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Original

Transcriptional regulation of bone sialoprotein gene by CO_2 laser irradiation

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Abstract: Bone sialoprotein (BSP), an early marker of osteoblast differentiation, has been implicated in the nucleation of hydroxyapatite during *de novo* bone formation. Low-power laser irradiation has a stimulating effect on cells and tissues. Although the carbon dioxide (CO_2) laser is a hard surgical laser, we have attempted to use it at low energy density to achieve biological alterations. To investigate the effects of CO_2 laser irradiation on BSP gene transcription, we used rat osteoblast-like ROS17/2.8 cells. BSP mRNA levels were increased at 12 h after irradiation with the CO_2 laser (2 W, 20 s). Transient transfection assays using various sizes of the rat BSP gene promoter linked to the luciferase reporter gene showed that CO_2 laser irradiation induced luciferase activity of a -116 to +60 BSP promoter construct (pLUC3) at 12 h in the cells. Transcriptional stimulation by CO_2 laser irradiation was abrogated in the pLUC3 construct containing a 2-bp mutation in the fibroblast growth factor 2 response element (FRE). Gel shift analyses showed that CO_2 laser irradiation increased the binding of nuclear protein to FRE. These studies demonstrate that CO_2 laser irradiation increases BSP transcription via FRE in the rat BSP gene promoter. (J Oral Sci 53, 51-59, 2011)

Keywords: bone sialoprotein; CO_2 laser irradiation; osteoblast; transcription.

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Introduction

Bone sialoprotein (BSP) is a mineralized tissue-specific non-collagenous glycosylated, phosphorylated and sulfated protein expressed almost exclusively in mineralized connective tissues (1-3). BSP is also expressed in breast, lung, thyroid and prostate cancers that metastasize to bone (4-7) and is associated with the formation of ectopic hydroxyapatite microcrystals in tumor tissues (8,9). Studies aimed at clarifying the transcriptional regulation of BSP have characterized the rat, mouse and human BSP gene promoters (10-12). These promoters have an inverted TATA box (-24 to -19) (13) and an inverted CCAAT box (-50 to -46), which are required for basal transcription (14). In addition, a cAMP response element (CRE; -75 to -68) (15), a fibroblast growth factor 2 response element (FRE; -92 to -85) (15,16), a pituitary-specific transcription factor-1 (Pit-1) motif (-111 to -105) (17), a homeodomain protein binding site (HOX; -199 to -192) (18,19), a transforming growth factor-β (TGF-β) activation element (-499 to -485) (20), and a glucocorticoid response element (-920 to -906), overlapping an AP-1 site (-921 to -915) (1,21) have also been characterized.

There are several kinds of hard laser, such as the neodymium:yttrium-aluminum-garnet (Nd:YAG), erbium:YAG (Er:YAG) and carbon dioxode (CO_2) lasers, and soft lasers such as the helium-neon and semiconductor lasers. These lasers are currently applied for a wide variety of dental procedures (22). Various effects, such as a bactericidal action in root canals and periodontal pockets, relief of pain, enhancement of wound healing, and stimulation of both fibroblast proliferation and macrophage phagocytosis have been reported (23,24). Low-power laser

irradiation exerts stimulatory effects on cell and tissues (25,26). After being absorbed, laser energy is transformed into heat, and therefore it would be expected that lasers would have useful effects such as anti-inflammation, pain elimination, wound healing and bone regeneration (27-31). Low-power lasers have also become well established in dentistry because of their anti-inflammatory, analgesic and regenerative effects, as well as a conditioning effect on tooth enamel (25,32). Early *in vivo* studies of bone regeneration after low-power laser treatment demonstrated increased bone deposition after tooth extraction, suggesting enhancing effects of this type of laser on ossification (33). These findings are supported by *in vitro* studies showing that low-power lasers significantly increased the proliferation, bone nodule formation and alkaline phosphatase activity of rat calvarial cells (34), and enhanced the osteogenic differentiation of murine mesenchymal stem cells (35). In the present study, we investigated the effects of a CO_2 laser at low energy density on BSP gene transcription in osteoblast-like cells.

