG-3'; GAPDH-R forward, 5'-AGATGTTGAAAGTGCGGT-3', and GAPDH-R reverse, 5'-ATTGAACTTGGCCTTAGT-3' using the SYBR Green qPCR Kit in a DNA Engine Opticon 2 continuous fluorescence detection system (MJ Research Inc.). The expected size of the PCR products for BSP and GAPDH were 73 and 167 bp, respectively. The amplification reactions were performed in 20 μl of final volume containing 1X SYBR Green Master Mix, 0.25 μM primer pair, and 10 ng of cDNA. To reduce variability between replicates, PCR premixes, which contain all of the reagents except for cDNA, were prepared and aliquoted into 0.2-ml thin wall strip tubes (MJ Research Inc.). The thermal cycling conditions were 40 cycles of the following protocol: 15 s of denaturation at 95 °C, 50 s of annealing at 64 °C, followed by 12 s of extension at 77 °C. Post-PCR melting curves confirmed the specificity of single-target amplification, and fold expression of the BSP relative to GAPDH was determined in triplicate (50).

Transient Transfection Assays—Exponentially growing UMR 106 cells were used for transfection assays. 24 h after plating, the cells at 50–70% confluence were transfected using a LipofectAMINE reagent. The transfection mixture included 1 μg of a luciferase (LUC) construct (containing 1 μg of pSV-β-galactosidase vector as an internal control). Two days post-transfection, the cells were deprived of serum for 12 h, and 3 μl 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) using a Luminescence reader BLR20 (Aloka) to measure the luciferase activity. The protein kinase inhibitors H89 (5 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 inhibition activity (52). Sodium orthovanadate (50 μM) and okadaic acid (50 μM) were used for tyrosine phosphatase and serine-threonine phosphatase inhibition, respectively (53). Forskolin (50 μM) and okadaic acid (50 μM) were used for p38 MAPK activation of adenylate cyclase (45). Oligonucleotide-directed mutagenesis for PDB was utilized to introduce dinucleotide substitutions using the QuikChange site-directed mutagenesis kit (Stratagene). All of the constructs were sequenced as described previously (42) to verify the fidelity of the mutagenesis.

Cell Mobility Shift Assays—Confluent UMR 106 cells in T-75 flasks incubated for 6 and 12 h with 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 protease inhibitors (the extraction buffer was 0.42 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM dl-dithiothreitol, 25% (v/v) glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, pH 7.9). Protein concentration was determined by the Bradford method (56). Double-stranded oligonucleotides encompassing the inverted CCAAT (nt -61 to -37, 5'-CCCTGGACCTGGATCTGGCAG-ACTGCTTAC-3') and FGF2 response element (FRE; nt -280 to -265, 5'-TCTTGGTGCCAGGG-3') were prepared by Bio-Synthesis, Inc., whereas consensus CRE (5'-AGAGATCTCGTACGAGTCTGAGTACGACTG-3') was purchased from Promega. For gel shift analysis the double-stranded oligonucleotides were end-labeled with [γ-32P]ATP and T4 polynucleotide kinase. Nuclear protein extracts (5 μg) were incubated for 20 min at room temperature in binding buffer containing 50 mM KCl, 0.1 mM radiolabeled double-stranded probe, 1 μCi/ml [γ-32P]ATP, 1 μg/ml poly(dI·dC), 10% glycerol, 5% sucrose, 0.1% Nonidet P-40, 5% glycerol, and 1 μg of 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 room temperature. For competition experiments unlabeled oligonucleotides for the CRE, mutation CRE (5’-CCCCACGCCGACGGCACCCG), FRE, and mutated FRE (5’-TTTTCTGCAAGAAACCACA) were used at 20- and 40-fold molar excess. After electrophoresis, the gels were dried, and the autoradiograms were prepared and analyzed using an image analyzer. Supershift experiments were performed with 1–2 μl of antibodies (Santa Cruz Biotechnology) against CRE-binding protein (CREB-1; sc-58), c-Jun (sc-44), c-Fos (sc-253), Pit-1 (sc-442), Oct-1 (sc-232), NFκB p50 (sc-7178), and phospho-CREB (Upstate Biotechnology) against CRE--binding protein (CREB-1; sc-58), c-Jun (sc-44), c-Fos (sc-253), Pit-1 (sc-442), Oct-1 (sc-232), NFκB p50 (sc-109), NFκB p65 (sc-109), NFκB p50 (sc-7178), and phospho-CREB (Upstate Biotechnology, Inc.; 00-519) separately to each gel shift reaction. The extracts were incubated for 5 h at 4 °C with the appropriate antibody before electrophoresis was performed under the same conditions as described above.

