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Nicotine suppresses bone sialoprotein gene expression

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Background and Objective: Tobacco smoking is a risk factor for periodontitis and osteoporosis. Nicotine is a major component of tobacco, and has been reported to inhibit proliferation and differentiation of osteoblasts. Bone sialoprotein (BSP) is a mineralized tissue-specific protein expressed by differentiated osteoblasts that appears to function in the initial mineralization of bone. The purpose of this study was to determine the effects of nicotine on bone metabolism.

Material and Methods: We used rat osteobast-like UMR106 and ROS 17/2.8 cells and rat stromal bone marrow RBMC-D8 cells. To determine the molecular basis of the transcriptional regulation of the BSP gene by nicotine, we conducted Northern hybridization, transient transfection analyses with chimeric constructs of the BSP gene promoter linked to a luciferase reporter gene and gel mobility shift assays.

Results: Nicotine (250 μg/mL) decreased the BSP mRNA levels at 12 and 24 h in UMR106 and ROS 17/2.8 cells. From transient transfection assays using various sized BSP promoter–luciferase constructs, nicotine decreased the luciferase activities of the construct, including the promoter sequence nucleotides −116 to +60, in UMR106 and RBMC-D8 cells. Nicotine decreased the nuclear protein binding to the cAMP response element (CRE), fibroblast growth factor 2 response element (FRE) and homeodomain protein-binding site (HOX) at 12 and 24 h.

Conclusion: This study indicates that nicotine suppresses BSP transcription mediated through CRE, FRE and HOX elements in the proximal promoter of the rat BSP gene.

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Tobacco smoking is associated with various oral lesions, such as oral cancer (1), leukoplakia (2,3) periodontal diseases (3–6) and alveolar bone loss (6). Furthermore, smoking has been suggested as a risk factor in postmenopausal osteoporosis (7) and bone fracture (8) and in increased bone loss and decreased intestinal calcium absorption (9). Nicotine is a major component of tobacco, and it has been reported that nicotine inhibits cell growth of various cell types, including skin fibroblasts (10), gingival fibroblasts (11), lung cancer cells (12) and osteosarcoma cells (13). Nicotine downregulates collagen gene expression, collagenase activity and DNA synthesis in cultured cardiac fibroblasts (14). In osteoblast-like cells, nicotine inhibits collagen synthesis and alkaline phosphatase activity, but stimulates DNA synthesis (15). Furthermore, nicotine has a direct effect on human bone cells in modulating proliferation, upregulation of c-fos oncoprotein and synthesis of osteopontin (16). Nicotine downregulates osteocalcin expression (17). However, molecular mechanisms of nicotine effects on mineralization and bone loss are not well understood.

Bone sialoprotein (BSP) is a sulphated and phosphorylated glycoprotein expressed almost exclusively in mineralized tissues (18,19). The temporospatial deposition of BSP into the extracellular matrix of bone (20) and its ability to nucleate hydroxyapatite crystal formation (21) indicate a

potential role for BSP in the initial mineralization of bone. Bone sialoprotein is also expressed in breast, lung, thyroid and prostate cancers (18,22,23). Thus, it has been suggested that BSP may be involved in the osteotropism of the metastatic cancer cells through its ability to bind to hydroxyapatite and to mediate cell attachment through cell surface integrins (18,22,23). Thus, regulation of BSP gene transcription appears to be important for the differentiation of osteoblasts, bone matrix mineralization and tumour metastasis.

To study the transcriptional regulation of the BSP gene, rat, human and mouse BSP gene promoters were cloned and sequenced (24–27). These promoters include an inverted TATA box (28) overlapping a vitamin D response element (29) and an inverted CCAAT box that is required for basal transcriptional activity (30,31). In addition, a cAMP response element (CRE; −75 to −68; 32–34), a fibroblast growth factor 2 response element (FRE; −92 to −85; 33,35), a pituitary-specific transcription factor-1 (Pit-1; −111 to −105) motif (36) and a homeodomain protein-binding site (HOX; −199 to −192; 37,38) have been characterized. Further upstream in the rat BSP promoter, a transforming growth factor-β activation element (TAE; −499 to −485; 39) and a glucocorticoid response element (GRE; −920 to −906), overlapping an activator protein 1 site (−921 to −915; 19,40) have also been identified.