Materials and Methods

Materials

Alpha minimum essential medium (α-MEM), fetal calf serum (FCS), lipofectamine, penicillin, streptomycin, and TrypLE™ Express were obtained from Invitrogen (Carlsbad, CA, USA). The protein kinase C inhibitor H7 and protein kinase A inhibitor H89 were from Seikagaku Corporation (Tokyo, Japan). Tyrosine kinase inhibitor herbimycin A (HA) was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). PGL3-basic luciferase plasmid, pSV-β-galactosidase control vector (β-Gal) and MAP kinase kinase (ERK1/2) inhibitor U0126 were purchased from Promega Co. (Madison, WI, USA). c-Jun N-terminal kinase (JNK) inhibitor SP600125 was from Calbiochem (San Diego, CA, USA). PP1 was from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). A Quikchange Site-directed Mutagenesis Kit was obtained from Stratagene (La Jolla, CA, USA). All chemicals used were of analytical grade. A CO_2 laser transmitter (GC NANOLASER GL-III) was obtained from GC (Tokyo, Japan).

Cell culture

Rat osteoblast-like ROS 17/2.8 cells were cultured at 37°C in 5% CO_2 and 95% air in α-MEM containing 10% FCS. The cells were grown to confluence in 60-mm tissue culture dishes and then cultured in α-MEM without serum for 12 h and then either left untreated or irradiated with the CO_2 laser at a dose of 0, 0.5, 1, 1.5, or 2 W for 20 s (power density: 0.5 w, 20 s; 0.357 J/cm^2, 1 w, 20 s; 0.715 J/cm^2, 1.5 w, 20 s; 1.07 J/cm^2, 2 w, 20 s; 1.43 J/cm^2), followed by incubation for 12 h, and followed the time course (3, 6, 12 and 24 h) after irradiation with the CO_2 laser (2 W, 20 s). Laser irradiation was performed at a distance of 300 mm (area of spot: 27.978 cm^2) from the probe to the cell layer. Total RNA was extracted with guanidinium thiocyanate as described previously (18). RNA was isolated from triplicate cultures and analyzed for the expression of BSP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA by Northern hybridization, as described below.

Northern hybridization

Twenty-five-microgram aliquots of total RNA were fractionated on a 1.2% agarose gel and transferred onto a Hybond N+ membrane. Hybridizations were performed at 42°C with ^{32}P-labeled rat BSP and GAPDH cDNA probes. Following hybridization, membranes were washed four times for 5 min each time 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, and 0.1% SDS. The hybridized bands, representing the rat BSP mRNA (two polyadenylated forms: 1.6 and 2.0 kilobases) and OPN mRNA were scanned in a Bio-imaging analyzer (Fuji BAS 2500, Tokyo, Japan).

Transient transfection assays

Exponentially growing ROS 17/2.8 cells were used for transient transfection assays. Twenty-four hours after plating, cells at 50-70% confluence were transfected using lipofectamine reagent. The transfection mixture included 1 μg of a luciferase (LUC) construct (pLUC1, -18~+60; pLUC2, -43~+60; pLUC3, -116~+60; pLUC4, -425~+60; pLUC5, -801~+60) and 2 μg of β-Gal vector as an internal control. Two days after transfection, the cells were deprived of FCS for 12 h and then irradiated with the CO_2 laser (2 W, 20 s), followed by culture for 12 h prior to harvest. The luciferase assay was performed in accordance with the supplier's protocol (picaGene, Toyo Inki, Tokyo, Japan) using a luminescence reader (Acuu FLEX Lumi 400; Aloka, Tokyo, Japan) to measure luciferase activity. The protein kinase inhibitors H7 (5 μM) and H89 (5 μM) were used to inhibit protein kinases C and A. HA (1 μM) and PP1 (10 μM) were used for tyrosine kinase and Src tyrosine kinase inhibition, respectively. U0126 (5 μM) was used as an ERK1/2 inhibitor, and SP600125 (10 μM) was used as a c-Jun N-terminal kinase (JNK) inhibitior. Oligonucleotide-directed mutagenesis by PCR was utilized to introduce dinucleotide substitutions using the Quikchange Site-directed Mutagenesis Kit. All constructs

were sequenced as described previously to verify the fidelity of the mutagenesis.