[Ca2+]i Determination—Confluent cells were preincubated with 2 μM fura-2/acetoxymethylester in α-MEM for 30 min at 37 °C. After they were loaded with fura-2/acetoxymethylester, the cells were detached from tissue culture flasks with the trypsin-EDTA solution, washed twice, and suspended in fresh α-MEM. Just before [Ca2+]i determination, the 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 spectrophotometer (Nihon Bunkou, Tokyo, Japan) with excitation at 340 and 380 nm and emission at 500 nm. [Ca2+]i was calculated from the measurement of the ratio of fluorescence intensities (57, 58). All of the experiments were performed three times with different cell batches.

Statistical Analysis—Triplicate samples were analyzed for each experiment, and the experiments were replicated to ensure consistency of the responses to PGE2. Significant differences between control and PGE2 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 PGE2 stimulation by conventional (Fig. 1A) and real time PCR (Fig. 1B). When osteoblastic UMR 106 cells were exposed to 3 μM, 300 nM, and 30 nM PGE2, 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 PGE2 effects on BSP transactivation, we applied real time PCR to examine the mRNA expression level of BSP. As Fig. 1B shows, PGE2 (3 μM, 300 nM, and 30 nM) can induce the mRNA expression of BSP.

Transient Transfection Analysis of Rat BSP Promoter Constructs—To determine the site of PGE2-regulated transcription in the 5′-flanking region of the BSP gene, various sized pro-
Fig. 3. Regulatory elements in the proximal rat BSP promoter. The positions of the inverted TATA and CCAAT boxes, a CRE, a FRE, Pit-1, a homebox-binding site (Hox), a transforming growth factor-β activation element (TAE) overlapping with AP2, glucocorticoid response elements (GRE) overlapping with AP2, 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 PGE2. Transient transfection analysis of pLUC3 in the presence or absence of PGE2 (3 μM) for 12 h in UMR 106 cells is shown together with the effects of the protein kinase C inhibitor (H7, 5 μM), cAMP-dependent protein kinase inhibitor (H89, 5 μM), tyrosine kinase inhibitor (herbimycin A, HA, 1 μM), Src kinase inhibitor (PP1, 10 μM), and MAP kinase kinase inhibitor U0126 (5 μM). The results obtained from three separate transfections were combined, and the values are expressed with standard errors. Significant differences compared with 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 (OKA, 50 nM), sodium orthovanadate (VANA, 50 μM), forskolin (FSK, 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 are expressed with standard errors. Significant differences in the relative luciferase activities obtained with pLUC3 are indicated at the following probability levels: #, p < 0.05; ***, 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 are expressed with standard errors. Significant difference compared with controls is shown at the p < 0.05 level (**).
pLUC3 promoter activity was increased 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
Fig. 9. PGE2 up-regulates a nuclear protein that recognizes the CRE and FRE. Radiolabeled double-stranded CRE (5′-CCCACACGCTGAGTCAGCGCCGC-3′) and FRE oligonucleotides (5′-TTTTCTGGTGAAACCCACA-3′) 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 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 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 unlabeled CRE (5′-CCCACACGCTGAGTCAGCGCCGC-3′, lanes 3 and 4), mutation CRE (m-CRE; CCCACACGCTGAGTCAGCGCCGC-3′, lanes 5 and 6), consensus CRE (AGAGATTGCCACCGGCCGC-3′, lanes 7 and 8), consensus CRE (AGAGATTGCCACCGGCCGC-3′, lanes 9 and 10), and inverted CCAAT (5′-GGTTTTCTGGTGAAACCCACA-3′, 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. 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 unlabeled FRE (5′-TTTTCTGGTGAAACCCACA-3′, lanes 3 and 4), mutation FRE (m-FRE; TTCTGCAGCCACCGCCCA; lanes 5 and 6), CRE (5′-CCCACACGCTGAGTCAGCGCCGC-3′, lanes 7 and 8), consensus CRE (AGAGATTGCCACCGGCCGC-3′, lanes 9 and 10), and inverted CCAAT (5′-CCCACACGCTGAGTCAGCGCCGC-3′, 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.