To elucidate the molecular mechanism of nicotine regulation of the BSP gene, we have analyzed the effects of nicotine on the expression of BSP in the rat osteobast-like UMR106 and ROS 17/2.8 cells and in rat stromal bone marrow RBMC-D8 cells. This study indicates that nicotine decreases BSP gene transcription mediated through CRE, FRE and HOX elements in the rat BSP gene promoter.

Material and methods

Material

α-Minimal essential medium (α-MEM), fetal calf serum (FCS), lipofectamine, penicillin and streptomycin and trypsin–EDTA were obtained from Invitrogen (Carlsbad, CA, USA). pSV-β-galactosidase control vector was purchased from Promega (Madison, WI, USA). Guanidium thiocyanate was purchased from Wako Pure Chemical (Tokyo, Japan). The (–)-nicotine(–)-1-methyl-2-(3-pyridyl)pyrrolidine was purchased from Sigma (St Louis, MO, USA). The protein kinase A (PKA) inhibitor, H89, and protein kinase C (PKC) inhibitor, H7, were from Seikagaku Corp. (Tokyo, Japan) and the tyrosine kinase inhibitor, herbimycin A (HA), was purchased from Wako Pure Chemical Industries, Ltd (Tokyo, Japan).

Cell culture

Rat osteoblast-like UMR106 cells (33), ROS 17/2.8 cells (35) and rat stromal bone marrow (RBMC-D8) cells (38) were used in this study. The cells were cultured at 37°C in air enriched with 5% CO_2 in α-MEM supplemented with 10% FCS. The cells were first grown to confluence in 60 mm tissue culture dishes in α-MEM containing 10% FCS, then cultured in α-MEM without serum, and stimulated with nicotine (250 μg/mL). Total RNA was isolated from triplicate cultures at various time and dose intervals and analyzed for the expression of BSP mRNA by Northern hybridization.

Northern hybridization

Total RNA from the UMR106 and ROS 17/2.8 cells was extracted with guanidium thiocyanate and, following purification, 20 μg aliquots of RNA were fractionated on a 1.2% agarose gel and transferred onto a Hybond N plus membrane, as described previously (35). Hybridizations were performed at 42°C with a ^{32}P-labeled rat BSP, osteopontin (OPN) and glyceraldehyde-3-phosophate dehydrogenase (GAPDH) cDNA probes. Following hybridization, membranes were washed four times for 5 min each at 21°C in 300 mM sodium chloride, 30 mM trisodium citrate pH 7.0 and 0.1% sodium dodecyl sulfate (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 were scanned in a Bio-imaging analyzer (Fuji BAS 2000, Tokyo, Japan).

Transient transfection assays

Exponentially growing UMR106 and RBMC-D8 cells were used for transfection assays. Twenty-four hours after plating, the cells at 40–60% confluence were transfected using a lipofectamine reagent. The transfection mixture included 1 μg of a luciferase (LUC) construct (pLUC1, −18 to +60; pLUC2, −43 to +60; pLUC3, −116 to +60; pLUC4, −425 to +60; and pLUC5, −801 to +60; 19) and 2 μg of pSV-β-galactosidase (β-Gal) vector as an internal transfection control. Two days post-transfection, the cells were deprived of serum for 12 h, and 250 μg/mL nicotine was added for 12 h prior to harvesting. The protein kinase inhibitors, H89 (5 μM) and H7 (5 μM), were used to inhibit protein kinases A and C, respectively. Herbimycin A (1 μM) was used for tyrosine kinase inhibiton. The luciferase assay was performed according to the manufacturer's protocol (PicaGene, Toyo Inki, Tokyo, Japan) using a luminescence reader (Acuu FLEX Lumi 400; Aloka, Tokyo, Japan) to measure the luciferase activity.