Gel mobility shift assays

Confluent ROS 17/2.8 cells were irradiated with the CO_2 laser (2 W, 20 s) and incubated for 3 to 12 h in α-MEM without FCS to prepare nuclear extracts, with the addition of extra proteinase inhibitors (the extraction buffer was 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol, 25% (v/v) glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A, and 1 μg/ml aprotinin, pH 7.9). For gel shift analysis, double-stranded oligonucleotides encompassing the inverted CCAAT (nt -61 to -37, 5'-CCGTGACCGTG**ATTGG**CTGCTGAGA), CRE (nt -84 to -59, 5'-CCCACAGCC**TGACGTCG** CACCGGCCG) and FRE (nt -98 to -79, 5'-TTTTCT**GGT GAGAA**CCCACA) in the rat BSP promoter were prepared by Bio-Synthesis, Inc. (Lewisville, TX). These double-stranded oligonucleotides were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Nuclear protein extracts (3 μg) were incubated for 20 min at room temperature with 0.1 pM radiolabeled double-stranded oligonucleotide in buffer containing 50 mM KCl, 0.5 mM EDTA, 10 mM Tris-HCl, pH 7.9, 1 mM dithiothreitol, 0.04% Nonidet P-40, 5% glycerol, and 1 μg of 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 200 V at room temperature. After electrophoresis, the gels were dried, and autoradiograms were prepared and analyzed using an image analyzer.

Statistical analysis

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

Results

Effects of CO_2 laser irradiation on BSP mRNA expression

To study the effect of the CO_2 laser on BSP transcription, we performed Northern hybridization analysis of total RNA extracted from osteoblastic ROS 17/2.8 cells. First, the dose-response relationship of BSP induction by the CO_2 laser was established by treating the ROS 17/2.8 cells with different power outputs from the laser for 20 s and then incubating them for 12 h. CO_2 laser irradiation increased the levels of BSP mRNA at 0.5, 1, 1.5 and 2 W and had a maximal effect at 2 W (Fig. 1A). To determine the time course of BSP mRNA expression, therefore, the cells were irradiated with the laser at 2 W for 20 s. It was found that laser irradiation up-regulated the level of BSP mRNA at 12 h after irradiation, whereas no effect on GAPDH was observed (Fig. 1B).

CO_2 laser stimulation of rat BSP gene transcription

To determine how CO_2 laser irradiation affects BSP transcription, transient transfection analyses were performed using chimeric constructs encompassing different regions of the rat BSP gene promoter ligated to a luciferase reporter gene (pLUC1~5) transfected into ROS 17/2.8 cells. After CO_2 laser irradiation (2 W, 20 s), luciferase activities of BSP promoter constructs (pLUC3; nts -116~+60, pLUC4; nts -425~+60, pLUC5; nts -801~+60) were increased at 12 h (Fig. 2). Included within the DNA sequence, which