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 nt −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 nt −108 and −85 in the BSP promoter (Fig. 7). Next we introduced mutations in the possible response elements encoded within nt −116 to +60 of plUC3. In addition, we examined whether these sites function in the large promoter construct (nt −1149 to +60; plUC7), as shown in Fig. 8. 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. 8). Furthermore, when both CRE and FRE sites were mutated, PGE2-induced luciferase activity was completely abolished (Fig. 8). These results suggest that the CRE and possibly the FRE are required as functional cis-elements for up-regulation of BSP transcription by PGE2.

Gel Mobility Shift Assays—To identify nuclear proteins whose binding to the CRE and FRE elements might be modulated by PGE2, double-stranded oligonucleotides were end-la-
beled 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 PGE₂ for 6 and 12 h. When the CRE and FRE were used as probes, the formation of FRE-protein complexes (Fig. 9, lanes 4–6) and slowly migrating CRE-protein complexes were increased by PGE₂ (Fig. 9, lanes 1–3). 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 double-stranded oligonucleotides (Fig. 11, lanes 3 and 4) reduced by the amount of complex formation dose-dependently. In contrast, mutated CRE, FRE, and inverted CCAAT (Fig. 10, lanes 5, 6, and 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 complex formation. To verify that the PGE₂ was operating through CRE and FRE, we also used gel mobility shift analyses to evaluate the potential effects of PGE₂ on the nearby inverted CCAAT and consensus CRE sites. When we used the inverted CCAAT sequence as a probe, the CCAAT-NP-Y protein complex (40, 41, 59) did not change after PGE₂ stimulation (Fig. 12, lanes 1–3). In comparison, CRE binding was increased by PGE₂ (Fig. 12, lanes 4–6). Notably, a stronger shift was obtained with the consensus CRE compared with 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), whereas incubation of nuclear extracts with anti-phospho-CREB antibody produced a visible supershift complex (Fig. 13, lane 5). CREF-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). Although 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 coincident 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 adenylyl 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 (61), and inositol phosphates, which cause the release of intracellular concentration of free calcium ([Ca²⁺]ᵢ) (58). Although 3 μM PGE₂ evoked an increase in [Ca²⁺]ᵢ in UMR 106 cells, 30 nM and lower concentrations of PGE₂ could not induce [Ca²⁺]ᵢ (data not shown), suggesting that PGE₂ stimulation of BSP transcription is independent of Ca²⁺ sig-
naling. In contrast, stimulation of BSP expression appears to utilize the cAMP-dependent protein-tyroline kinase pathway because 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 signaling 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 UMR 106 cells, PGE2 (3 μM and 30 nM) stimulated BSP promoter activity (pLUC3) −3.8- and 2.2-fold (Fig. 2, A and B), comparable with 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 UMR 106 cells. From transient transfection assays we initially located the PGE2-responsive region to the proximal promoter (nt −116 and −43; Fig. 2) of the BSP gene, which encompasses an inverted CCAAT box (nt −50 and −46), a putative CRE (nt −75 and −68), a PFG2 response element (FRE; nt −92 and −85), and a Pit-1 (nt −111 and −105) motif (Fig. 3). The results of luciferase analyses using fine 5′ deletion constructs between nt −116 to −43 in the BSP promoter show that the PGE2 effects are targeted to a region encompassed by nt −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 PGE2-induced transcription with constructs −84BSPLUC and −60BSPLUC (Fig. 7) indicate that the CCAAT is not a target of PGE2 regulation. In comparison, mutations in the CRE and FRE sites suggest that they are required for the induction of BSP expression by the PGE2. The involvement of the CRE 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 PGE2 (Fig. 9). How-

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Prostaglandin E₂ Stimulates Bone Sialoprotein (BSP) Expression through cAMP and Fibroblast Growth Factor 2 Response Elements in the Proximal Promoter of the Rat BSP Gene

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*J. Biol. Chem.* 2003, 278:28659-28667.
doi: 10.1074/jbc.M300671200 originally published online May 23, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M300671200

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