Gel mobility shift assays

Confluent UMR106 and RBMC-D8 cells stimulated for 6, 12 and 24 h with nicotine (250 μg/mL) in α-MEM without serum were used to prepare nuclear extracts. Nuclear proteins were extracted by the method of Dignam *et al*. (41) 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 leupeptin, 2 μg/mL pepstatin A and 1 μg/mL aprotinin, pH 7.9). Protein concentration was determined by the Bradford assay (42). Double-stranded oligonucleotides encompassing (shown in bold) the inverted CCAAT (nucleotides −61 to −37,

5′-CCGTGACCGTG**ATTGG**CTGCT GAGA), CRE (nucleotides −84 to −59, 5′-CCCACAGCC**TGACGTCG**CACC GGCCG), FRE (nucleotides −98 to −79, 5′-TTTTCT**GGTGAGAA**CCC ACA) and HOX (nucleotides −204 to −179, 5′- TCCTCAGCCT**TCAATTA-AA**TCCCACA) in the rat BSP promoter were prepared by Bio-Synthesis, Inc. (Lewisville, TX, USA). Consensus CRE (5′-AGAGATTGCC**TGACGT-CA**GAGAGCTAG) was purchased from Promega. For gel shift analysis, the 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 150 V at room temperature. After electrophoresis, the gels were dried, and the autoradiograms were prepared and analyzed using an image analyzer.

Statistical analysis

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

Results

Stimulation of BSP mRNA expression in UMR106 cells

To study the regulation of BSP expression by nicotine, we used UMR106 and ROS 17/2.8 cells, which have been shown to have osteoblastic characteristics and to express BSP mRNA constitutively (33,35). First, a dose–response relation for nicotine regulation of BSP was established by treating the UMR106 cells with different concentrations of nicotine for 12 h and measuring the BSP mRNA levels by Northern blot analysis. Nicotine decreased BSP mRNA at 250 and 750 μg/mL (Fig. 1A). Nicotine (250 μg/mL) was used to determine a time course of BSP mRNA expression. Nicotine (250 μg/mL) downregulated BSP mRNA accumulation markedly in UMR106 and ROS 17/2.8 cells (Fig. 1B,C). An inhibition was evident 3 h after the addition of nicotine, with maximal effects obtained at 12 and 24 h in UMR106 cells (Fig. 1B). While nicotine inhibited BSP mRNA expression at 24 h, increased OPN mRNA levels were observed 6, 12 and 24 h after the addition of nicotine in ROS 17/2.8 cells (Fig. 1C). These results suggest that the effects of nicotine were not a non-specific toxic effect.