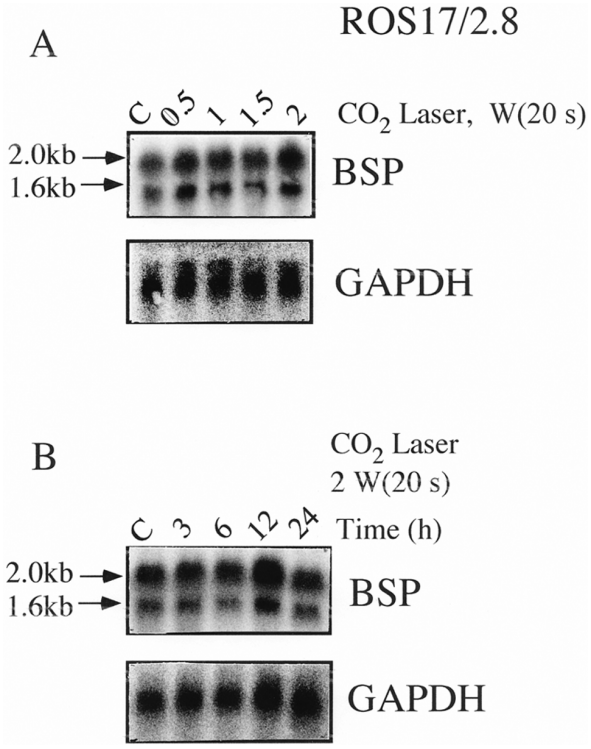

Fig. 1 Effect of CO_2 laser irradiation on BSP mRNA levels in ROS17/2.8 cells. A: Dose response effects of CO_2 laser irradiation (2 W, 20 s) on BSP mRNA levels. B: Time course effects of laser irradiation on BSP expression. Total RNA was extracted with guanidinium thiocyanate, and Northern hybridization was performed with ^{32}P-labeled rat BSP and GAPDH cDNA probes. The experiments were performed in triplicate for each data point. Results of representative hybridization analysis of control and CO_2 laser-treated cells are shown.

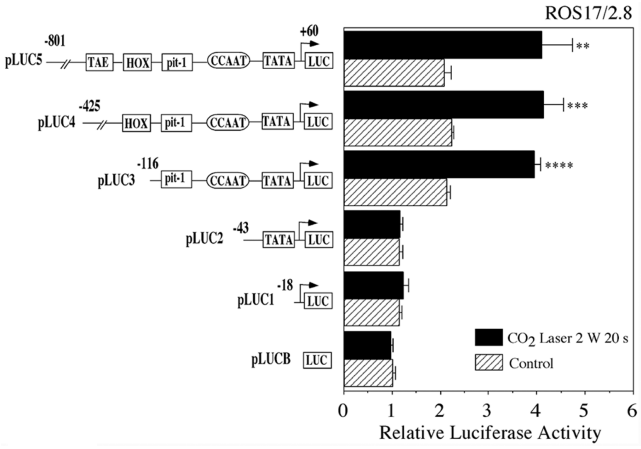

Fig. 2 Effect of CO_2 laser irradiation on BSP promoter activity in ROS 17/2.8 cells. Transient transfection of ROS 17/2.8 cells that were untreated or CO_2 laser (2 W)-irradiated for 20 s and cultured for 12 h was performed to determine the transcriptional activities of chimeric constructs that included various regions of the rat BSP promoter ligated to a luciferase reporter gene. The data for transcriptional activity obtained from four separate transfections with the constructs pLUC basic (pLUCB) and pLUC1 to pLUC5 have been combined, and the values are expressed with standard errors. **($P < 0.05$), ***($P < 0.02$), ****($P < 0.01$).

is unique in these regions, is an inverted CCAAT box (nts -50 to -46), a CRE motif (nts -75 to -68), a Runx2(1) motif (nts -84 to -79), a FRE motif (nts -92 to -85) and a Pit-1 motif (nts -111 to -105) (Fig. 3). To determine more precisely the target sites between nts -116 and -43 in the rat BSP gene promoter through which the CO_2 laser effect is mediated, we prepared a series of 5'-deletion constructs. The transcriptional activities of -84, -108 and -116BSPLUC in ROS17/2.8 cells were stimulated by the CO_2 laser (2 W, 20 s) at 12 h (Fig. 4). To determine the signaling pathways mediating the effects of the CO_2 laser, we tested the effects of several protein kinase inhibitors on the BSP transcription elicited by the laser. The tyrosine kinase inhibitor HA, the Src tyrosine kinase inhibitor PP1, and the ERK1/2 inhibitor U0126 abolished the effects of CO_2 laser irradiation on pLUC3 transcriptional activity (Fig. 5). To confirm the functional elements, we performed luciferase analyses using mutation constructs. After introducing 2-bp mutations into the CCAAT, CRE, FRE and Pit-1 elements within pLUC3, the basal activities of these constructs were lower than the basal transcriptional activity of pLUC3. The transcriptional stimulation elicited by the CO_2 laser (2 W, 20 s) was abrogated in the M-FRE construct (Fig. 6).