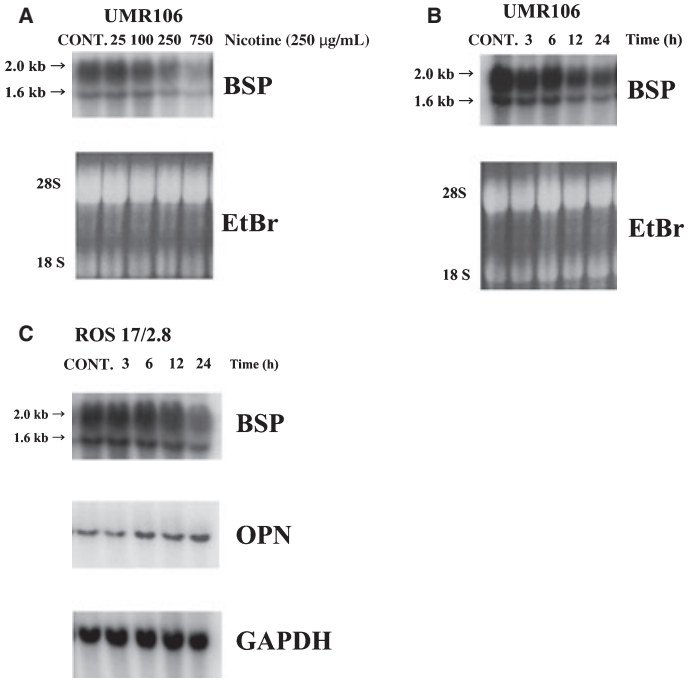

Fig. 1. Northern hybridization analysis of nicotine effects on BSP mRNA expression. (A) Dose–response analysis of the effect of nicotine on BSP mRNA levels in osteoblastic cell line UMR106 treated for 12 h. (B) Twenty-four hour time course revealed a reduction of BSP mRNA in UMR106 cells treated with 250 μg/mL nicotine. (C) The 24 h time course revealed a decrease in BSP mRNA and increase in OPN mRNA levels in ROS 17/2.8 cells treated with 250 μg/mL nicotine. Total RNA was isolated from triplicate cultures harvested after incubation for 3, 6, 12 and 24 h and used for Northern hybridization analysis using a ^{32}P-labeled rat BSP, OPN and GAPDH DNA probe. No effect was observed on ribosomal RNA (28S and 18S) in UMR106 cells or on GAPDH in ROS 17/2.8 cells.

Transient transfection analysis of rat BSP promoter constructs

Transient transfections of chimeric constructs encompassing different regions of the rat BSP promoter ligated to a luciferase reporter gene (pLUC1–pLUC5) were performed in UMR106 and RBMC-D8 cells. The transcriptional activity of pLUC3, which encompasses nucleotides −116 to +60, was reduced ~0.57-fold after 12 h treatment with 250 μg/mL nicotine in UMR106 cells (Fig. 2A). Nicotine also decreased transcription of pLUC4 (−425 to +60) and pLUC5 (−801 to +60). In shorter constructs (pLUC1, −18 to +60; pLUC2, −43 to +60), luciferase activities were not influenced by nicotine (Fig. 2A). When transcriptional activity in response to 250 μg/mL nicotine was analyzed in normal RBMC-D8 cells (38,43), the transcriptional activities of pLUC3, pLUC4 and pLUC5 were decreased (Fig. 2B). Within the DNA sequence that is unique to pLUC3 and pLUC4, an inverted CCAAT box (nucleotides