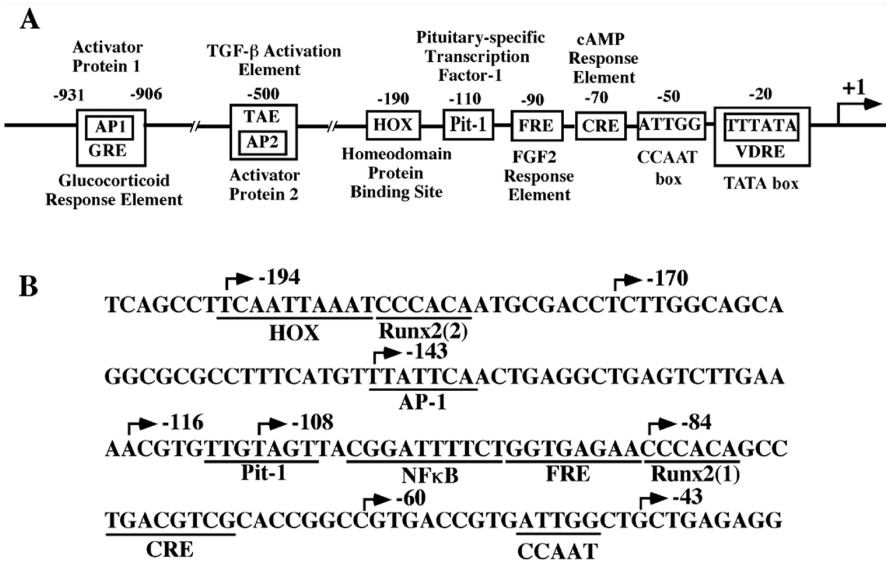

Fig. 3 Regulatory elements in the proximal promoter of the rat BSP gene. A: Diagram showing the positions of the inverted TATA and CCAAT boxes, vitamin D response element (VDRE) that overlaps the inverted TATA box, CRE (cAMP response element), FRE (FGF2 response element), Pit-1 (mediating the stimulatory effects of parathyroid hormone, PTH), the homeodomain protein-binding site (HOX), TAE (TGF-β activation element) overlapping with AP2, and GRE (glucocorticoid response element) overlapping with AP1. B: The nucleotide sequence of the rat BSP gene proximal promoter, encompassing an inverted CCAAT box, and the CRE, Runx2(1), FRE, NFκB, AP1, Runx2(2) and HOX elements is shown from -201 to -35.

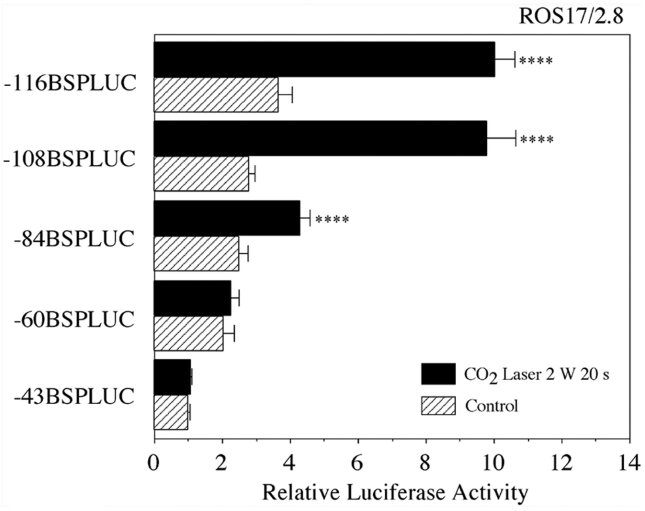

Fig. 4 Fine 5'-deletion mapping of the nts -116 to -43 elements in the BSP promoter. A series of rat BSP promoter 5'-deletion constructs were analyzed for relative promoter activity after transfection into ROS17/2.8 cells, which were subsequently irradiated with the CO_2 laser (2 W, 20 s) and incubated for 12 h. The results of transcriptional activity obtained from four separate transfections with the 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. ****($P < 0.01$).