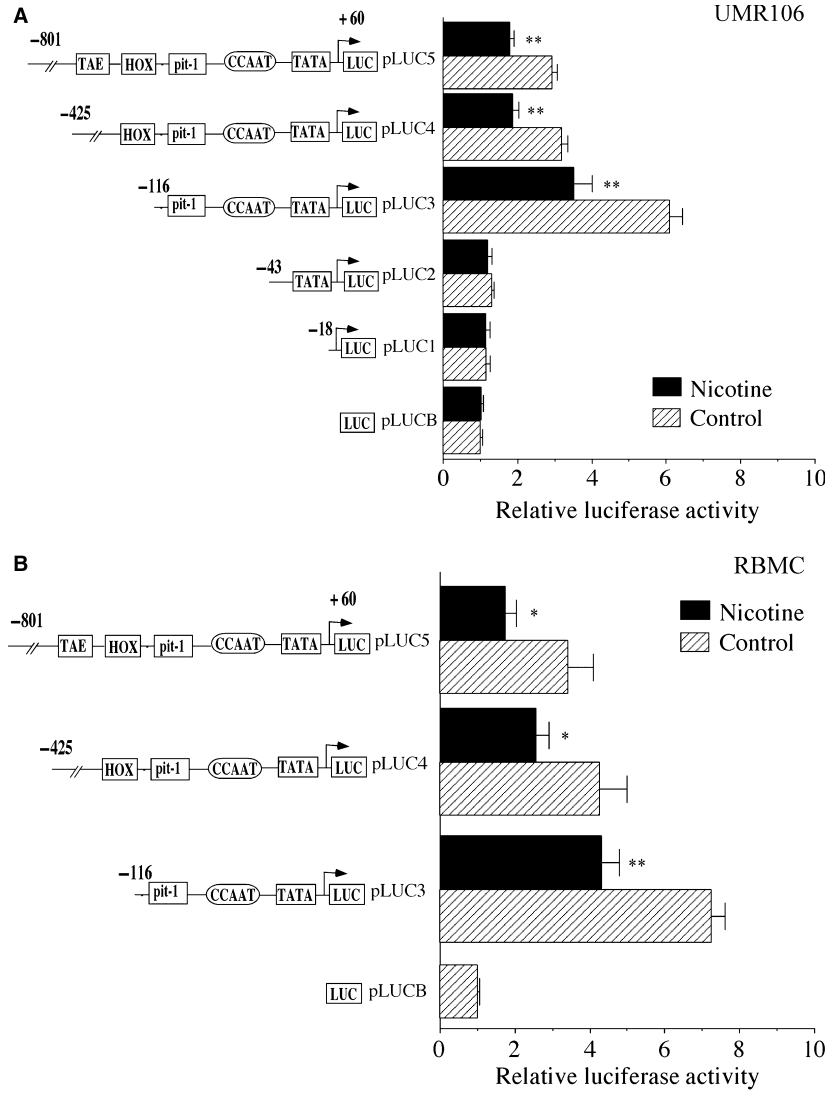

Fig. 2. Nicotine regulates BSP promoter activity in UMR106 (A) and RBMC-D8 cells (B). Transient transfections of UMR106 and RBMC-D8 cells in the presence or absence of nicotine (250 μg/mL) for 12 h were used to determine transcriptional activities of chimeric constructs that included various regions of the BSP promoter ligated to a luciferase reporter gene. The results of transcriptional activities obtained from four separate transfections with constructs, pLUC basic (pLUCB) and pLUC1–pLUC5, have been combined, and the values are expressed with standard errors. Significant differences from control: $*p < 0.1$; $**p < 0.01$.

−50 to −46), CRE (nucleotides −75 to −68), FRE (nucleotides −92 to −85), Pit-1 (nucleotides −111 to −105) and HOX (nucleotides −194 to −185) sites are present (Fig. 3). Since protein kinases mediate nicotine signaling activities, we also investigated the effects of the PKC inhibibitor, H7, the PKA inhibitor, H89, and the tyrosine kinase inhibitor, herbimycin A (HA). Nicotine-reduced pLUC3 promoter activity was inhibited by H7, H89 and HA (data not shown), indicating an involvement of PKC, PKA and tyrosine kinase in the signaling pathway.

Gel mobility shift assays

To determine whether nuclear protein binding to response elements in pLUC3 and pLUC4 might be modulated by nicotine, double-stranded oligonucleotides were end-labeled and incubated with equal amounts (3 μg) of nuclear proteins extracted from confluent RBMC-D8 cells that were either not treated (control) or treated with 250 μg/mL nicotine for 6, 12 and 24 h. When CRE and consensus CRE were used in RBMC-D8 cells as probes, the formation of CRE–protein (Fig. 4, lanes 5–8) and consensus CRE–protein complexes (Fig. 4, lanes 9–12) was increased by nicotine (250 μg/mL) at 6 h, and declined at 12 and 24 h. When we used the inverted CCAAT sequence as a probe, the DNA and nuclear factor-Y protein complex (30,31) did not change after stimulation by nicotine (Fig. 4, lanes 1–4). When FRE and HOX were used as probes, the formation of FRE–protein complexes (Fig. 4, lanes 13–16) and HOX–protein complexes (Fig. 4, lanes 17–20) in RBMC-D8 cells was increased by nicotine (250 μg/mL), reaching maximal levels at 6 h, and declining at 12 and 24 h.