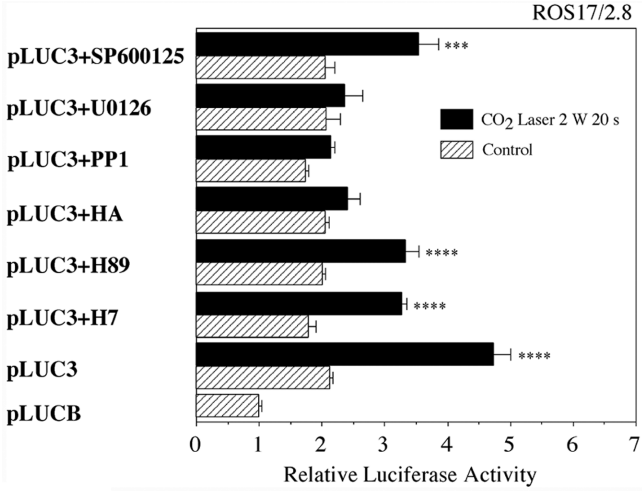

Fig. 5 Effects of kinase inhibitors on transcriptional activation by CO_2 laser irradiation. Transient transfection analysis of pLUC3 in ROS 17/2.8 cells irradiated with the CO_2 laser (2 W, 20 s) and incubated for 12 h is shown together with the effects of a PKC inhibitor (H7, 5 μM), PKA inhibitor (H89, 5 μM), tyrosine kinase inhibitor (herbimycin A; HA, 1 μM), MEK inhibitor (U0126, 5 μM), Src tyrosine kinase inhibitor (PP1, 10 μM), and JNK inhibitor SP600125 (10 μM). The results of transcriptional activity obtained from four separate transfections with the constructs were combined and the values expressed with standard errors. Significant differences from control: ***($P < 0.02$), ****($P < 0.01$).

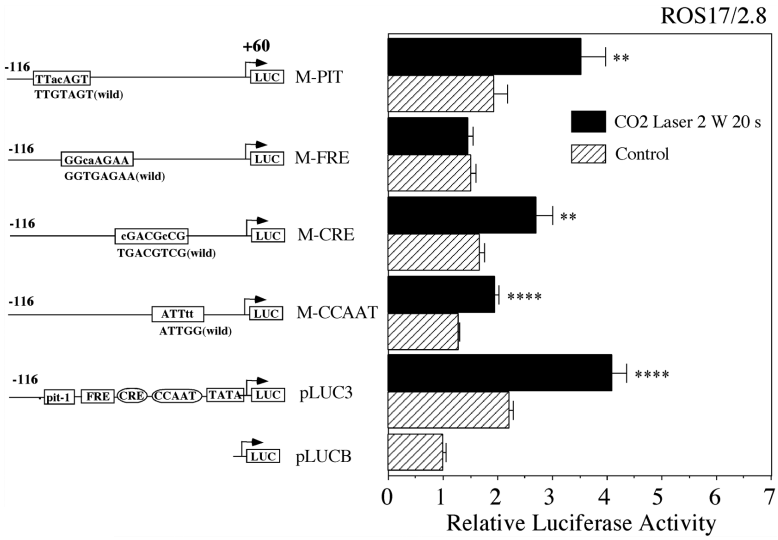

Fig. 6 Site mutation analysis of luciferase activities. Dinucleotide substitutions were made within the context of the pLUC3 promoter fragment. M-CCAAT (ATTtt), M-CRE (cGACGcCG), M-FRE (GGcaAGAA) and M-PIT (TTacAGT) constructs were analyzed for relative promoter activity after transfection into ROS17/2.8 cells, and examined for regulatory effects after irradiation with the CO_2 laser (2 W, 20 s) and incubation for 12 h. The data for transcriptional activity obtained from four separate transfections with the constructs were combined, and the values expressed with standard errors. **($P < 0.05$), ****($P < 0.01$).

Gel mobility shift assays

To identify nuclear proteins that bind to the CCAAT, CRE and FRE elements and mediate the effects of the CO_2 laser on transcription, double-stranded oligonucleotides were end-labeled and incubated with equal amounts (3 μg) of nuclear proteins extracted from confluent ROS 17/2.8 cells that were either untreated (control) or treated with the CO_2 laser (2 W, 20 s) for 3, 6 and 12 h. When we used the inverted CCAAT elements as probes, the DNA-protein complexes were not changed by laser irradiation (Fig. 7, lanes 1-4). When FRE was used as a probe, irradiation with the laser increased the binding of nuclear proteins to FRE at 3 h, and this was followed by a return to the basal level at 6 h and a further increase at 12 h (Fig. 7, lanes 9-12). Irradiation with the laser did not induce formation of the CRE-protein complex (Fig. 7, lanes 5-8). To further characterize the proteins in the complex formed with the FRE, we used antibodies against several transcription factors. Anti-Dlx5 and anti-Msx2 antibodies disrupted FRE-binding proteins (Fig. 8, lanes 4 and 5), while anti-Runx2 and anti-Smad1 antibodies did not reduce FRE-protein complex formation (Fig. 8, lanes 6 and 7).

Discussion

In this study, we showed that CO_2 laser irradiation enhanced transcription of the rat BSP gene by targeting FRE in its proximal promoter. BSP is a valuable marker of osteogenic differentiation and bone formation, and the RGD cell attachment sequence facilitates attachment of normal bone or cancer cells to mineralized tissue surfaces. BSP is also highly expressed in osteotropic cancer cells and plays a role in tumor metastasis to bone (3,8,9). BSP and OPN, as members of the family of small integrin binding proteins (SIBLINGs), are the major constituents

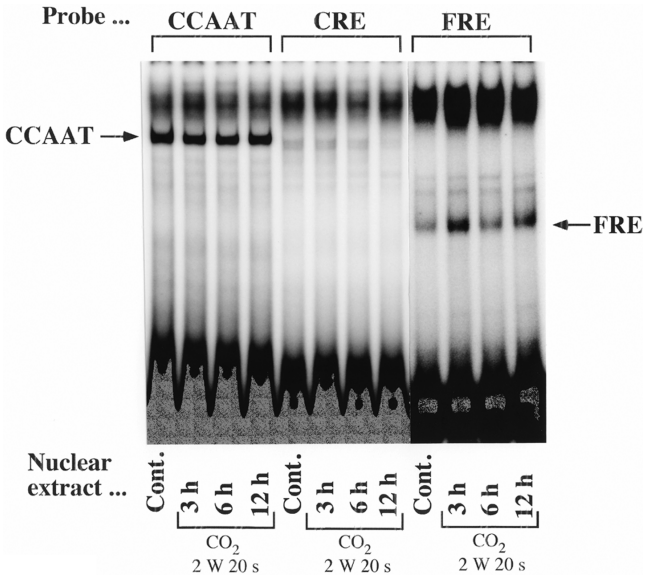

Fig. 7 Increase in the binding of nuclear proteins to the FRE induced by CO_2 laser irradiation. Radiolabeled double-stranded inverted CCAAT (-61 CCGTGACCGTG**ATT GG**CTGCTGAGA -79), CRE (-84 CCCACAAGCC**T GACGTCG**CACCGGCCG -59) and FRE (-98 TT TTCT**GGTGAGAA**CCCACA -79) oligonucleotides were incubated with nuclear protein extracts (3 μg) obtained from ROS17/2.8 cells that were untreated or irradiated (lanes 1, 5 and 9) with the CO_2 laser (2 W, 20 s) and incubated for 3 h (lanes 2, 6 and 10), 6 h (lanes 3, 7 and 11), or 12 h (lanes 4, 8 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 image analyzer.