Discussion

Smoking is a risk factor in many diseases, in particular oral cancer and periodontitis (1–3). Smokers have been shown to have 4–5% lower bone mineral density (44). Nicotine is a major component of tobacco, and it has been reported that the effects of nicotine decrease expression of collagen (10,14), osteocalcin (17) and osteopontin (16). Bone sialoprotein is a mineralized tissue-specific protein expressed by newly formed osteoblasts coincident with mineralization, which provides a valuable maker for osteogenic differentiation and bone formation (23). However, until now there has been no investigation of the effect of nicotine on BSP expression.

In this study, we have identified CRE, FRE and HOX as nicotine response elements in the rat BSP gene promoter that mediate the action of nicotine on BSP transcription. Nicotine (250 μg/mL) decreased BSP mRNA levels (Fig. 1) in UMR106 and ROS 17/2.8 cells. and downregulated BSP promoter activities (pLUC3, pLUC4 and pLUC5) in UMR106 and RBMC-D8 cells (Fig. 2A,B). From transient transfection assays, we located the nicotine response region to

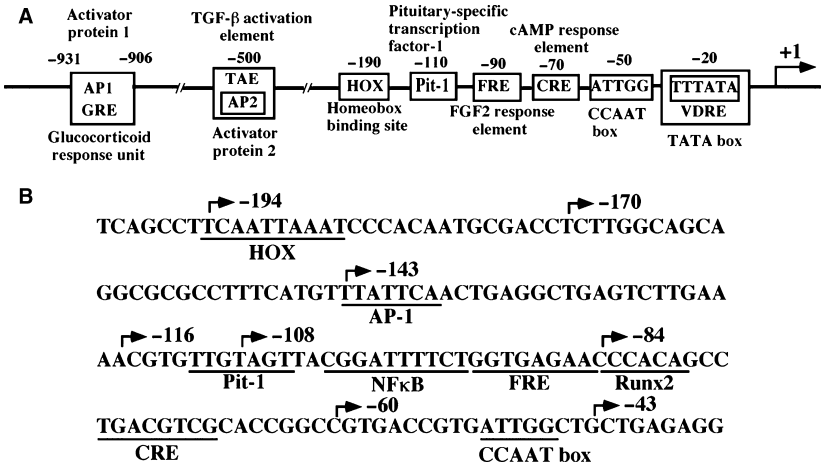

Fig. 3. Regulatory elements in the proximal rat BSP promoter. (A) The position of the inverted TATA and CCAAT boxes, a CRE, a FRE, Pit-1, a homeobox-binding site (HOX), a TGF-β activation element (TAE) overlapping with activator protein 2 (AP-2), glucocorticoid response elements (GRE) overlapping with activator protein 1 (AP-1), 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). (B) The nucleotide sequences of the rat BSP gene promoter encompassing an inverted CCAAT box, CRE, runt homeodomain protein 2 (Runx 2), FRE, nuclear factor-κB (NF-κB), Pit-1, AP-1 and HOX are shown from nucleotides −201 to −35.