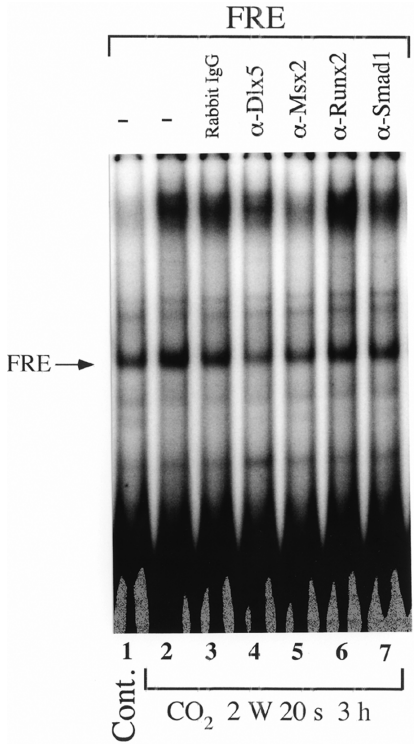

Fig. 8 Specific binding of nuclear proteins to the FRE. Radiolabeled double-stranded FRE was incubated with nuclear protein extracts (3 μg) obtained from ROS17/2.8 cells that were untreated (lane 1) or irradiated with the CO_2 laser (2 W, 20 s) and cultured for 3 h (lanes 2-7). Supershift experiments were performed with 0.4 μg of antibodies against IgG (lane 3), Dlx5 (lane 4), Msx2 (lane 5), Runx2 (lane 6) and Smad1 (lane 7) added separately to each gel shift reaction.

of non-collagenous proteins in bone matrix, and expression of their gene products has been shown to be under the control of calcitropic hormones and cytokines (3,8). In the present study, CO_2 laser irradiation increased the level of BSP mRNA and transcriptional activity of the BSP gene (Fig. 1 and 2), thus supporting the possibility that CO_2 laser irradiation could be utilized to regulate osteoblastic function in the local bone environment.

Using transient transfection assays, we localized the CO_2 laser-responsive region to the proximal promoter (pLUC3, -116 to -43) of the rat BSP gene, which encompasses an inverted CCAAT box, and the CRE, Runx2(1), FRE and Pit-1 motifs (Fig. 3). However, no further increase in transcription of the pLUC4 and pLUC5 constructs was observed (Fig. 2), suggesting that CO_2 laser irradiation influences BSP transcription through the response element in pLUC3 that contains the FRE. Using fine 5'-deletion constructs between nts -116 to -43 in the rat BSP promoter, -84BSPLUC, containing CRE and Runx2(1), was identified as one of the targets of CO_2 laser irradiation. Luciferase activity of -108BSPLUC was further enhanced by laser irradiation, suggesting that the responsive region lies not only in -84BSPLUC but also in the -108BSPLUC construct (Figs. 3 and 4). Transcriptional activity was totally abrogated by mutation of FRE in the pLUC3 construct (Fig. 6), thus further confirming that FRE is the functional element responsible for the effect of CO_2 laser irradiation on BSP transcription.

The involvement of FRE was further supported by gel shift analyses (Fig. 7). Transcription factors that bind specifically to FRE were demonstrated by supershift assays, which indicated that anti-Dlx5 and anti-Msx2 antibodies interacted with FRE-binding proteins (Fig. 8).

Animal studies have demonstrated that low-power laser irradiation enhances the functional attachment of titanium implants to bone and promotes bone healing and mineralization (36). As the regeneration of bone defects is a key problem in regenerative medicine, the biostimulatory effects of low-power lasers could be an attractive form of co-treatment for repair of such defects (37). The CO_2 laser is easily absorbed by water, and therefore irradiated deep tissues are rarely injured (38). Although the CO_2 laser is a hard surgical laser, we have attempted to use it at low energy density to achieve biological alterations (31). Laser irradiation has been reported to stimulate the proliferation of osteoblast-like cells (39), and a similar effect of low-output CO_2 laser irradiation on fibroblasts has also been reported (40).

In this study, we have shown that the CO_2 laser can enhance BSP gene transcription through the tyrosine kinase, Src tyrosine kinase and ERK1/2 signaling pathways, and identified FRE in the rat BSP gene promoter as the target of CO_2 laser-mediated enhancement of BSP gene transcription.

Acknowledgments

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