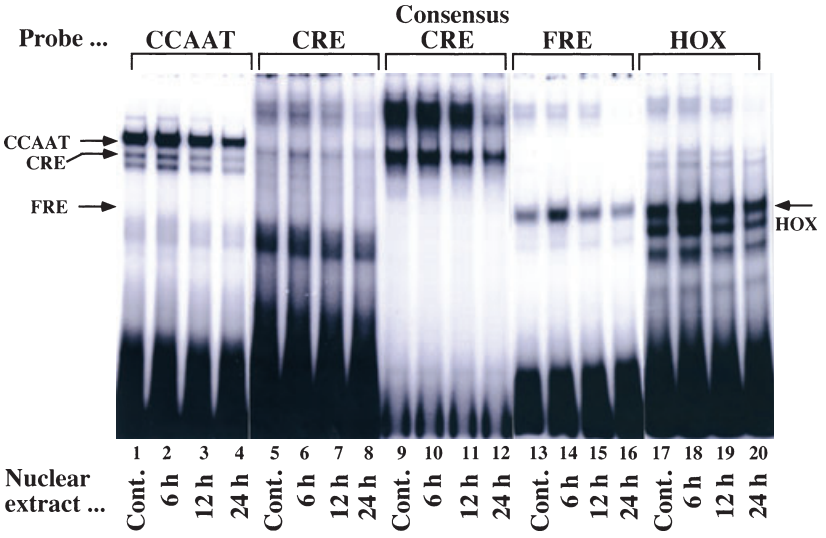

Fig. 4. Gel mobility shift analysis of inverted CCAAT, CRE, consensus CRE, FRE and HOX oligonucleotides. Radiolabeled double-stranded inverted CCAAT (−61 CCGTGACCGTG**ATTGG**CTGCTGAGA −37), CRE (−84 CCCACAGCC**TGACGTCG**CACCGGCCG −59), consensus CRE (AGAGATTGCC**TGACGTCA**GAGAGCTAG), FRE (−98 TTTTCT**GGTGAGAA**CCCACA −79) and HOX oligonucleotides (−204 TCCTCAGCCT**TCAATTAAA**TCCCACA −179) were incubated for 20 min at 21°C with nuclear protein extracts (3 μg) obtained from RBMC-D8 cells incubated without (control; lanes 1, 5, 9, 13 and 17) or with nicotine (250 μg/mL) for 6 h (lanes 2, 6, 10, 14 and 18), 12 h (lanes 3, 7, 11, 15 and 19) and 24 h (lanes 4, 8, 12, 16 and 20). The 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.

the proximal promoter (nucleotides −425 to −43) of the rat BSP gene, which encompasses an inverted CCAAT box, CRE, runt homeodomain protein 2, FRE, Pit-1 and HOX elements (Fig. 3). Previously, we have identified several response elements that mediate the effects of hormones, growth factors and cytokines in the rat BSP gene promoter, and most of them exist in pLUC3 and pLUC4 (19,29,32–36,38,39,45). Therefore, we investigated the binding of nuclear proteins to the possible response elements in pLUC3 and pLUC4 by gel shift analyses. The formation of CRE–protein and consensus CRE–protein complexes in RBMC-D8 cells was increased at 6 h and decreased at 12 and 24 h by nicotine (250 μg/mL; Fig. 4). When FRE and HOX were used as probes, the formation of FRE–protein and HOX–protein complexes was increased by nicotine (250 μg/mL) at 6 h, and declined at 12 and 24 h (Fig. 4). Although nicotine suppressed cellular proliferation, it increased alkaline phosphatase (ALP) activity in UMR 106-01 cells (46). The suppressive effect of nicotine on proliferation was biphasic, with a decrease at 2 h, recovery to near control levels at 6 h and a maximal decrease at 48 h. This biphasic effect of nicotine was similar to our gel shift results. In contrast, nicotine inhibited ALP activity, but stimulated DNA synthesis in osteoblast-like cells (15). These results suggest that the effects of nicotine on osteoblast-like cells could be dependent on the stage of differentiation of the osteoblasts. Nicotine upregulated c-fos mRNA levels and osteopontin protein synthesis in human osteoblast-like cells (16). Nicotine stimulated the deposition of Ca^{2+} in ROB-C26 cells, which are characteristic of the early stage of cell differentiation, and decreased the deposition of Ca^{2+} in MC3T3-E1 cells, which are characteristic of the late stage of differentiation of osteoblasts (47). Nicotine (10^{-3} M) inhibited mineralized bone nodule formation and BSP mRNA levels in Saos2 cells *in vitro* (48). These results might support the results of our gel shift assays, in which the formation of CRE–protein, FRE–protein and HOX–protein complexes increased at the early time point (6 h) and decreased thereafter.

In conclusion, we have identified CRE, FRE and HOX elements in the rat BSP proximal gene promoter that mediate the effects of nicotine on BSP

transcription. Since BSP is expressed by differentiated osteoblasts and nicotine is an important risk factor for periodontal disease, it is conceivable that these response elements may contribute to the effects of nicotine on bone metabolism, which closely relate to BSP expression and bone formation.

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